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Metabolic Evidence for the Involvement of a Δ^4-Palmitoyl-Acyl Carrier Protein Desaturase in Petroselinic Acid Synthesis in Coriander Endosperm and Transgenic Tobacco Cells[1]

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We have previously demonstrated that the double bond of petroselinic acid (18:1Δ^{6cis}) in coriander (*Coriandrum sativum* L.) seed results from the activity of a 36-kD desaturase that is structurally related to the Δ^9-stearoyl-acyl carrier protein (ACP) desaturase (E.B. Cahoon, J. Shanklin, J.B. Ohlrogge [1992] Proc Natl Acad Sci USA 89: 11184–11188). To further characterize the biosynthetic pathway of this unusual fatty acid, ^{14}C-labeling experiments were conducted using developing endosperm of coriander. Studies were also performed using suspension cultures of transgenic tobacco (*Nicotiana tabacum* L.) that express the coriander 36-kD desaturase, and as a result produce petroselinic acid and Δ^4-hexadecenoic acid. When supplied exogenously to coriander endosperm slices, [1-^{14}C]palmitic acid and stearic acid were incorporated into glycerolipids but were not converted to petroselinic acid. This suggested that petroselinic acid is not formed by the desaturation of a fatty acid bound to a glycerolipid or by reactions involving acyl-coenzyme As (CoA). Instead, evidence was most consistent with an acyl-ACP route of petroselinic acid synthesis. For example, the exogenous feeding of [1-^{14}C]lauric acid and myristic acid to coriander endosperm slices resulted in the incorporation of the radiolabels into long-chain fatty acids, including primarily petroselinic acid, presumably through acyl-ACP-associated reactions. In addition, using an in vitro fatty acid biosynthetic system, homogenates of coriander endosperm incorporated [2-^{14}C]malonyl-CoA into petroselinic acid, of which a portion was detected in a putative acyl-ACP fraction. Furthermore, analysis of transgenic tobacco suspension cultures expressing the coriander 36-kD desaturase revealed significant amounts of petroselinic acid and Δ^4-hexadecenoic acid in the acyl-ACP pool of these cells. Also presented is evidence derived from [U-^{14}C]nonanoic acid labeling of coriander endosperm, which demonstrates that the coriander 36-kD desaturase positions double bonds relative to the carboxyl end of acyl-ACP substrates. The data obtained in these studies are rationalized in terms of a biosynthetic pathway of petroselinic acid involving the Δ^4 desaturation of palmitoyl-ACP by the 36-kD desaturase followed by two-carbon elongation of the resulting Δ^4-hexadecenoyl-ACP.

Seed oils of most species of the Umbelliferae (or Apiaceae), Garryaceae, and Araliaceae are characterized by their high content of the unusual monounsaturated fatty acid petroselinic acid (18:1Δ^{6cis}) (Kleiman and Spencer, 1982). Prior to a recent report from this laboratory (Cahoon et al., 1992), little was known of the biosynthetic origin of the *cis*Δ^6 double bond of this fatty acid. Such information is of possible biochemical and biotechnological significance. Because petroselinic acid is the likely product of a previously uncharacterized desaturase (Cahoon et al., 1992), data regarding its synthesis may contribute to an understanding of fatty acid desaturation mechanisms in plants. In addition, petroselinic acid can be used as a chemical precursor of the industrially valuable compounds lauric acid (12:0) and adipic acid (6:0 dicarboxylic) and is therefore a potential target for oilseed modification research (Murphy, 1992).

Double bonds of plant fatty acids may result from enzymic activity involving several different substrate types. The most common desaturation mechanism in plants involves the insertion of double bonds into acyl moieties esterified to glycerolipids. Examples of this include the desaturation of oleic acid to linoleic acid on PC (Gurr et al., 1969; Slack et al., 1978; Stymne and Appelqvist, 1978) and the desaturation of palmitic acid to *trans*Δ^3-hexadecenoic acid on phosphatidylglycerol (Bartels et al., 1967). In addition, fatty acid unsaturation may arise from reactions that use saturated acyl chains bound to ACP. The primary example of such a pathway is the desaturation of stearoyl-ACP to oleoyl-ACP through the activity of the Δ^9-stearoyl-ACP desaturase (Nagai and Bloch, 1968; Jaworski and Stumpf, 1974). Acyl-CoA esters may also serve as substrates for fatty acid desaturases. Although such reactions have yet to be conclusively demonstrated in higher plants, the desaturation of eicosanoyl-CoA is the proposed biosynthetic route of Δ^5-eicosenoic acid in seeds of meadowfoam (*Limanthes alba*) (Pollard and Stumpf, 1980; Moreau et al., 1981). Finally, Shibahara et al. (1990) have proposed a mechanism in pulp of kaki (*Diospyros kaki*) in which the double bonds of oleic acid and *cis*-vaccenic acid can be enzymically shifted between the Δ^9 and Δ^{11} positions.

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Abbreviations: ACP, acyl carrier protein; PC, phosphatidylcholine. Fatty acid nomenclature: Δ^x indicates that the double bond of a fatty acid is located at the *x*th carbon atom relative to the carboxyl end of the acyl chain (e.g. 18:1Δ^6); ω^y indicates that the double bond of a fatty acid is located at the *y*th carbon atom relative to the methyl terminus of the acyl chain (e.g. 18:1ω^{12}).

With regard to the biosynthesis of petroselinic acid, we have previously identified a 36-kD peptide in Umbelliferae endosperm that displays immunological cross-reactivity with antibodies against the Δ^9-stearoyl-ACP desaturase of avocado (Cahoon et al., 1992). This polypeptide is absent in tissues that do not synthesize petroselinic acid, including leaves and roots of coriander (*Coriandrum sativum* L.), an Umbelliferae species. In addition, expression of a coriander endosperm cDNA for the 36-kD peptide in tobacco (*Nicotiana tabacum* L.) resulted in the production of petroselinic acid and Δ^4-hexadecenoic acid (16:1Δ^4) in transgenic callus. Furthermore, translation of the coriander cDNA indicated that the 36-kD peptide is synthesized with a plastid transit peptide and, in its mature form, possesses 70% amino acid identity with the Δ^9-stearoyl-ACP desaturase of castor. These results lead us to propose that petroselinic acid is the product of an acyl-ACP desaturase that is related to the Δ^9-stearoyl-ACP desaturase (Cahoon et al., 1992). However, expression of the 36-kD desaturase in *Escherichia coli* resulted in the recovery of nearly all of the recombinant protein as an insoluble aggregate. Upon urea solubilization, this protein possessed no detectable in vitro desaturase activity with saturated C_{14}, C_{16}, and C_{18} acyl-ACPs. Therefore, we were unable to prove directly that the 36-kD peptide of coriander endosperm is an acyl-ACP desaturase rather than, for example, an acyl-CoA desaturase. The chain length of the fatty acid substrate of the 36-kD desaturase was also not determined. In this communication, results are presented from a variety of ^{14}C-labeling studies using endosperm of coriander and suspension cells of transgenic tobacco that express the cDNA for the coriander 36-kD desaturase. These results demonstrate the involvement of an acyl-ACP desaturation pathway in the synthesis of petroselinic acid. Also presented are details of the double bond positioning properties of the coriander 36-kD desaturase and a proposed biosynthetic pathway for petroselinic acid.

MATERIALS AND METHODS

Plant Material

Experiments were conducted using either endosperm or endosperm homogenates obtained from developing seeds of coriander (*Coriandrum sativum* L.). Plants were grown and mericarps were harvested as described (Cahoon and Ohlrogge, 1994). Studies were also performed with suspension cells derived from calli of tobacco (*Nicotiana tabacum* L.) transformed with a cDNA for the 36-kD (or type II) desaturase of coriander endosperm as previously described (Cahoon et al., 1992). The coriander cDNA was inserted behind the cauliflower mosaic virus 35S promoter in the plant expression vector pBI121 (Clontech, Palo Alto, CA). Transgenic and wild-type suspension cells were maintained in liquid medium (Linsmaier and Skoog, 1965) with constant shaking at 100 rpm at 28°C. The medium of transgenic cells also contained kanamycin (100 mg/L) and carbenicillin (250 mg/L).

Labeling of Coriander Endosperm Slices with 1-^{14}C-Fatty Acids

Transverse slices (approximately 1–2 mm thickness) of coriander endosperm (40–100 mg fresh weight) were incubated in loosely capped 13 × 100 mm tubes containing 3 μCi of the ammonium salts of [1-^{14}C]lauric acid (12:0) (57 mCi/mmol, Amersham, Arlington Heights, IL), myristic acid (14:0) (55 mCi/mmol) (American Radiolabeled Chemicals, St. Louis, MO), palmitic acid (16:0) (58 mCi/mmol, New England Nuclear), stearic acid (18:0) (55 mCi/mmol, American Radiolabeled Chemicals), or oleic acid (18:1Δ^9) (52 mCi/mmol, Research Products International, Mt. Prospect, IL) dispersed in 250 μL of 0.1 M potassium phosphate (pH 7.2). Labeling studies were also performed using 1 μCi of [U-^{14}C]nonanoic acid (9:0) that was synthesized as described below. Sample tubes were rotated at 100 rpm and maintained at room temperature. Endosperm slices were incubated in lauric acid and myristic acid for 3.5 h, palmitic acid, oleic acid, and nonanoic acid for 6 h, and stearic acid for 10 h. At each time point label was removed and endosperm was washed twice with ice-cold water. Lipids were then extracted as described for radiolabeling studies reported in the accompanying paper (Cahoon and Ohlrogge, 1994). To assess the ability of endosperm to synthesize petroselinic acid after extended labeling periods such as that used in the stearic acid feeding experiment, coriander endosperm slices were preincubated for 8.5 h in 50 mM Mes, pH 5.0. At the end of this period, endosperm slices were supplied with 3 μCi of [1-^{14}C]acetate and incubated for an additional 1.5 h, at which time the tissue was homogenized and lipids were extracted as described above.

Elongation and/or desaturation of the labeled fatty acids was determined by a combination of argentation and reverse-phase TLC of fatty acid methyl esters prepared from recovered lipids. Extracted glycerolipids were transesterified by incubation for 45 min at room temperature in 1.5 mL of 0.5 M sodium methoxide in methanol supplemented with 20% (v/v) toluene. At the end of this period, 1.5 mL of 125 mM sulfuric acid was added and fatty acid methyl esters were extracted three times with 3 mL of hexane. Fatty acid methyl esters were subsequently separated by 15% argentation TLC with development in toluene at −20°C as described (Morris et al., 1967; Cahoon and Ohlrogge, 1994). To further characterize the distribution of radioactivity in fatty acid methyl esters, labeled bands on argentation TLC plates were eluted from scrapings using hexane:ethyl ether (2:1, v/v). The recovered fatty acid methyl esters were further resolved by reverse-phase TLC using KC18 (200-μm layer) plates (Whatman, Maidstone, England) developed sequentially to 75 and 100% of their length (20 cm) in acetonitrile:methanol:water (65:35:0.5, v/v). In addition, to characterize the distribution of label in molecules, monounsaturated products of [1-^{14}C]-lauric acid and myristic acid that co-migrated with petroselinic acid were cleaved by permanganate-periodate oxidation (Christie, 1982). Chain lengths of oxidation products were determined using reverse-phase TLC as described above. This method was also used to determine double bond positions of monounsaturated products of [U-^{14}C]nonanoic acid. In this case, oxidation products were analyzed by reverse-phase TLC using a solvent system of acetonitrile:methanol:water (75:25:0.5, v/v). Radiolabeled fatty acid methyl esters and oxidation products were visualized by autoradiography of TLC plates, and radioactivity was quantified by liquid scintillation counting of TLC scrapings in a nonaqueous complete cocktail.

In Vitro Synthesis of Petroselinic Acid from [2-^{14}C]Malonyl-CoA

The synthesis of petroselinic acid from [2-^{14}C]malonyl-CoA was studied using crude homogenates of endosperm dissected from the pericarp and seed coat of coriander mericarps. In these experiments, the highest activity was obtained from endosperm at latter stages of mid-development (22–24 DAF under our growth conditions), and all assays were performed using freshly collected plant material. Endosperm was ground with a glass Elvehjem homogenizer in an ice-cold buffer composed of 100 mM Tris-HCl, pH 7.5, 1 mM Na_2EDTA, 2.5 mM DTT, 1 mM $MgCl_2$, 1 mM KCl, 1 mM isoascorbate, and 0.1% (w/v) BSA (fraction V, Sigma). The homogenate was passed through two layers of Miracloth (Calbiochem). The approximate protein concentration of homogenates was 7.5 mg/mL as estimated using the method of Bradford (1976) and a BSA standard. [2-^{14}C]Malonyl-CoA was either purchased (55 mCi/mmol, Amersham) or chemically synthesized from [2-^{14}C]malonate (56 mCi/mmol, American Radiolabeled Chemicals) using the method of Rutkowski and Jaworski (1978) but without dilution of specific activity.

Reactions were performed in 13 × 100 mm glass screw-cap test tubes. Typical reaction mixtures consisted of 5 μL of 100 mM ascorbate, 7.5 μL of 25 mM NADPH, 5 μL of 50 mM NADH (NADPH and NADH were freshly prepared in 100 mM Tricine, pH 8.0), 1 μL of catalase (800,000 units/mL), 7.5 μL of 1 mg/mL recombinant spinach ACP-I, 5 μL of 100 mM ATP, 80 μL of 100 mM Pipes (pH 6.0), 34 μL of water, and endosperm homogenate (200–300 μg of protein). The reaction mixture was brought to a total volume of 245 μL with tissue homogenization buffer. Reactions were started with the addition of 2.4 to 3.1 nmol of [2-^{14}C]malonyl-CoA (275,000–350,000 dpm) in a 5-μL volume. This assay was based in part on McKeon and Stumpf's description (1981) for measurement of Δ^9-stearoyl-ACP desaturase activity. Upon addition of the radiolabeled malonyl-CoA, the loosely capped reaction tubes were rotated at 100 rpm and maintained at 25°C. At time points of 7 or 20 min, 40 μL of glacial acetic acid and 4.5 mL of acetone were added to each tube and the contents were dried under N_2. The reaction products were subsequently methyl esterified by heating in 10% (w/v) boron trichloride/methanol (Alltech) at 90°C for 35 min. Fatty acid methyl esters were then analyzed by argentation TLC as described above. To confirm the identity of [^{14}C]methyl petroselinic acid, this fatty acid was recovered from argentation TLC plates and subsequently analyzed by reverse-phase TLC as either an intact moiety or as products of permanganate-periodate oxidation using methods described above.

The O_2 dependence of [2-^{14}C]malonyl-CoA incorporation into petroselinic acid was assessed using the assay conditions described above except that reaction tubes were purged with N_2 prior to and after addition of the radiolabeled substrate. In addition, tubes were completely sealed for the duration of the incubation period. The Fd requirement of petroselinic acid synthesis from [2-^{14}C]malonyl-CoA was determined by supplementing assay components described above with 15 μL of 1 mM spinach Fd (Sigma) and 3.1 μL of Fd:NADPH reductase (10 units/mL) (Sigma) in place of a corresponding amount of water. In both experiments, reactions were carried out for 20 min. Assays were stopped and products were analyzed as described above.

As one method of characterizing the radiolabeled products of in vitro reactions, assay mixtures were extracted according to the method of Bligh and Dyer (1959). At time points of 7 or 20 min after addition of [2-^{14}C]malonyl-CoA, reactions were stopped with 50 μL of glacial acetic acid and 4 mL of chloroform:methanol (1:1, v/v). Two phases were formed with the addition of 1.5 mL of H_2O. After centrifugation, the lower chloroform layer was removed and the upper aqueous-methanolic phase was extracted twice with 2 mL of hexane:isopropanol (7:2, v/v). The hexane extract was pooled with the recovered chloroform layer, and the combined extracts were dried under N_2. Lipids obtained in the organic phase were resolved by silica TLC using sequential developments in polar and nonpolar solvent systems as described (Cahoon and Ohlrogge, 1994). In addition, the free fatty acid band on TLC plates was scraped into 1.5 mL of boron trichloride/methanol and heated at 90°C for 15 min. The resulting fatty acid methyl esters were analyzed by argentation TLC as described above. The aqueous-methanolic phase from the Bligh and Dyer extract was saponified as described by Browse and Slack (1985). The recovered fatty acids were converted to methyl ester derivatives by reaction in 10% (w/v) boron trichloride/methanol and were then analyzed by argentation TLC. Saturated fatty acid methyl esters were purified from argentation TLC plates and further resolved by reverse-phase TLC as described above.

To analyze the acyl-ACP products of in vitro fatty acid biosynthetic assays, a modified version of the method of Mancha et al. (1975) was employed. The procedure used was essentially the same as that described by Roughan and Nishida (1990) except that stopped reactions were extracted three times with 2 mL of 50% isopropanol-saturated petroleum ether prior to ammonium sulfate precipitation of acyl-ACPs, and the precipitated acyl-ACPs were washed three times with 4 mL of chloroform:methanol (1:2, v/v). For analysis of acyl-ACPs, reactions were typically carried out for 7 min. Petroleum ether extracts were dried under N_2 and converted to fatty acid methyl esters by reaction in 10% (w/v) boron trichloride/methanol as described above. Fatty acid methyl esters from petroleum ether extracts and from the sodium methoxide transesterified acyl-ACPs were resolved by argentation TLC as described above.

Radioactivity in TLC-separated products of these studies was visualized by autoradiography and quantified as described above.

Metabolism of [1-^{14}C]Acyl-ACPs and -CoAs by Coriander Endosperm Homogenates

The ability of coriander endosperm homogenates to convert saturated [1-^{14}C]acyl-ACPs and -CoAs to petroselinic acid was determined using essentially the same assay conditions and product analysis as described above for studies of malonyl-CoA metabolism. In addition, some assays (as described in "Results and Discussion") were supplemented with 10 μL of 1 mM spinach Fd (Sigma) and 8 μL of Fd:NADPH reductase (Sigma) (10 units/mL) and/or 10 μL of 2 mM malonyl-CoA in place of a corresponding volume of water.

Reactions were carried out with 15,000 dpm (6.8 nCi or 117 to 124 pmol) of [1-^{14}C]acyl-ACP and -CoA as described below. To assess the ability of homogenates to synthesize petroselinic acid in vitro, reactions using [2-^{14}C]malonyl-CoA (2.4–3.1 nmol) as the substrate were conducted in parallel.

Radiolabeled acyl-ACPs were prepared from *E. coli* ACP and [1-^{14}C]myristic acid, [1-^{14}C]palmitic acid, and [1-^{14}C]-stearic acid according to the method of Rock and Garwin (1979). [1-^{14}C] Stearoyl-CoA was synthesized enzymically as described by Taylor et al. (1989). [1-^{14}C]Palmitoyl-CoA (56 mCi/mmol) was purchased from Amersham.

Analysis of the Long/Medium-Chain Acyl-ACP Pool of Transgenic Tobacco Suspension Cultures

To characterize the composition of long/medium-chain acyl-ACP pools of tobacco suspension cells transformed with the 36-kD coriander desaturase (Cahoon et al., 1992), 2.5 mL of transgenic and wild-type cultures were incubated with shaking in 25 μCi of [1-^{14}C]acetate (52 mCi/mmol, New England Nuclear) in 13-mL polypropylene screw-cap tubes. (Suspension cells had been subcultured in fresh media 4 d prior to use in these experiments.) After a 12-min labeling period, 100% (w/v) TCA was added to suspension cultures to a final concentration of 5% (w/v). Sample tubes were immediately vortexed and frozen in liquid N_2. After they were thawed on ice, labeled cells were homogenized with a Polytron PT10/35 (Brinkman), and the homogenate was centrifuged at 10,000*g* for 20 min at 5°C. The resulting pellet was washed with 2.5 mL of 1% (w/v) TCA and centrifuged again at 10,000*g* for 20 min. The supernatant was discarded and the pellet was centrifuged at 10,000*g* for an additional 5 min to remove traces of TCA. Proteins including free and acylated ACPs in the TCA pellet were resuspended in 1.25 mL of 50 mM Hepes, pH 7.8,containing 10 mM *N*-ethylmaleimide. The pH of the resuspended pellet was adjusted as necessary to approximately pH 6.5 with 1 M Tris, pH 9.0, and debris was removed by centrifugation of the sample at 10,000*g* for 10 min.

Proteins in the resuspended TCA pellet were separated on a 13% native polyacrylamide gel containing 1 M urea and blotted to nitrocellulose as previously described (Post-Beitenmiller et al., 1991). Radiolabeled long/medium-chain acyl-ACPs were identified on western blots by their mobility equivalent to that of [1-^{14}C]palmitic acid or myristic acid esterified to a mixture of tobacco ACP isoforms. Radioactivity on western blots was detected by phosphorimaging. Using this gel system, long/medium-chain acyl-ACPs migrated as essentially one band. Regions of the nitrocellulose containing ^{14}C-long/medium chain acyl-ACPs were cut from western blots with scissors. Acyl-ACPs on the cut nitrocellulose pieces were subsequently transesterified by reaction for 45 min in 0.5 M sodium methoxide in methanol as described above. Approximately 1.5 mL of sodium methoxide was used per 25 to 30 mg of nitrocellulose. In preliminary studies using this procedure, approximately 80% of [1-^{14}C]palmitoyl-ACP blotted onto nitrocellulose was recovered as fatty acid methyl esters.

Fatty acid methyl esters derived from the long/medium-chain acyl-ACP pool of wild-type and transgenic tobacco cells were further analyzed by argentation TLC. Samples were loaded onto 15% argentation TLC plates, which were sequentially developed to heights of 10, 14, 18, and 20 cm in toluene at −20°C. Radioactivity in separated fatty acid methyl esters was detected by phosphorimaging of TLC plates and mobilities were compared with those of radiolabeled standards. To more completely characterize the identity of 16:1Δ^4 in the acyl-ACP pool of transgenic tobacco calli, the methyl ester derivative of this radiolabeled fatty acid was recovered from argentation TLC plates and analyzed by reverse-phase TLC as an intact or permanganate-periodate-oxidized moiety using methods described above.

Synthesis of [U-^{14}C]Nonanoic Acid

[U-^{14}C]Nonanoic acid (9:0) was synthesized from the methyl ester of [U-^{14}C]oleic acid (900 mCi/mmol, New England Nuclear) for use in radiolabeling studies described above. [U-^{14}C]Oleic acid (a gift from Dr. Edward Emken, U.S. Department of Agriculture Northern Regional Research Center, Peoria, IL) was converted to a methyl ester derivative using 10% (w/v) boron trichloride in methanol and purified from contaminating radiolabeled material using argentation TLC. Radiolabeled methyl oleic acid was subsequently converted to [U-^{14}C]nonanoic acid and methyl azelaic acid using permanganate-periodate oxidation as described by Christie (1982). The products were separated by silica TLC using a solvent system of hexane:ethyl ether:acetic acid (60:40:1, v/v). Radiolabeled nonanoic acid, which migrated above methyl azelaic acid, was recovered from silica gel with three washings of scrapings with 2 mL of chloroform:ethyl ether:methanol (1:1:1, v/v) (Kates, 1972). The resulting [U-^{14}C]nonanoic acid was found to be radiochemically pure by reverse-phase TLC analysis.

RESULTS AND DISCUSSION

Metabolism of Exogenous ^{14}C-Fatty Acids by Coriander Endosperm Slices

Results of a previous study in this laboratory demonstrated that the double bond of petroselinic acid of Umbelliferae endosperm arises from the activity of a 36-kD peptide that is structurally related to the Δ^9-stearoyl-ACP desaturase (Cahoon et al., 1992). The identity of the substrate of the 36-kD desaturase, however, was not determined in that report. Based on what is known of the synthesis of other unsaturated fatty acids in plants, the double bond of petroselinic acid most likely results from a reaction(s) involving a saturated acyl chain bound to either (a) a glycerolipid, (b) CoA, or (c) ACP. In this regard, kinetics of [1-^{14}C]acetate radiolabeling of carrot and coriander endosperm shown in the accompanying paper (Cahoon and Ohlrogge, 1994) gave no indication that petroselinic acid is synthesized via the desaturation of saturated acyl chains esterified to glycerolipids, including PC. As another method of determining whether a saturated fatty acid bound to a glycerolipid (or CoA) is the precursor of petroselinic acid, the metabolism of 1-^{14}C-long-chain fatty acids by coriander endosperm was examined. Such studies using whole plant tissues and green algae have been previously conducted to demonstrate the in vivo activity of desa-

turases that use fatty acids bound to glycerolipids or CoA as substrates (e.g. Gurr et al., 1969; Pollard and Stumpf, 1980; Stymne and Stobart, 1986).

In the present study, incubation of coriander endosperm slices in a buffered solution containing [1-^{14}C]stearic acid for 10 h resulted in the incorporation of radioactivity into all major glycerolipid classes including PC, which contained approximately 17% of the incorporated label (data not shown). Despite this, no Δ^6 desaturation of [1-^{14}C]stearic acid to petroselinic acid was detected (Table I). Thus, these results suggest that the Δ^6 desaturation of stearic acid bound to a glycerolipid is not the biosynthetic route of petroselinic acid. Furthermore, it is generally assumed that fatty acids are incorporated into glycerolipids as CoA esters (Roughan and Slack, 1982). As such, the inability to synthesize petroselinic acid from exogenous stearic acid would suggest the absence of a Δ^6-stearoyl-CoA desaturation pathway in coriander endosperm.

As with [1-^{14}C]stearic acid, radiolabeled palmitic acid was not metabolized to petroselinic acid when it was provided exogenously to slices of coriander endosperm (Table I). The lack of palmitic acid incorporation into petroselinic acid would suggest that petroselinic acid does not derive from a palmitoyl-CoA desaturation/elongation pathway.

In addition to radiolabeled C_{16} and C_{18} saturated fatty acids, the metabolism of [1-^{14}C]oleic acid by coriander endosperm slices was examined. In this regard, Shibahara et al. (1990) have provided evidence suggesting that the Δ^9 double bond of oleic acid can be shifted to the Δ^{11} position to form *cis*-vaccenic acid in pulp of kaki. However, when supplied exogenously to coriander endosperm, [1-^{14}C]oleic acid was not converted to petroselinic acid (Table I). As such, it is unlikely that metabolic shifting of the double bond of oleic acid is associated with petroselinic acid formation in coriander endosperm.

To further characterize petroselinic acid biosynthesis, coriander endosperm slices were incubated in buffered solutions containing [1-^{14}C]lauric acid and myristic acid. Several previous studies have demonstrated that medium-chain fatty acids such as lauric acid and myristic acid can be converted to long-chain derivatives including palmitic acid and oleic acid by green algae and intact tissues of higher plants (Kannangara et al., 1973; Norman et al., 1985; Norman and St. John, 1986). These modification reactions presumably occur via acyl-ACP-associated reactions, since this is the principal (if not only) route of C_{16} and C_{18} saturated fatty acid synthesis and Δ^9-stearic acid desaturation in most plants. In contrast to medium-chain fatty acids, exogenously supplied long-chain fatty acids (e.g. palmitic acid and stearic acid) are not readily modified by acyl-ACP-associated reactions by plant tissues (Roughan et al., 1987). Consistent with an acyl-ACP route of metabolism, radiolabeled lauric acid and myristic acid were converted to a number of long-chain derivatives including palmitic acid and oleic acid by coriander endosperm (Table I). However, the major elongation product of both [1-^{14}C]-lauric acid and myristic acid was petroselinic acid, which accounted for approximately 60% of the fatty acids derived from both medium-chain precursors.

Analysis of [^{14}C]petroselinic acid following permanganate-periodate oxidation indicated that lauric acid and myristic acid were incorporated as intact moieties. In this regard, radioactivity in petroselinic acid was found not to be randomized as might be expected if the lauric acid and myristic acid had been first β-oxidized and the radioactivity reincorporated into fatty acids. Instead, radiolabel was detected only in the lauric acid product of oxidized petroselinic acid derived from [1-^{14}C]lauric acid and only in the methyl adipic acid product of oxidized petroselinic acid formed from [1-^{14}C]myristic acid (data not shown). Significant amounts of [1-^{14}C]lauric acid and myristic acid were also converted to a hexadecenoic acid (16:1) moiety that was identified as a Δ^6 isomer by argentation TLC analysis and by permanganate-periodate oxidation. Interestingly, this fatty acid comprised approximately 10 to 15% of the elongation products of [1-^{14}C]lauric acid and myristic acid but comprised ≤2.5% of the ^{14}C-fatty acids resulting from [1-^{14}C]acetate labeling of coriander endosperm for a similar time period (Cahoon and Ohlrogge, 1994). The basis for this difference is not known. However, because

Table I. *Metabolism of exogenous [1-^{14}C]medium- and long-chain fatty acids by slices of coriander endosperm*

Three microcuries (0.2 mM) of each fatty acid was supplied as an ammonium salt to endosperm slices in a buffered solution. [1-^{14}C]Lauric acid (12:0) and myristic acid (14:0) were incubated with endosperm slices for 3.5 h, palmitic acid (16:0) and oleic acid (18:1Δ^9) were incubated for 6 h, and stearic acid (18:0) was incubated for 10 h.

1-^{14}C-Fatty Acid	Endosperm Fresh Weight	^{14}C Incorporated into Glycerolipids	Percent of Incorporated Label Elongated/ Desaturated	Percent Distribution of ^{14}C in Elongated/Desaturated Fatty Acids							
				14:0	16:0	16:1Δ^6	18:0	18:1Δ^6	18:1Δ^9	18:2	Other
	mg	*dpm* × 10^{-4}									
12:0	65	218	15.9	4.6	12.0	11.4	<1.0	60.3	5.0	1.6	4.1[a]
14:0	66	174	9.2		16.7	14.1	<1.0	58.0	8.3[b]	2.3	<1%
16:0	66	61.4	0			n.d.[c]	n.d.	n.d.	n.d.	n.d.	n.d.
18:0	92	10.4	0					n.d.	n.d.	n.d.	n.d.
18:1Δ^9	60	133	30.2					n.d.		100	n.d.

[a] Includes a 14:1 isomer that comigrated with petroselinic acid on argentation TLC plates and was tentatively identified as 14:1Δ^6. Also includes a 16:1 isomer that comigrated with oleic acid on argentation TLC plates. [b] Total radioactivity in fatty acid methyl esters comigrating with oleic acid on argentation TLC plates and therefore may include 16:1 isomers as detected in products of [1-^{14}C]lauric acid labeling. [c] n.d., Not detected.

desaturases, including the stearoyl-CoA desaturase (Bloomfield and Bloch, 1960; Holloway et al., 1963) and presumably the stearoyl-ACP desaturase (Cheesebrough and Cho, 1990), typically introduce double bonds into only one specific position of fatty acid substrates regardless of chain length, it is assumed that the Δ^6 double bond of petroselinic acid and Δ^6-hexadecenoic acid result from the same enzymic reaction.

In summary, [1-^{14}C]long-chain fatty acids (e.g. stearic acid, palmitic acid, oleic acid) were incorporated into glycerolipids but were not converted to petroselinic acid in detectable levels by coriander endosperm. This suggests that petroselinic acid is not formed via the desaturation of an acyl moiety esterified to a glycerolipid or CoA. In contrast to the metabolic fate of long-chain fatty acids, [1-^{14}C]medium-chain fatty acids were converted in relatively high levels to petroselinic acid. Because these fatty acids, unlike long-chain fatty acids, presumably enter the acyl-ACP track of de novo fatty acid synthesis, these results suggest the involvement of acyl-ACP-linked reactions in petroselinic acid biosynthesis.

In Vitro Synthesis of Petroselinic Acid from [2-^{14}C]Malonyl-CoA

To further characterize the biosynthetic origin of petroselinic acid, an in vitro system for the synthesis of this fatty acid was developed using crude homogenates of coriander endosperm and [2-^{14}C]malonyl-CoA. Included in this assay were a variety of potential fatty acid biosynthetic and desaturation cofactors, including NADPH, NADH, ACP, and catalase. Depending on the experiment, assays were carried out for 7 or 20 min. The major radiolabeled acyl products of both 7- and 20-min reactions were palmitic acid and stearic acid, which typically accounted for 55 to 70% of the radioactivity recovered in fatty acids. In addition, 20 to 35% of the label incorporated into fatty acids was typically associated with petroselinic acid. The remainder of the labeled fatty acid (5–10%) was present as oleic acid, and no radioactivity was detected in dienoic fatty acids or in any hexadecenoic acid (16:1) isomers (e.g. Δ^4- or Δ^6-hexadecenoic acid) that might potentially comigrate with oleic acid or petroselinic acid on argentation TLC plates. The identity of [^{14}C]petroselinic acid was confirmed by the mobility of its methyl ester derivative on argentation and reverse-phase TLC plates and by reverse-phase TLC analysis of its permanganate-periodate oxidation products. Using the latter method, a nearly proportional distribution of radiolabel was detected between the lauric acid and methyl adipic acid oxidation products, indicating that the [^{14}C]petroselinic acid formed in the in vitro assay resulted from de novo fatty acid biosynthesis rather than from the elongation of a preformed, nonradioactive fatty acid (data not shown). Finally, the conversion of [2-^{14}C]malonyl-CoA to petroselinic acid appeared to be dependent on the developmental stage of coriander endosperm as well as on maintaining high protein concentrations during tissue homogenization and in the subsequent assays (see "Materials and Methods").

The incorporation of [2-^{14}C]malonyl-CoA into petroselinic acid was reduced by more than 3-fold when the assay was conducted under O_2-limiting conditions (Table II). The apparent dependence of petroselinic acid synthesis on molecular

Table II. *O_2 and Fd dependence of petroselinic acid and oleic acid synthesis from [2-^{14}C]malonyl-CoA by homogenates of coriander endosperm*

Control (or complete) assays contained Fd and Fd:NADPH reductase and were performed as described in "Materials and Methods." O_2 dependence ($+N_2$) of petroselinic acid and oleic acid synthesis was examined by carrying out reactions in tightly capped tubes that had been purged with N_2 prior to and after addition of substrate ([2-^{14}C]malonyl-CoA). Fd dependence (−Fd/FNR) of petroselinic acid and oleic acid synthesis from [2-^{14}C]malonyl-CoA was studied by omitting spinach Fd and Fd:NADPH-reductase (FNR) from assays. Shown is the distribution of ^{14}C recovered in fatty acid methyl esters obtained by transesterification and subsequent argentation TLC analysis of the products of 20-min assays.

Treatment	Fatty Acid Products	Percent Distribution of ^{14}C in Products
Complete	SatFA[a]	56 (5300)[b]
	18:1Δ^6	29 (2740)
	18:1Δ^9	15 (1460)
−Fd/FNR	SatFA	65 (5430)
	18:1Δ^6	28 (2340)
	18:1Δ^9	7 (620)
$+N_2$	SatFA	85 (9790)
	18:1Δ^6	8 (960)
	18:1Δ^9	7 (830)

[a] SatFA, Saturated fatty acids. [b] Numbers in parentheses indicate dpm of ^{14}C recovered in each acyl moiety.

O_2 is consistent with the known catalytic properties of plant fatty acid desaturases (Jaworski, 1987). Unexpectedly, the addition of Fd and Fd:NADPH reductase had only a small effect on petroselinic acid synthesis from [2-^{14}C]malonyl-CoA. In contrast, the presence of these cofactors in assays resulted in a 2-fold stimulation of oleic acid production.

To determine the exact form (e.g. glycerolipid or ACP esters) of fatty acids produced in vitro from [2-^{14}C]malonyl-CoA, reactions were extracted according to the method of Bligh and Dyer (1959). Using this procedure, the radiolabel in the total lipid extract (or chloroform layer) of in vitro assays after 7 and 20 min was detected primarily as free or unesterified fatty acids (Table III). More than 70% of the radioactivity in free fatty acids of a 7-min assay was associated with petroselinic acid. Saponification of the aqueous-methanolic upper phase of Bligh and Dyer extracts revealed a much different distribution of radiolabeled fatty acids. In this phase, ≥90% of the radioactivity recovered in acyl moieties of 7- and 20-min reactions was present in saturated fatty acids, most likely in the water-soluble form of CoA or ACP esters. In contrast, petroselinic acid accounted for less than 8 and 4% of the radiolabeled fatty acids recovered in the aqueous-methanolic phase of extracts of 7- and 20-min reactions, respectively.

Products of in vitro assays were also analyzed using the method of Mancha et al. (1975) as modified by Roughan and Nishida (1990). Using this method, acyl-ACPs are recovered exclusively in an ammonium sulfate pellet obtained after methanol:chloroform precipitation. Analysis of acyl-ACPs of a 7-min reaction in this manner revealed a preponderance of

Table III. *Glycerolipid and fatty acid products of [2-^{14}C]malonyl-CoA metabolism by crude homogenates of coriander endosperm*

Reactions products of 7- and 20-min assays were analyzed following extraction according to the method of Bligh and Dyer (1959). Aqueous-methanolic phase and organic phase refer to reaction products recovered in the upper and lower phases, respectively, of the Bligh and Dyer extract.

Glycerolipid/Fatty Acid Products	Reaction Time	
	7 min	20 min
	dpm	
Aqueous-methanolic phase		
16:0	2130	5570
18:0	700	4690
18:1Δ^6	250	510
18:1Δ^9	100	200
Organic phase		
FFA[a]	760	1760
SatFA[b] + 18:1Δ^9	<50	n.a.[c]
18:1Δ^6	720	n.a.
PC	140	210
DAG[d]	80	<50
TAG[e]	60	180
Other	<50	<50
Total lipid		
SatFA	n.a.	540
18:1Δ^6	n.a.	1550
18:1Δ^9	n.a.	330

[a] FFA, Free fatty acid. [b] SatFA, Saturated fatty acids. [c] n.a., Not analyzed. [d] DAG, Diacylglycerol. [e] TAG, Triacylglycerol.

radioactivity associated with saturated fatty acids (Table IV). This result was consistent with the radiolabeled fatty acid composition of the aqueous-methanolic phase of Bligh and Dyer extracts. In addition, approximately 15 to 20% of the total [2-^{14}C]malonyl-CoA incorporated into petroselinic acid in a 7-min reaction was present in the putative acyl-ACP fraction. The majority of [^{14}C]petroselinic acid, however, was recovered in the initial isopropanol saturated-petroleum ether wash, most likely in the form of free fatty acid as indicated by the results of the Bligh and Dyer extraction described above. The relatively low recovery of petroselinic acid as an acyl-ACP ester is consistent with the presence of high levels of petroselinoyl-ACP thioesterase activity in coriander endosperm extracts as reported by Dörmann et al. (1994). This is also consistent with the detection of the majority of radiolabeled petroselinic acid as free fatty acid in these assays.

In Vitro Metabolism of [1-^{14}C]Acyl-ACPs and -CoAs by Coriander Homogenates

The metabolic evidence presented above and previously reported data demonstrating that the double bond of petroselinic acid arises from the activity of a Δ^9-stearoyl-ACP desaturase-like peptide (Cahoon et al., 1992) are consistent with an acyl-ACP route of petroselinic acid biosynthesis. To test this directly, the ability of crude coriander homogenates to convert [1-^{14}C]saturated acyl-ACPs to petroselinic acid was examined. Because it was known from earlier experiments that coriander endosperm can incorporate exogenous [1-^{14}C]myristic acid into petroselinic acid, saturated acyl-ACPs of 14 carbon atoms and longer were used as potential substrates. However, under conditions sufficient for petroselinic acid synthesis from [2-^{14}C]malonyl-CoA and for oleic acid synthesis from [1-^{14}C]stearoyl-ACP, no conversion of radiolabeled acyl-ACPs (or -CoAs) to petroselinic acid was detected (results not shown). Also, in the case of assays carried out with [1-^{14}C]myristoyl-ACP and -palmitoyl-ACP, the addition of unlabeled malonyl-CoA resulted in nearly complete elongation of the acyl-ACP substrates to stearic acid; however, no detectable amount of radiolabeled petroselinic acid was formed. Furthermore, the presence of Fd and Fd:NADPH reductase in assays resulted in significant enhancement of oleic acid synthesis but had no apparent influence on petroselinic acid production from [1-^{14}C]acyl-ACPs (data not shown).

Analysis of the Fatty Acid Composition of the Long/Medium-Chain Acyl-ACP Pool of Transgenic Tobacco

To provide further evidence for the involvement of acyl-ACP-associated reactions in the synthesis of petroselinic acid, the long/medium-chain acyl-ACP pools of cell-suspension cultures of tobacco transformed with a cDNA for the coriander 36-kD desaturase (Cahoon et al., 1992) were examined. These cells, which were derived from transgenic callus, produce approximately 4 wt% petroselinic acid and 5 wt% Δ^4-hexadecenoic acid (16:1Δ^4) (neither fatty acid is detectable in control callus). Because tobacco likely does not contain a

Table IV. *Products of the metabolism of [2-^{14}C]malonyl-CoA by homogenates of coriander endosperm*

Reaction products of 7-min assays were analyzed using the extraction method of Mancha et al. (1975) with modifications as described by Roughan and Nishida (1990). Fatty acids and glycerolipids recovered from the initial 50% (v/v) isopropanol-saturated petroleum ether extract were converted to fatty acid methyl ester derivatives. Results shown were obtained following argentation TLC separation of fatty acid methyl esters and represent data from three independent experiments.

Fatty Acid	Petroleum Ether Extract	$(NH_4)_2SO_4$ Pellet/Acyl-ACP Pool
	dpm recovered	*dpm recovered*
Experiment I		
SatFA[a]	110 (3)[b]	3300 (97)
18:1Δ^6	1360 (86)[c]	220 (14)
18:1Δ^9	240 (59)[d]	170 (41)
Experiment II		
SatFA	180 (4)	3870 (96)
18:1Δ^6	700 (81)	160 (19)
18:1Δ^9	150 (65)	80 (35)
Experiment III		
SatFA	60 (1)	5840 (99)
18:1Δ^6	600 (79)	160 (21)
18:1Δ^9	100 (62)	60 (38)

[a] SatFA, Saturated fatty acids. [b] Percent of total dpm of ^{14}C recovered in saturated fatty acids. [c] Percent of total dpm of ^{14}C recovered in petroselinic acid. [d] Percent of total dpm of ^{14}C recovered in oleic acid.

petroselinoyl-ACP-specific thioesterase such as that present in Umbelliferae endosperm (Dörmann et al., 1994), it was expected that petroselinic acid might be more easily detectable in the acyl-ACP pool of these cells than in homogenates of coriander endosperm as described above. The method used in this experiment also provided a more direct means of analyzing acyl-ACPs than that of Mancha et al. (1975) used above in studies of [2-^{14}C]malonyl-CoA metabolism by coriander endosperm homogenates. To enhance the sensitivity of acyl-ACP analyses, wild-type and transgenic tobacco suspension cells were first incubated in [1-^{14}C]acetate. Proteins recovered after radiolabeling of tobacco cells and subsequent TCA precipitation were resolved on native polyacrylamide gels containing 1 M urea and blotted to nitrocellulose. Phosphorimaging of the resulting western blots indicated the presence of radiolabel associated primarily with a protein band that comigrated with a tobacco long/medium-chain acyl-ACP standard (Fig. 1A). Examination of fatty acid methyl esters derived from acyl-ACPs of the transgenic tobacco revealed the presence of a radiolabeled band that comigrated with a methyl petroselinic acid standard on argentation TLC plates (Fig. 1B). The major monounsaturated moiety in these samples, however, was a fatty acid methyl ester identified as methyl Δ^4-hexadecenoic acid based on its mobility on argentation and reverse-phase TLC and on the chain lengths of its permanganate-periodate oxidation products (data not shown). Neither methyl petroselinic acid nor Δ^4-hexadecenoic acid was present in fatty acid methyl esters prepared from acyl-ACPs of wild-type tobacco cells. Interestingly, at least 60% of the radioactivity found in the long/medium-chain acyl-ACP pool of transgenic cells was in the form of Δ^4-hexadecenoic acid and petroselinic acid despite the fact that these acyl moieties together compose ≤10 wt% of the total fatty acids of these cells.

In summary, the presence of petroselinic acid in the acyl-ACP pool of transgenic tobacco cells provides direct evidence that the synthesis of this fatty acid involves acyl-ACP-associated reactions. In addition, because tobacco cultures expressing the 36-kD desaturase contain Δ^4-hexadecenoic acid in their long/medium-chain acyl-ACP pool, it is unlikely that this fatty acid is a partial β-oxidation product of petroselinic acid. As discussed below, a more likely explanation is that Δ^4-hexadecenoyl-ACP is the direct precursor of petroselinic acid.

Metabolism of [U-^{14}C]Nonanoic Acid by Coriander Endosperm Slices

We have previously reported (Cahoon et al., 1992) that the expression of a cDNA for the 36-kD desaturase of coriander endosperm in tobacco resulted in the production of petroselinic acid as well as Δ^4-hexadecenoic acid in transgenic callus. The occurrence of both of these fatty acids in transgenic tobacco can be interpreted in two ways. First, the 36-kD desaturase may position the double bonds with respect to the methyl end of acyl-ACPs and is therefore functionally an ω^{12}

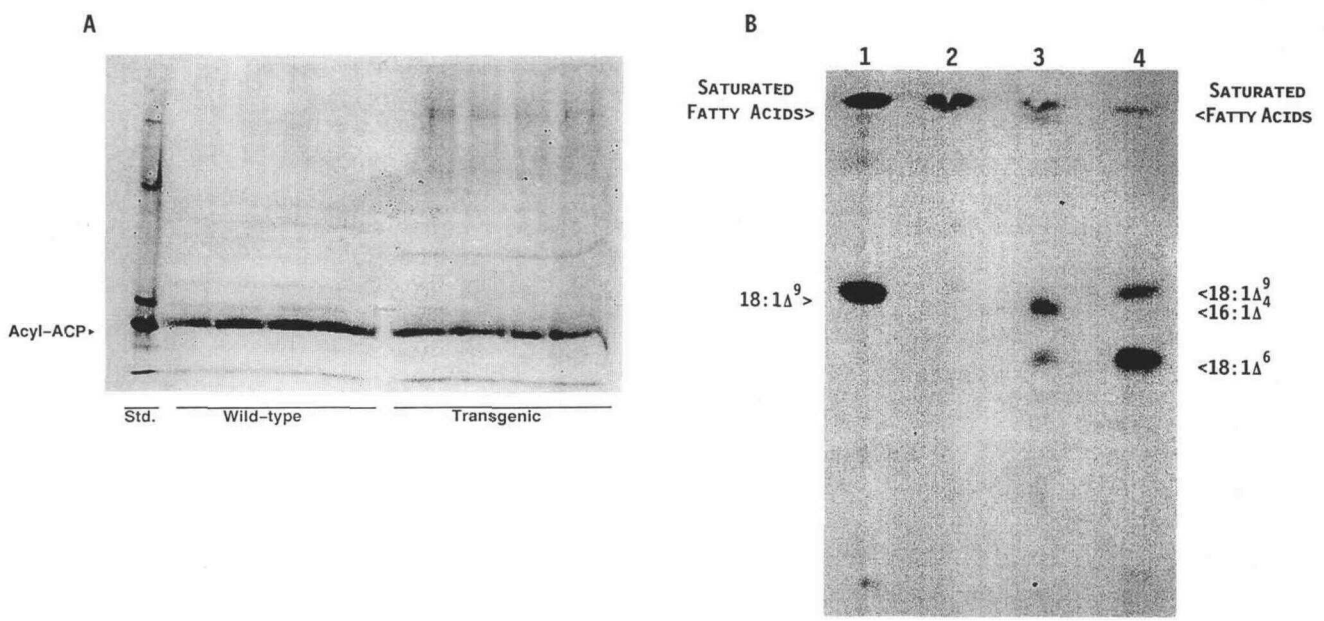

Figure 1. Analysis of long/medium-chain acyl-ACPs of suspension cultures of wild-type tobacco and transgenic tobacco expressing a cDNA for the coriander 36-kD desaturase. Shown in A is a phosphorimage of a nitrocellulose blot of TCA-precipitated proteins of wild-type and transgenic tobacco cells resolved by native PAGE following [1-^{14}C]acetate labeling of suspension cultures. In the standard (Std.) lane is a mixture of tobacco ACP isoforms esterified to [1-^{14}C]myristic acid. Regions of blots comigrating with the acyl-ACP standard were transesterified, and the resulting fatty acid methyl esters were separated by argentation TLC as shown on the phosphorimage in B. Lanes 2 and 3 are methyl esters derived from acyl-ACPs of wild-type and transgenic suspension cells, respectively. Lanes 1 and 4 are methyl esters of ^{14}C-fatty acid standards. Shown in lane 1 are [1-^{14}C]methyl palmitic acid and oleic acid. Standards in lane 4 were prepared by [1-^{14}C]-acetate labeling of coriander endosperm and consist of [^{14}C]methyl petroselinic acid, oleic acid, and saturated fatty acids.

(or n-12) desaturase. Such an enzyme would be capable of inserting a double bond at the Δ^6 (or ω^{12}) carbon of stearoyl-ACP to form petroselinic acid and at the Δ^4 (or ω^{12}) carbon of palmitoyl-ACP to form Δ^4-hexadecenoic acid. Alternatively, the position of carbon atoms from the carboxyl (or Δ) end of acyl-ACP substrates may dictate where the 36-kD desaturase introduces double bonds. In this regard, it would be unlikely that such an enzyme could possess the dual properties of a Δ^4 and a Δ^6 desaturase. Therefore, the co-occurrence of petroselinic acid and Δ^4-hexadecenoic acid in transgenic tobacco would suggest that petroselinic acid is an elongation product of Δ^4-hexadecenoic acid.

To determine whether the 36-kD desaturase positions the double bonds with respect to the carboxyl (Δ) or methyl (ω) end of acyl chains, the metabolism of [U-^{14}C]nonanoic acid (9:0) supplied exogenously to slices of coriander endosperm was examined. Based on results of radiolabeling experiments described above using exogenous medium-chain fatty acids, it would be expected that nonanoic acid would be readily elongated by slices of coriander endosperm. In addition, because nonanoic acid contains an odd number of carbon atoms, the mode of double bond positioning by the 36-kD desaturase can be directly assessed. For example, if this enzyme functions as an ω^{12} (or n-12) desaturase, monounsaturated products would possess double bonds at odd-numbered carbon atoms (e.g. 17:1Δ^5 or 19:1Δ^7). Conversely, given that petroselinic acid (18:1Δ^6) is the major fatty acid of coriander endosperm, double bond placement with respect to the carboxyl end of fatty acids would likely result in monounsaturated acyl chains with unsaturation primarily at the Δ^6 position (e.g. 17:1Δ^6 or 19:1Δ^6).

As detailed in Table V, the major products of nonanoic acid labeling were 17:1Δ^6 and 19:1Δ^6, which together accounted for nearly 60% of the total elongation/desaturation products. The identities of these radiolabeled molecules was determined by their mobility on argentation and reverse-phase TLC following conversion to methyl esters. In addition, the free fatty acid generated from the permanganate-periodate oxidation of the double bonds of the 17:1 and 19:1 moieties displayed mobility on reverse-phase TLC plates equivalent to that of C_{11} and C_{13} moieties, respectively (data not shown). This result is consistent with the location of the double bond at the Δ^6 position of both the 17:1 and 19:1 products. Among the other fatty acids formed from [U-^{14}C]-nonanoic acid were [^{14}C]17:0 and 15:1Δ^6.

Overall, the detection of radiolabeled 17:1Δ^6 and 19:1Δ^6 (rather than 17:1Δ^5 or 19:1Δ^7 monounsaturated fatty acids) conclusively demonstrates that the 36-kD desaturase does not function as an ω^{12} desaturase but instead positions double bond insertion from the carboxyl end of acyl chains. In this regard, the 36-kD desaturase of coriander endosperm is similar to the Δ^9-stearoyl-CoA desaturase of rat liver (Holloway et al., 1963) and yeast (Bloomfield and Bloch, 1960) and the Δ^9-stearoyl-ACP desaturase of soybean (Cheesbrough and Cho, 1990), which can introduce double bonds at the Δ^9 carbon atom of both C_{18} and C_{16} substrates.

Table V. *Products of [U-^{14}C]nonanoic acid labeling of coriander endosperm slices*

Labeling was conducted over a 6-h period with 53 mg of tissue and approximately 1 μCi of [U-^{14}C]nonanoic acid. Fatty acid products were analyzed by combination of argentation and reverse-phase TLC following transesterification of the total lipid extract in 0.5 M sodium methoxide in methanol. Double bond positions were determined by reverse-phase TLC analyses of permanganate-periodate oxidation products.

Elongation/Desaturation Products of [U-^{14}C]Nonanoic Acid	Percent of Total Elongated/ Desaturated Fatty Acid
15:0	4.5
15:1Δ^6	4.9
17:0	18.3
17:1Δ^6	46.4
17:1$\Delta^{4/9a}$	10.6
19:1Δ^6	10.4
Other[b]	4.8
Total ^{14}C elongated/desaturated	369,000 dpm

[a] Consists of mixture of Δ^4 and Δ^9 isomers. [b] Includes $\leq$1.3% each of 11:0, 13:0, 15:1$\Delta^{4/9}$, 19:0, and 19:1$\Delta^{4/9}$.

Synopsis of Metabolic Studies of Petroselinic Acid Synthesis

From the data presented above and from previously reported results (Cahoon et al., 1992), we propose that petroselinic acid is formed by the Δ^4 desaturation of palmitoyl-ACP followed by elongation of the resulting Δ^4-hexadecenoyl-ACP to petroselinoyl-ACP. The rationale for this conclusion is as follows. Petroselinic acid can be synthesized from [1-^{14}C]myristic acid by slices of coriander endosperm (Table I). This would suggest that the Δ^6 double bond of petroselinic acid can arise from a Δ^2, Δ^4, or Δ^6 desaturation step at a point between the C_{14} and C_{18} stages of de novo fatty acid synthesis by one of the following pathways:

(a) 14:0-ACP $\rightarrow$ 14:1Δ^2-ACP $\rightarrow$ 16:1Δ^4-ACP $\rightarrow$ 18:1Δ^6-ACP

(b) 16:0-ACP $\rightarrow$ 16:1Δ^4-ACP $\rightarrow$ 18:1Δ^6-ACP

(c) 18:0-ACP $\rightarrow$ 18:1Δ^6-ACP

However, [1-^{14}C]myristic acid can also be converted to Δ^6-hexadecenoic acid by coriander endosperm (Table I). This product cannot result from a Δ^2 desaturation step as in pathway a (i.e. Δ^2 desaturation of myristoyl-ACP followed by two-carbon elongation would produce Δ^4-hexadecenoyl-ACP rather than Δ^6-hexadecenoyl-ACP). Assuming that the same desaturase is involved in the insertion of the double bond of both petroselinic acid and Δ^6-hexadecenoic acid, pathway a would be an unlikely route of petroselinic acid formation. In addition, transgenic tobacco calli expressing the coriander 36-kD desaturase produce not only petroselinic acid but also Δ^4-hexadecenoic acid (Cahoon et al., 1992), which, like petroselinic acid, is detectable in the acyl-ACP pool of these cells. Δ^4-Hexadecenoic acid and petroselinic acid, however, likely cannot arise from pathways b and c, respectively. Results of nonanoic acid labeling, for example, demonstrate that the 36-kD desaturase does not function as an ω^{12} desaturase. Such a catalytic property would be necessary for this enzyme to synthesize both Δ^4-hexadecenoic acid and petroselinic acid directly. Furthermore, desaturases typ-

ically introduce double bonds into specific positions of fatty acid substrates regardless of chain length (e.g. Bloomfield and Bloch, 1960; Halloway et al., 1963; Cheesebrough and Cho, 1990; Higashi and Murata, 1993). As such, it is unlikely that the 36-kD desaturase acts as both a Δ^4-palmitoyl-ACP desaturase (pathway b) and a Δ^6-stearoyl-ACP desaturase (pathway c). More consistent with the double bond-positioning properties of the 36-kD desaturase and the fatty acid and acyl-ACP profile of transgenic tobacco is that a double bond is first introduced at the Δ^4 position of palmitoyl-ACP via the activity of the 36-kD desaturase. Subsequent elongation of 16:1Δ^4-ACP would result in the synthesis of petroselinoyl-ACP. As such, we propose that pathway b as shown above is the most likely route of petroselinic acid formation in Umbelliferae endosperm. The fact that ^{14}C-short- and ^{14}C-medium-chain fatty acids (e.g. nonanoic acid, lauric acid, and myristic acid) can be converted to other Δ^6 monounsaturated fatty acids in addition to petroselinic acid when supplied exogenously to coriander endosperm (Tables I and V) suggests that the 36-kD desaturase does not have an absolute specificity for palmitoyl-ACP.

If petroselinic acid is synthesized from Δ^4-hexadecenoyl-ACP, the efficiency of this elongation reaction would appear to be quite different between coriander endosperm and transgenic tobacco calli. In coriander endosperm, for example, the mass ratio of petroselinic acid to Δ^4-hexadecenoic acid in the total lipid extract is on the order of 500:1 (72 wt% petroselinic acid:0.1–0.2 wt% Δ^4-hexadecenoic acid) (Cahoon and Ohlrogge, 1994). This ratio in transgenic tobacco callus, however, is approximately 0.8:1 (4 wt% petroselinic acid:5 wt% Δ^4-hexadecenoic acid) (Cahoon et al., 1992). This substantial difference might indicate that some component(s) of Δ^4-hexadecenoyl-ACP elongation in coriander endosperm is absent in transgenic tobacco callus. This component may, for example, be a β-keto-acyl-ACP synthase that is specialized for the metabolism of Δ^4-hexadecenoyl-ACP. Whatever its nature, the enzyme(s) involved in Δ^4-hexadecenoyl-ACP elongation in Umbelliferae endosperm is likely to be an essential factor for achieving high levels of petroselinic acid production in transgenic plants.

Finally, a puzzling aspect of this study has been the inability to detect in vitro synthesis of petroselinic acid by crude homogenates of coriander endosperm using radiolabeled acyl-ACP substrates. This result is somewhat surprising given the high degree of structural similarity between the 36-kD desaturase and the Δ^9-stearoyl-ACP desaturase (Cahoon et al., 1992). Activity of the latter enzyme can be readily detected in extracts of most plant tissues, including coriander endosperm (as described above), using radiolabeled stearoyl-ACP in the presence of added NADPH, Fd, and Fd:NADPH reductase. It cannot be ruled out that the acyl-ACP desaturase assay used in our studies lacked an essential cofactor(s). For example, Fd may not be the preferred electron donor for the 36-kD desaturase. However, this is not consistent with the plastid localization of this enzyme and its structural similarity to the Δ^9-stearoyl-ACP desaturase (i.e. the in vitro activities of all plastid desaturases characterized to date, including the Δ^9-stearoyl-ACP desaturase, are stimulated by Fd [Jaworski, 1987; Schmidt and Heinz, 1990]). It is also possible that the 36-kD desaturase may be inactive with fatty acids esterified to *E. coli* ACP. Instead, this enzyme might be capable of using only fatty acid substrates bound to higher plant ACP or, more specifically, to Umbelliferae endosperm ACPs. However, plant and *E. coli* ACP typically have similar in vitro activity in studies of plant fatty acid biosynthetic reactions (Guerra et al., 1986), and the 36-kD desaturase is functional in vivo in tobacco, a "non-Umbelliferae" species. Another possibility is that the 36-kD desaturase operates in close association with one or more fatty acid biosynthetic enzymes. In such a scenario, growing acyl chains may be channeled through an elongation/desaturation pathway that is inaccessible in vitro to exogenous acyl-ACPs. The existence of metabolic channels or "metabolons" has been proposed for a number of biosynthetic pathways; however, such entities are often elusive to biochemical characterization (Srere, 1987).

CONCLUSIONS

1. [1-^{14}C]Stearic acid, palmitic acid, and oleic acid supplied exogenously to coriander endosperm slices were incorporated into glycerolipids but not converted to petroselinic acid. This suggests that petroselinic acid is not formed by desaturation reactions involving fatty acids esterified to glycerolipids or CoA or, in the case of oleic acid, by reactions involving the shifting of Δ^9 double bonds.

2. [1-^{14}C]Lauric acid and myristic acid provided exogenously to coriander endosperm slices were apparently modified by acyl-ACP-associated reactions and incorporated into petroselinic acid in high levels. To a lesser extent, both radiolabels were also converted to Δ^6-hexadecenoic acid.

3. Crude homogenates of coriander endosperm were capable of incorporating [2-^{14}C]malonyl-CoA into petroselinic acid in an O_2-dependent manner. The resulting radiolabeled petroselinic acid was detected primarily as free fatty acid and in lower levels as putative acyl-ACP esters.

4. Analysis of transgenic tobacco suspension cells expressing the coriander 36-kD desaturase following [1-^{14}C]acetate labeling revealed significant amounts of petroselinic acid and Δ^4-hexadecenoic acid in the long/medium-chain acyl-ACP pool. Neither fatty acid was detected in the total lipids or acyl-ACPs of wild-type tobacco cultures.

5. [U-^{14}C]Nonanoic acid (9:0) supplied exogenously to coriander endosperm was incorporated into Δ^6 isomers of 15:1, 17:1, and 19:1, demonstrating that the 36-kD desaturase positions double bonds relative to the carboxyl terminus of fatty acid substrates.

6. Considered in total, the metabolic data reported in this communication are most consistent with a biosynthetic pathway of petroselinic acid involving the Δ^4 desaturation of palmitoyl-ACP followed by two-carbon elongation of the resulting Δ^4-hexadecenoyl-ACP. Relative to coriander endosperm, elongation of Δ^4-hexadecenoyl-ACP appears to be a limiting reaction in the synthesis of petroselinic acid by transgenic tobacco.

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