Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2510–2514, March 1991
Botany

Stearoyl-acyl-carrier-protein desaturase from higher plants is structurally unrelated to the animal and fungal homologs

(fatty acid desaturation/cDNA clone/lipid unsaturation/fatty acid synthase)

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Communicated by N. E. Tolbert, December 17, 1990 (received for review October 5, 1990)

ABSTRACT **Stearoyl-acyl-carrier-protein (ACP) desaturase (EC 1.14.99.6) was purified to homogeneity from avocado mesocarp, and monospecific polyclonal antibodies directed against the protein were used to isolate full-length cDNA clones from *Ricinus communis* (castor) seed and *Cucumis sativus* (cucumber). The nucleotide sequence of the castor clone pRCD1 revealed an open reading frame of 1.2 kilobases encoding a 396-amino acid protein of 45 kDa. The cucumber clone pCSD1 encoded a homologous 396-amino acid protein with 88% amino acid identity to the castor clone. Expression of pRCD1 in *Saccharomyces cerevisiae* resulted in the accumulation of a functional stearoyl-ACP desaturase, demonstrating that the introduction of this single gene product was sufficient to confer soluble desaturase activity to yeast. There was no detectable identity between the deduced amino acid sequences of the castor Δ^9-stearoyl-ACP desaturase and either the Δ^9-stearoyl-CoA desaturase from rat or yeast or the Δ^{12} desaturase from *Synechocystis*, suggesting that these enzymes may have evolved independently. However, there was a 48-residue region of 29% amino acid sequence identity between residues 53 and 101 of the castor desaturase and the proximal border of the dehydratase region of the fatty acid synthase from yeast. Stearoyl-ACP mRNA was present at substantially higher levels in developing seeds than in leaf and root tissue, suggesting that expression of the Δ^9 desaturase is developmentally regulated.**

Fatty acid biosynthesis in plants occurs in the chloroplasts of green tissue and in the plastids of nonphotosynthetic tissues (1). In most plants, the primary products of fatty acid synthesis are palmitoyl-acyl-carrier protein (ACP) (16:0-ACP) and stearoyl-ACP (18:0-ACP). The soluble plastid enzyme, stearoyl-ACP desaturase (EC 1.14.99.6), introduces the first double bond into stearoyl-ACP between carbons 9 and 10 to produce oleoyl-ACP (18:1$^{\Delta 9}$-ACP). Additional double bonds are introduced into oleic acid or palmitic acid after incorporation of the fatty acids into lipids. Since most plants lack any other desaturase that acts on 18:0, the level of stearoyl-ACP desaturase activity is a key determinant of the overall level of fatty acid desaturation.

The plant stearoyl-ACP desaturase is unusual in that it is the only soluble desaturase identified to date. All other desaturases identified in plants, algae, animals, and fungi are integral membrane proteins. Therefore, it has become a focus of biochemical investigation of plant lipid desaturation. Though quite active in crude preparations of various tissues including safflower seeds and avocado mesocarp, the purification of stearoyl-ACP desaturase to homogeneity has until recently proved difficult. Partially purified preparations have been used to establish *in vitro* cofactor requirements for ferredoxin, ferredoxin NADPH reductase, and molecular oxygen and to determine kinetic constants for the enzyme (2). The mechanisms of the plant and animal Δ^9-stearoyl-ACP desaturases are thought to be identical; however, the reaction mechanism is as yet unknown.

As first steps toward understanding the reaction mechanism and the regulation of stearoyl-ACP desaturation, we have undertaken isolation and characterization of cDNA clones† encoding the Δ^9-stearoyl-ACP desaturase from castor (*Ricinus communis*) and from cucumber (*Cucumis sativus*) and the expression of a functional desaturase enzyme in yeast (*Saccharomyces cerevisiae*).

MATERIALS AND METHODS

Affinity Ligand Synthesis. [9,10-^{3}H]Stearic acid (2396 GBq/mmol) was custom-synthesized by Amersham by the reductive tritiation of oleic acid with tritium gas. ACP was purified from *Escherichia coli* as described (3). Stearoyl-CoA was synthesized as described (4). *N*-Hexadecyliodoacetamide was synthesized with 64% yield as follows. Iodoacetic anhydride [Aldrich; 40 ml at 2.5% (wt/vol)] in diethyl ether was added dropwise with continuous stirring to 100 ml of 0.5% hexadecylamine (Aldrich) in diethyl ether. After 40 min, 140 ml of petroleum ether was added and the resulting precipitate was collected on a glass fiber filter, washed with 40 ml of diethyl ether/petroleum ether [1:1 (vol/vol)], and air-dried. A nonhydrolyzable acyl-ACP was prepared by adding 30 mg of *N*-hexadecyl iodoacetamide in 6 ml of 2-propanol to 30 mg of reduced ACP in 6 ml of 10 mM sodium phosphate (pH 7.0) followed by stirring for 2.5 hr at 40°C. The reaction was brought to 10 mM 2-mercaptoethanol and desalted into 10 mM sodium phosphate (pH 7.0) by passage over a 1.0 × 30 cm Sephadex G-25 column. The acyl-ACP was bound to 4 ml of Affi-Gel 15 (Bio-Rad) according to the manufacturer's instructions.

Stearoyl-ACP Desaturase Assay. Desaturase activity was measured as the ferredoxin-dependent release of ^3H_2O from [9,10-^{3}H]stearoyl-CoA (5) by using the assay conditions of McKeon and Stumpf (2). Assays (40 μl) were incubated at 25°C for 20 min, terminated by the addition of 1.3 ml of ethanol, and clarified by centrifugation at 16,000 × *g* for 5 min. Residual substrate was removed by the addition of 40 mg of Norit A, followed by clarification as before, and the ^3H_2O release was measured by scintillation counting of the supernatant fraction.

Purification of Stearoyl-ACP Desaturase. In a typical preparation, 1 kg of avocado mesocarp tissue was homogenized in 4 liters of acetone (−20°C) in a Waring Blendor, collected by vacuum filtration, reextracted once in the same manner, and dried under vacuum. The acetone powder was suspended at a 1:6 (wt/vol) ratio in 40 mM Tris·HCl, pH 8.0/2 mM

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Abbreviation: ACP, acyl-carrier protein.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M59857 for castor and M59858 for cucumber).

benzamidine/2 mM ε-aminocaproic acid/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride at 4°C. The resulting slurry was centrifuged at 27,000 × g for 1 hr. The turbid aqueous phase was filtered through two layers of Miracloth (CalBiochem) and clarified by centrifugation at 100,000 × g for 30 min. The extract was applied to a 1 × 5 cm acyl-ACP affinity column and washed with 30–50 column volumes of 40 mM Tris·HCl (pH 8.0), and material was eluted with 1.2 M NaCl in the same buffer. The acyl-ACP affinity column eluent was desalted using a 1.5 × 25 cm Sephadex G-25 column equilibrated with 25 mM [bis-(2-hydroxyethyl)amino]-tris(hydroxymethyl)methane (Bistris)·iminodiacetic acid (pH 7.1) and applied to a Mono P HR 5/20 FPLC column (Pharmacia). The column was eluted with 10% (vol/vol) Polybuffer 74·iminodiacetic acid (pH 4.0) (Pharmacia).

Peptide Sequencing. Avocado desaturase (1 mg) was digested to completion with 20 μg of trypsin (type XIII, Sigma). The resulting peptide fragments were resolved by C_{18} reversed-phase HPLC and subjected to automated Edman degradation on a gas-phase sequenator (Applied Biosystems, type 470).

Production of Antibodies. Polyclonal antibodies against pure avocado desaturase were raised in a New Zealand White rabbits and the anti-desaturase antibodies were purified using a 1 × 2 cm column containing 1 mg of avocado desaturase coupled to Affi-Gel 15 (6).

Isolation and Characterization of cDNA Clones. λgt11 cDNA libraries from developing seed of *R. communis* (cv. Baker 296) and 7-day-old cucumber cotyledons (*C. sativus* cv. Long Green Improved) were generously provided by S. Blakeley and D. Dennis (Queens University, Kingston, ON) and J. Greenler and W. Becker (University of Wisconsin, Madison). The cDNA libraries were screened as described (7) except that immunodetection was by a colorimetric method (8). The inserts from immunopositive plaques were cloned into pBluescript. Single-stranded sequencing was performed on both strands by the dideoxynucleotide method (9) using synthetic oligonucleotides as primers.

Expression of cDNAs in *S. cerevisiae*. The *Eco*RI fragment from pRCD1 was ligated into the yeast expression vector pYcDE-2, a derivative of pMAC561 (10), in the forward and reverse orientations and used to transform yeast strain AB1380 (*MATα, ψ⁺ ade2-1, lys2-1, trp1, ura3, his5, can1-100*) to tryptophan prototrophy. Desaturase activity was measured in extracts from late logarithmic phase cultures grown at 30°C in synthetic complete medium minus tryptophan (11). The extracts were subjected to SDS/PAGE (12) followed by Western blot analysis (8) using anti-desaturase antibodies.

Northern Blot Analysis. Total RNA was purified using a guanidinium thiocyanate extraction method (13) subjected to denaturing formaldehyde/agarose gel electrophoresis and transferred to a nylon membrane (Zeta-Probe, Bio-Rad) according to Maniatis *et al.* (14). The 1.6-kilobase (kb) *Eco*RI fragment from pCD1 (i.e., the castor cDNA) and a 3.3-kb *Sma* I fragment from a constitutive β-tubulin gene, *Tub-2* from *Colletotrichum graminicola* (15), were isolated from low-melting-temperature agarose gels and labeled with [α-^{32}P]dCTP. The filters were hybridized for 12 hr at 58°C as described (16), except that the hybridization solution also contained 5% (wt/vol) dextran sulfate.

RESULTS

Purification and Properties of Stearoyl-ACP Desaturase. McKeon and Stumpf (2) reported that ACP was a useful affinity ligand for the purification of the safflower desaturase. Because the avocado desaturase did not bind to this matrix, we prepared a derivatized form in which a synthetic alkyl chain was attached to ACP by a stable thio ether linkage. In contrast to underivatized ACP, the avocado desaturase bound strongly to this matrix, enabling affinity purification from crude extracts. Use of the ^{3}H release assay, rather than the more commonly used argentation thin layer chromatography method, permitted the routine assay of large numbers of fractions.

After gel-permeation chromatography or Mono P FPLC, the enzyme was judged to be essentially pure by the absence of contaminating bands on heavily loaded SDS/PAGE (Fig. 1). Purification from the acetone powder extract was estimated to be ≈750-fold, suggesting that the enzyme represents ≈0.1% of the total soluble cellular protein. The properties of the purified stearoyl desaturase from avocado were indistinguishable from those described for safflower (2). Both enzymes (*i*) are soluble dimers of ≈70 kDa, (*ii*) are able to convert stearate to oleate using either ACP or CoA as the acyl carrier, (*iii*) require ferredoxin, ferredoxin NADPH reductase, and NADPH for activity, and (*iv*) are stimulated 5- to 10-fold by the presence of catalase (data not presented). The apparent specific activity of the purified desaturase was 65 milliunits/mg (with stearoyl-CoA). Attempts to obtain N-terminal sequence information from the purified avocado desaturase by gas-phase Edman degradation were unsuccessful, suggesting that the N terminus is blocked. However, partial amino acid sequence was determined for two internal tryptic peptides. The peptide sequences were DETGASP and DYADILE. Desaturase activity in partially purified preparations was removed by the addition of antibodies raised against the purified protein (data not presented). Antibodies recognized single bands of similar molecular mass on Western blots of crude extracts from avocado mesocarp, developing safflower, and castor seeds (Fig. 1), indicating that plant stearoyl-ACP desaturases from distantly related species possess common antigenic determinants.

Isolation of cDNA Clones. Antibodies raised against avocado stearoyl-ACP desaturase were used to identify clones from libraries representing mRNA from developing castor seeds and cucumber cotyledons. The largest of the clones from each library were subcloned into pBluescript to produce plasmids pRCD1 (castor) and pCSD1 (cucumber), and the DNA sequences were determined. Because of space limitations, only the castor DNA sequence has been presented (Fig. 2).

The 1643-base-pair cDNA clone pRCD1 contains a 1188-base-pair open reading frame between nucleotides 49 and 1237. The deduced amino acid sequence contains two regions

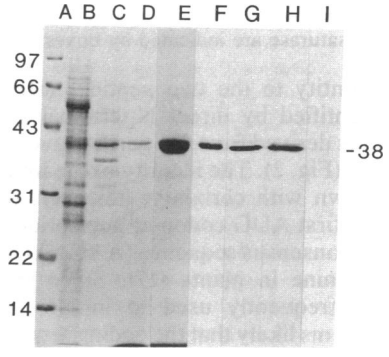

FIG. 1. SDS/PAGE (lanes A–E) of the purification of avocado stearoyl-ACP desaturase stained with Coomassie brilliant blue. Lanes: A, molecular mass standards; B, mesocarp crude extract (35 μg); C, acyl-ACP affinity column eluent (10 μg); D, Mono P eluent (1 μg); E, Sephadex G-75 eluent (15 μg). Western blot analysis (lanes F–I) of 25 μg of crude extracts from seeds of various species using anti-avocado stearoyl-ACP desaturase antibodies (lanes F–H) or preimmune antibodies (lane I). Lanes: F and I, avocado mesocarp; G, immature safflower seed; H, stage 3 castor seed. Molecular masses in kDa are shown.

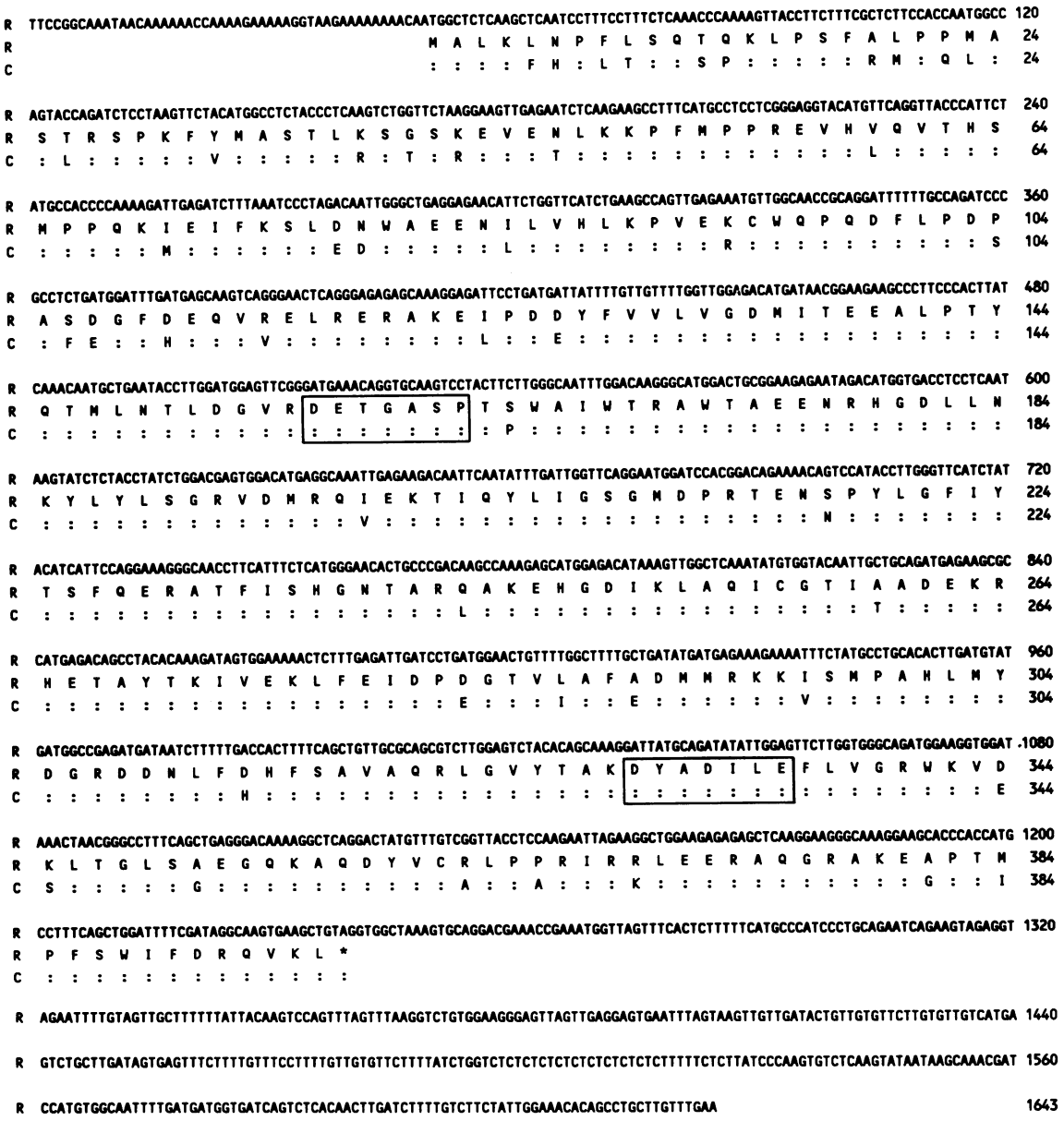

FIG. 2. Composite nucleotide sequence and deduced amino acid sequence of cDNAs from the *R. communis* (castor) stearoyl-ACP desaturase pRCD1 (labeled R) and the deduced amino acid sequence of the *C. sativus* (cucumber) stearoyl-ACP desaturase pCSD1 (labeled C). Identical amino acids are indicated by colons. Regions of amino acid sequence corresponding to the directly determined sequences of tryptic peptides of the avocado desaturase are indicated by boxes.

of sequence identity to the two peptides (DETGASP and DYADILE) identified by direct N-terminal sequencing of tryptic fragments derived from the purified avocado stearoyl-ACP desaturase (Fig. 2). The identity of the initiator methionine is not known with certainty; however, the sequence surrounding the first AUG codon at nucleotide 49 conforms perfectly to the consensus sequence (AACAAUGGC) for the initiating methionine in plants (17). Since the first AUG codon is most frequently used to initiate translation in eukaryotes, it seems likely that the coding sequence begins at nucleotide 49. The deduced amino acid sequence of the 1188-base-pair open reading frame corresponds to a protein of 396 amino acids and a predicted molecular mass of 45.4 kDa. The deduced amino acid sequence of this open reading frame is generally hydrophilic, lacking clusters of hydrophobic residues commonly found in membrane-associated proteins.

A comparison of the derived amino acid sequences of the castor and cucumber desaturase cDNAs (Fig. 2) shows that they possess open reading frames identical in size. Further, a comparison of the nucleotide sequence of the two clones reveals a contiguous stretch of high homology (>80%) in the region corresponding to bases 49–1239 of the castor clone, with no discernable homology in the flanking nucleotide sequences, consistent with initiation at base 49 of the castor clone (data not presented). The deduced amino acid sequence of the cucumber desaturase was 88% identical to the castor sequence (Fig. 2). When conservative amino acid substitutions (K/R, F/T/W, N/Q, S/T, E/D, and V/I/L) were taken into account, the sequence similarity increased to 98%. This high degree of sequence similarity between enzymes from two distantly related families suggests that the sequence is highly conserved among higher plants.

Expression of Castor Stearoyl-ACP Desaturase in Yeast. To confirm the identity of the pRCD1 clone, it was introduced, in both orientations, into the yeast expression vector pYcDE-2 under the control of the *ADH-1* promotor. Soluble extracts derived from cultures of either untransformed yeast or yeast containing the expression plasmid with either orientation were tested for the desaturase polypeptide by prob-

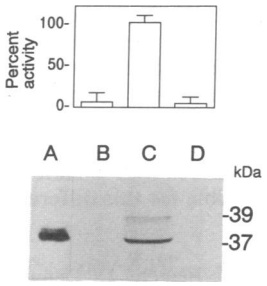

FIG. 3. Expression of *R. communis* stearoyl-ACP desaturase in *S. cerevisiae*. (*Upper*) Desaturase activity (where 100% represents 17 microunits of desaturase activity). Values represent mean ± SD of two experiments ($n = 5$). (*Lower*) Western blot analysis of 25 μg of crude protein extract separated by SDS/PAGE on a 12% gel, transferred to nitrocellulose, and probed with anti-avocado desaturase antibodies. Lanes: A, stage 3 castor seeds; B–D, yeast strain AB1380; B, untransformed; C, transformed with pYcDE-2 containing the insert from pRCD1 in the forward orientation; D, transformed with pYcDE-2 containing the insert from pRCD1 in the reversed orientation.

ing Western blots with anti-desaturase antibodies (Fig. 3 *Lower*). Only cultures carrying the plasmid with the insert in the correct orientation were immunopositive. The polypeptide produced in yeast migrated in SDS/PAGE with an apparent molecular mass of ≈37 kDa. This suggests that the transit peptide was proteolytically removed from the polypeptide in yeast.

The extracts were also assayed for stearoyl-ACP desaturase activity. Active enzyme was detected in extracts from yeast expressing the RCD1 open reading frame but not in controls (Fig. 3 *Upper*). Thus, the identity of the polypeptide as stearoyl-ACP desaturase was unambiguously established.

Gene Expression. Northern blot analysis of RNA derived from various tissues of castor revealed a single ≈17-kb band that hybridized with the pRCD1 probe (Fig. 4*A*). RNA from developing seeds showed a much stronger hybridization signal with the pRCD1 probe than did RNA derived from leaves or roots, where little signal could be seen, indicating that the transcript represents a substantially greater proportion of the total mRNA in developing seeds. When duplicate

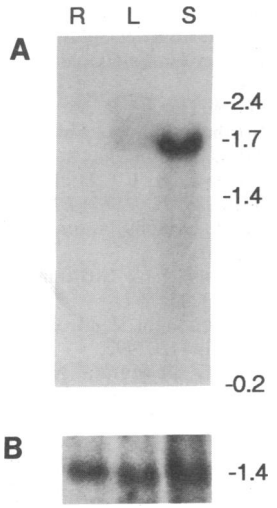

FIG. 4. Northern blot analysis of total RNA from *R. communis*. RNA (15 μg) was electrophoresed through a 1.5% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane, and probed with the 1.6-kb *Eco*RI fragment of pRCD1 (*A*) or the 3.3-kb *Sma* I fragment from the *Tub-2* gene (*B*). RNA was isolated from tissues as follows. Lanes: R, root; L, leaves; S, stage 3 developing seeds. Numbers to the right indicate size in kb.

blots were hybridized with a probe encoding a constitutive tubulin gene *Tub-2* (15), a signal of similar intensity was observed in all lanes. Thus, the accumulation of desaturase mRNA is, indeed, tissue specific.

DISCUSSION

Several independent lines of evidence indicate that clone pRCD1 encodes the stearoyl-ACP desaturase from castor. (*i*) The clone was selected using an antibody that was specific for stearoyl-ACP desaturase. (*ii*) The derived amino acid sequence of the pRCD1 and pCSD1 open reading frames contained two regions (after predicted trypsin cleavage sites) with 100% identity to directly determined sequences from two 7-amino acid regions of tryptic peptides derived from the purified avocado stearoyl-ACP desaturase. (*iii*) Expression of pRCD1 in yeast, an organism that does not possess a soluble stearoyl-ACP desaturase, conferred soluble ferredoxin-dependent Δ^9 desaturation activity indistinguishable from that found in crude extracts of plant tissues.

The fact that the Δ^9 desaturase is localized within chloroplasts indicates that it should possess a transit peptide. The N-terminal regions of the castor and cucumber sequences are rich in hydroxylated and hydrophobic residues and low in positively charged residues, characteristics associated with chloroplast transit peptides (18). The pure avocado protein had an apparent molecular mass of 38 kDa by SDS/PAGE. Similarly, Western blot analysis of crude extracts from developing castor seeds revealed an immunodetectable polypeptide of 38 kDa. However, the predicted molecular mass of the clone encoded by pRCD1 is 45.4 kDa. Thus, the 38-kDa mature protein is presumably produced from a precursor by proteolytic cleavage of 7.4 kDa, or ≈65 amino acids, which is similar to the median length of 60 residues for transit peptides of stromal proteins (18). However, several attempts to identify the N-terminal residue of the mature peptide by direct N-terminal amino acid sequencing failed. It is interesting to note that when expressed in yeast, pRCD1 encoded a protein with a monomer molecular mass of ≈37 kDa, with a minor species appearing at 39 kDa. This indicates that the transit peptide is proteolytically removed in yeast.

Western blot analysis suggests that stearoyl-ACP desaturases from various distantly related species possess common antigenic determinants. To examine the extent of sequence conservation of stearoyl-ACP desaturase among higher plants, a full-length cDNA was also isolated from a distantly related plant species, cucumber. Strict conservation of amino

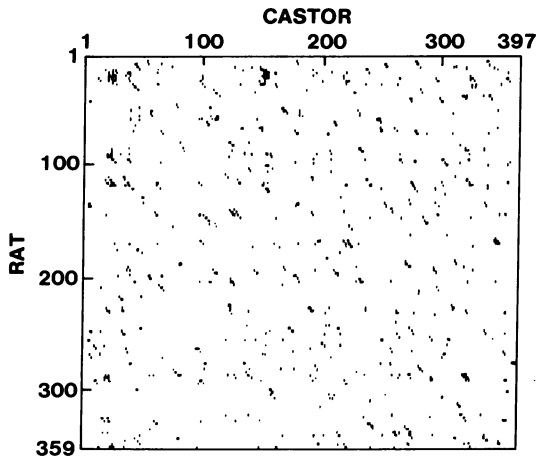

FIG. 5. Amino acid sequence comparison of Δ^9-stearoyl desaturases from rat and castor. Each dot indicates that five out of seven amino acid residues were identical or represented by conservative substitutions.

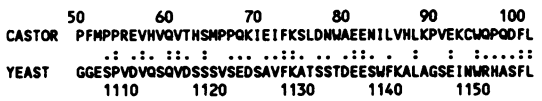

FIG. 6. Amino acid sequence comparison of regions of sequence identity between castor stearoyl-ACP desaturase and yeast fatty acid synthase β subunit. Two dots indicate identity, and one dot indicates a conservative substitution. Numbers represent amino acid residues beginning at the initiator methionine.

acid sequence between castor and cucumber is evident in the fact that the overall amino acid sequence is more conserved (88%) than the overall nucleotide sequence (81%, data not presented). The N-terminal region of the castor and cucumber sequences are relatively divergent, showing only 71% sequence identity over the first 60 amino acids. By contrast, the remainder of the polypeptide sequences are highly similar with 91% identity over the remaining 336 amino acids. This suggests that the structural requirements for a functional transit peptide are substantially less rigid than those that contribute to catalytic activity.

Animals and fungi contain a functionally homologous enzyme, the Δ^9-stearoyl-CoA desaturase, for which the nucleotide and deduced amino acid sequences of the corresponding cDNA or genomic clones have been reported (19, 20). These enzymes share three regions of sequence identity, consistent with a common origin. By contrast, comparisons between the amino acid sequences of the plant Δ^9 desaturases and either the animal or the fungal Δ^9 desaturases by using diagonal plots failed to reveal any sequence identity (Fig. 5). The lack of any apparent sequence similarity is striking in the light of the very high degree of conservation of amino acid identity within the two plant species investigated. We therefore suggest that the plant Δ^9 desaturase is evolutionarily unrelated to the animal and fungal enzymes. The lack of structural similarity reflects the facts that the fatty acid substrates are on different carriers (ACP and CoA), the enzymes utilize different electron donors (ferredoxin vs. cytochrome b_5), and the plant enzyme is soluble whereas the animal and fungal enzymes are integral membrane proteins. Diagonal plot comparison of the amino acid sequence of the castor Δ^9 desaturase with the *Synechocystis* Δ12 desaturase (21) showed a similar lack of homology. By contrast, a search of release 12.0 of the Swiss-Prot data base and release 22.0 of the National Biomedical Research Foundation–Protein Identification Resource data base revealed a 48-residue region (residues 53–101 of the castor enzyme) with 29% sequence identity in a region beginning at residue 1105 of the yeast fatty acid synthetase β subunit (Fig. 6). If conservative substitutions were ignored, the similarity rose to 67%. This region of sequence similarity lies at the distal border of a 276-residue domain of unknown function within the yeast enzyme and extends into the first 12 residues of the dehydratase region (22). The facts that the plant enzyme binds acyl-ACP and the yeast complex binds acyl-ACP-fatty acid synthase raise the possibility that the region of similarity may be involved in this binding.

Aside from the isolation of several genes for ACP and a gene for glycerol-3-phosphate acyltransferase, the cDNA clones reported here represent, to our knowledge, the only other genes involved in higher plant lipid metabolism cloned (for review, see ref. 23). Since lipid synthesis takes place in all cells but is strongly induced in the cells of developing seeds of many species, it is expected that a unique set of regulatory elements may be involved in controlling the expression of genes for lipid biosynthetic enzymes. Results presented here indicate that transcripts from the desaturase gene(s) represent a substantially greater proportion of mRNA in seeds than in other tissues. The availability of these desaturase cDNA clones will enable investigation of the mechanisms responsible for this differential accumulation of mRNA.

We are grateful to S. Blakely, D. Dennis, J. Greenler, and W. Becker for providing the cDNA libraries; D. Panaccione and R. Hanau for the β-tubulin clone Tub-2; E. Cahoon and J. Ohlrogge for providing [1-^{14}C]stearoyl-ACP; and W. Bernhard and T. Johnson for providing purified spinach ferredoxin. We are also grateful to J. Leykham (Michigan State University Macromolecular Structure Facility) for assistance with amino acid sequence analysis, W. Hitz for suggesting use of alkyl-ACP as an affinity ligand, and C. Mullins for technical assistance. This work was supported in part by grants from the Department of Agriculture/National Science Foundation/Department of Energy Plant Science Center Program, the National Science Foundation (Grant DCB-8916311), the State of Michigan Research Excellence Fund Program, and the Department of Energy (Grant DE-AC02-76ER0-1338).

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