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Biochemistry

An oleate 12-hydroxylase from *Ricinus communis* L. is a fatty acyl desaturase homolog

(ricinoleic acid/castor/FAH12/transgenic plants)

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ABSTRACT **Recent spectroscopic evidence implicating a binuclear iron site at the reaction center of fatty acyl desaturases suggested to us that certain fatty acyl hydroxylases may share significant amino acid sequence similarity with desaturases. To test this theory, we prepared a cDNA library from developing endosperm of the castor-oil plant (*Ricinus communis* L.) and obtained partial nucleotide sequences for 468 anonymous clones that were not expressed at high levels in leaves, a tissue deficient in 12-hydroxyoleic acid. This resulted in the identification of several cDNA clones encoding a polypeptide of 387 amino acids with a predicted molecular weight of 44,407 and with ≈67% sequence homology to microsomal oleate desaturase from *Arabidopsis*. Expression of a full-length clone under control of the cauliflower mosaic virus *35S* promoter in transgenic tobacco resulted in the accumulation of low levels of 12-hydroxyoleic acid in seeds, indicating that the clone encodes the castor oleate hydroxylase. These results suggest that fatty acyl desaturases and hydroxylases share similar reaction mechanisms and provide an example of enzyme evolution.**

Ricinoleic acid (D-12-hydroxyoctadec-*cis*-9-enoic acid) is a hydroxylated fatty acid identified as a constituent of the seed storage oil in at least 12 genera from 10 families of higher plants (reviewed in ref. 1). The agriculturally important source of ricinoleate is castor-oil plant (*Ricinus communis* L.), in which ricinoleic acid is restricted to the seed triacylglycerides, where it constitutes 85–90% of the fatty acids (2). In addition to its well-known purgative property, castor oil, by virtue of its ricinoleate content, has many industrial uses, including the manufacture of nylon, paints and varnishes, resins, lubricants, and cosmetics (2).

In vivo labeling studies indicated that, in the developing endosperm of castor, ricinoleate is synthesized by direct hydroxyl substitution of an oleic acid moiety rather than via an unsaturated, keto or epoxy intermediate (3). The hydroxylase activity, which is thought to be associated with the endoplasmic reticulum (4, 5), is labile and has not been purified. *In vitro* ricinoleate synthesis by microsomal membranes requires oleoyl-CoA (or oleoylphosphatidylcholine), molecular oxygen, and NADH (4, 5). Enzyme assays (5) and radioisotope labeling studies (6) indicate that the substrate for the oleate hydroxylase is oleic acid esterified to either phosphatidylcholine or another phospholipid. The NADH is required to reduce cytochrome b_5, the intermediate electron donor for the hydroxylase reaction (7). The involvement of iron in catalysis was suggested by observations that the hydroxylase activity was sensitive to cyanide and azide, and dialysis against metal chelators reduced activity, which could be restored by addition of $FeSO_4$ (4). Carbon monoxide does not inhibit hydroxylation, indicating that a cytochrome P-450 is not involved (4, 5).

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The castor oleate hydroxylase has many superficial similarities to the microsomal fatty acyl desaturases (8). In particular, the microsomal $\omega - 6$ oleate desaturase acts on oleate esterified to the *sn*-2 position of phosphatidylcholine (9) and requires molecular oxygen and reduced cytochrome b_5 (7, 10). Recent Mossbauer studies of the castor stearyl-acyl carrier protein (ACP) desaturase have shown that this soluble enzyme contains a catalytically active μ-oxo-bridged diiron cluster (Fe—O—Fe) (11). This cofactor is also found in the hydroxylase component of the bacterial enzyme methane monooxygenase. Based on the fact that the Fe—O—Fe cluster is now known to be involved in both hydroxylation and desaturation (12), and in view of the superficial similarities between the castor hydroxylase and the microsomal $\omega - 6$ oleate desaturase, we have speculated that modifications of the active site of desaturases or hydroxylases containing Fe—O—Fe clusters could alter the outcome of the reaction, thereby converting a desaturase to a hydroxylase and vice versa (1). Furthermore, plants in which ricinoleic acid occurs are found throughout the plant kingdom; yet close relatives of these plants do not contain ricinoleate. This suggests that the ability to synthesize ricinoleic acid has arisen several times independently and that a relatively minor genetic change was associated with acquisition of hydroxylase activity. This raised the possibility that the oleate hydroxylase may exhibit significant amino acid sequence similarity to a desaturase.

A putative iron-binding motif, (D/E)-E-X-R-H, has been identified in the castor stearyl-ACP desaturase primary structure by comparison with other soluble enzymes, such as methane monooxygenase, that contain the Fe—O—Fe cluster (11). Recently, cDNA clones have been isolated for membrane-bound desaturases encoding microsomal and plastic $\omega - 3$ and $\omega - 6$ desaturates of several plant species (reviewed in ref. 13). Of great interest is the identification of repeated histidine-rich motifs in all of these sequences, the membrane-bound stearoyl-CoA desaturase from animals and fungi, and in two membrane-bound monooxygenases (9, 13). These motifs, H-X-X-H-H or H-X-X-X-H-H, may be the functional equivalents in membrane-bound Fe—O—Fe proteins of the (D/E)-E-X-R-H motif in the soluble Fe—O—Fe proteins.

On the basis of the foregoing considerations, we hypothesized that it might be possible to identify a cDNA for the castor oleate hydroxylase by making several assumptions. First, the hydroxylase should contain a pair of histidine-rich motifs. Second, the hydroxylase gene would not be expressed in leaves, which lack ricinoleate. Third, because of the similarities of the reactions catalyzed by the hydroxylase and the castor stearyl-ACP desaturase, we assumed that the mRNAs for the hydroxylase should be present in developing seeds at similarly high

Abbreviation: TMS, trimethylsilyl.

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levels to that of the desaturase, estimated to be about 0.1% of total mRNA (14).

A cDNA library prepared from developing castor endosperm was enriched for clones expressed at moderate levels in developing endosperm but without detectable expression in leaves by a differential screening approach, and partial nucleotide sequence data were obtained for 468 of these clones (15). Three clones with identical overlapping nucleotide sequences satisfied the proposed criteria. The evidence that these clones encode an oleate hydroxylase is reported here.‡

MATERIALS AND METHODS

Nucleic Acid Manipulations. Total RNA was purified from castor developing stages III–V (16), cellular endosperm, and embryo. Poly(A)$^+$ RNA was enriched by two rounds of chromatography on oligo(dT) cellulose (17).

A cDNA library of 1.4×10^6 primary transformants was constructed from poly(A)$^+$ RNA in the vector pYES2.0 by using a kit ("Librarian IV"; Invitrogen) according to the manufacturer's instructions. A second cDNA library was constructed in the vector Lambda ZAP II (Stratagene) according to the manufacturer's instructions. Filter replicas of this library were hybridized with labeled first-strand cDNA from developing seed RNA and leaf RNA, and moderately abundant, apparently seed-specific clones were retained for partial DNA sequencing (15). All nucleotide sequences were determined by dye-terminator cycle sequencing on an Applied Biosystems 373A sequenator according to the manufacturer's instructions.

For Southern blots, filters were hybridized in 7% SDS/0.25 M Na_2HPO_4/1 mM EDTA/1% bovine serum albumin at 65°C overnight, then washed sequentially in 2×, 1×, and 0.5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS at 65°C for 15 min each before being exposed to film. Unless otherwise indicated, all other filter hybridizations were carried out at 65°C in 4× SET (0.6 M NaCl/0.12 M Tris·HCl, pH 7.4/8 mM EDTA)/0.1% sodium pyrophosphate/0.2% SDS/0.1% heparin/5% dextran sulfate. Blots were washed three times in 2× SSC/0.1% SDS at room temperature, exposed to x-ray film, and then to a phosphorimaging screen (Molecular Dynamics).

Northern Blot Analysis. Poly(A)$^+$ RNA (3 μg) and RNA standards (0.16–1.77 kb ladder, GIBCO/BRL) were electrophoresed through an agarose gel containing formaldehyde (17). Following electrophoresis, RNA was transferred from the gel to a nylon membrane (Hybond N, Amersham) and fixed to the filter by exposure to UV light for 2 min. The 700-bp insert of clone pCRS677 was labeled by random priming and hybridized to the filter as described above.

Gas Chromatography. To prepare fatty acid methyl esters, samples of 25 seeds or 0.2 g of young leaf were treated in 2 ml of 1 M anhydrous methanolic HCl for 1–2 h at 80°C. Upon cooling, 1/10th volume water was added and the fatty acid methyl esters were extracted into 1 ml of petroleum ether. The ether phase was recovered and dried under a stream of nitrogen, and fatty acid methyl esters were redissolved in 200 μl of acetonitrile. Bis(trimethylsilyl)trifluoracetamide (trimethylsilyl = TMS) (100 μl, Supelco) was added and hydroxyl groups were derivatized at 70°C for 15 min, dried under nitrogen, and redissolved in 100 μl of chloroform.

Fatty acid methyl esters were analyzed by gas chromatography by using a Hewlet–Packard 5830 Series II instrument. Samples (2 μl) were injected onto an SP 2340 fused silica capillary column (60 m; 0.20-μm i.d.; Supelco) using a 15:1 split. Oven temperature was held at 195°C for 18 min, increased to 230°C (25°C min^{-1}), held at 230°C for 5 min, and then reduced to 195°C (25°C min^{-1}). For standard fatty acid analysis the flame ionization detector was selected. Injector and detector were kept at a constant 220°C.

Mass Spectrometry of Fatty Acid Samples. Samples were chromatographed as described above, except that the injection temperature was 300°C and the column was connected to a mass spectrometer (Hewlet–Packard). Ions were scanned for m/z values between 30 and 500.

Plant Transformation. Two independent constructs were used to place the insert from pFL2 under transcriptional control of the cauliflower mosaic virus *35S* promoter in binary Ti plasmids. For plasmids pA4 and pB6, pFL2 was linearized with *Xba* I, blunt-ended with the Klenow fragment of DNA polymerase I, and then digested with *Bam*HI to release the 1.3-kb insert, which was then ligated into the binary Ti vector pBI121, which had been digested with *Sac* I blunt-ended with T4 DNA polymerase, then digested with *Bam*HI. For plasmid p9/18.3, pFL2 was digested with *Xba* I and then partially digested with *Sac* I. A gel band of ≈1.45 kb that contained the insert was isolated from a gel and ligated into the *Xba* I and *Sac* I sites of the plasmid pSLJ4K1 (18). The resulting plasmid was then digested with *Eco*RI and *Hin*dIII, and the insert fragment ligated into the *Eco*RI and *Hin*dIII sites of pBI121 to produce p9/18.3. This resulted in replacement of the *35S* promoter-β-glucuronidase-*nos* terminator fragment of pBI121 with the *35S* promoter-pFL2 insert-*nos* terminator fragment derived from pSLJ4K1.

The three clones (A4, B6, and 9/18.3) were transformed into *Agrobacterium tumefaciens* strains GV3101 and LBA4404 by electroporation. *Nicotiana tabacum* SR-1 leaf explants were transformed according to Newman *et al.* (19), except that leaves were maintained on No. 3 medium for 3 days prior to inoculation.

RESULTS

Isolation and Sequencing of cDNA Clone pFL2. An average of ≈400 bp of nucleotide sequence was obtained from the presumptive 5′ end of 468 anonymous clones from a cDNA library prepared from developing castor endosperm and enriched for seed-specific clones by differential screening (15). Two clones were identified which had open reading frames with significant similarity to a microsomal ω − 3 linoleate desaturase from *Arabidopsis*. A third, shorter clone was subsequently identified by comparison to the microsomal ω − 6 desaturase of *Arabidopsis*. The partial sequence of these clones, pCRS290, pCRS677, and pCRS834, indicated that they were independent clones representing the same gene (14). One clone was used as a hybridization probe to isolate a putative full-length cDNA clone designated pFL2, and the complete nucleotide sequence of the insert in pFL2 was obtained for both strands.

The clone pFL2 encodes a 1161-bp open reading frame which is preceded by a 186-bp 5′ untranslated region and followed by a 101-bp 3′ untranslated region, including a short (9-bp) poly(A) tail. The open reading frame encodes a 387-amino acid protein with a predicted molecular weight of 44,407 Da that we have designated FAH12. Alignment of the deduced amino acid sequences of FAH12 and the *Arabidopsis* FAD2 microsomal oleate 12-desaturase (20) shows that these sequences have 67% identity and are colinear except for a 4-amino acid insertion near the amino terminus of the predicted FAH12 protein (Fig. 1). The FAH12 sequence includes three histidine-rich motifs (at residues 108–113, 145–149, and 319–323) that are conserved among all membrane-bound desaturases and thought to be important in the active site of these enzymes, possibly as binding sites for the iron cofactors (9, 12, 13, 20).

Comparison of the deduced amino acid sequence of FAH12 with that of a range of plant membrane-bound desaturases

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. U22378).

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FAD2   MGAGGRMP----VPTSSKKSETDTTKRVPCEKPPFSVGDLKKAIPPHCFKRSIPRSFSYL
       ::.:::::.     .:.::....  ::.: .:::::..:::::.:::::::.::. ::::::.
FAH12  MGGGGRMSTVITSNNSEKKGGSSHLKRAPHTKPPFTLGDLKRAIPPHCFERSFVRSFSYV
              10        20        30        40        50        60
        60        70        80        90       100       110
FAD2   ISDIIIASCFYYVATNYFSLLPQPLSYLAWPLYWACQGCVLTGIWVIAHECGHHAFSDYQ
         :. ..  :: .:::.:. .. ::::.:: .::  :::.:::.:::.:::::::::.::
FAH12  AYDVCLSFLFYSIATNFFPYISSPLSYVAWLVYWLFQGCILTGLWVIGHECGHHAFSEYQ
              70        80        90       100       110       120
       120       130       140       150       160       170
FAD2   WLDDTVGLIFHSFLLVPYFSWKYSHRRHHSNTGSLERDEVFVPKQKSAIKWYGKYLNNPL
         ::.:::: :: ::::::::::::::::::.:::::::::::::: :: :.::.:: :::
FAH12  LADDIVGLIVHSALLVPYFSWKYSHRRHHSNIGSLERDEVFVPKSKSKISWYSKYSNNPP
              130       140       150       160       170       180
       180       190       200       210       220       230
FAD2   GRIMMLTVQFVLGWPLYLAFNVSGRPYDGFACHFFPNAPIYNDRERLQIYLSDAGILAVC
       ::.. :.. ..:::::::::::::::::: ::::. : .::...::::::..:: ::.:.
FAH12  GRVLTLAATLLLGWPLYLAFNVSGRPYDRFACHYDPYGPIFSERERLQIYIADLGIFATT
              190       200       210       220       230       240
       240       250       260       270       280       290
FAD2   FGLYRYAAAQGMASMICLYGVPLLIVNAFLVLITYLQHTHPSLPHYDSSEWDWLRGALAT
       : ::. . :.:.: .. .:::::::::: :::.:::::::::..:.:.:::::::::..:
FAH12  FVLYQATMAKGLAWVMRIYGVPLLIVNCFLVMITYLQHTHPAIPRYGSSEWDWLRGAMVT
              250       260       270       280       290       300
       300       310       320       330       340       350
FAD2   VDRDYGILNKVFHNITDTHVAHHLFSTMPHYNAMEATKAIKPILGDYYQFDGTPWYVAMY
       ::::::.:::::::::.:::::::::.:.:::.:::::::::::.:.::..::::.: :..
FAH12  VDRDYGVLNKVFHNIADTHVAHHLFATVPHYHAMEATKAIKPIMGEYYRYDGTPFYKALW
              310       320       330       340       350       360
       360       370       380
FAD2   REAKECIYVEPDREGDKKGVYWYNNKL
       :::::::.:::: .. ..::.::.::
FAH12  REAKECLFVEPDEGAPTQGVFWYRNKY
              370       380
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FIG. 1. Comparison of amino acid sequences of microsomal oleate desaturase (FAD2) from *Arabidopsis* and the castor oleate hydroxylase (FAH12). Identical amino acids are indicated by two dots and conservative differences are indicated by a single dot. The histidine motifs are highlighted.

(Fig. 2) indicates that FAH12 is more closely related to FAD2 than to other desaturases. For simplicity, only sequences from *Arabidopsis* and castor were used for the comparison (a representative of each membrane-bound desaturase gene that has been cloned is available from *Arabidopsis*). The other sequence for a putative membrane-bound desaturase from castor (FAD7) is included as an indicator of the level of divergence of a given gene between these two species. The FAH12 and FAD2 sequences are considerably more divergent (67% identity) than are the FAD7 sequences of castor and Arabidopsis (83% identity when the divergent putative transit peptides are excluded). Inclusion of desaturase sequences from other organisms in the analysis confirms that the divergence between FAH12 and FAD2 of *Arabidopsis* is at the limits of that expected for interspecific divergence of a single gene (data not shown).

Southern Analysis. A Southern blot of genomic DNAs probed at high stringency with pFL2 indicated that this clone corresponds to a single-copy gene in castor (Fig. 3). No signal was apparent on Southern blots of *Arabidopsis* genomic DNA probed at high stringency (14), consistent with the absence of any known oleate 12-hydroxylase enzyme in this species.

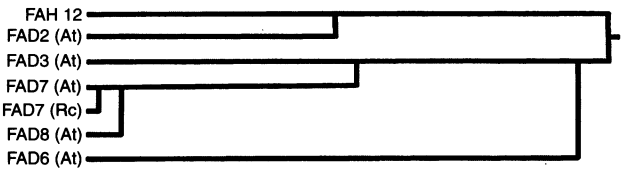

FIG. 2. Dendrogram derived by multiple alignment of deduced amino acid sequences of FAH12 and various fatty acyl desaturases. At, *Arabidopsis thaliana*; Rc, *Ricinus communis*. FAD2 and FAD3 are microsomal $\omega - 6$ and $\omega - 3$ desaturases, respectively. FAD6 is a plastid $\omega - 6$ desaturase. FAD7 and FAD8 are plastid $\omega - 3$ desaturases.

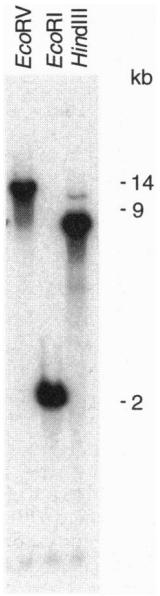

FIG. 3. Southern blot of genomic DNA from castor (5 μg per lane) probed with the ^{32}P-labeled insert of clone pFL2. The positions of DNA size standards (kb) are shown on the right.

Additional bands could be detected on the castor blots under less-stringent hybridization conditions (14), indicating the existence of *FAH12*-related sequences. Indeed, we have recently isolated a related castor clone that is expressed at similar levels in seeds and leaves and may, therefore, represent the castor *FAD2* desaturase (P.B. and C.S., unpublished data).

Seed-Specific Expression of *FAH12* in Castor. Ricinoleic acid occurs only in the seeds of castor. In contrast, as a general rule, fatty acid desaturases active in seeds are also represented by the same gene or a functionally equivalent isozyme in vegetative tissues. Therefore, we examined the expression pattern of *FAH12* by Northern blot analysis. The *FAH12* probe hybridized to a single band of $\approx$1.6 kb in developing seed RNA (Fig. 4*A*), without any visible hybridization to leaf RNA. Upon overexposure (Fig. 4*B*), a band of similar size was detected in leaf RNA, in addition to a second, larger band in seed RNA. There was a 268-fold difference in signal intensity between the primary seed and leaf bands when the blot was exposed to a phosphorimager screen. This strong seed-specific expression of *FAH12* was also evident from the observation that 1/560th of the clones from a seed cDNA library hybridized at high stringency to the *FAH12* gene. These observations are consistent with the abundance of oleate 12-hydroxylase activity in developing seeds and the apparent lack of ricinoleic acid in other tissues. No desaturase or other putative desaturase homolog which would have these characteristics is known in castor.

Analysis of Transgenic Tobacco Plants. Transgenic tobacco plants containing the *FAH12* cDNA behind the cauliflower mosaic virus *35S* promoter were produced and seed was obtained from each primary transgenic plant to produce a set

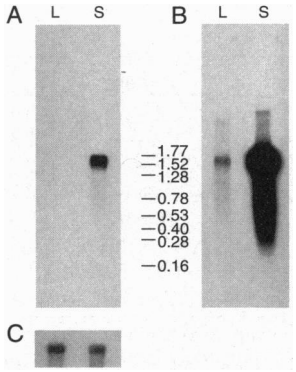

FIG. 4. Northern blot analysis of *FAH12* expression in castor. A ^{32}P-labeled probe corresponding to $\approx$700 bp of the 3′ end of clone pFL2 was hybridized to 3 μg of poly(A)$^+$ RNA from leaves (L) and developing seeds (S) of castor. (*A*) Blot exposed to film for 30 min. (*B*) Same blot exposed for 16 h. (*C*) Same blot rehybridized to a ^{32}P-labeled probe made from the *Colletotrichum graminicola* β-tubulin gene *TUB2* at 58°C (21). The positions of RNA size standards (kb) are shown between *A* and *B*.

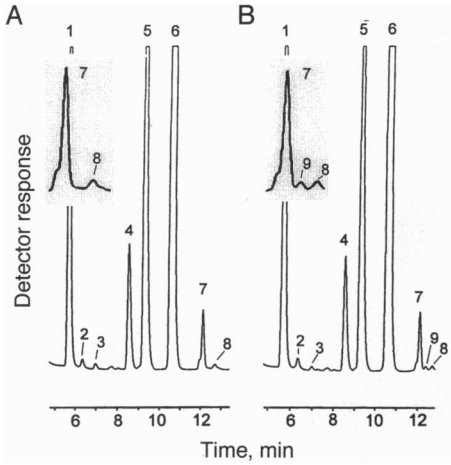

FIG. 5. Gas chromatograms of TMS-derivatized fatty acid methyl esters from tobacco seed lipids. (*A*) Tobacco transformed with pBI121. (*B*) Tobacco transformed with pA4. The number 9 corresponds to a novel peak at 12.4 min. (*Insets*) Region of the chromatograms around 12.4 min at higher resolution. Peak identification: 1, 16:0; 2, not identified; 3, not identified; 4, 18:0; 5, $18:1^{\Delta 9}$; 6, $18:2^{\Delta 9,12}$; 7, $18:3^{\Delta 9,12,15}$; 8, 20:0; 9, $18:1^{\Delta 9}$-OH.

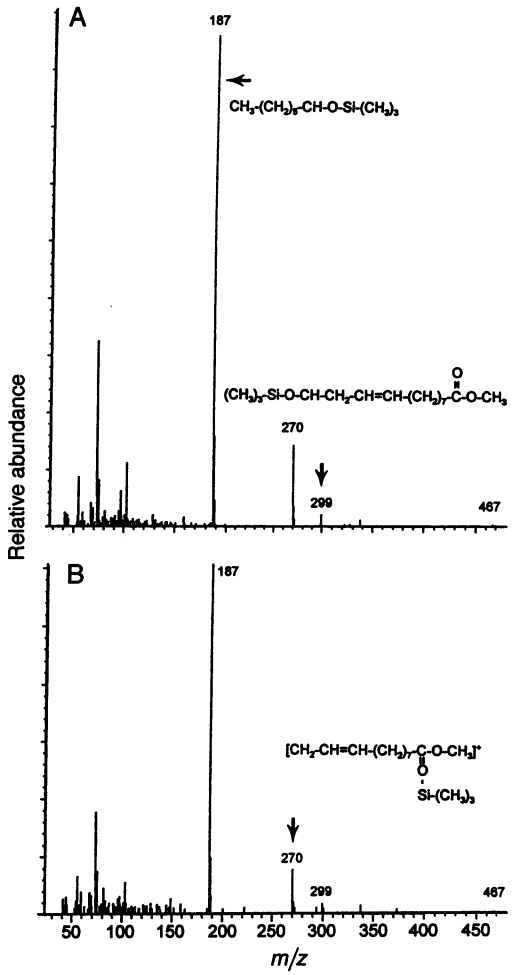

FIG. 6. Mass spectrum of TMS-methylricinoleate (*A*) and the peak at 12.4 min on the chromatogram in Fig. 5*B* (*B*). The structure of the two fragments at *m/z* 187 and 299 is shown in *A*. A rearrangement product of the fragment at *m/z* 299 produces the fragment at *m/z* 270 shown in *B*.

of T2 families. The presence of an intact *FAH12* gene in the genome of T2 plants was verified by selecting for kanamycin resistance and by using DNA from selected plants as a template for PCR amplification of the gene with specific primers. Southern blot analysis established that the number of copies in the genome of transgenic plants ranged from 2 to 4 (results not shown).

Fatty acids were extracted from mature seeds from T2 plants, and individual species were resolved as TMS-esters by gas chromatography. In four of seven independent transgenic lines, a novel peak was observed at the position of the chromatogram where TMS-methylricinoleate eluted (Fig. 5). This peak was absent in the chromatograms of both wild-type seeds (results not presented) and seeds of plants transformed with the pBI121 vector (Fig. 5). Mass spectrometry of the novel peak showed that it produced an equivalent mass spectrum to that of derivatized ricinoleate (Fig. 6). Three characteristic ions with *m/z* values 187, 270, and 299 correspond to the three major fragmentation or rearrangement products of TMS-methylricinoleate (Fig. 6). Thus, on the basis of chromatographic retention and mass spectrum the novel peak was unambiguously identified as TMS-derivatized methylricinoleate. Although clearly detectable, accumulation of ricinoleate in the seeds of transgenic tobacco plants was very limited, representing ≈0.1% of the total seed fatty acids (Table 1).

Leaf tissue was harvested from individual plants of T2 families, and fatty acid composition was determined by gas chromatography (Table 1). No accumulation of ricinoleate could be detected, even when relatively large amounts of tissue were used and individual peaks were further analyzed by mass spectrometry. Analysis of shoots from T1 plants gave a similar result. Except for the presence of ricinoleate, comparison of fatty acid composition between transgenic and control plants did not reveal significant differences.

DISCUSSION

Because we and others have not been able to purify the castor oleate hydroxylase, we undertook to isolate a cDNA clone by

Table 1. Fatty acid composition of transgenic and control tobacco plants

Line	Source	Fatty acid, mol %						
		16:0	16:3	18:0	18:1	18:2	18:3	18:1-OH
Control	Seed	10.8 ± 0.1	—	2.5	11.1 ± 0.1	74.0 ± 0.1	1.2	0
TFT1	Seed	10.4 ± 0.1	—	2.3	11.1 ± 0.1	74.4 ± 0.1	1.1 ± 0.1	0.1 ± 0.1
TFT2	Seed	10.6 ± 0.1	—	2.7 ± 0.1	11.8	73.4 ± 0.3	1.1	0.1
TFT3	Seed	11.0 ± 0.1	—	2.7 ± 0.3	11.0 ± 0.1	73.8 ± 0.3	1.0 ± 0.1	0.1 ± 0.1
Control	Leaf	12.6 ± 0.3	8.7 ± 0.3	0.9 ± 0.1	1.2 ± 0.1	12.7 ± 0.3	55.6 ± 2.1	0
TFT1	Leaf	10.2 ± 3.5	9.1 ± 4.2*	1.0 ± 0.6	0*	8.7 ± 4.7	53.7 ± 2.3	0
TFT2	Leaf	12.9 ± 1.0	9.2 ± 1.4	1.1 ± 0.3	1.6 ± 0.3	12.0 ± 1.8	57.2 ± 3.1	0
TFT3	Leaf	13.0 ± 1.1	7.7 ± 1.6	1.1 ± 0.2	1.3 ± 0.2	9.7 ± 1.2	60.9 ± 2.8	0

Values presented are the mean ± SD (n = 3).
*The 16:3 and 18:1 peaks overlapped in some of these measurements.

exploiting the high-throughout capabilities of automated DNA sequenators to examine anonymous clones from a castor endosperm cDNA library for the presence of deduced amino acid sequence homology to known fatty acyl desaturases. The basis of this strategy was concurrent studies of the stearyl-ACP desaturase, which had suggested that hydroxylases and desaturases shared a similar reaction mechanism (1, 11–13), and by the striking, superficial similarities between the oleate hydroxylase from castor and several microsomal desaturases (5). At the time we were engaged in this work, the first plant microsomal desaturase sequence had just become available for the *FAD3* gene of Arabidopsis, an $\omega - 3$ linoleate desaturase (26). By partially sequencing 468 cDNAs from castor, we identified two overlapping clones from a gene with low but significant similarity to the *Arabidopsis FAD3* desaturase. The gene was very strongly expressed in developing seeds but weakly or not at all in leaves. Therefore, we tested the function of the gene by expressing it in transgenic plants.

Seeds from four of seven independent transgenic tobacco plants contained a fatty acid that comprised only 0.1% of the total seed fatty acids and that eluted from chromatograms of the same position as a ricinoleic acid standard. GC/MS of TMS derivatives of this fatty acid produced a characteristic fragmentation pattern that unambiguously identified the peak as TMS-methylricinoleate. Thus, we conclude that the *FAH12* cDNA encodes the oleate hydroxylase from castor.

Of the known desaturases, the oleate hydroxylase is most closely related to the microsomal $\omega - 6$ desaturase from *Arabidopsis* (20). This suggests that the two enzymes are derived from a common ancestral enzyme or that the hydroxylase has evolved from the $\omega - 6$ desaturase. We have recently isolated a cDNA clone for a putative $\omega - 6$ desaturase from castor (unpublished results) which is expressed throughout the plant and has approximately the same degree of sequence similarity to the castor oleate hydroxylase as to the $\omega - 6$ desaturase from *Arabidopsis*. Therefore, it appears that it will be necessary to obtain additional examples of sequence information for these two enzymes to use comparative sequence information to identify amino acid residues that may play a role in diverting the catalytic cycle towards desaturation or hydroxylation.

Ricinoleic acid could not be detected in the leaves of any of the transgenic plants. Northern analysis of transgenic tobacco leaves showed high abundance and correct size of the hydroxylase mRNA (results not shown). Preliminary attempts to measure enzyme activity in leaf extracts failed. Therefore, additional studies will be required to determine if a functional enzyme accumulates at the appropriate subcellular location. It is possible that the hydroxylase may be active in transgenic plants but that ricinoleic acid is rapidly catabolized and, therefore, does not accumulate. There is evidence of high phospholipase A activity in castor and other plants toward oxygenated acyl groups (6, 22). Ricinoleic acid, which resembles hydroperoxides formed in conditions of membrane oxidative damage, might be selectively removed and degraded in tobacco leaves by enzymes involved in maintaining membrane integrity. These factors may also limit the buildup of ricinoleic acid in the seed; however, detectable accumulation proves that the castor hydroxylase is at least partially active during seed development. Among seed-specific factors which may be limiting, discrimination of tobacco acyltransferases against CoA- or lipid-bound ricinoleoyl groups may play a role in restricting their transfer to specific positions of the glycerol backbone. Diacylglycerol acyltransferases may have poor affinity for ricinoleoyl containing molecules and prevent accumulation of the unusual fatty acid in the triacylglycerol pool. Such selectivity has been demonstrated in recent studies of acyltransferases in several plant species (23–25). The accumulation of ricinoleic acid in seeds of transgenic tobacco but not in leaves (despite use of the constitutive cauliflower mosaic virus *35S* promoter) is consistent with the normal pattern of accumulation in castor and of unusual plant fatty acids in general. Thus, it is possible that expression of the transgene reveals a homeostatic mechanism normally masked by the transcriptional control of the hydroxylase gene in castor.

The high degree of chemical diversity represented in the collective chemical constituents of angiosperms has arisen by the rapid evolution of a large number of enzymes. The results presented here provide an example of how a new enzymatic function may arise by modifications of a preexisting enzyme.

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