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Purification of a Jojoba Embryo Fatty Acyl-Coenzyme A Reductase and Expression of Its cDNA in High Erucic Acid Rapeseed

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The jojoba (*Simmondsia chinensis*) plant produces esters of long-chain alcohols and fatty acids (waxes) as a seed lipid energy reserve. This is in contrast to the triglycerides found in seeds of other plants. We purified an alcohol-forming fatty acyl-coenzyme A reductase (FAR) from developing embryos and cloned the cDNA encoding the enzyme. Expression of a cDNA in *Escherichia coli* confers FAR activity upon those cells and results in the accumulation of fatty alcohols. The FAR sequence shows significant homology to an Arabidopsis protein of unknown function that is essential for pollen development. When the jojoba FAR cDNA is expressed in embryos of *Brassica napus*, long-chain alcohols can be detected in transmethylated seed oils. Resynthesis of the gene to reduce its A plus T content resulted in increased levels of alcohol production. In addition to free alcohols, novel wax esters were detected in the transgenic seed oils. In vitro assays revealed that *B. napus* embryos have an endogenous fatty acyl-coenzyme A: fatty alcohol acyl-transferase activity that could account for this wax synthesis. Thus, introduction of a single cDNA into *B. napus* results in a redirection of a portion of seed oil synthesis from triglycerides to waxes.

Long-chain primary alcohols are found throughout the biological world. They occur both as free alcohols and, more commonly, in a combined state. Waxes (oxygen esters of fatty alcohols and fatty acids) can be major components of biological systems and fill a variety of functions. They are found on the surfaces of plants and animals and provide protection against various stresses such as desiccation, wetting, and pathogen attack. Many microorganisms, such as *Acinetobacter* sp., *Euglena gracilis,* and some marine algae, store waxes as energy reserves. Bees produce and secrete wax to form a structure in the hive in which larvae are protected and food is stored. The large spermaceti organ in the head of the sperm whale is filled with a mixture of waxes and triglycerides. It is thought that the whale regulates its buoyancy during deep dives by thermally altering the physical state, and thus the density, of this mixture. Fatty alcohols are also precursors of ether lipids, which are found in a number of animal tissues, especially nervous system tissues, in which they can be a major component of the lipid fraction.

The biochemistry of fatty alcohol synthesis has been examined in diverse organisms. In several cases it has been demonstrated that the alcohol is formed by a four-electron reduction of fatty acyl-coenzyme A (CoA) (Kahn and Kolattukudy, 1973; Kolattukudy and Rogers, 1978; Bishop and Hajra, 1981; Wu et al., 1981; Kolattukudy and Rogers, 1986). NADH or NADPH is used as a cofactor to accomplish this reduction. Although the alcohol-generating FAR reactions proceed through an aldehyde intermediate, a free aldehyde is not released (Kolattukudy, 1970). Thus, the alcohol-forming FARs are distinct from those enzymes that carry out two-electron reductions of fatty acyl-CoA and yield free fatty aldehyde as a product (Wang and Kolattukudy, 1995a; Reiser and Somerville, 1997; Vioque and Kolattukudy, 1997). A further distinction is that the alcohol-forming FARs are thought to be integral membrane proteins, whereas those that carry out two-electron reductions are either soluble enzymes or have a peripheral membrane association. Alcohol-forming FARs have been purified from pea leaves (Vioque and Kolattukudy, 1997) and from duck uropygial glands (Wang and Kolattukudy, 1995b). Both of these proteins have apparent masses of approximately 58 kD. The aldehyde-generating proteins that have been identified so far have masses of approximately 30 kD (Wang and Kolattukudy, 1995a; Reiser and Somerville, 1997; Vioque and Kolattukudy, 1997).

Jojoba (*Simmondsia chinensis*) is a native of deserts of the American Southwest, and is unusual among higher plants in that its seed storage lipids are waxes rather than triglycerides. These waxes, which can comprise up to 60% of the dry weight of the almond-sized seed, are esters of long-chain (mostly C20, C22, and C24), monounsaturated fatty acids and alcohols (Miwa, 1971). The waxes are produced in developing embryos during seed formation (Ohlrogge et al., 1978; Pollard et al., 1979; Wu et al., 1981). In jojoba, as in many other oil seed plants, oleic acid (C18:1) is synthesized in plastids by soluble enzymes via acyl-carrier protein (ACP)-linked intermediates. Oleic acid is exported from the plastid and converted to a CoA ester. A membrane-associated fatty acyl-CoA elongase (FAE) system then produces the C20:1, C22:1, and C24:1 acyl-CoAs, which are the precursors of wax synthesis. Long-chain acyl-CoA is reduced to alcohol by FAR, and the wax storage lipid is formed by a fatty acyl-CoA: fatty alcohol acyltransferase (wax synthase, WS) activity. The fatty acid

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and alcohol compositions of the waxes indicate that the jojoba FAR has a preference for very-long-chain acyl-CoA substrates and that C18:1 acyl-CoA is a relatively poor substrate.

In contrast to the extensive biochemical characterizations of alcohol-forming FAR and WS, the genetic and evolutionary aspects of these enzymes are far less clear. To our knowledge, genes associated with an alcohol-forming FAR have not previously been reported and, except for the case of jojoba embryos (Lardizabal et al., 2000), neither proteins nor genes associated with a WS have been identified. In this report we describe the purification of a membrane-associated, NADPH-dependent alcohol-forming FAR from developing jojoba embryos, as well as the cloning of a cDNA that encodes FAR. The identity of the cDNA clone was confirmed by detection of FAR activity and free alcohols in bacteria expressing the clone. In addition, we show that expression of the jojoba cDNA in developing embryos of *Brassica napus* results in not only the synthesis of long-chain alcohols in the transformed tissues, but also in the appearance of novel wax esters. Conversion of the fatty alcohols to waxes in the transgenic plants is not always complete, and the overall level of alcohol and wax production is relatively low. In an accompanying paper, we show that efficient conversion of fatty alcohol into wax esters in transgenic plants can be accomplished by co-expression of a cDNA encoding a jojoba wax synthase enzyme (Lardizabal et al., 2000). The accession number for the jojoba FAR reductase mRNA is AF149917; the accession number for the altered codon mRNA is AF149918.

MATERIALS AND METHODS

Enzyme Assays

FAR activity was assayed by measuring the formation of tetracosenol from [1-^{14}C]tetracos-cis-15-enoyl-CoA (C24:1-CoA) (custom synthesis, Amersham-Pharmacia Biotech, Uppsala). Assay mixtures contained 17 μM C24:1-CoA (10 Ci/mol), 15 mM NADPH, 0.5 M NaCl, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH, pH 7.5, 1 mM dithiothreitol (DTT), 1 mM EDTA, 10% (w/v) glycerol, and 0.3% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) in a final volume of 250 μL. For analysis of column fractions, 2 mM NADPH was used and the NaCl concentration varied from 0.3 to 0.6 M. Incubations were performed at 30°C for 30 min and were stopped by the addition of 250 μL of isopropanol:acetic acid (4:1, v/v). Neutral lipids were extracted using a method modified from Hara and Radin (1978). Hexane:isopropanol (3:2, v/v, 4 mL), which also contained cold carriers (oleyl alcohol and oleic acid, both at 12.5 μg/mL, and "jojoba bean oil" [Sigma-Aldrich, St. Louis] at 6.25 μg/mL) were added, followed by 2 mL of 6.7% (w/v) sodium sulfate.

Radioactivity present in the organic phase was determined using liquid scintillation counting, whereas a second portion was used for thin-layer chromatography (TLC) analysis (silica gel-G, developed with hexane:diethyl ether:acetic acid [70:30:2, v/v]). Radioactivity on the TLC plates was localized and quantitated with a radioanalytic scanner (Scanalytics, Billerica, MA). The jojoba embryo WS activity was completely inhibited by 0.3% (w/v) CHAPS, thus simplifying quantitation of FAR activity. WS assays of extracts of developing *B. napus* embryos were performed as described in Lassner et al. (1996) using the neutral lipid extraction procedure and the TLC system described here for the FAR assays.

Enzyme Purification and Protein Sequencing

Developing seeds of jojoba were harvested from plantations in Arizona during the period of maximal wax synthesis. Embryos were dissected and a microsomal membrane fraction was isolated essentially as described in Lassner et al. (1996). The protein concentration of membrane preparations was typically between 7 and 9 mg/mL. Solid CHAPS was added to the suspension to a final concentration of 2% (w/v). After gentle stirring on ice for 1 h, the CHAPS and NaCl concentrations were adjusted to 0.75% (w/v) and 0.4 M, respectively, and the sample was centrifuged (200,000*g* for 1 h). The supernatant fraction was used immediately for chromatography. All chromatography solutions contained 25 mM HEPES-NaOH, pH 7.5, 20% (w/v) glycerol, 0.75% (w/v) CHAPS, and 1 mM EDTA (buffer A). Additionally, NaCl was added to the buffer A solution at the following levels: 0.1 M (buffer B), 0.4 M (buffer C), 0.5 M (buffer D), and 1.0 M (buffer E).

The 200,000*g* supernatant fraction was applied to a Blue A agarose (Amicon, Beverly, MA) column that had been equilibrated with buffer C. After washing with buffer C and then with buffer D, FAR activity was eluted with buffer E. Pooled material from the Blue A column was concentrated approximately 8-fold via ultrafiltration in a pressure cell fitted with a YM30 membrane (Amicon). The concentrated sample (in 4-mL aliquots) was applied to a Sephacryl S-100 HR (Amersham-Pharmacia Biotech) column (2.5 × 90 cm) that had been equilibrated with buffer D. The S-100 column was also used to estimate the size of the solubilized FAR compared with the elution pattern of molecular mass standards (Bio-Rad Laboratories, Hercules, CA) chromatographed under the same column and buffer conditions. Fractions that contained high levels of FAR activity were pooled and concentrated (approximately 20-fold) via ultrafiltration as before. The NaCl concentration was reduced to 0.1 M by dilution with buffer A and the sample was loaded onto a palmitoyl-CoA-agarose (Sigma) column (1.0 × 1.5 cm) that had been equilibrated with buffer B. The column was then washed sequentially with: buffer B, buffer B containing 10 mM NADH, buffer B, and buffer B containing 15 mM NADPH.

Proteins present in the various samples were resolved by SDS-PAGE using a 10% to 15% (w/v) acrylamide gradient. Silver staining was performed according to the method of Blum et al. (1987). Protein concentrations were determined as described by Bradford (1976) using bovine serum albumin as a standard. The protein sequencing strategy and methodology were essentially as described in Knutzon et al. (1995).

cDNA Cloning

RNA isolation and cDNA library construction were previously described (Lassner et al., 1996). For use as a template for PCR amplification, first-strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Life Technologies/Gibco-BRL, Cleveland) according to the manufacturer's directions using the primer CCAAGCTTCTGCAGGAGCTCTTTTTTTTTTTTTTT.

At an early stage of protein purification, the N-terminal amino acid sequence GATEKSIKSTMKDMGIERA was obtained from a 32-kD polypeptide that appeared to track with FAR activity during chromatography. We performed 3′ RACE (Frohman et al., 1988) using the primers ATGAARGAYATGGGNATHGA (based on the amino acid sequence MKDMGIE from the 32-kD peptide mentioned above) and CCAAGCTTCTGCAGGAGCTC to PCR amplify a 1-kb DNA fragment from a first-strand cDNA template. The 1-kb PCR product was purified by agarose gel electrophoresis and cloned in pCR1000 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). This clone was used as a probe to isolate full-length cDNAs from a library constructed using the procedure of Alexander (1987). DNA sequence analysis was performed using MacVector Software (IBI/Kodak, Rochester, NY). TopPredII software (Claros and Van Heijne, 1994) was used to determine potential transmembrane domains. The deduced protein sequence was used to search non-redundant databases using the BLAST service provided by the National Center for Biotechnology Information (Altschul et al., 1990).

Antibody Production and Immunoblot Analysis

The PCR primers TCTAGAGGATCCCAATGGTTCAAGGGTAAGTAC and GGATCCGAATTCTTAGTTAAGAACGTGCTCTACGAC were used to amplify a region of the FAR cDNA encoding amino acids 410 to 493, a hydrophilic region of the protein. The PCR product was cloned as a *Bam*HI-*Eco*RI fragment into pGEX2T, and the protein was prepared as described in Lassner et al. (1996). The protein was used to produce antiserum in rabbits (Berkeley Antibody Company, Richmond, CA). Immunoblot analysis was performed by running Novex 12% Tris-Gly gels and electroblotting the proteins to nitrocellulose membranes. The blots were probed using the antiserum, alkaline-phosphatase-conjugated goat anti-rabbit IgG, and western blue staining according to the manufacturer's protocols (Promega, Madison, WI).

Expression of FAR in *Escherichia coli*

In vitro mutagenesis (Kunkel et al., 1987) was used to introduce an *Nde*I site at the ATG of the reductase open reading frame, and the resulting reductase DNA fragment was cloned into pET3A (Rosenberg et al., 1987). The derivative plasmid, pCGN7800, was transformed into *E. coli* BL21(DE3). Neutral lipids were extracted from the recombinant *E. coli* using the solvents described in the enzyme assay procedures, separated by TLC, and stained with iodine. Lipids that co-migrated with the fatty alcohol standards were eluted from the silica and further analyzed by reverse phase TLC and gas chromatography (GC). The sample was injected onto a Supelcowax 10 column (30 m × 0.32 mm, Supelco, Bellefonte, PA) mounted on a GC (model 5890, Hewlett-Packard, Palo Alto, CA). Helium was used a carrier gas with an on-column flow of 5.5 mL/min, and a split flow of 15 mL/min. The injector temperature was 260°C and the flame ionization detector was at 270°C. The sample was analyzed using the following temperature program: 190°C for 15 min, followed by a temperature increase of 5°C/min to a temperature of 250°C and held at 250°C for 3 min. Cells of the recombinant *E. coli* were harvested by centrifugation, and FAR enzyme assays were performed as described above after lysing the cells in assay buffer using lysozyme and CHAPS.

Plant Expression

The primers GGCGCGCCGGTACCTCTAGACCTGGCGATTCAACGTGGTC and CTGCAGGGATCCGTCGACTTGCTTCTTGTGAATTGAGA were used to amplify approximately 1 kb of DNA flanking the 5′ end of a *B. napus* oleosin gene, and the primers CTGCAGTAAATTACGCCATGACTATTTTCAandGCGCGCCGGTACCTCTAGAGGTTCTGCCATGTCTCAACGTTCA were used to amplify approximately 700 nucleotides of DNA flanking the 3′ end of the gene (Lee and Huang, 1991). The 5′ and 3′ flanking regions were assembled to form an oleosin expression cassette in BssHII-digested pBC- (Stratagene, La Jolla, CA). The primers GTCGACAAAATGGAGGAAATGGGAAGCATT and GTCGACTTAGTTAAGAACGTGCTCTACGAC were used to PCR amplify the coding region of the FAR cDNA and to introduce *Sal*I cloning sites flanking the start and stop codons. The FAR coding region was inserted into the *Sal*I site of the oleosin expression cassette, and the resulting plasmid was cloned into an *Agrobacterium tumefaciens* binary vector (McBride and Summerfelt, 1990) to yield pCGN7643. Plasmid pCGN7643 was used to tranform *B. napus* cv Reston as described by Radke et al. (1987) The FAR gene was resynthesized to reduce the A + T content of the gene, while retaining the correct protein sequence using the method of Bambot and Russell (1993). The resynthesized gene was cloned into the oleosin expression cassette and binary vector to yield plant transformation vector pCGN7677.

Oil Analysis

GC analysis to determine the alcohol content of seed oils was performed on transmethylated oil using the conditions described in the *E. coli* expression procedures. The identity of the fatty acid methyl ester and alcohol peaks were verified using GC-mass spectrometry (MS) (analysis performed by Alpha Chemical and Biomedical Laboratories, Santa Rosa, CA). Lipids were extracted from seeds by grinding in hexane. High-temperature GC analysis to examine the wax and alcohol content was performed using a triglyceride column (Chrompack, Raritan, NJ) using the following temperature program: 190°C for 3 min, followed

by a temperature increase of 15°C/min to a temperature of 300°C for 2 min, followed by a temperature increase of 2°C/min to a temperature of 360°C for 3 min. The column flow rate was 2.1 mL/min helium, the split flow rate was 6 mL/min, the injector temperature was 360°C, and the flame-ionization detector temperature was 370°C. The identity of the waxes were verified using GC-MS (analysis performed by Alpha Chemical and Biomedical Laboratories). The oil was analyzed by silver-phase HPLC, and the C40:2 wax was purified using preparative silver-phase HPLC. The identity of the purified C40:2 wax was confirmed by comparing the infrared spectrum and the proton NMR spectrum to the spectra of an authentic standard (analysis performed by Alpha Chemical and Biomedical Laboratories). The molecular mass of the $(M + H)^+$ ion of C40:2 wax isolated from the plant oil and an authentic standard were determined to be 588.59154 D (the theoretical mass of a C40:2 wax ester is 588.592357 D) by high-resolution MS (analysis performed by Mass Search, Modesto, CA).

RESULTS

Purification of the Jojoba Embryo FAR

A microsomal membrane fraction enriched in FAR activity was isolated from developing embryos of jojoba and used as the starting material for enzyme purification. Separation of the microsomal membrane fraction on linear Suc density gradients revealed that the vast majority of the membranes in that fraction have a density typically associated with the endoplasmic reticulum (ER) and that FAR activity co-migrated with that fraction (data not shown). This presumed ER fraction also possesses high levels of WS and FAE activities required for wax synthesis in jojoba embryos (Lassner et al., 1996; Lardizabal et al., 2000). Although an equivalent amount of activity partitions with a wax pad that forms upon the initial centrifugation step, we were unable to solubilize the activity from this fraction.

We used the detergent CHAPS to solubilize the jojoba FAR from membranes. Although the presence of high levels of CHAPS in assay solutions resulted in an apparent loss of enzyme activity, this inhibition could be completely reversed by simple dilution of the detergent to below its critical micellar concentration (CMC). The nominal CMC of CHAPS is approximately 0.5% (w/v), but it does vary with buffer and salt conditions (Chattopadhyay and Harikumar, 1996). We routinely diluted the CHAPS to 0.3% (w/v) in our assays. The jojoba embryo FAR activity is strictly dependent on NADPH, and NADH is not utilized by the enzyme (Pollard et al., 1979).

Representative chromatographic separations obtained during FAR purification are shown in Figure 1. The inclusion of CHAPS at greater than CMC in all column buffers was essential to prevent aggregation of the enzyme. Conversely, the use of too high a CHAPS concentration (e.g. 1%) resulted in a rapid loss of enzyme activity. Chromatography on Blue A agarose provided the majority of enrichment of FAR activity (Fig. 1A). Only the material eluted by 1.0 M NaCl was utilized for further purification. Size exclusion chromatography (Fig. 1B) was included to improve resolution during the subsequent palmitoyl-CoA-agarose chromatography. The FAR activity elution profile

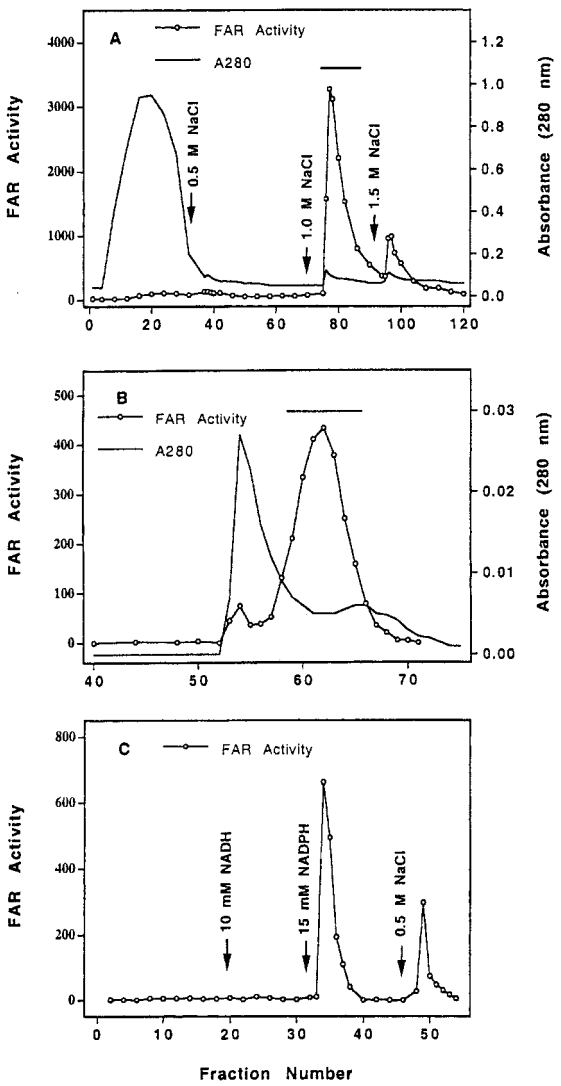

Figure 1. Chromatographic enrichment of jojoba FAR activity. A, Blue A agarose chromatography. Solubilized jojoba microsomal membranes were applied to a Blue A agarose column in a buffer containing 0.4 M NaCl. FAR activity was eluted using a buffer containing 1.0 M NaCl. FAR activity is shown as picomoles of alcohol formed per minute per fraction. Relative protein concentrations of the various fractions are shown as the A_{280} value of the UV monitor. The horizontal bar indicates fractions pooled for subsequent chromatography. Arrows indicate when buffer solutions containing other than 0.4 M NaCl were applied to the column. B, Sephacryl S-100 chromatography. Pooled fractions from the Blue A agarose column were concentrated and applied to the column. Activity, relative protein, and fractions pooled for the next chromatographic step are indicated as in A. C, Palmitoyl-CoA agarose chromatography. Pooled fraction from the size exclusion column step were applied to a palmitoyl-CoA-agarose column. After washing with equilibration buffer and then buffer containing NADH, FAR activity was eluted with buffer containing NADPH. The effect of subsequent washing with a buffer containing 0.5 M NaCl is also shown. Arrows indicate when various solutions were applied to the column.

from the size-exclusion column corresponded to an apparent mass of 49 kD compared with the elution of protein standards run under the same conditions. Palmitoyl-CoA is not a substrate for the jojoba FAR, however, it does act as an inhibitor of the reduction of longer chain length acyl-CoA (data not shown). The solubilized enzyme binds to a palmitoyl-CoA-agarose matrix and can be affinity eluted by NADPH (Fig. 1C). Consistent with the reductant specificity of the enzyme, NADH was ineffective at releasing activity from this matrix. The enrichment of FAR specific activity obtained during purifications varied, but was typically 150- to 250-fold relative to a cell-free, homogenized embryo preparation.

Figure 2 shows the polypeptide profiles of samples taken from various stages of a representative purification experiment. SDS-PAGE analysis revealed that two heavily stained polypeptides (relative molecular masses of 56 and 54 kD) were present in the NADPH eluted fractions from the palmitoyl-CoA-agarose column (Fig. 2, lane 6). Other minor bands visible in this gel were not always detected in other preparations and, when present, did not consistently track with FAR activity during chromatographic separations. Several peptides generated using either trypsin or cyanogen bromide were isolated from the two electrophoretically separated proteins and their N-terminal sequences determined. Most of the sequences obtained from peptides of the 54-kD protein were also found in peptides isolated from the 56-kD polypeptide. Additionally, immunoblot analysis of a jojoba embryo cell-free extract showed that only the 56-kD polypeptide was present (data not shown). The smaller polypeptide appeared to be an artifact of protein purification.

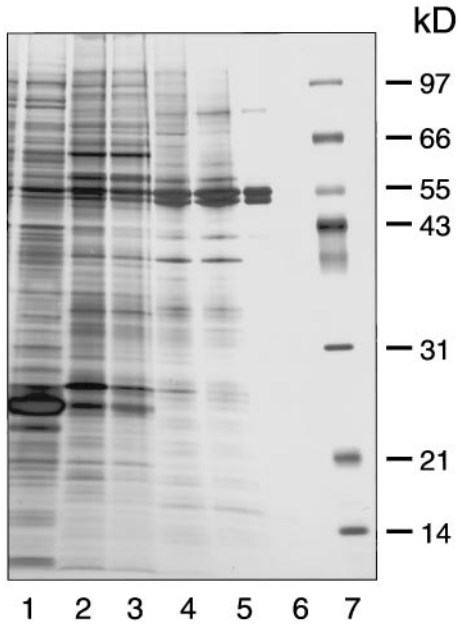

Figure 2. SDS-PAGE showing purification of jojoba FAR. Proteins were resolved on a 10% to 15% polyacrylamide gradient gel and stained with silver (Blum et al., 1987). The samples loaded onto the gel were taken from a representative purification sequence. Lane 1, Cell-free homogenate; lane 2, microsomal membranes; lane 3, detergent-solubilized 200,000*g* supernatant fraction; lane 4, Blue A agarose column (1.0 M NaCl elution); lane 5, size-exclusion column, pooled retained fractions; lane 6, palmitoyl-CoA agarose column (NADPH elution); and lane 7, molecular mass standards.

Cloning of the Jojoba FAR cDNA

During optimization of the FAR purification procedures, samples containing the 54- and 56-kD proteins and several others were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane for N-terminal amino acid sequencing. No sequence data were obtained from either the 54- or 56-kD proteins, indicating they had been blocked either in vivo or during purification. A relatively minor 32-kD protein was also analyzed. The sequence GATEKSIKSTMKDMGIERA was obtained from this band and was used to design a degenerate oligonucleotide for use in cloning. A combination of PCR amplification and cDNA library screening was used to isolate a 1.7-kb cDNA clone. The deduced mass of the protein encoded by this cDNA clone was 56.2 kD. The sequence encoding the peptide used for cloning was near the center of the cDNA. Subsequently, all of the peptide sequences obtained from the 56- and 54-kD proteins were found to be present in the deduced amino acid sequence of this cDNA. The 32-kD protein apparently was generated by proteolysis of either the 56- or 54-kD protein. The 32-kD protein was not observed in samples containing FAR activity after optimization of the purification protocol.

Figure 3 shows the deduced amino acid sequence encoded by the 1.7-kb cDNA. The deduced protein has a pI of 8.76 and hydropathy analysis using the TopPredII program (Claros and von Heijne, 1994) suggests that there are one or two transmembrane domains. The sequence lacks the motif GXGXXG/A found in many nucleotide-binding enzymes (Wierenga et al., 1985); however, it does contain the similar motif (IVF) X(ILV) TGXTGFL(GA) suggested by Aarts et al. (1998). A query of the public data banks with the jojoba FAR sequence using the BLAST search program (Altschul et al., 1990) revealed significant homology with a family of Arabidopsis and *B. napus* proteins related to ms2 (GenBank accession nos. 1491638, 1171027, 1491615, 3549681, and AB007651) (Aarts et al., 1993, 1997). Arabidopsis ms2 is a tapetum-specific protein of unknown enzymatic activity that is essential for pollen formation (Aarts et al., 1997). The other family members have unknown expression patterns, and are proteins of unknown function. There are also *Caenorhabditis elegans* and *Drosophila melanogaster* proteins of unknown function with significant (E value <1e-05) homology to the jojoba FAR (GenBank accession nos. 3786433 and 2827491). Querying the dBEST database with the jojoba FAR shows that related expressed sequence tags are found in cotton, rice, and corn.

Expression of Functional FAR in *E. coli*

The 1.7-kb cDNA was expressed in *E. coli* using a T7 RNA-polymerase-based system. Enzyme assays showed the presence of FAR activity in the transformed cells that was not present in the control cells (data not shown).

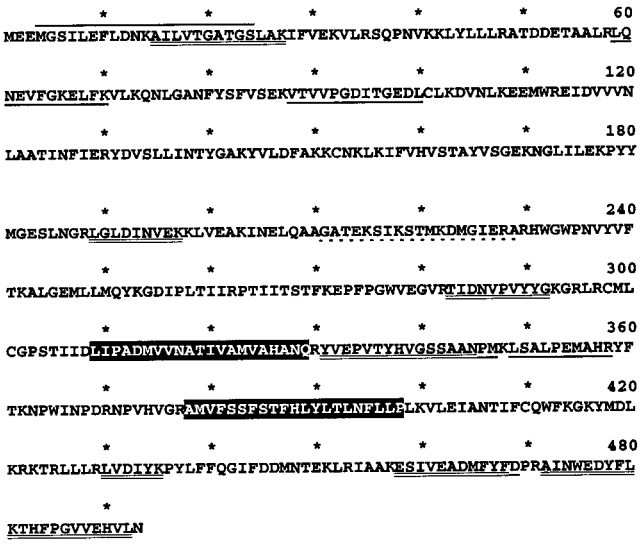

Figure 3. Amino acid sequence of the jojoba FAR. The predicted amino acid sequence of the jojoba FAR protein was translated from the cDNA. Tryptic peptide sequences derived from both the 54- and 56-kD proteins are double-underlined. Tryptic peptide sequences derived only from the 56-kD protein are single-underlined. A sequence derived from a cyanogen bromide peptide is shown as an overline. The N-terminal sequence of a 32-kD protein used to design a primer to isolate the cDNA clone is underlined with a dashed line. Two potential transmembrane helixes are shaded.

Figure 4 shows TLC separation of lipids extracted from those cells. The cells transformed with the FAR construct contained fatty alcohols, while the control *E. coli* cells did not. Further analysis (GC and reversed-phase TLC) of material eluted from this region of the TLC plate revealed that these were primarily C16:0 and C18:1 (cis-vaccenic) alcohols (data not shown). Assays of jojoba embryo extracts indicated that the FAR is capable of reducing C16:0-ACP, suggesting that the alcohol accumulating in these *E. coli* cells may be derived from acyl-ACP. No waxes were detected in the *E. coli* cells expressing FAR using either radiochemical assays or lipid analyses.

Seed Oils of Transgenic Plants Expressing the Jojoba FAR Contain Wax Esters

The jojoba FAR cDNA was placed under the control of oleosin regulatory sequences (Lee and Huang, 1991). Oleosin is an oil body protein that is highly expressed in developing *B. napus* embryos. The oleosin promoter-FAR gene fusion was transferred to a binary *A. tumefaciens* vector and used to generate transgenic high erucic acid rapeseed (HEAR) plants. HEAR (*B. napus* cv Reston) was chosen as the host due to the presence of C20:1 and C22:1 fatty acids in its seed lipids. Developing HEAR embryos have an active FAE system that produces the long-chain acyl-CoA substrates preferred by the jojoba FAR. The seed lipids from 19 transgenic HEAR plants were transmethylated and analyzed by GC. Eleven of the samples from these transgenic plants were found to contain up to 0.16 weight % of their lipids as fatty alcohols (Fig. 5A), whereas the control samples were completely devoid of such alcohols. When single seeds from the best event were analyzed, up to 0.56 weight % of the lipids were determined to be fatty alcohols. As anticipated, C22:1 was the predominant alcohol species detected. Its identity was confirmed using GC-MS.

Although GC analysis of transmethylated oil demonstrated the presence of fatty alcohols in transgenic plants, it did not indicate if the alcohols were present in a free or a combined state. High-temperature GC and GC-MS of underivatized oil from HEAR transformants containing the native FAR cDNA sequence failed to detect any free alcohols, but, rather, suggested the presence of a series of wax esters with chain lengths of 38, 40, 42, and 44 carbons and two, three, and four double bonds (data not shown, but see later sections and Lardizabal et al., 2000). These compounds were not detected in oil from control plants. The most abundant putative wax ester, C40:2, was purified by TLC and silver-phase HPLC and subjected to infrared spectrometry, proton NMR, methanolysis and GC analysis, and high-resolution MS. The data demonstrated that this compound was indeed a wax ester comprised of a C22:1 alcohol and a C18:1 fatty acid. Thus, HEAR embryos contain an endogenous activity capable of esterifying at least a portion of the fatty alcohol product of the introduced FAR.

Resynthesis of the FAR cDNA Increases Expression in Transgenic Plants

Although alcohols were produced in transgenic HEAR embryos, the amount that accumulated was very low. Im-

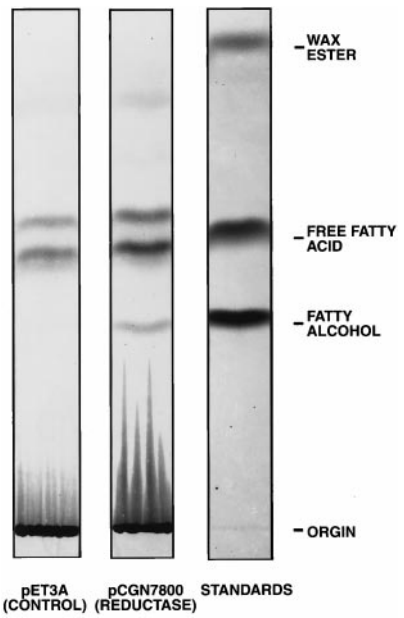

Figure 4. TLC analysis of *E. coli* lipids. Lipids were extracted from *E. coli*, analyzed using normal phase TLC, and visualized by staining with iodine. The standards lane contains a wax ester (jojoba oil), a free fatty acid (oleic acid), and a fatty alcohol (oleyl alcohol). The lane labeled pET3A contains lipids from *E. coli* transformed with the empty expression vector pET3A. The lane labeled pCGN7800 contains lipids from *E. coli* transformed with the pET3A containing the jojoba FAR.

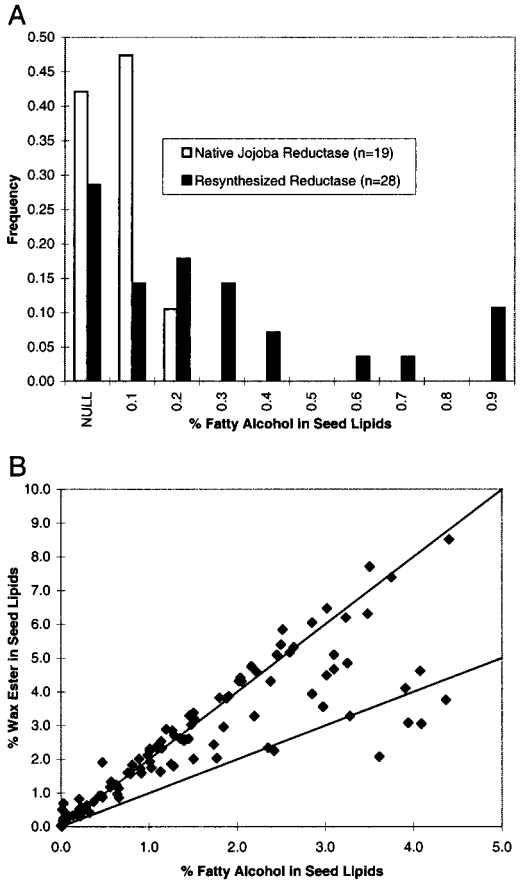

Figure 5. Fatty alcohols and wax esters in transgenic HEAR seed oils. A, Alcohol content of the pooled seeds from plants transformed with the native and resynthesized FAR genes. Pooled seeds from transgenic plants containing either the native jojoba FAR or the synthetic gene designed to reduce AT content were analyzed by transmethylation of the seed oil and GC. The frequency of independent transgenic plants whose seed alcohol content fell in the various ranges was plotted against the amount of fatty alcohol present in the seed oil. Gene resynthesis increased both the frequency of transgenic plants with detectable fatty alcohol in their oil and the quantity of fatty alcohol found in the seed oil. B, Wax versus alcohol content of individual seeds. The wax ester and fatty alcohol content of single seeds from transgenic plants containing the synthetic FAR were determined as described in "Materials and Methods." The upper line represents complete esterification of the fatty alcohol into wax esters, and the lower line represents 50% esterification of the fatty alcohols into wax esters. As the fatty alcohol content of the seed increases, the endogenous wax synthase fails to esterify all of the fatty alcohol into wax esters.

munoblots and enzymatic assays of extracts from developing embryos indicated that very little FAR was being produced. RNA blots probed with a FAR-specific probe revealed a smear of hybridizing material of small size with little full-length message (data not shown). Subsequent probing of the RNA filter with a napin probe (Kridl et al., 1991) suggested that the napin RNA was intact. Examination of the FAR cDNA showed that portions of the coding region contained approximately 75% A + T content (Fig. 6). Transgenes with high A + T content have been associated with aberrant transcript processing and RNA instability (De Rocher et al., 1998; Diehn et al., 1998). Therefore, the cDNA sequence was altered to lower its A + T content without changing the amino acid composition of the encoded protein. The resynthesized coding region was placed under control of oleosin regulatory sequences, and transgenic HEAR plants were generated. Analysis of transmethylated seed lipids from pools of seeds from these plants showed that the gene resynthesis resulted in an increase in the proportion of plants with detectable levels of fatty alcohol in their seed oils, and an increase in the quantity of fatty alcohols relative to plants with the native cDNA (Fig. 5A). Oils from pooled seeds of several primary transformants contained as much as 0.9% fatty alcohol by weight. When single seeds from the individual with the highest alcohol content were analyzed, the alcohol content ranged up to 4.4 weight % of seed lipids (Fig. 5B).

Analysis of Underivatized Seed Oil of Transgenic HEAR Expressing the Resynthesized FAR Gene

The presence of wax esters in seeds from plants containing the resynthesized FAR cDNA was demonstrated by high-temperature GC of the underivatized oil. Unlike the oils from the plants containing the FAR gene prior to resynthesis, free fatty alcohols were detected in the oil. The wax and alcohol contents of oil from a number of seeds were determined. The wax was quantified by using high-temperature GC and comparing the area of the wax peaks against that of an internal wax standard (C35:1 wax composed of C18:1 alcohol and C17:0 fatty acid). The total fatty acid and fatty alcohol content of the same oil was quantified by GC analysis of the transmethylated oil. Figure 5B shows that in seed oils with less than 1% alcohol, most of the alcohol is esterified into wax esters. In seed oils with greater than 1% alcohol, the ratio of wax to alcohol is variable. In some of the oils, nearly all of the alcohol is

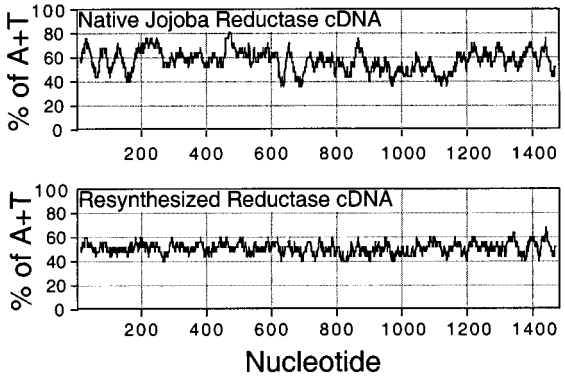

Figure 6. A + T content of the native jojoba FAR open reading frame (ORF) and a synthetic ORF. The A + T content of 25 nucleotide windows of the jojoba FAR ORFs were plotted against their position in the ORFs. A, The A + T content of the native jojoba FAR ORF. Several regions of the ORF have A + T contents exceeding 75%. B, The A + T content of a synthetic FAR ORF designed to reduce the A + T content without changing the protein encoded by the native FAR ORF.

converted to wax, and in some, less than half of the alcohol is converted to wax esters.

HEAR Embryos Possess WS Activity

Enzyme assays of extracts from developing HEAR embryos revealed the presence of a low level of endogenous WS activity. Specifically, when the extracts were incubated with oleyl alcohol and [1-^{14}C]16:0-CoA, a radiolabeled product was formed that co-migrated with a wax standard upon TLC analysis. This activity is minor compared with the WS activity detected in jojoba. Cell-free extracts of developing jojoba embryos have WS activities that range from 500 to 900 pmol min^{-1} mg^{-1} protein. The activity detected in HEAR embryos was measured to be about 15 to 25 pmol min^{-1} mg^{-1} protein. We have not attempted to further characterize the HEAR activity in terms of its substrate preferences or cellular location.

DISCUSSION

We have purified from jojoba embryos a membrane-associated, alcohol-forming FAR involved in wax formation and cloned its cDNA. Analyses of *E. coli* expressing the FAR cDNA demonstrate that the product of the FAR is free alcohol, and that the enzyme itself has no capacity to form wax esters from those alcohols.

The jojoba FAR cDNA is predicted to encode a protein with a molecular mass of 56.2 kD. This is in good agreement with the apparent molecular mass estimated from SDS-PAGE of the purified protein. Thus, FAR from jojoba, pea (Vioque and Kolattukudy, 1997), and duck (Wang and Kolattukudy, 1995b) all have very similar molecular masses. It will be interesting to compare the sequence of the jojoba FAR with those of the other FAR proteins once they become available. Although two bands of 56 and 54 kD were present in the purified jojoba FAR preparation, immunoblot analysis suggests that the 54-kD band was an artifact (data not shown). Additionally, no evidence of a second class of FAR transcript was found during the cloning process. The isolation of a cyanogen-bromide-generated peptide whose amino acid sequence begins near the amino terminus of the deduced protein sequence (Fig. 3) suggests that the FAR does not have a cleaved signal peptide. As has been noted for several proteins associated with lipid metabolism, the jojoba FAR has a basic pI, a characteristic suggested to be important in interactions with the acidic phospholipid membrane (Coleman, 1990). The 49-kD size estimate obtained by size exclusion chromatography of the solubilized enzyme suggests that FAR is a monomeric enzyme.

The jojoba FAR sequence shows extensive homology to the Arabidopsis tapetum-specific protein ms2. Several recent publications demonstrate the presence of neutral lipids in two novel organelles, elaioplasts and tapetosomes, found in tapetal cells (Ting et al., 1998; Hernandez-Pinzon et al., 1999). Elaioplasts are plastids that contain TAG and sterol esters. The elaioplast sterol esters are deposited on the surface of mature pollen grains. Tapetosomes are lipid bodies that contain oleosin-like proteins and neutral lipids including TAG and wax esters. TAG is the most abundant tapetosome lipid. An attractive hypothesis is that the ms2 protein forms fatty alcohols that are constituents of the tapetosome wax esters. The tapetosome lipids are not deposited in mature pollen, but are broken down as the tapetum degrades. The oxidation of the tapetosome neutral lipids could provide an energy source for developing pollen, much as neutral lipids can serve as an energy reserve in germinating seeds. Because TAG is more abundant than wax esters in the tapetosome, it remains cryptic why an alcohol-forming reductase would be necessary for pollen development if the waxes are simply energy sources. It is possible that wax esters or fatty alcohols are essential precursors for the formation of pollen constituents.

The evolutionary origin of wax synthesis in jojoba seeds has been obscure. The discovery of at least four related genes in Arabidopsis and expressed sequence tags from other plant species suggests that FAR-like genes are ubiquitous in plants. In addition to their role in pollen formation, these FAR-like enzymes may be responsible for the formation of the alcohol component of the wax esters found in the cuticular lipids of Arabidopsis and other species. We do not know if any of the FAR-related proteins from other plant species are seed expressed, or if jojoba recruited a FAR expressed in tapetal, epidermal, or other tissues for the formation of the alcohol component of its seed wax esters. The identification of related proteins in animals as diverse as *C. elegans* and *D. melanogaster* suggests that these proteins may be ubiquitous in animals, and might form fatty alcohols that animals use for the formation of wax esters, ether lipids, or other compounds.

We know of no examples in which free long-chain alcohols accumulate to high levels within living cells, and it is likely that high levels of fatty alcohols are detrimental to cell viability. Thus, the capacity to esterify or otherwise modify any alcohols produced seems essential. It is intriguing that HEAR, a plant that does not produce waxes in its seed oil, possesses an endogenous wax synthesis capability. It is tempting to speculate that an acyl-transferase enzyme, perhaps involved in triglyceride or phospholipid synthesis, is also capable of catalyzing wax formation. For example, diacylglycerol acyltransferase, the only microsomal enzyme unique to triglyceride (versus phospholipid) synthesis, combines diacylglycerol (an alcohol) with fatty acyl-CoA. However, the data do not exclude the possibility that an enzyme with a dedicated wax synthesis activity, such as those assumed to be associated with the formation of cuticular lipids, may be present in these HEAR cells. Regardless of the origin, the presence of WS activity in HEAR seeds suggests a pathway for the evolution from TAG to waxes as seed storage lipids. After recruiting a reductase for the formation of fatty alcohols in developing embryos of jojoba, natural selection would favor the evolution of greater wax synthase activity in the embryos to reduce the free alcohol composition of the seeds. Other adaptation steps could include the ability to oxidize the wax esters to release their energy for seed germination (Moreau and Huang, 1977) and the loss of TAG synthesis,

possibly via a reduction of diacylglycerol acyltransferase activity.

We have shown that the introduction of a single foreign cDNA into HEAR can result in a redirection of the storage oil synthesis pathways from TAG to wax. Although the fatty alcohol product of the introduced FAR can be esterified to form waxes, this conversion is often incomplete. In an accompanying paper (Lardizabal et al., 2000), we show the effects of combining a WS and a fatty acid elongation enzyme with FAR to increase both the alcohol formation and wax ester content of transgenic plant oils.

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LITERATURE CITED

Aarts MGM, Dirkse WG, Stiekema WJ, Pereira P (1993) Transposon tagging of a male sterility gene in *Arabidopsis*. Nature **363:** 715–717

Aarts MGM, Hodge R, Kalantidis K, Florack D, Wilson ZA, Mulligan BJ, Stiekema WJ (1997) The *Arabidopsis MALE STERILITY 2* protein shares similarity with reductases in elongation/condensation complexes. Plant J **12:** 615–623

Alexander DC (1987) An efficient vector-primer cDNA cloning system. Methods Enzymol **154:** 41–64

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol **215:** 403–410

Bambot SB, Russell AJ (1993) Efficient total gene synthesis of 1.35-kb hybrid alpha-lytic protease gene using the polymerase chain reaction. PCR Methods Applic **2:** 266–271

Bishop JE, Hajra AK (1981) Mechanism and specificity of formation of long-chain alcohols by developing rat brain. J Biol Chem **256:** 9542–9550

Blum H, Beier H, Gross HS (1987) Improved silver staining of plant proteins, RNA, and DNA in polyacrylamide gels. Electrophoresis **8:** 93–99

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **71:** 248–254

Chattopadhyay A, Harikumar KG (1996) Dependence of critical micelle concentration of a zwitterionic detergent on ionic strength: implications in receptor solubilization. FEBS Lett **391:** 199–202

Claros MG, von Heijne G (1994) TopPred II: an improved software for membrane protein structure predictions. Comput Appl Biosci **10:** 685–686

Coleman J (1990) Characterization of the *Escherichia coli* gene for 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (*plsC*). Mol Gen Genet **232:** 295–303

De Rocher EJ, Vargo-Gogola TC, Diehn SH, Green PJ (1998) Direct evidence for rapid degradation of *Bacillus thuringiensis* toxin mRNA as a cause of poor expression in plants. Plant Physiol **117:** 1445–1461

Diehn SH, Chiu W, De Rocher EJ, Green PJ (1998) Premature polyadenylation at multiple sites within a *Bacillus thuringiensis* toxin gene-coding region. Plant Physiol **117:** 1433–1443

Frohman MA, Dush MK, Martin GR (1988) Rapid production of full length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA **85:** 8998–9002

Hara A, Radin NS (1978) Lipid extraction of tissues with a low-toxicity solvent. Anal Biochem **90:** 420–426

Hernandez-Pinzon I, Ross JHE, Barnes KA, Damant AP, Murphy DJ (1999) Composition and role tapetal lipid bodies in the biogenesis of the pollen coat of *Brassica napus*. Planta **208:** 588–598

Kahn AA, Kolattukudy PE (1973) Control of synthesis and distribution of acyl moieties in etiolated *Euglena gracilis*. Biochem **12:** 1939–1948

Knutzon DS, Lardizabal KD, Nelsen JS, Bleibaum JL, Davies M, Metz JG (1995) Cloning of a coconut endosperm cDNA encoding a 1-acyl-*sn*-glycerol-3-phosphate acyltransferase that accepts medium-chain-length substrates. Plant Physiol **109:** 999–1006

Kolattukudy PE (1970) Reduction of fatty acids to alcohols by cell-free preparations of *Euglena gracilis*. Biochemistry **9:** 1095–1102

Kolattukudy PE, Rogers L (1978) Biosynthesis of fatty alcohols, alkane-1,2-diols and wax esters in particulate preparations from the uropygial glands of white-crowned sparrows (*Zonotrichia Leucophyrus*). Arch Biochem Biophys **191:** 244–258

Kolattukudy PE, Rogers L (1986) Acyl-CoA reductase and acyl-CoA: fatty acyl transferase in the microsomal preparation from the bovine meibomian gland. J Lipid Res **27:** 404–411

Kridl JC, McCarter DW, Rose RE, Scherer DE, Knutzon DS, Radke SE, Knauf VC (1991) Isolation and characterization of an expressed napin gene from *Brassica rapa*. Seed Sci Res **1:** 209–219

Kunkel TA, Roberts JD, Zakour RA (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol **154:** 367–382

Lardizabal KD, Metz JG, Sakamoto T, Hutton WC, Pollard MR, Lassner MW (2000) Purification of a jojoba embryo wax synthase, cloning of its cDNA, and production of high levels of wax in seeds of transgenic Arabidopsis. Plant Physiol **122:** 645–655

Lassner MW, Lardizabal K, Metz JG (1996) A jojoba β-ketoacyl-CoA synthase cDNA complements the canola fatty acid elongation mutation in transgenic plants. Plant Cell **8:** 281–292

Lee K, Huang AHC (1991) Genomic nucleotide sequence of a *Brassica napus* 20-kilodalton oleosin gene. Plant Physiol **96:** 1395–1397

McBride KE, Summerfelt KR (1990) Improved binary vectors for *Agrobacterium*-mediated plant transformation. Plant Mol Biol **14:** 269–276

Miwa TK (1971) Jojoba oil wax esters and derived fatty acids and alcohols: Gas chromatographic analyses. J Am Oil Chem Soc **48:** 259–264

Moreau RA, Huang AC (1977) Gluconeogenesis from storage wax in the cotyledons of jojoba seedlings. Plant Physiol **60:** 329–333

Ohlrogge JB, Pollard MR, Stumpf PK (1978) Studies on biosynthesis of waxes by developing jojoba seed tissues. Lipids **13:** 203–210

Pollard MR, McKeon T, Gupta LM, Stumpf PK (1979) Studies on biosynthesis of waxes by developing jojoba seed. II. The demonstration of wax biosynthesis by cell-free homogenates. Lipids **14:** 651–662

Radke SE, Andrews BM, Moloney MM, Crouch ML, Kridl JC, Knauf VK (1987) Transformation of *Brassica napus* L. using *Agrobacterium tumefaciens*: developmentally regulated expression of a reintroduced napin gene. Theor Appl Genet **75:** 685–694

Reiser S, Somerville C (1997) Isolation of mutants of *Acinetobacter calcoaceticus* deficient in wax ester synthesis and complementation of one mutation with a gene encoding a fatty acyl coenzyme A reductase. J Bacteriol **179:** 2969–2975

Rosenberg AH, Lade BN, Chui D, Lin S, Dunn JJ, Studier FW (1987) Vectors for selective expression of cloned DNA's by T7 RNA polymerase. Gene **56:** 125–135

Ting JTL, Wu SSH, Ratnayake C, Huang AHC (1998) Constituents of the tepetosomes and eleaioplasts in *Brassica campestris* tapetum and their degradation and retention during microsporogenesis. Plant J **16:** 541–551

Vioque J, Kolattukudy PE (1997) Resolution and purification of an aldehyde-generating and an alcohol-generating fatty acyl-CoA reductase from pea leaves (*Pisum sativum* L.). Arch Biochem Biophys **340:** 64–72

Wang X, Kolattukudy PE (1995a) Solubilization and purification of aldehyde-generating fatty acyl-CoA reductase from green alga *Botryococcus braunii*. FEBS Lett **370:** 15–18

Wang X, Kolattukudy PE (1995b) Solubilization, purification and characterization of fatty acyl-CoA reductase from duck uropygial gland. Biochem Biophys Res Commun **208:** 210–215

Wierenga RK, De Maeyer MCH, Hol WG (1985) Interaction of pyrophosphate moieties with α-helices in dinucleotide binding proteins. Biochemistry **24:** 1346–1357

Wu X-Y, Moreau RA, Stumpf PK (1981) Studies of biosynthesis of waxes by developing jojoba seed. III. Biosynthesis of wax esters from acyl-CoA and long-chain alcohols. Lipids **6:** 897–902