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Edgar Benjamin Cahoon

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John B. Ohlrogge

Major professor

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SYNTHESIS AND METABOLISM OF Δ^6MONOUNSATURATED FATTY ACIDS IN DEVELOPING SEED OF UMBELLIFERAE SPECIES AND *THUNBERGIA ALATA*

By

Edgar Benjamin Cahoon

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ABSTRACT

SYNTHESIS AND METABOLISM OF Δ^6MONOUNSATURATED FATTY ACIDS IN DEVELOPING SEED OF UMBELLIFERAE SPECIES AND *THUNBERGIA ALATA*

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Studies were conducted to characterize the synthesis and metabolism of the unusual fatty acids petroselinic acid and Δ^6hexadecenoic acid. Petroselinic acid, the *cis*Δ^6 isomer of octadecenoic acid (18:1), composes up to 85 wt% of the seed oil of Umbelliferae (Apiaceae), Araliaceae, and Garryaceae species, and Δ^6hexadecenoic acid (16:1Δ^6) accounts for more than 80 wt% of the seed oil of *Thunbergia alata*. Both fatty acids have potential economic value.

Results of ^{14}C-radiolabeling studies performed with seed endosperm of coriander (*Coriandrum sativum*), an Umbelliferae species, suggested that petroselinic acid is formed through an acyl-acyl carrier protein (ACP) desaturation pathway. Consistent with this, antibodies raised against the avocado Δ^9stearoyl (18:0)-ACP desaturase detected a 36 kDa peptide on western blots that was specific to tissues that synthesize petroselinic acid. A cDNA for this peptide was isolated from a coriander endosperm cDNA library and introduced into tobacco. The resulting transgenic calli produced nearly 5 wt% each of petroselinic acid and Δ^4hexadecenoic acid (16:1Δ^4), neither of which was detected in control calli. Further metabolic studies with coriander endosperm and transgenic tobacco cell cultures revealed that the 36 kDa peptide is functionally a Δ^4palmitoyl (16:0)-ACP desaturase. As such, the biosynthetic pathway of petroselinic acid likely involves the Δ^4 desaturation of palmitoyl-ACP followed by two-carbon elongation of the resulting 16:1Δ^4-ACP. Extending these results to the biosynthesis of Δ^6hexadecenoic acid, the activity of a soluble Δ^6palmitoyl-ACP desaturase was detected in extracts of *T. alata* endosperm. A cDNA for the Δ^6palmitoyl-ACP desaturase was isolated from a *T. alata* endosperm cDNA library using PCR with oligonucleotides designed against

conserved regions of $\Delta^9$18:0-ACP desaturases of various species. Amino acid sequences deduced from cDNAs for the coriander $\Delta^4$16:0-ACP desaturase and the *T. alata* $\Delta^6$16:0-ACP desaturase share significant identity with the $\Delta^9$18:0-ACP desaturase. These results indicate that variations in the primary structures of acyl-ACP desaturases can result in enzymes with altered substrate recognition and double bond positioning properties.

In addition, incubation of endosperm of the Umbelliferae carrot (*Daucus carota*) and coriander in [1-^{14}C]acetate revealed that petroselinic acid readily enters phosphatidylcholine (PC) prior to deposition in triacylglycerol. This result indicates that PC can participate in the metabolism of an unusual fatty acid in developing seeds.

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ABBREVIATIONS

ACP.....acyl carrier protein

ATP.....adenosine triphosphate

CaMV 35S.....cauliflower mosaic virus 35S promoter

cDNA.....complimentary deoxyribonucleic acid

CDP.....cytidine diphosphate

CoA.....coenzyme A

CPT.....CDP-choline:DAG choline phosphosphotransferase

DAF.....days after flowering

DAG.....diacylglycerol

DAGAT.....diacylglycerol acyltransferase

DGDG.....digalacatosyl diacylglycerol

dNTP.....dideoxy nucleotide triphosphate

DTT.....dithiothreitol

EDTA.....ethylenediamine tetraacetic acid

EGTA.....ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid

FA.....fatty acid

Fd.....ferredoxin

FFA.....free (or unesterified) fatty acid

FNR.....ferredoxin:NADPH reductase

G-3-P.....glycerol-3-phosphate

GC-MS.....gas chromatography-mass spectrometry

GPAT.....glycerol-3-phosphate acyltransferase

IPTG.....isopropyl-β-D-thiogalactopyranoside

LPA.....lysophosphatidic acid

LPAT.....lysophosphatidic acid acyltransferase

Mes.....2-(N-morpholino) ethanesulfonic acid

MGDG.....monogalactosyl diacylglycerol

mRNA.....messenger ribonucleic acid

NADH.....β-nicotinamide dinucleotide (reduced)

NADPH.....β-nicotinamide dinucleotide phosphate (reduced)

PA.....phosphatidic acid

PC.....phosphatidylcholine

PCR.....polymerase chain reaction

PE.....phosphatidylethanolamine

PI.....phosphatidylinositol

Pipes.....piperazine-N,N-bis(2-etanesulfonic acid)

PMSF.....phenylmethyl sulfonyl fluoride

PVPP.....polyvinylpolypyrrolidone

R_f.....retention factor

RNA.....ribonucleic acid

SatFA.....saturated fatty acid

SDS/PAGE.....sodium dodecyl sulfate/polyacrylamide gel electrophoresis

TAG.....triacylglycerol

TCA.....trichloroacetic acid

TLC.....thin-layer chromatography

T_m.....gel-to-liquid cyrstalline phase transition temperature

Tris.....tris(hydroxymethyl)amino methane

FATTY ACID NOMENCLATURE

x:*y*.....*x* is the number of carbon atoms in the fatty acid or acyl chain:*y* is the number of double bonds in the fatty acid, *e.g.*, 18:1 is a fatty acid that contains eighteen carbon atoms and one double bond.

Δ^x.....Indicates that the double bond(s) is positioned at the *x*th carbon atom relative to the carboxyl end of an unsaturated fatty acid, *e.g.*, $18{:}1\Delta^6$ is an eighteen carbon fatty acid that contains one double bond at the sixth carbon atom relative to the carboxyl end of the acyl chain.

ω^x.....Indicates that the double bond(s) is positioned at the *x*th carbon atom relative to the methyl end of an unsaturated fatty acid, *e.g.*, $18{:}1\omega^{12}$ is an eighteen carbon fatty acid that contains one double bond at the twelfth carbon atom relative to the methyl end of the acyl chain. (Note: $18{:}1\Delta^6$ and $18{:}1\omega^{12}$ are the same fatty acid.)

9:0.....nonanoic acid

10:0.....decanoic acid

12:0.....lauric acid

14:0.....myristic acid

16:0.....palmitic acid

$16{:}1\Delta^4$.....Δ^4hexadecenoic acid

$16{:}1\Delta^6$.....Δ^6hexadecenoic acid

$16{:}1\Delta^9$.....palmitoleic acid

17:0.....heptadecanoic acid

18:0.....stearic acid

$18:1\Delta^{6}$.....petroselinic acid

$18:1\Delta^{8}$.....Δ^{8}octadecenoic acid

$18:1\Delta^{9}$.....oleic acid

$18:1\Delta^{11}$.....*cis*-vaccenic acid

$18:1\Delta^{9}$,12-OH.....ricinoleic acid (Note: 12-OH, hydroxyl residue positioned at the twelth carbon atom from the carboxyl end of the acyl chain.)

$18:1\Delta^{9}$, 12-epoxy.....vernolic acid (Note: 12-epoxy, epoxy residue positioned at the twelth carbon atom from the carboxyl end of the acyl chain.)

$18:2\Delta^{9,12}$.....linoleic acid

$18:3\Delta^{6,9,12}$.....γ-linolenic acid

$18:3\Delta^{9,12,15}$.....α-linolenic acid

$20:1\Delta^{5}$.....Δ^{5}eicosenoic acid

$22:1\Delta^{13}$.....erucic acid

CHAPTER 1

Introduction

Seed oils of plants can contain a diversity of unusual fatty acids. The term "unusual" indicates that a given fatty acid is found in only a limited number of families or species. In addition, the chemical structures of unusual fatty acids of seed oils often differ significantly from those of the common C_{16} and C_{18} acyl moieties such as palmitic acid (16:0) and oleic acid (18:1Δ^9). In fact, more than 200 different unusual fatty acid structures have been identified in plants (Badami and Patil, 1981; van de Loo *et al.*, 1993). Structural divergence in these acyl moieties can include variations in carbon chain lengths as found in medium-chain fatty acids (C_8-C_{14}) such as lauric acid (12:0) and decanoic acid (10:0) and in very long chain fatty acids ($\geq C_{20}$) such as erucic acid (22:1Δ^{13}). Unusual fatty acids can also contain functional groups such as hydroxyl residues and epoxide rings as present, for example, in ricinoleic acid (18:1Δ^9,12-OH) and vernolic acid (18:1Δ^9,12-epoxy), respectively.

A more subtle variation observed in the chemical structures of certain unusual fatty acids involves the positioning of double bonds in monounsaturated acyl chains. Present in various amounts in nearly all seed oils is oleic acid which has a double bond at the Δ^9 carbon atom. However, monounsaturated fatty acids with double bonds at positions other than the Δ^9 carbon are known to exist as major components of the seed oil of a few families or species of plants. This thesis examines two examples of such acyl chains, the *cis*Δ^6 monounsaturated fatty acids petroselinic acid and Δ^6hexadecenoic acid (Figure 1.1). Oils containing these unusual fatty acids are of potential economic value. Despite this, the synthesis and metabolism of petroselinic acid and Δ^6hexadecenoic acid have received little previous characterization.

Natural Occurrence of Petroselinic Acid and Δ^6Hexadecenoic Acid

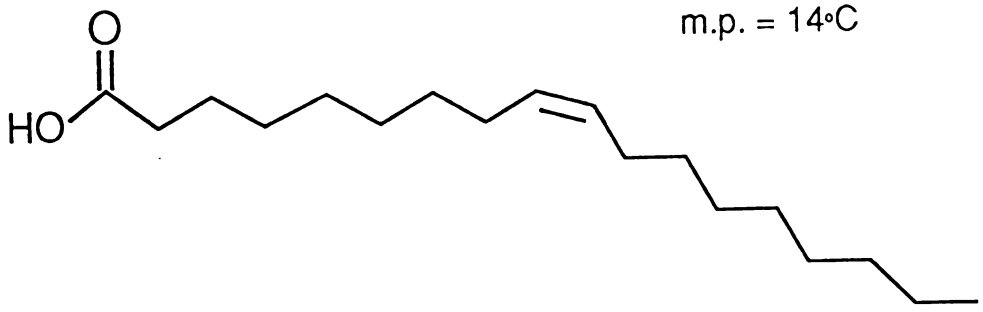
Petroselinic acid was first identified in the seed oil of parsley (*Petroselinum* sp.), an Umbelliferae (or Apiaceae) species, by Vongerichten

Figure 1.1. Comparison of the chemical structures and melting points of oleic acid (A), petroselinic acid (B), and Δ^6hexadecenoic acid (C).

A

Oleic Acid (18:1Δ^9)

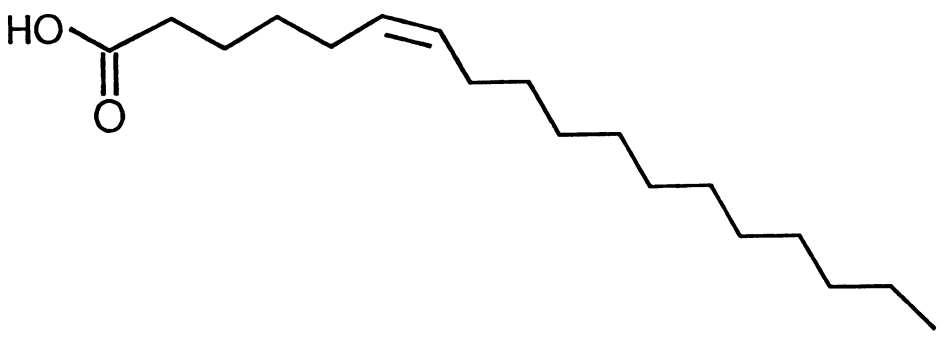
m.p. = 14°C



B

Petroselinic Acid (18:1Δ^6)

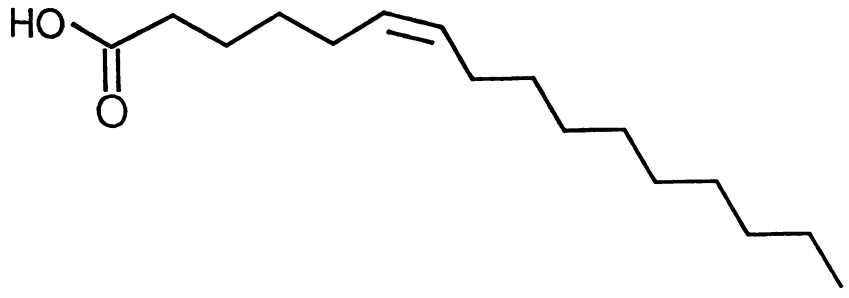
m.p. = 30°C



C

Δ^6 Hexadecenoic Acid (16:1Δ^6)

m.p. = ?



and Köhler (1909) and was later detected in the seed oil of English ivy (*Hedera helix*), an Araliaceae species, by Palazzo and Tamburello (1914). Since these early reports, petroselinic acid has been identified in a wide range of Umbelliferae, Araliaceae, and Garryaceae species where it can compose up to 85 wt% of the seed oil (Kleiman and Spencer, 1982). Petroselinic acid has also been found in the seed oil of a few genera of other families including the Cornaceae (Kleiman and Spencer, 1982; Breuer *et al.*, 1987) and Simarubaceae (Spencer *et al.*, 1970). Furthermore, small amounts of petroselinic acid have been detected in human hair (Weitkamp *et al.*, 1947) and other human tissues (Ohlrogge *et al.*, 1981).

Petroselinic acid is apparently a seed-specific fatty acid in plants as only trace amounts have been found in leaves and roots of at least two Umbelliferae species examined (Ellenbracht *et al.*, 1980; Dutta and Appelqvist, 1991). In addition, cell cultures of a number of Umbelliferae species have been established, and the use of such systems for the study of petroselinic acid synthesis has been proposed (Ellenbracht *et al.*, 1980). However, in the case of callus cultures of parsley (*Petroselinum crispum*) (Ellenbracht *et al.*, 1980) and embryogenic cultures of carrot (*Daucus carota*) (Dutta and Appelqvist, 1989), petroselinic acid was found to compose ≤1% of the total fatty acid. In contrast to these results, levels of petroselinic acid are "strongly increased" in triacylglycerol during somatic embryogenesis of the Umbelliferae anise (*Pimpinella anisum*), as reported in a recent meeting abstract (Theimer *et al.*, 1993). Disregarding this preliminary report, the virtual lack of petroselinic acid in carrot embryo cultures may suggest that synthesis of this fatty acid in Umbelliferae seed is associated with endosperm, as this is the predominant cell type, rather than embryo, in this tissue.

In contrast to petroselinic acid, Δ^6hexadecenoic acid is a relatively obscure fatty acid. This is due in part to its more recent discovery as a major component of a plant seed oil (Spencer *et al.*, 1971). The most significant natural source of Δ^6hexadecenoic acid is seed of *Thunbergia alata* (or black-eyed susan vine), which is generally classified

in the Acanthaceae family (Cronquist, 1988). The oil of this tissue contains more than 80 wt% Δ^6hexadecenoic acid (Spencer *et al.*, 1971). Whether the occurrence of this fatty acid is limited to the seed of *T. alata* has not been determined (though, given what is known of the typical tissue distribution of unusual fatty acids, this is presumed to be the case). Trace amounts of Δ^6hexadecenoic acid have also been detected in seed extracts of plants including English ivy (Grobois, 1971) and *Pricamnia selowii* (Spencer *et al.*, 1970). In addition, Δ^6hexadecenoic acid has been identified in lipids of human skin (Shinohara, 1970) and adipose tissue (Jacob and Grimmer, 1968).

Physical, Nutritional, and Industrial Properties of Petroselinic Acid

The presence of unsaturation at the Δ^6 carbon atom instills petroselinic acid with properties distinct from those of its Δ^9 isomer oleic acid. Perhaps the most dramatic divergence in the physical properties of petroselinic acid and oleic acid is the difference in melting points of these fatty acids. Depending on the reference, the melting point of petroselinic acid is either 30°C (*The Merck Index*) or 33°C (*The Lipid Handbook*). As a result, petroselinic acid and tripetroselinin (m.p. 28°C, *The Lipid Handbook*), a triacylglycerol containing exclusively petroselinic acid, exist as solids at room temperature. In contrast, the melting point of oleic acid is approximately 14°C (*The Lipid Handbook*) or well below room temperature. The high melting point of tripetroselinin has implications for nutritional uses of this oil. For example, tripetroselinin and possibly other oils rich in petroselinic acid could be used in the manufacture of unsaturated margarines without requiring hydrogenation. This process is currently used to convert conventional polyunsaturated plant oils to a solid form as is necessary for margarine production. Hydrogenation of polyunsaturated oils, however, results in extensive conversion of *cis* double bonds to the *trans* configuration (Weidermann, 1978). *Trans* unsaturated fatty acids fed to humans have been shown to result in lipoprotein levels that favor the onset of cardiovascular disease (Zack

and Katan, 1992; Siguel and Lermann, 1993). Therefore, a margarine produced from petroselinic acid-rich oils may have more favorable health-related properties than one produced from conventional plant oils.

Because of the location of its double bond at the Δ^6 carbon atom, petroselinic acid can also serve as a precursor of two valuable industrial chemicals: lauric acid (12:0) and adipic acid (6:0 dicarboxylic). These compounds are formed by the oxidative cleavage of petroselinic acid at its double bond through methods such as ozonolysis. Lauric acid, the C_{12} product of this cleavage, is used extensively in the production of detergents and surfactants and is derived commercially from palm kernel and coconut. Neither palm (*Elaeis guineensis*) nor coconut (*Cocos nucifera*) is grown for oil production in the U.S. or other nations of temperate climate (Battey *et al.*, 1989). An oilseed that produces large amounts of petroselinic acid and that was adapted to climatic conditions of the U.S. could therefore serve as a potential domestic source of lauric acid. In addition, adipic acid, the C_6 product of petroselinic acid oxidation, is a precursor of nylon 6,6. This polymer is the most extensively manufactured nylon and is used primarily in the production of carpet fibers (Putscher, 1984). Though adipic acid is derived commercially from cyclohexane (Danly and Campbell, 1985), a petroleum by-product, plant oils rich in petroselinic acid could provide a renewable source of this compound.

Furthermore, it has been reported that triacylglycerols containing petroselinic acid as well as other C_{18} monounsaturated fatty acids with double bonds between the Δ^2 to Δ^7 positions are resistant to hydrolysis by porcine pancreatic lipase (Heimermann *et al.*, 1973). In contrast, triacylglycerols containing oleic acid are more readily digested by this enzyme. A similar phenomenon has also been observed with lipases of cotyledons of germinating *Brassica napus* seeds (Hills *et al.*, 1990). In this case, it was speculated that differences in rates of lipase hydrolysis of oleic acid and petroselinic acid may relate to the different *syn-/anti-* orientations of the double bonds of these fatty acids.

(Because its unsaturation is positioned at an even-numbered carbon, the double bond of petroselinic acid exists in an *anti-* orientation relative to the carboxyl group. Conversely, the Δ^9 double bond oleic acid is present in a *syn-* configuration.) If it is assumed that human pancreatic lipase functions in the same manner as its porcine counterpart, then plant oils rich in petroselinic acid would likely not be hydrolyzed as well as conventional vegetable oils (*i.e.*, those containing oleic acid and its polyunsaturated derivatives) in the small intestine. As a result, petroselinic acid-rich oils could be potentially useful as low-caloric edible oils.

A number of other physical properties and possible economic uses of petroselinic acid have been reviewed previously by Placek (1963).

In contrast to petroselinic acid, the melting point and other physical properties of Δ^6hexadecenoic acid have not been reported. However, like petroselinic acid, Δ^6hexadecenoic acid can be oxidatively cleaved at its double bond to produce adipic acid for nylon synthesis. The other compound formed in this process, decanoic acid (or capric acid) (10:0), is used in the manufacture of artificial fruit flavorings and perfumes (*The Merck Index*). It is also likely that oils rich in Δ^6hexadecenoic acid are resistant to hydrolysis by pancreatic lipase and, as such, could serve as a component of low-caloric edible oils. In addition, the use of Δ^6hexadecenoic acid in cosmetics and skin creams has been patented (Steifel, 1977). These formulations apparently have excellent skin-care properties, and as Δ^6hexadecenoic acid is a natural component of skin lipids (Shinohara, 1970), the likelihood of allergic response to such cosmetics is minimal (Steifel, 1977).

Biosynthesis of Petroselinic Acid and Δ^6Hexadecenoic Acid

Prior to the research presented in this thesis, no studies of Δ^6hexadecenoic acid biosynthesis had been reported. In addition, the only previous work that directly addressed the metabolic origin of petroselinic acid was a proceedings paper by Grobois and Mazliak (1978). In these studies, ^{14}C-acetate was provided to intact English ivy seeds and membrane

fractions of this tissue. The radiolabel was incorporated into petroselinic acid which was associated primarily with polar lipids. From these results, it was speculated that petroselinic acid may arise from reactions involving phospholipid precursors.

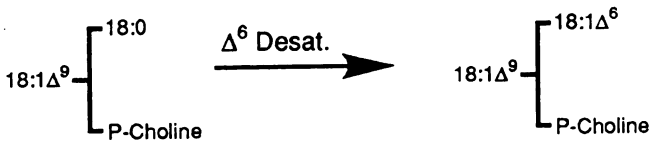
With such little background information available, attempts to characterize the biosynthesis of petroselinic acid and Δ^6hexadecenoic acid must take into consideration all potential pathways (as summarized in Figure 1.2). Based on what is known of the synthesis of other unsaturated fatty acids, one possibility is that the double bonds of petroselinic acid and Δ^6hexadecenoic acid result from aerobic desaturation of an acyl moiety bound to either a glycerolipid (Browse and Somerville, 1991) or coenzyme A (CoA) (Cook, 1985). Fatty acids formed via glycerolipid-linked desaturation include, for example, linoleic acid, which can be derived from the Δ^{12} (or n-6) desaturation of oleic acid esterified to phosphatidylcholine in plant and algal cells (Gurr *et al.*, 1969; Slack *et al.*, 1978). In addition, acyl-CoA desaturation is the primary route of monounsaturated fatty acid (particularly oleic acid) biosynthesis in animals (Cook, 1985). Though such reactions have yet to be conclusively demonstrated in plants, evidence has been presented for the involvement of an acyl-CoA desaturase in the formation of Δ^5eicosenoic acid (20:1Δ^5) in meadowfoam (*Limanthes alba*) seed (Pollard and Stumpf, 1980; Moreau *et al.*, 1981).

If produced through either acyl-CoA or glycerolipid-linked desaturation, the likely precursors of petroselinic acid and Δ^6hexadecenoic acid would be esters of stearic acid and palmitic acid, respectively. Seed oils of species which synthesize petroselinic acid and Δ^6hexadecenoic acid typically contain 70 to 85 wt% of these fatty acids. Therefore, such species would not only require a likely Δ^6 desaturase but also a mechanism for the release of large amounts of stearic acid and palmitic acid from the *de novo* reactions of fatty acid synthesis, in order to generate the appropriate acyl-CoA or glycerolipid substrate for the desaturase. The stearic acid or palmitic acid needed as precursors in the

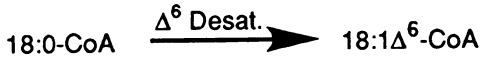
Figure 1.2. Potential pathways for the biosynthesis of petroselinic acid.

Potential Pathways of Petroselinic Acid ($18:1\Delta^{6cis}$) Biosynthesis

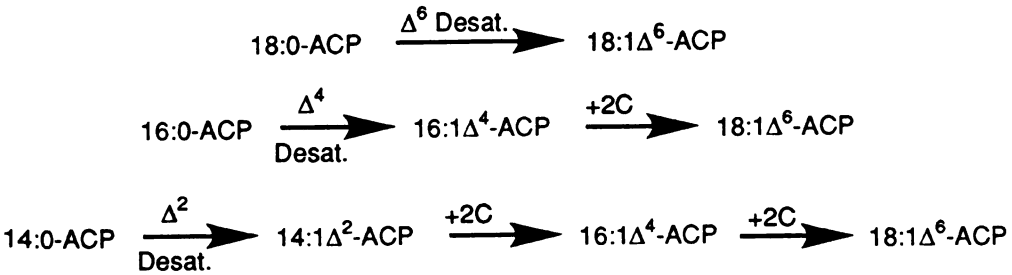
A) Desaturation on a Glycerolipid



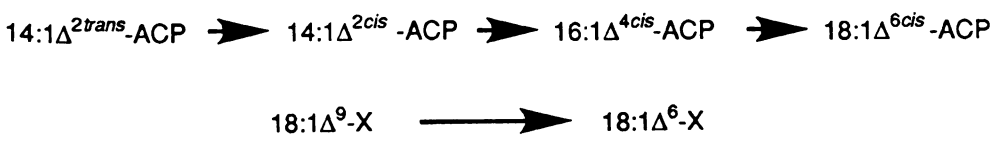
B) Desaturation of an Acyl-CoA



C) Desaturation/Elongation of Acyl-ACP



D) Isomerization of an Unsaturated Precursor



above biosynthetic scenario could result from an acyltransferase or thioesterase specialized for the efficient removal of these moieties from acyl carrier protein (ACP).

Routes of petroselinic acid and Δ^6hexadecenoic acid synthesis involving desaturation of an acyl moiety bound to a glycerolipid or CoA would therefore seemingly require both a unique desaturase and a unique acyl-ACP thioesterase or acyltransferase. The degree of metabolic specialization necessary for such a pathway is not without precedent. For example, oil of meadowfoam is composed of approximately 60 wt% of Δ^5eicosenoic acid (Pollard and Stumpf, 1980). It has been proposed that this fatty acid arises from the activity of a Δ^5eicosanoyl-CoA desaturase, as alluded to above (Pollard and Stumpf, 1980; Moreau *et al.*, 1981). The eicosenoyl-CoA substrate is believed to originate from palmitoyl-CoA or stearoyl-CoA that is elongated by reactions separate from those of *de novo* fatty acid synthesis. The pathway for Δ^5eicosenoic acid formation would therefore need an unprecedented desaturase, a non-ACP-track fatty acid elongation system, and a means of releasing significant amounts of palmitic acid or stearic acid from pools of acyl-ACPs.

The involvement of aerobic desaturation in the synthesis of petroselinic acid and Δ^6hexadecenoic acid could also entail reactions that use acyl-ACPs as substrates. The only previously identified acyl-ACP desaturase is the Δ^9stearoyl (18:0)-ACP desaturase, which catalyses the production oleoyl-ACP from 18:0-ACP. This enzyme was originally identified in photoauxotrophic cultures of *Euglena gracilis* (Nagai and Bloch, 1965), and its activity was subsequently detected in tissue and chloroplast extracts of higher plants (Nagai and Bloch, 1968; Jacobson *et al.*, 1974; Jaworski and Stumpf, 1974; McKeon and Stumpf, 1982). In contrast to all other known desaturases, the $\Delta^9$18:0-ACP desaturase displays soluble activity (Nagai and Bloch, 1968). In addition, this enzyme uses reduced ferredoxin as its electron donor rather than cytochrome b_5, in contrast to the membrane-bound desaturases of animals and the endoplasmic reticulum-localized desaturases of plants (Nagai and

Bloch, 1968). The soluble nature of the $\Delta^9$18:0-ACP desaturase has facilitated its purification from tissues of higher plants (McKeon and Stumpf, 1982; Cheesbrough and Cho, 1990; Kinney *et al.*, 1990; Shanklin and Somerville, 1991; Thompson *et al.*, 1991) and a number of cDNAs encoding this enzyme have recently been isolated (Kinney *et al.*, 1990; Shanklin and Somerville, 1991; Shanklin *et al.*, 1991; Thompson *et al.*, 1991; Nishida *et al.*, 1992; Sato *et al*, 1992; Slocombe *et al.*, 1992; Taylor *et al.* 1992).

If petroselinic acid and Δ^6hexadecenoic acid result from the activity of an analogous enzyme, the most direct pathways would involve the Δ^6desaturation of 18:0-ACP and palmitoyl (16:0)-ACP, respectively. Tissues that synthesize petroselinic acid, however, also produce oleic acid. Therefore, in order to form the large amounts of petroselinic acid typically found in seed oils, a $\Delta^6$18:0-ACP desaturase would potentially have to "outcompete" the $\Delta^9$18:0-ACP desaturase for their common substrate (assuming that these enzymes occur together in plastids and in comparable amounts). This competition could be effectively avoided if the double bond of petroselinic acid were inserted prior to the C_{18} stage of *de novo* fatty acid synthesis. Alternatively, a Δ^6 desaturase could have a lower K_m for 18:0-ACP than the $\Delta^9$18:0-ACP desaturase.

A further possibility is that petroselinic acid and Δ^6hexadecenoic acid are formed by isomerization of double bonds of pre-existing monounsaturated fatty acids. For example, Shibahara *et al.* (1990) have presented data suggesting that the Δ^9 double bond of oleic acid can be enzymatically shifted to the Δ^{11} position to form *cis*-vaccenic acid in pulp of kaki (*Diospyros kaki*). This reaction is apparently reversible as the Δ^{11} double bond of *cis*-vaccenic acid can also be moved to the Δ^9 carbon to produce oleic acid. These results were obtained by monitoring the metabolism of deuterated fatty acids using GC-MS. However, the mechanism through which double bond migration occurs, including the exact form of the substrate, has yet to be determined. If a similar reaction is associated with petroselinic acid synthesis, it could be envisioned

that the double bond of oleic acid is isomerized to the Δ^6 position. In the case of Δ^6hexadecenoic acid, such a biosynthetic pathway is more difficult to propose. Seeds typically contain only trace amounts of C_{16} monounsaturated fatty acids (Harwood, 1980). Therefore, a potential precursor of Δ^6hexadecenoic acid would likely be absent in developing seed of *Thunbergia alata*.

Another form of double bond isomerization is observed in the anaerobic reaction leading to the synthesis of palmitoleic acid ($16:1\Delta^9$) and *cis*-vaccenic acid ($18:1\Delta^{11}$) in *Escherichia coli*. The double bond of these fatty acids has been shown to arise from an isomerization step that occurs between the C_8 and C_{10} stages of *de novo* fatty acid biosynthesis. More specifically, the double bond of the *trans*Δ^2decenoyl (10:1)-ACP intermediate is shifted to the *cis*Δ^3 configuration by the activity of the 3-hydroxydecanoyl-ACP dehydrase (Bloch, 1970). The resulting *cis*Δ^3-decenoyl-ACP is subsequently elongated to yield palmitoleic acid and *cis*-vaccenic acid. Because double bonds of petroselinic acid and Δ^6hexadecenoic acid are located at even-numbered carbon positions, the *E. coli* pathway of unsaturated fatty acid formation cannot be easily related to the synthesis of these fatty acids. Instead, schemes involving, for example, the isomerization of a *trans*Δ^2enoyl-ACP intermediate to a *cis*Δ^2 moiety could be proposed. In the case of petroselinic acid, such a pathway would likely entail the conversion of *trans*Δ^2tetradecenoyl (14:1)-ACP to *cis*Δ^2tetradecenoyl-ACP followed by four-carbon elongation.

Though petroselinic acid and Δ^6hexadecenoic acid could arise through other biosynthetic scenarios, those described above are the most likely routes based on what is known for the synthesis of other unsaturated fatty acids in various organisms.

Metabolism of Petroselinic Acid and Δ^6Hexadecenoic Acid

As with the synthesis of petroselinic acid and Δ^6hexadecenoic acid, the routes through which these fatty acids are incorporated into glycerolipids of developing seeds have not been determined. With regard to Δ^6hexadecenoic acid, there have been no previous reports of the levels

of this fatty acid in specific glycerolipid classes of *Thunbergia alata* seed. Previous studies, however, have indicated that amounts of petroselinic acid are disproportionately distributed between the storage lipid triacylglycerol and polar or membrane-type glycerolipids in Umbelliferae seed. In the report by Prasad *et al.* (1987), petroselinic acid was found to compose approximately 70 wt% of the seed oils of carrot and coriander but only 16 to 22 wt% of the fatty acids of the total phospholipid fraction. Similar results have been reported for developing carrot seed (Dutta and Appelqvist, 1991). In this case, triacylglycerols contained >75 wt% petroselinic acid , whereas polar lipids contained <15 wt% of this fatty acid. This difference in amounts of petroselinic acid in polar and storage lipids was observed despite the fact that polar lipids synthesis occurs simultaneous to periods of rapid petroselinic acid formation and deposition into triacylglycerols (Dutta and Appelqvist, 1991). It would thus appear that Umbelliferae seed possess some mechanism for limiting the accumulation of petroselinic acid in membrane-type glycerolipids while concentrating this fatty acid into a storage form in triacylglycerols.

This phenomenon is similar to that which has been previously described for the metabolism of other unusual fatty acids (as reviewed by Browse and Somerville, 1991; Stymne *et al.*, 1990), particularly acyl moieties whose chemical structures differ significantly from those of common fatty acids such as oleic acid. For example, in seeds that synthesize high levels of medium-chain (Slabas *et al.*, 1982; Bafor *et al.*, 1990) and very-long chain fatty acids (Gurr *et al.*, 1974; Pollard *et al.*, 1980), these unusual acyl chains typically compose less than a few weight percent of the fatty acids of polar lipids but can account for 50 to 90 wt% of the fatty acids of triacylglycerols. In theory, the ability of seeds to limit the accumulation of unusual fatty acids in polar lipids insures that "proper" membrane function is not disrupted by the divergent chemical structures of these acyl moieties (Stymne *et al.*, 1990). In this regard, the melting point of petroselinic acid is approximately 30°C.

Therefore, T_ms of polar lipids that contain large amounts of this fatty acid would likely be quite elevated compared to those containing primarily oleic acid and linoleic acid. As a result, bilayers containing glycerolipids rich in petroselinic acid might exist in the non-physiological gel phase at normal ambient temperatures. Furthermore, because additional double bonds are not inserted into petroselinic acid, the presence of large amounts of this fatty acid in polar lipids might reduce the ability of cells to adjust levels of membrane unsaturation in response to changes in their environment.

The mechanism through which developing seeds limit amounts of unusual fatty acids in membrane-type glycerolipids while accumulating these acyl moieties in triacylglycerols (TAG) has been the subject of considerable speculation in the literature. One idea is that enzymes involved in polar lipid and TAG metabolism have different substrate specificities. In this regard, phosphatidylcholine (PC) is usually the most abundant polar lipid in seeds (Harwood, 1980). In addition, PC and TAG are both derived from diacylglycerol (DAG). The conversion of DAG to PC and TAG is catalyzed by the enzymes CDP-choline:DAG choline phosphotransferase (choline phosphotransferase) and DAG acyltransferase, respectively. As such, the incorporation of unusual fatty acids into PC could be avoided if choline phosphotransferase discriminates against DAG rich in unusual acyl moieties. Through the selective use of DAG species containing common fatty acids for PC synthesis, a pool of DAG enriched in unusual fatty acids would remain for incorporation into TAG. In contrast, results reported by Bafor *et al.* (1990) have indicated that the opposite scenario is involved in the deposition of medium-chain fatty acids such as decanoic acid (10:0) into TAG of developing seeds of *Cuphea lanceolata*. In these studies, diacylglycerol acyltransferase rapidly and preferentially converted DAG containing medium chain fatty acids into TAG. By channeling DAG rich in medium chain fatty acids into the synthesis of storage lipids, these molecules were made unavailable for conversion to

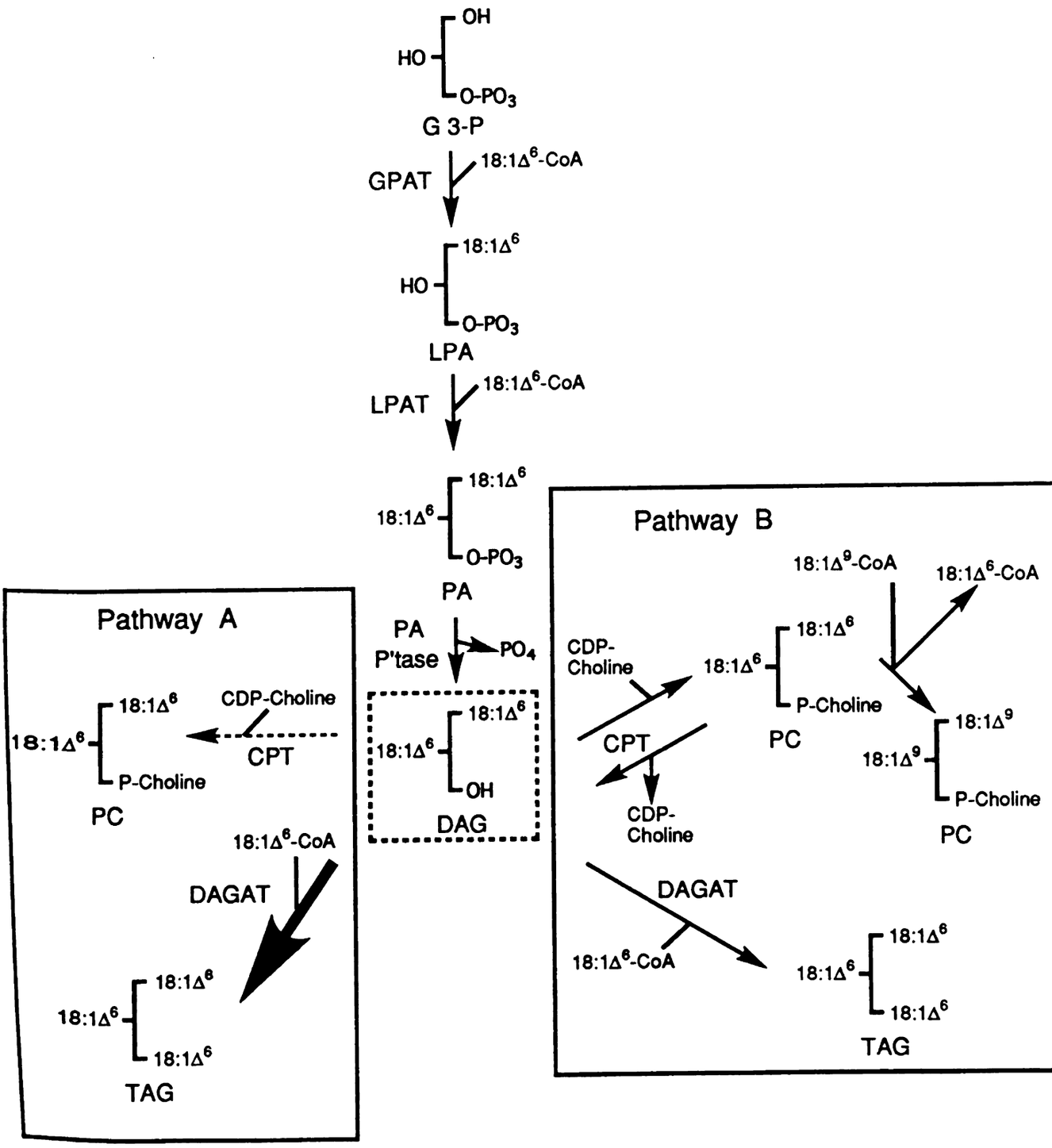
PC. Such a mechanism thus effectively precludes the accumulation of medium-chain fatty acids in membranes.

Based on the above discussion, the unequal distribution of petroselinic acid in polar and storage lipids could be explained by the fact that choline phosphotransferase uses DAG species containing common fatty acids in preference to those containing petroselinic acid (see Figure 1.3). Alternatively, DAG acyltransferase may rapidly shunt DAG rich in petroselinic acid into TAG, leaving behind molecules containing reduced amounts of this unusual fatty acid for subsequent incorporation into PC. Either scenario could be used to explain results reported by Dutta *et al.* (1992) regarding the metabolism of petroselinic acid. In these studies, ^{14}C-glycerol-3-phosphate provided to carrot seed microsomes was converted primarily to phosphatidic acid and TAG in the presence of petroselinoyl-CoA. Little of the radiolabel, however, was detected in PC. This finding suggests that, through some mechanism, movement of petroselinic acid into polar lipids such as PC is partially prevented (as shown in Figure 1.3, Pathway A).

Another possibility is that petroselinic acid is not excluded from polar lipids (as shown in Figure 1.3, Pathway B). Instead, an "editing" mechanism might exist which prevents high levels of petroselinic acid accumulation in polar lipids. Such a scenario could arise, for example, if choline phosphotransferase is incapable of distinguishing DAG moieties that contain petroselinic acid from those containing oleic acid. Grobois and Mazliak (1979), for example, reported that large amounts of radiolabeled petroselinic acid appear in polar lipids following incubation of English ivy seeds in ^{14}C-acetate. In addition, PC has been shown to readily participate in the movement of fatty acids into TAG in seeds of species (*e.g.*, safflower, soybean, and linseed) that synthesize primarily C_{18} polyunsaturated fatty acids such as linoleic acid ($18:2\Delta^{9,12}$) and α-linolenic acid ($18:3\Delta^{9,12,15}$)(Slack *et al.*, 1978). In such seeds, oleic acid first enters PC where it can serve as a substrate for one or

Figure 1.3. Potential pathways for the metabolism of petroselinic acid. Diacylglycerol (DAG) is formed through reactions of the "Kennedy" pathway (Kennedy, 1961). Alternatives routes of DAG metabolism may explain why petroselinic acid is typically found in much lower amounts in phospholipids such as phosphatidylcholine (PC) than in triacylglycerols (TAG): (1) DAG rich in petroselinic acid may be partially excluded from incorporation into PC (Pathway A). This partial exclusion may result from the substrate specificities of choline phosphotransferase (CPT) or diacylglycerol acyltransferase (DAGAT) (as described in the text). (2) Alternatively, DAG rich in petroselinic acid may be incorporated into PC and TAG at comparable rates (Pathway B). Petroselinic acid may then be removed or "edited" from PC by the reverse reaction of CPT or by phospholipases that cleave this fatty acid directly from the glycerol backbone. In the latter case, petroselinic acid may be replaced by CoA esters of common fatty acids (*e.g.*, oleic acid, 18:1Δ^9) from the acyl-CoA pool. G 3-P, glycerol-3-phosphate; GPAT, G 3-P acyltransferase; LPA, Lysophosphatidic acid; LPAT, LPA acyltransferase; PA, phosphatidic acid; PA P'tase, PA phosphatase; DAG, Diacylglycerol; CPT, choline phosphotransferase; PC, Phosphatidylcholine; DAGAT, DAG acyltransferase; TAG, Triacylglycerol.

Potential Pathways of Petroselinic Acid Metabolism



two desaturation steps. The resulting polyunsaturated acyl moieties may then be incorporated into TAG. A similar route of metabolism has also been described for the unusual fatty acids ricinoleic acid in castor (*Ricinus communis*) endosperm (Bafor *et al.*, 1991) and γ-linolenic acid ($18:3\Delta^{6,9,12}$) in borage (*Borago officinalis*) seed (Griffiths *et al.*, 1988). However, as with C_{18} polyunsaturated fatty acids, the synthesis of these fatty acids occurs on PC from oleic acid or linoleic acid (Bafor *et al.*, 1991; Stymne and Stobart, 1986).

The removal of fatty acids from PC for subsequent storage in TAG can occur by release of the intact glycerol backbone from PC and subsequent incorporation of the resulting DAG into TAG as has been shown by experiments of Slack *et al.* (1978). The conversion of PC to DAG can potentially result from the reverse reaction of choline phosphotransferase (Slack *et al.*, 1983) or by phospholipase C activity. Results of other studies have also indicated that fatty acids synthesized on PC can be directly removed from the glycerol backbone of this lipid and be exchanged with other fatty acids in the acyl-CoA pool (Stymne and Stobart, 1987). The released fatty acids are subsequently re-incorporated as CoA esters onto glycerol backbones targeted for TAG synthesis. This direct removal of fatty acids from PC may occur through activity of phospholipases (*e.g.*, phospholipases A_1, A_2, or B). In this regard, Banas *et al.* (1992) have demonstrated the existence of phospholipases in plant extracts that cleave unusual oxygenated fatty acids from PC. This activity likely prevents the accumulation of unusual fatty acids such as ricinoleic acid and vernolic acid in PC following their synthesis on this lipid (Stymne, 1993). Based on the above examples, if petroselinic acid does readily enter PC and other polar lipids in developing Umbelliferae endosperm, accumulation of this fatty acid could be restricted by conversion of phospholipids rich in petroselinic acid to DAG or by the direct removal of petroselinic acid from the glycerol backbone of phospholipids.

A final consideration regarding the metabolism of petroselinic acid and Δ^6hexadecenoic acid in developing seeds is the substrate specificities

of acyltransferases that give rise to DAG. This molecule is formed through reactions of the Kennedy pathway (Kennedy, 1961) involving the successive incorporation of two acyl-CoA moieties onto glycerol-3-phosphate and the subsequent removal of the phosphate residue from the *sn*-3 carbon of the glycerol backbone. The first acylation step is catalyzed by glycerol-3-phosphate acyltransferase (GPAT). This enzyme has a rather broad substrate specificity in seeds of species examined to date (Ichihara, 1984; Griffiths *et al.*, 1985; Löhden and Frentzen, 1992; Eccleston and Harwood, 1990) and can incorporate CoA esters of unusual fatty acids (Sun *et al.*, 1988). In contrast, the substrate specificity of lysophosphatidic acid acyltransferase (LPAT), the enzyme that catalyzes the second acylation step of the Kennedy pathway, is typically more defined. Studies reported by Ichihara *et al.* (1987), for example, have shown that LPAT of safflower seed microsomes displays a marked preference for fatty acids such as oleic acid and linoleic acid that contain Δ^9 unsaturation. Significantly less activity was detected in the presence of saturated substrates or unsaturated substrates such as petroselinic acid with double bonds at positions other than the Δ^9 carbon.

The narrow substrate specificity of LPAT may explain that rapeseed TAG, for example, contains significant amounts of erucic acid in only the *sn*-1 and *sn*-3 positions of TAG. As a result, the oil of this plant can, in theory, contain no more than 67 mol% erucic acid. However, TAG of certain species are composed of significantly more than 67 mol% of a given unusual fatty acid. Examples of this include the seed oil of many *Cuphea* species and the kernel oil of palm which can contain 80 to 90% medium chain fatty acids (*The Lipid Handbook*). This composition suggests that LPAT of such tissues is specialized for the incorporation of these unusual acyl chains. In the case of both palm kernel and *Cuphea* embryo extracts, LPAT has been shown to catalyze the incorporation of medium chain acyl-CoAs (Oo and Huang, 1989; Bafor *et al.*, 1990). This was particularly true when the acceptor molecule for the acyltransferase was lysophosphatidic acid that contained a medium chain fatty acid. Such results are of

relevance to genetic engineering studies of seed oils. In this regard, transgenic *Brassica* species have recently been developed that synthesize significant amounts (>25 mol% of the total seed oil) of lauric acid (Voelker *et al.*, 1992). Levels of lauric acid accumulation in excess of 67 mol% in oils of these plants, however, may also require the introduction of an LPAT such as present in *Cuphea* embryo and palm kernel that is specialized for the metabolism of this fatty acid.

TAG of species that produce petroselinic acid and Δ^6hexadecenoic acid can contain 70 to 85 mol% of these unusual fatty acids. In addition, Gunstone (1991) has demonstrated using ^{13}C-NMR that petroselinic acid can compose nearly 50% of the *sn*-2 position of TAG in several Umbelliferae species. The contributions of acyltransferases to levels of petroselinic acid in Umbelliferae seed oils has been examined by Dutta *et al.* (1992). In these studies, the ability of acyltransferases of carrot seed microsomes to incorporate oleoyl-CoA and petroselinoyl-CoA onto glycerol backbones was compared. In the case of GPAT, the rate of oleoyl-CoA incorporation was nearly twice that of petroselinoyl-CoA. LPAT also incorporated oleoyl-CoA at rates significantly higher than that for petroselinoyl-CoA. However, relative to those of rapeseed and safflower, microsomes of carrot seed were the only plant extract examined that could introduce petroselinoyl-CoA onto a petroselinoyl-lysophosphatidic acid acceptor. Still, the rate of this esterification was four-times lower than that observed for oleoyl-CoA. These findings suggest that acyltransferases may contribute somewhat to the total levels and stereospecific distribution of petroselinic acid found in Umbelliferae TAG. However, other factors may be of equal or greater importance. Such factors may include, for example, the relative levels of petroselinoyl-CoA present in acyl-CoA pools that are available for incorporation on to glycerol backbones *in vivo*.

Rationale

As described above plant oils containing petroselinic acid and Δ^6hexadecenoic acid have potential industrial and nutritional value, yet

there are currently no established oilseed crops that accumulate large amounts of these fatty acids. Such crops, however, could be produced by introducing genes encoding enzymes specialized for the synthesis and metabolism of petroselinic acid and Δ^6hexadecenoic acid into existing oilseeds such as a rapeseed and sunflower. Limiting this endeavor is the lack of understanding of pathways of biosynthesis and metabolism of these unusual fatty acids. Studies presented in this thesis are an attempt to provide such information.

In addition to the biotechnological implications of this work, aspects of the synthesis and metabolism of petroselinic acid and Δ^6hexadecenoic acid are of general significance to plant lipid biochemistry. For example, studies of the biosynthetic origin of the double bonds of these fatty acids would likely provide new information regarding desaturation mechanisms in plants. Also, characterization of pathways through which petroselinic acid and Δ^6hexadecenoic acid are incorporated into TAG of developing seeds may be useful for understanding the metabolism of other unusual fatty acids.

Studies of petroselinic acid biosynthesis presented in this thesis were conducted primarily with developing endosperm of the Umbelliferae coriander (*Coriandrum sativum*) (Figures 1.4A, 1.5). This tissue accumulates petroselinic acid in its seed oil to levels of nearly 75 wt% of the total fatty acid (Kleiman and Spencer, 1982). In addition, unlike many Umbelliferae, coriander is an annual rather than a biennial. As such, seeds can be harvested year-round from this plant without requiring vernalization of roots. Furthermore, the endosperm of coriander is larger than that produced by many other annual Umbelliferae, which makes this tissue particularly attractive for biochemical studies. Studies in Chapter 4 were performed with endosperm of the Umbelliferae wild carrot (*Daucus carota*) (Figure 1.5) as well as with endosperm of coriander. Petroselinic acid composes at least 70 wt% of the seed oil of carrot (Kleiman and Spencer, 1982). In addition, the campus of Michigan State University has extensive natural stands of wild carrot that typically

Figure 1.4. Examples of plants that accumulate petroselinic acid (A) or Δ^6hexadecenoic acid (B) in their seed oil. Shown in *A* are flowers of coriander (*Coriandrum sativum*), an Umbelliferae species. The seed oil of this plant can contain petroselinic acid at levels of more than 75 wt% of the total fatty acid. Shown in *B* are vines and flowers of *Thunbergia alata*. Δ^6Hexadecenoic acid can compose more than 80 wt% of the seed oil of this plant.

(A)

(B)

Figure 1.5. Mericarps (fruits) and dissected endosperm of carrot (*Daucus carota*) and coriander (*Coriandrum sativum*) (left to right). Studies in Chapters 2, 3, and 4 were conducted using endosperm (white tissue above) of either of these plants.

bloom in August. The ready availability of developing seeds of this plant was a major reason for its use in studies detailed in Chapter 4. Experiments described in Chapter 5 were conducted with endosperm of *Thunbergia alata* (Figure 1.4B), which is the only plant identified to date that accumulates large amounts of Δ^6hexadecenoic acid in its seed oil. Advantageous qualities of this plant include its perennial growth habit (under greenhouse conditions) and the fact that it flowers bountifully throughout its life cycle.

Finally, the chapters that follow are presented as publication units. However, studies in Chapters 2 and 3 are complementary as a portion of the research presented in the latter chapter provided the basis for experiments described in Chapter 2.

Literature Cited

Badami RC, Patil KB (1981) Structure and occurrence of unusual fatty acids in minor seed oils. Prog Lipid Res **19**: 119-153

Bafor M, Jonsson L, Stobart AK, Stymne S (1990) Regulation of triacylglycerol biosynthesis in embryos and microsomal preparations from the developing seeds of *Cuphea lanceolata*. Biochem J **272**: 31-38

Bafor M, Smith MA, Jonsson L, Stobart K, Stymne S (1991) Ricinoleic acid biosynthesis and triacylglycerol assembly in microsomal preparations from developing castor-bean (*Ricinus communis*) endosperm. Biochem J **280**: 507-514

Banas A, Johansson I, Stymne S (1992) Plant microsomal phospholipases exhibit preference for phosphatidylcholine with oxygenated acyl groups. Plant Sci **84**: 137-144

Battey JF, Schmid KM, Ohlrogge JB (1989) Genetic engineering for plant oils: Potential and limitations. Trends Biotech **7**: 122-125

Bloch K (1970) β-Hydroxydecanoyl thioester dehydrase. *In* PD Boyer, ed, The Enzymes, Third Edition, Volume 5. Academic Press, New York, p 441-464

Breuer B, Stuhlfauth T, Fock H, Huber H (1987) Fatty acids of some Cornaceae, Aquifoliqaceae, Hammelidaceae, and Styracaceae. Phytochemistry **26**: 1441-1445

Browse J, Somerville C (1991) Glycerolipid synthesis: Biochemistry and regulation. Annu Rev Plant Physiol Plant Mol Biol **42**: 467-506

Cheesbrough TM, Cho SH (1990) Purification and characterisation of soyabean stearoyl-ACP desaturase. *In* PJ Quinn, JL Harwood, eds, Plant Lipid Biochemistry, Structure and Utilization. Portland Press, London, p 129-130

Cook HW (1985) Fatty acid desaturation and chain elongation in eucaryotes. *In* DE Vance, JE Vance, eds, Biochemistry of Lipids and Membranes. Benjamin/Cummings Publishing Company, Menlo Park, CA, p 181-212

Cronquist A (1988) The Evolution and Classification of Flowering Plants, Second Edition. New York Botanical Garden, Bronx, NY

Danly DE, Campbell CR (1985) Adipic acid. *In* Kirk-Othmer Concise Encyclopedia of Chemical Technology. John Wiley & Sons, New York, p 37-38

Dutta PC, Appelqvist L-A (1989) The effects of different culture conditions on the accumulation of depot lipids notably petroselinic acid during somatic embryogenesis in *Daucus carota* L. Plant Sci **64**:167-177

Dutta PC, Appelqvist L-A (1991) Lipids and fatty acid patterns in developing seed, leaf, root, and in tissue culture initiated from embryos of *Daucus carota* L. Plant Sci **75**: 177-183

Dutta PC, Appelqvist L-A, Stymne S (1992) Utilization of petroselinate (C $18:1^{\Delta 6}$) by glycerol acylation enzymes in microsomal preparations of developing embryos of carrot (*Daucus carota* L.), safflower (*Carthamus tinctorius* L.) and oil rape (*Brassica napus* L.). Plant Sci **81**: 57-64

Eccleston VS, Harwood JL (1990) Acylation reactions in developing avocado fruits. *In* PJ Quinn, JL Harwood, eds, Plant Lipid Biochemistry, Structure and Utilization. Portland Press, London, p 178-180

Griffiths G, Stobart AK, Stymne S (1985) The acylation of *sn*-glycerol 3-phosphate in microsomal preparations from the developing cotyledons of safflower (*Carthamus tinctorius*) seed. Biochem J **230**: 379-388

Griffiths G, Stobart AK, Stymne S (1988) Δ^6- and Δ^{12}-dependent activities and phosphatidic acid formation in microsomal preparations from the developing cotyledons of common borage (*Borago officinalis*). Biochem J **252**: 641-647

Grobois M (1971) Biosynthèse des acides gras au cours du développement du fruit et de la graine du lierre. Phytochemistry **10**: 1261-1273

Grobois M, Mazliak P (1979) Ultrastructural sites involved in petroselinic acid (C18:1Δ^6) biosynthesis during ivy seed (*Hedera helix*) development. *In* L-A Appelqvist, C Lijenberg, eds, Advances in the Biochemistry and Physiology of Plant Lipids. Elsevier/North-Holland Biomedical Press, Amsterdam, p 409-414

Gunstone FD (1991) The ^{13}C-NMR spectra of six oils containing petroselinic acid and of aquilegia and meadowfoam oil which contain Δ5 acids. Chem Phys Lipids **58**: 159-167

Gurr MI, Blades J, Appleby RS, Smith CG, Robinson MP, Nichols BW (1974) Studies on seed-oil triglycerides. Triglyceride biosynthesis and storage in whole seeds and oil bodies of *Crambe abyssinica*. Eur J Biochem **43**: 281-290

Gurr MI, Robinson MP, James AT (1969) The mechanism of formation of polyunsaturated fatty acids by photosynthetic tissue. The tight coupling of oleate desaturation with phospholipid biosynthesis in *Chlorella vulgaris*. Eur J Biochem **9**: 70-78

Harwood JL (1980) Plant acyl lipids: Structure, distribution, and analysis. *In* PK Stumpf, ed, The Biochemistry of Plants. A Comprehensive Treatise. Vol 4. Lipids: Structure and Function. Academic Press, New York, p 1-55

Heimermann WH, Holman RT, Gordon DT, Kowalyshyn DE, Jensen RG (1973) Effect of double bond position in octadecenoates upon hydrolysis by pancreatic lipase. Lipids **8**: 45-47

Hills MJ, Kiewitt I, Mukherjee KD (1990) Lipase from *Brassica napus* L. discriminates against *cis*-4 and *cis*-6 unsaturated fatty acids and secondary and tertiary alcohols. Biochem Biophys Acta **1042**: 237-240

Ichihara K (1984) Sn-glycerol-3-phosphate acyltransferase in a particulate fraction from maturing safflower seeds. Arch Biochem Biophys **232**: 685-698

Ichihara K, Asahi T, Fujii S (1987) 1-Acyl-*sn*-glycerol-3-phosphate acyltransferase in maturing safflower seeds and its contribution to the non-random fatty acid distribution of triacylglycerol. Eur J Biochem **167**:339-347

Jacob J, Grimmer G (1968) Structure and amount of positional isomers of monounsaturated fatty acids in human depot fat. J Lipid Res **9**: 730-732

Jacobson BS, Jaworski JG, Stumpf PK (1974) Fat metabolism in higher plants. LXII. Stearoyl-acyl carrier protein desaturase from spinach chloroplasts. Plant Physiol. **54**: 484-486

Jaworski JG (1987) Biosynthesis of monoenoic and polyenoic fatty acids. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants: A Comprehensive Treatise, Vol 9, Lipids: Structure and Function. Academic Press, New York, p 159-174

Jaworski JG, Stumpf PK (1974) Fat metabolism in higher plants: Properties of a soluble stearoyl-acyl carrier protein desaturase from maturing *Carthamus tinctorius*. Arch Biochem Biophys **162**: 158-165

Kennedy EP (1961) Biosynthesis of complex lipids. Fed Proc Am Soc Exp Biol **20**: 934-940

Kinney AJ, Hitz WD, Yadav NS (1990) Stearoyl-ACP desaturase and a β-ketoacyl-ACP synthetase from developing soybean seeds. *In* PJ Quinn, JL Harwood, eds, Plant Lipid Biochemistry, Structure and Utilization. Portland Press, London, p 126-128

Kleiman R, Spencer GF (1982) Search for new industrial oils: XVI. Umbelliflorae-seed oils rich in petroselinic acid. J Am Oil Chem Soc **59**: 29-38

Löhden I, Frentzen M (1992) Triacylglycerol biosynthesis in developing seeds of *Tropaeolum majus* L. and *Limanthes douglasii* R.Br. Planta **188**: 215-224

McKeon T, Stumpf PK (1981) Stearoyl-acyl carrier protein desaturase from safflower seeds. Methods Enzymol **71**: 275-281

McKeon TA, Stumpf PK (1982) Purification and characterization of stearoyl-acyl carrier protein desaturase and the acyl-acyl carrier protein thioesterase from maturing seeds of safflower. J Biol Chem **257**: 12141-12147

Moreau RA, Pollard MR, Stumpf PK (1981) Properties of a Δ5-fatty acyl-CoA desaturase in the cotyledons of developing *Limanthes alba*. Arch Biochem Biophys **209**: 376-384

Nagai J and Bloch K (1965) Synthesis of oleic acid by *Euglena gracilis*. J Biol Chem **240**: PC3702-PC3703

Nagai J, Bloch K (1968) Enzymatic desaturation of stearyl acyl carrier protein. J Biol Chem **243**: 4626-4633

Nishida I, Beppu T, Matsuo T, Murata N (1992) Nucleotide sequence of a cDNA encoding a precursor to stearoyl-(acyl-carrier-protein) desaturase from spinach, *Spinacia oleracea*. Plant Mol Biol **19**: 711-713

Ohlrogge JB (1988) Molecular approaches to the study and modification of oilseed fatty acid synthesis. *In* TH Applewhite, ed, Proceedings World Conference on Biotechnology for the Fats and Oils Industry. American Oil Chemists Society, Champaign, IL, p 87-92

Ohlrogge JB, Emken EA, Gulley RM (1981) Human tissue lipids: Occurrence of fatty acid isomers from dietary hydrogenated oils. J Lipid Res **22**: 955-960

Oo K-C, Huang AHC (1989) Lysophosphatidate acyltransferase activities in the microsomes from palm endosperm, maize scutellum, and rapeseed cotyledon of maturing seeds. Plant Physiol **91**: 1288-1295

Palazzo FC, Tamburello A (1914) The iso-oleic acid of the seeds of ivy. Atti R Acad Lincei **23**: 352-356

Placek LL (1963) A review on petroselinic acid and its derivatives. J Am Oil Chem Soc **4**: 319-329

Pollard MR, Stumpf PK (1980) Biosynthesis of C_{20} and C_{22} fatty acids by developing seeds of *Limanthes alba*. Chain elongation and Δ5 desaturation. Plant Physiol **66**: 649-655

Prasad RBN, Rao YN, Rao SV (1987) Phospholipids of palash (*Butea monosperma*), papaya (*Carica papaya*), jangli badam (*Sterculia foetida*), coriander (*Coriandrum sativum*) and carrot (*Daucus carota*) seeds. J Am Oil Chem Soc **64**: 1424-1427

Putscher RE (1984) Polyamides. *In* Encyclopedia of Chemical Technology. Vol 18. John Wiley & Sons, New York, p 328-371

Sato A, Becker CK, Knauf VC (1992) Nucleotide sequence of a complimentary DNA clone encoding stearoyl-acyl carrier protein desaturase from *Simmondsia chinensis*. Plant Physiol **99**: 362-363

Shanklin J, Somerville C (1991) Stearoyl-acyl carrier protein desaturase from higher plants is structurally unrelated to the animal and fungal homologs. Proc Natl Acad Sci USA **88**: 2510-2514.

Shanklin J, Mullins C, Somerville C (1991) Sequence of a complementary DNA from *Cucumis sativus* L. encoding the stearoyl-acyl-carrier protein desaturase. Plant Physiol **97**: 467-468

Shibahara A, Yamamoto K, Takeoka M, Kinoshita A, Kajimoto G, Nakayama T, Noda M (1990) Novel pathways of oleic and *cis*-vaccenic acid biosynthesis by an enzymatic double-bond shifting reaction in higher plants. FEBS Lett **264**: 228-230

Shinohara T (1970) Chemical constituents of human skin lipids. IV. Monounsaturated acids in the free and ester-type fatty acids of human skin-surface lipids. Kagaku Keisatu Kenkyusho Hokoku **23**: 18-23

Siguel EN, Lermann RH (1993) Trans-fatty acid pattern in patients with angiographically documented coronary artery disease. Am J of Cardiology **71**: 916-920

Slabas AR, Roberts PA, Ormesher J, Hammond EW (1982) *Cuphea procumbens*. A model system for studying the mechanism of medium-chain fatty acid biosynthesis in plants. Biochem Biophys Acta **711**:411-420

Slack CR, Roughan PG, Balasingham N (1978) Labeling of glycerolipids in the cotyledons of developing oilseeds by [1-^{14}C]acetate and [2-^{3}H]glycerol. Biochem J **179**:421-433

Slack CR, Campbell LC, Browse JA, Roughan PG (1983) Some evidence for the reversibility of choline phosphotransferase-catalysed reaction in developing linseed cotyledons in vivo. Biochem Biophys Acta **754**: 10-20

Slocombe SP, Cummins I, Jarvis RP, Murphy DJ (1992) Nucleotide sequence and temporal regulation of a seed-specific *Brassica napus* cDNA encoding a stearoyl-acyl carrier protein (ACP) desaturase. Plant Mol Biol **20**: 151-155

Spencer GF, Kleiman R, Earle FR, Wolff IA (1970) The *trans*-6 fatty acids of *Picramnia sellowii* seed oil. Lipids **5**: 285-287

Spencer GF, Kleiman R, Miller RW, Earle FR (1971) Occurrence of *cis*-6-hexadecenoic acid as the major component of *Thunbergia alata* seed oil. Lipids **6**: 712-714

Steifel WK (1977) Skin treatment with emollient cis-6-hexadecenoic acid or derivatives. US Patent #4036991

Stymne S (1992) Biosynthesis of "uncommon" fatty acids and their incorporation into triacylglycerols. *In* N Murata, C Somerville, eds, Biochemistry and Molecular Biology of Membrane and Storage Lipids of Plants. American Society of Plant Physiologists, Rockville, MD, p 150-158

Stymne S, Bafor M, Jonsson L, Wiberg E, Stobart K (1990) Triacylglycerol assembly. *In* PJ Quinn, JL Harwood, eds, Plant Lipid Biochemistry, Structure and Utilization. Portland Press, London, p 191-197

Stymne S, Stobart AK (1986) Biosynthesis of γ-linolenic acid in cotyledons and microsomal preparations of the developing seeds of common borage (*Borago officinalis*) Biochem J **240**: 385-393

Stymne S, Stobart AK (1987) Triacylglycerol biosynthesis. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants: A Comprehensive Treatise, Vol 9, Lipids: Structure and Function. Academic Press, New York, p 175-211

Sun C, Cao Y-Z, Huang AHC (1988) Acyl coenzyme A preference of the glycerol phosphate pathway in the microsomes from the maturing seeds of palm, maize, and rapeseed. Plant Physiol **88**: 56-60

Taylor MA, Smith SB, Davies HV, Burch LR (1992) The primary structure of a cDNA clone of the stearoyl-acyl carrier protein desaturase gene from potato (*Solanum tuberosum* L.). Plant Physiol **100**: 533-534

Theimer RR, Schöpf UFM, Siegert HRJ, Radetzky R (1993) Changes in triacylglycerol storage and composition in cell cultures and somatic embryos of *Pimpinella anisum* L. Abstracts of Biotechnological Aspects of Plant Lipids, Carmona, Spain, May 26-29, 1993, p 35

The Lipid Handbook (1986) FD Gunstone, JL Harwood, FB Padley, eds, Chapman and Hall, New York

The Merck Index, Tenth Edition (1983) Merck & Company, Rahway, NJ

Thompson GA, Scherer DE, Foxall-Van Aken S, Kenny JW, Young HL, Shintani DK, Kridl JC, Knauf VC (1991) Primary structures of the precursor and mature forms of stearoyl-acyl carrier protein desaturase from safflower embryos and requirement of ferredoxin for enzyme activity. Proc Natl Acad Sci USA **88**: 2578-2582

Weitkamp AW, Smiljanic AM, Rothman S (1947) The free fatty acids of human hair fat. J Am Chem Soc **69**: 1936-1939

Wiedermann LH (1978) Margarine and margarine oil, formulation and control. J Am Oil Chem Soc **55**: 823-829

van de Loo FJ, Fox BG, Somerville C (1993) Unusual fatty acids. *In* TS Moore Jr, ed, Lipid Metabolism in Plants. CRC Press, Boca Raton, FL, p 91-126

Voelker TA, Worrell AC, Anderson L, Bleibaum J, Fan C, Hawkins DJ, Radke SE, Davies HM (1992) Fatty acid biosynthesis redirected to medium chains in transgenic oilseed plants. Science **257**: 72-74

Vongerichten E, Köhler A (1909) Über Petroselinsäure, eine neue Ölsäure. Ber Dtsch Chem Ges **42**: 1638-1639

Zock PL, Katan MB (1992) Hydrogenation alternatives: Effects of *trans* fatty acids and stearic acid versus linoleic acid serum lipids and lipoproteins in human. J Lipid Res **33**: 399-410

CHAPTER 2[1]

Expression of a Novel Coriander Desaturase Results in Petroselinic Acid Production in Transgenic Tobacco

Abstract

Little is known about the metabolic origin of petroselinic acid ($18:1\Delta^{6cis}$), the principal fatty acid of the seed oil of most Umbelliferae, Araliaceae, and Garryaceae species. To examine the possibility that petroselinic acid is the product of an acyl-acyl carrier protein (ACP) desaturase, western blots of coriander (*Coriandrum sativum* L.) and other Umbelliferae seed extracts were probed with antibodies against the Δ^9stearoyl-ACP desaturase of avocado. In these extracts, proteins of 39 and 36 kDa were detected. Of these, only the 36 kDa peptide was specific to tissues that synthesize petroselinic acid. A cDNA encoding the 36 kDa peptide was isolated from a coriander cDNA library, placed under control of the cauliflower mosaic virus 35S promoter, and introduced into tobacco via *Agrobacterium tumefaciens*-mediated transformation. Expression of this cDNA in transgenic tobacco callus was accompanied by the accumulation of petroselinic acid and Δ^4hexadecenoic acid, both of which were absent in control callus. These results demonstrate the involvement of a 36 kDa putative acyl-ACP desaturase in the biosynthetic pathway of petroselinic acid and the ability to produce fatty acids of unusual structure in transgenic plants by the expression of a gene for a novel desaturase.

[1]Material in this chapter was published previously [Cahoon EB, Shanklin J, Ohlrogge JB (1992) Proc Natl Acad Sci USA 89: 11184-11188].

Introduction

Petroselinic acid (18:1$\Delta^{6\underline{cis}}$) is an unusual fatty acid that occurs primarily in seeds of Umbelliferae (or Apiaceae), Araliaceae, and Garryaceae species (Kleiman and Spencer, 1982). This fatty acid composes as much as 85% of the total fatty acid of Umbelliferae seeds but is virtually absent in leaves and other tissues of these plants (Ellenbracht *et al.*, 1980; Kleiman and Spencer, 1982; Dutta and Appelqvist, 1991). The structure of petroselinic acid differs from that of oleic acid (18:1$\Delta^{9\underline{cis}}$), a common plant fatty acid, by the position of its double bond. Because of the presence of unsaturation at the Δ^6 carbon atom, petroselinic acid is of potential industrial significance. Through chemical cleavage at its double bond, petroselinic acid can be used as a precursor of lauric acid (12:0), which is a component of detergents and surfactants, and adipic acid (6:0 dicarboxylic), which is the monomeric component of nylon 6,6 (Murphy, 1992).

The pathway for petroselinic acid biosynthesis has not been previously determined. Monounsaturated fatty acids of plants typically derive from the desaturation of C_{16} and C_{18} saturated fatty acids bound to acyl carrier protein (ACP) or to glycerolipids (Browse and Somerville, 1991; Jaworski, 1987). Our preliminary results from a variety of [^{14}C] labelling studies suggest that petroselinic acid is the product of an acyl-ACP desaturase (see Chapter 3). The only such enzyme to have been identified in plants is the Δ^9stearoyl-ACP (18:0-ACP) desaturase (EC 1.14.99.6) which catalyzes the conversion of 18:0-ACP to 18:1Δ^9-ACP (Nagai and Bloch, 1968; Jaworski and Stumpf, 1974). This reaction is readily assayable in tissue extracts of most plants using [^{14}C]18:0-ACP and cofactors including ferredoxin, NADPH, and ferredoxin-NADPH reductase (Jaworski and Stumpf, 1974; McKeon and Stumpf, 1981). However, the *in vitro* synthesis of petroselinic acid from [^{14}C]acyl-ACPs, including [1-^{14}C]18:0-ACP (or from [1-^{14}C]18:0-CoA), has yet to be detected using seed extracts of the Umbelliferae species coriander and carrot (see Chapter 3). Lack of a direct assay complicates any attempt to characterize the

biosynthetic pathway or to purify the acyl-ACP desaturase believed to be involved in petroselinic acid synthesis.

As an alternative approach, the possibility that this proposed acyl-ACP desaturase is antigenically related to $\Delta^9$18:0-ACP desaturase was examined. In this study, western blots of extracts of tissues that synthesize petroselinic acid were probed with antibodies raised against the $\Delta^9$18:0-ACP desaturase of avocado (Shanklin and Somerville, 1991) to identify any related peptides. These antibodies were also used to isolate cDNAs from a coriander endosperm expression library to directly explore the possible involvement of an acyl-ACP desaturase in the synthesis of petroselinic acid.

Materials and Methods

Western Blot Analysis

Plant tissues were homogenized in 50 mM potassium phosphate (pH 7.2), 2 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM sodium metabisulfite, 5 mM EDTA, 5 mM isoascorbate (5 ml/g fresh weight) with a Polytron PT10/35 (Brinkman), passed through two layers of miracloth (Calbiochem), and mixed with SDS/PAGE sample buffer.

Protein extracts of transgenic tobacco calli were obtained by homogenization of tissue with an Elvehjem homogenizer in 2 ml of a solution of 0.7 M sucrose, 0.5 M Tris, 50 mM EDTA, 0.1 M potassium chloride, pH 9.4, containing 2% (v/v) β-mercaptoethanol and 2 mM PMSF added just prior to use. The homogenate was mixed thoroughly with 2 ml of phenol and centrifuged at 3000x *g* for 10 min. The upper phenol phase was recovered, and proteins were precipitated with the addition of 10 ml of 0.1 M ammonium acetate in methanol, and overnight incubation at -20°C. The protein pellet obtained following centrifugation was washed sequentially with methanolic ammonium acetate and acetone then air-dried prior to addition of SDS/PAGE sample loading buffer.

Proteins of plant extracts were separated by SDS/PAGE (Laemmli, 1970) using 11% (w/v) acrylamide gels. Proteins were transferred from

gels and fixed to nitrocellulose as described (Post-Beittenmiller *et al.*, 1989). Western blot analyses were performed using polyclonal, immunoaffinity-purified antibodies raised against the $\Delta^9$18:0-ACP desaturase of avocado (Shanklin and Somerville, 1991) and colorimetric detection as described (Jabben *et al.*, 1989).

cDNA Library Construction

Total RNA was isolated from the endosperm and embedded embryo of developing seed of coriander (*Coriandrum sativum* L.) cremocarps (fruits) collected at stages ranging from early through mid-development. Tissue samples were ground in liquid nitrogen, transferred to a Elvehjem tissue grinder, and homogenized for an additional 2 min in a buffer (3.5 ml/g tissue) preheated to 80°C consisting of 0.2 M sodium borate, 30 mM EGTA, 1% SDS, 1% deoxycholate, 2% polyvinylpyrollidone 40,000, 10 mM dithiothreitol (freshly added), pH 8.5. The remainder of the total RNA purification was performed as described by Hall *et al.* (1978). Poly A^+ RNA isolation from coriander total RNA and subsequent cDNA library construction were performed by Stratagene, Inc. cDNA was synthesized using an oligo-dT primer and inserted into the EcoRI site of the λ-ZAP II vector (Stratagene).

Isolation and Characterization of cDNA Clones

The coriander endosperm cDNA library was subjected to immunological screening as described (Sambrook *et al.*, 1989) using an immunoaffinity-purified polyclonal antibody against $\Delta^9$18:0-ACP desaturase of avocado (Shanklin and Somerville, 1991). Immunopositive clones were purified to homogeneity, and pBluescript SK(-) phagemid was excised as described (Short *et al.*, 1988). Nucleotide sequence was obtained for both strands of DNA by dideoxy chain-termination using Sequenase 2.0 (U.S. Biochemical).

To obtain a full-length Type II clone, the coriander cDNA endosperm library was re-screened using an [α-^{32}P]dCTP random-labelled DNA probe derived from a 394 bp *Nco*I restriction fragment of a partial Type II cDNA

clone. Library screening was performed as described (Sambrook *et al.*, 1989).

Expression of cDNAs in *E. coli*

Polymerase chain reaction (PCR) primers were designed from the 5′ and 3′ ends of Type I and II cDNA sequences encompassing the mature peptide (native protein minus the plastid transit peptide). Type I cDNA primers contained flanking *Xba*I and *Nde*I restriction site recognition sequences (5′ primer, 5′TGGTCTAGACATATGGCCTCTACTCTTGGCATC3′ and 3′ primer, 5′ACCTCTAGACATATGTACAGACCACAATAAA3′). Type II cDNA primers were designed with flanking *Eco*RI and *Nde*I restriction sites (5′ primer, 5′TAGGAATTCATATGGCTTCAACTCTTCAT3′ and 3′ primer, 5′ACCGAATTCATATGATGATCTGACG3′). PCRs consisted of 50-100 ng of Type I or TypeII cDNAs contained in pBluescript SK(-), 0.25 μM of primers, 0.16 mM dNTPs, 1x reaction buffer, and 1 U Taq DNA polymerase (Boehringer Mannheim) in a 25 μl reaction volume. Reactions were performed with a Perkin-Elmer/Cetus thermal cycler with a program of 5 min at 94°C; 30 cycles of 1.1 min at 94°C, 2 min at 55°C, 3 min at 72°C; followed by 10 min at 72°C.

The Type I and Type II-derived PCR products were digested with *Xba*I and *Eco*RI, respectively, and ligated into the corresponding sites of pBluescript KS(+). Following amplification in *E. coli* strain DH5α, plasmids were isolated, digested with *Nde*I, gel-purified, and inserted into the *Nde*I site of the pET3a *E. coli* expression vector (Studier *et al.*, 1990). Type I and II-containing plasmids were introduced into the *E. coli* strain BL21 and grown under carbenicillin selection as described (Studier *et al.*, 1990).

The Type II sequence contained an indigenous *Nde*I restriction site within its translational stop codon. As a result, the protein expressed from this cDNA included at its C-terminus three additional amino acids encoded by the pET3a vector.

Cells containing the expression plasmids lacking insert or with Type I or Type II mature peptide-encoding sequence were grown to A_{600}=0.6,

induced with isopropyl β-D-thiogalactopyranoside (IPTG) (0.4 mM final concentration), and grown for an additional 4 h. Protein extracts from these cells were separated by SDS/PAGE and analyzed by Coomassie staining or by western blotting as described above. In the case of BL21 cells with Type I-containing plasmids, washed pelleted cells were lysed by four cycles of freezing in liquid nitrogen followed by thawing in a 25°C water bath. Cell debris was pelleted and the soluble extract was used for $\Delta^9$18:0-ACP desaturase assays as described (McKeon and Stumpf, 1981).

Expression of Type II cDNA in Tobacco

PCR primers were designed from 5′ and 3′ terminal sequences of the full-length Type II cDNA. These primers included a flanking *Bam*HI restriction site on the 5′ primer and flanking *Bam*HI and *Sac*I restriction sites on the 3′ primer (5′ primer, 5′TAGGATCCATGGCCATGAAACTGAAT3′ and 3′primer, 5′ACGGATCCGAGCTCTCGACGACCACTCATATG3′). PCRs were performed as described above except Vent DNA polymerase (New England Biolabs) was used. The PCR product was gel purified, digested with *Bam*HI, and cloned into pBluescript KS(+). The insert was excised by cleavage with *Bam*HI and *Sac*I, gel-purified, and ligated into the *Bam*HI and *Sac*I sites of pBI121 (Jefferson *et al.*, 1987) behind the cauliflower mosaic virus (CaMV) 35S promoter. The resulting plasmid pEC301 was subsequently introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation (Mersereau *et al.*, 1990). The transformed *Agrobacterium* cells were grown at 27°C under kanamycin selection and co-cultivated with leaf disks of tobacco (*Nicotiana tabacum* L.) as described (Rogers *et al.*, 1986).

Fatty Acid Analysis of Transgenic Tobacco

Fatty acid methyl esters were prepared from transgenic tobacco calli by heating of tissue at 90°C for 40 min in 10% (w/v) boron trichloride/methanol (Alltech) supplemented with 15% toluene (v/v). Resulting methyl esters were analyzed by gas chromatography using a Hewlett-Packard 5890 gas chromatograph with a 50 m x 0.25 mm i.d. CP-Si188 column (Chrompack) and flame ionization detection. Oven temperature was programmed from 155°C (60 min hold) to 175°C at 2.5°C/min with a column

head pressure of 7.5 psi He. Samples of monounsaturated fatty acid methyl esters for gas chromatography-mass spectroscopy (GC-MS) analyses were purified by argentation TLC (Morris *et al.*, 1967). TLC plates were prepared by saturation of silica gel K6 (Whatman) TLC plates in 15% (w/v) silver nitrate/acetonitrile. Fatty acid methyl esters were separated by development of argentation TLC plates in toluene at -20°C. Double bonds of purified monounsaturated fatty acids were converted to thiomethyl adducts by reaction with dimethyl disulfide (Aldrich) in the presence of I_2 as described (Yamamoto *et al.*, 1991) except that derivitization was performed for two hours. Derivatized samples were analyzed by GC-MS with a Hewlett-Packard 5890 GC coupled to a Hewlett-Packard 5970A mass selective detector (MSD) using a 15 m x 0.25 mm i.d. DB-17 column (J&W Scientific) with the oven temperature programmed from 185°C to 230°C at 10°C/min. The MSD inlet temperature was 280°C, and the ionizing potential of the MSD was 70 eV.

Results

Immunodetection of Two Putative Acyl-ACP Desaturases in Umbelliferae Seed Extracts

A primary goal of our research was to determine the metabolic origin of the *cis*Δ^6 double bond of petroselinic acid using seeds of the Umbelliferae species coriander and carrot. In experiments described in Chapter 3, [1-^{14}C]stearic acid (18:0) or [1-^{14}C]palmitic acid (16:0) fed to endosperm slices of coriander and carrot was incorporated into glycerolipids but not desaturated. However, crude homogenates of coriander endosperm were capable of the *de novo* synthesis of petroselinic acid from [2-^{14}C]malonyl-CoA. The majority of the resulting [^{14}C]petroselinic acid was detected as free fatty acid. A smaller portion of the recovered petroselinic acid was associated with protein, presumably representing the acyl-ACP fraction. These results, therefore, suggested that petroselinic acid derives from an acyl-ACP rather than a glycerolipid-type desaturase. Despite this, we were unable to demonstrate

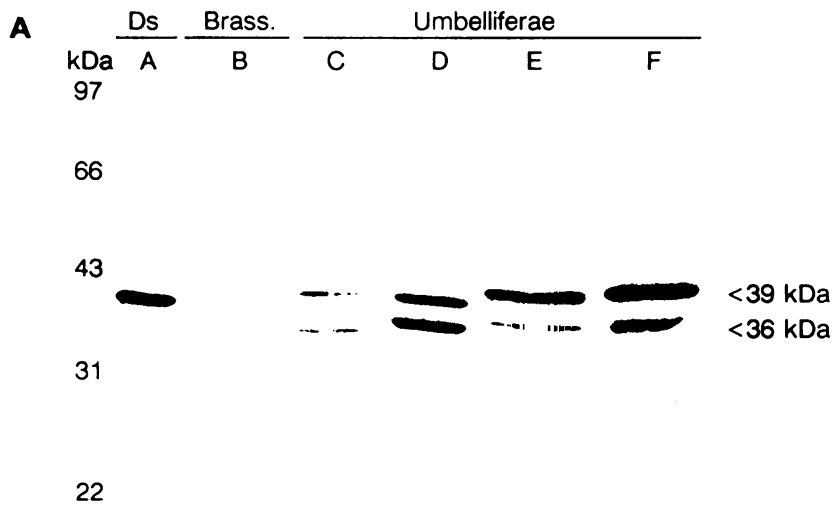
the *in vitro* synthesis of petroselinic acid from [1-^{14}C]stearoyl- or [1-^{14}C]palmitoyl-ACP as well as [1-^{14}C]stearoyl-CoA (Chapter 3).

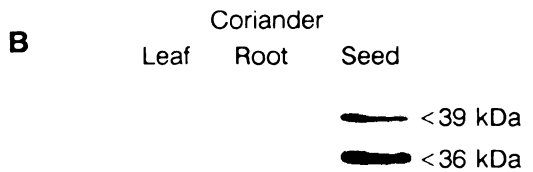
Using an alternate approach, we investigated the possibility that the proposed acyl-ACP desaturase associated with petroselinic acid synthesis is related to the $\Delta^9$18:0-ACP desaturase. This enzyme was recently purified to homogeneity from avocado (Shanklin and Somerville, 1991), safflower (Thompson *et al.*, 1991), and soybean (Cheesbrough and Cho, 1990; Kinney *et al.*, 1990) and corresponding cDNAs isolated from castor (Shanklin and Somerville, 1991), cucumber (Shanklin *et al.*, 1991), safflower (Thompson *et al.*, 1991), and soybean (Kinney *et al.*, 1990). A polyclonal antibody raised against the $\Delta^9$18:0-ACP desaturase of avocado (Shanklin and Somerville, 1991) was used to probe western blots of extracts of developing seeds of coriander and several other Umbelliferae species. In these extracts, two immunoreactive proteins of an apparent mass on SDS/PAGE of 39 and 36 kDa were detected (Figure 2.1A, Lanes C-F). In contrast, tissues that do not synthesize petroselinic acid, *e.g.*, vegetative tissues of coriander (Figure 2.1B) and seeds of species outside of the Umbelliferae, Araliaceae, or Garryaceae families (Figure 2.1A, Lane B), contained only a 39 kDa protein with antigenic recognition by anti-$\Delta^9$18:0-ACP desaturase antibodies. From these results, it was hypothesized that the 39 kDa peptide is a $\Delta^9$18:0-ACP desaturase, and the additional 36 kDa peptide, seen only in tissues which contain petroselinic acid, is an acyl-ACP desaturase associated with the synthesis of this fatty acid.

Isolation of Putative Coriander Acyl-ACP Desaturase cDNA Clones

As an initial step in determining the function of the 39 and 36 kDa peptides identified on western blots, clones encoding acyl-ACP desaturases were isolated from a cDNA expression library prepared from coriander endosperm. Screening of the library with antibodies against avocado $\Delta^9$18:0-ACP desaturase yielded two classes of clones, designated Type I and Type II, as determined by partial nucleotide sequencing. Translation of the 5′ nucleotide sequence revealed that both Type I and Type II cDNAs contained considerable amino acid sequence similarity with those of

Figure 2.1. Western blot analyses of seed extracts of Brassicaceae (Brass.) and Umbelliferae spp. (*A*) and leaf, root, and seed extracts of coriander (*Coriandrum sativum* L.) (*B*). Blot (*A*) lanes are: A, castor recombinant $\Delta^9$18:0-ACP desaturase (Ds) (600 ng) (see Shanklin and Somerville, 1991); B, crambe (*Crambe abyssinica* L.); C, coriander; D, wild carrot (*Daucus carota* L.); E, sweet cicely (*Myrrhis odorata* L.); and F, angelica (*Angelica archangelica* L.) seed extracts (50-60 μg). (Lane B is a Cruciferae species. Lanes C-F are Umbelliferae species). Blot (*B*) lanes are: leaf (110 μg), root (110 μg), and seed (60 μg) extracts of coriander.


A
Ds
Brass.
Umbelliferae
kDa
A
B
C
D
E
F
97
66
43
31
22
<39 kDa
<36 kDa


B
Coriander
Leaf
Root
Seed
<39 kDa
<36 kDa

previously isolated $\Delta^9$18:0-ACP desaturase cDNAs of castor (Shanklin and Somerville, 1991), cucumber (Shanklin and Somerville, 1991), and safflower (Thompson *et al.*, 1991). However, a region near the amino-terminus of translated Type II clones exhibited marked divergence with a similar region in translated Type I (data not shown) and $\Delta^9$18:0-ACP desaturase clones (Figure 2.2). This divergence included the absence of a sequence encoding 15 amino acids in the Type II clone relative to the Type I and to the previously reported $\Delta^9$18:0-ACP desaturase cDNAs (Shanklin and Somerville, 1991; Thompson *et al.*, 1991).

The $\Delta^9$18:0-ACP desaturase is found in the plastid of plant cells (Jaworski, 1987). A similar localization would be expected for other acyl-ACP desaturases. However, the longest Type I and II cDNA clones obtained through antibody screening of the expression library lacked nucleotide sequence encoding for an entire plastid transit peptide. A full-length Type II clone (Figure 2.2) was isolated by screening the coriander cDNA library with a nucleotide probe derived from a 394 bp *Nco*I restriction fragment of a partial Type II cDNA. The full-length 1309 bp cDNA clone contained a methionine codon at nucleotide 7 with surrounding bases that differed by only one nucleotide from a plant consensus translational start site (Lütcke *et al.*, 1987). The open-reading frame of the Type II cDNA consisted of 1155 nucleotides encoding a 385-amino acid peptide with a calculated molecular weight of 43.8 kDa. By homology with the deduced amino acid sequence of castor (Shanklin and Somerville, 1991), cucumber (Shanklin and Somerville, 1991), and safflower (Thompson *et al.*, 1991) $\Delta^9$18:0-ACP desaturase cDNAs, the encoding sequence of the mature protein likely begins at nucleotide 115 of the Type II cDNA. The 108 bases preceding the start of the mature peptide encoded a 36 amino acidsequence with properties similar to that of plastid transit peptides (Keegstra *et al.*, 1989). The transit peptide encoded by the Type II cDNA was 3 amino acids longer than those encoded by the $\Delta^9$18:0-ACP desaturase cDNAs reported to date (Shanklin and Somerville, 1991; Thompson *et al.*, 1991) and possessed a markedly different amino acid sequence composition.

Figure 2.2. Nucleotide sequence of the coriander Type II acyl-ACP desaturase cDNA (TII) and a comparison of the deduced amino acid sequences of TII and castor $\Delta^9$18:0-ACP desaturase cDNA (Cas) (Shanklin and Somerville, 1991). Identical amino acids are indicated by colons. Amino acids which are absent relative to either of the two sequences are indicated by dashed lines. Alignment of the TII nucleotide sequence is maintained with a dotted line. The underlined alanine (amino acid 37) indicates the likely amino-terminus of the mature peptide encoded by the Type II cDNA.

```
TII     GCAAAAATGGCCATGAAACTGAATGCCCTCATGACTCTTCAGTGCCCAAAAAGGAACATGTTTACGAGAATTGCCCCTCCT    81
TII           M  A  M  K  L  N  A  L  M  T  L  Q  C  P  K  R  N  M  F  T  R  I  A  P  P     25
CAS              M  A  L  K  :  N  P  F  L  S  Q  T  Q  K  L  P  S  F  A  L  :  :           22

TII  CAAGCAGGGAGAGTGAGATCAAAGGTGTCCATGGCTTCAACTCTTCATGCTAGCCCACTGGTGTTCGACAAGCTGAAGGCTGGG  165
TII   Q  A  G  R  V  R  S  K  V  S  M  A  S  T  L  H  A  S  P  L  V  F  D  K  L  K  A  G    53
CAS   M  :  S  T  R  S  P  :  F  Y  :  :  :  :  :  K  S  G  S  K  E  V  E  N  :  :  K  P    50

TII  AGGCCT.........GAGGTG.....................................GATGAATTGTTCAACTCTCTGGAGGGT  204
TII   R  P  -  -  -  E  V  -  -  -  -  -  -  -  -  -  -  -  -  D  E  L  F  N  S  L  E  G    66
CAS   F  M  P  P  R  :  :  H  V  Q  V  T  H  S  M  P  P  Q  K  I  :  I  :  K  :  :  D  N    78

TII  TGGGCCAGGGACAACATCCTTGTGCACCTGAAATCCGTAGAGAACTCATGGCAGCCGCAAGACTATCTGCCCGATCCCACATCC  288
TII   W  A  R  D  N  I  L  V  H  L  K  S  V  E  N  S  W  Q  P  Q  D  Y  L  P  D  P  T  S    94
CAS   :  :  E  E  :  :  :  :  :  :  :  P  :  :  K  C  :  :  :  :  :  F  :  :  :  :  A  :   106

TII  GATGCATTTGAAGATCAAGTCAAGGAGATGAGAGAACGGGCCAAGGACATCCCTGATGAATACTTTGTTGTTCTTGTTGGAGAC  372
TII   D  A  F  E  D  Q  V  K  E  M  R  E  R  A  K  D  I  P  D  E  Y  F  V  V  L  V  G  D   122
CAS   :  G  :  D  E  :  :  R  :  L  :  :  :  :  :  E  :  :  :  D  :  :  :  :  :  :  :  :   134

TII  ATGATCACTGAAGAGGCACTCCCAACTTACATGTCTATGCTTAACAGATGTGATGGCATTAAGGATGACACTGGCGCTCAACCT  456
TII   M  I  T  E  E  A  L  P  T  Y  M  S  M  L  N  R  C  D  G  I  K  D  D  T  G  A  Q  P   150
CAS   :  :  :  :  :  :  :  :  :  :  Q  T  :  :  :  T  L  :  :  V  R  :  E  :  :  :  S  :   162

TII  ACTTCTTGGGCCACTTGGACCAGGGCTTGGACTGCTGAGGAGAACCGCCATGGCGATCTTCTCAACAAGTATCTTTATCTCTCT  540
TII   T  S  W  A  T  W  T  R  A  W  T  A  E  E  N  R  H  G  D  L  L  N  K  Y  L  Y  L  S   178
CAS   :  :  :  :  I  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :   190

TII  GGCCGAGTTGATATGAGGATGATTGAGAAGACTATTCAATATCTTATCGGCTCTGGAATGGATACAAAAACAGAGAACTGTCCC  624
TII   G  R  V  D  M  R  M  I  E  K  T  I  Q  Y  L  I  G  S  G  M  D  T  K  T  E  N  C  P   206
CAS   :  :  :  :  :  :  Q  :  :  :  :  :  :  :  :  :  :  :  :  :  :  P  R  :  :  :  S  :   218

TII  TACATGGGCTTCATCTACACATCTTTCCAGGAAAGAGCCACATTCATCTCCCATGCCAACACAGCCAAACTTGCTCAACACTAC  708
TII   Y  M  G  F  I  Y  T  S  F  Q  E  R  A  T  F  I  S  H  A  N  T  A  K  L  A  Q  H  Y   234
CAS   :  L  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  :  G  :  :  :  R  Q  :  K  E  H   246

TII  GGTGACAAGAACCTAGCTCAAGTGTGTGGCAACATTGCTTCTGACGAGAAACGCCATGCCACCGCCTACACCAAAATCGTGGAG  792
TII   G  D  K  N  L  A  Q  V  C  G  N  I  A  S  D  E  K  R  H  A  T  A  Y  T  K  I  V  E   262
CAS   :  :  I  K  :  :  :  I  :  :  T  :  :  A  :  :  :  :  :  E  :  :  :  :  :  :  :  :   274

TII  AAGCTTGCGGAGATTGACCCAGACACCACTGTTATCGCATTTTCTGACATGATGAGGAAGAAAATACAAATGCCAGCTCATGCA  876
TII   K  L  A  E  I  D  P  D  T  T  V  I  A  F  S  D  M  M  R  K  K  I  Q  M  P  A  H  A   290
CAS   :  :  F  :  :  :  :  :  G  :  :  L  :  :  A  :  :  :  :  :  :  :  S  :  :  :  :  L   302

TII  ATGTACGATGGCTCCGATGATATGCTTTTCAAGCACTTCACAGCCGTTGCTCAGCAGATTGGAGTCTACTCTGCATGGGATTAC  960
TII   M  Y  D  G  S  D  D  M  L  F  K  H  F  T  A  V  A  Q  Q  I  G  V  Y  S  A  W  D  Y   318
CAS   :  :  :  :  R  :  :  N  :  :  D  :  :  S  :  :  :  :  R  L  :  :  :  T  :  K  :  :   330

TII  TGTGACATAATTGATTTTCTGGTGGATAAATGGAACGTTGCGAAGATGACAGGGCTGTCGGGTGAAGGGAGAAAGGCTCAAGAA 1044
TII   C  D  I  I  D  F  L  V  D  K  W  N  V  A  K  M  T  G  L  S  G  E  G  R  K  A  Q  E   346
CAS   A  :  :  L  E  :  :  :  G  R  :  K  :  D  :  L  :  :  :  :  A  :  :  Q  :  :  :  D   358

TII  TATGTTTGTAGCTTGGCTGCTAAGATCAGGAGAGTTGAGGAGAAGGTTCAAGGCAAGGAGAAGAAAGCTGTGTTGCCTGTGGCT 1128
TII   Y  V  C  S  L  A  A  K  I  R  R  V  E  E  K  V  Q  G  K  E  K  K  A  V  L  P  V  A   374
CAS   :  :  :  R  :  P  P  R  :  :  :  L  :  :  R  A  :  :  R  A  :  E  :  P  T  -  M  P   385

TII  TTCAGCTGGATTTTCAACCGTCAGATCATCATATGAGTGGTCGTCGACATTCAATATTAGACTTTTCAATTATGCTTATGCTTT 1212
TII   F  S  W  I  F  N  R  Q  I  I  I  *                                                   385
CAS   :  :  :  :  :  D  :  :  V  K  L  *

TII  TCCTTTTGATGTTATTATGTTTATGCTTATGCTATCGGTCGGTGTTTGTTGTCAGATCTGGTTATGTAAAACTTATATTTAAAT 1296

TII  GAATGTTGGATTT                                                                        1309
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In addition to the absence of 15 amino acids near its amino-terminus, the Type II cDNA-encoded peptide differed from that of $\Delta^9$18:0-ACP desaturase (Shanklin and Somerville, 1991; Thompson *et al.*, 1991) by the presence of one additional amino acid near its carboxyl-terminus. Excluding missing amino acids, the overall amino acid sequence identity of the mature Type II-encoded peptide and the mature castor $\Delta^9$18:0-ACP desaturase (Shanklin and Somerville, 1991) was 70%.

Expression of Type I and Type II Coriander cDNAs in *E. coli*

In an attempt to establish the identity of the Type I and Type II coriander clones, the mature peptide-encoding sequences of both cDNAs were engineered into the pET3a vector and expressed in the *E. coli* strain BL21 (Studier *et al.*, 1990). Protein encoded by the Type I cDNA was readily detectable in Coomassie-stained SDS-polyacrylamide gels of crude *E. coli* protein extracts (data not shown). This protein displayed immunological cross-reactivity with anti-$\Delta^9$18:0-ACP desaturase antibodies and exhibited identical mobility on SDS/PAGE as the 39 kDa protein detected in coriander seed extracts (data not shown). In addition, $\Delta^9$18:0-ACP desaturase activity could be detected in soluble extracts of *E. coli* expressing this protein (data not shown). The Type I cDNA was therefore identified as encoding a $\Delta^9$18:0-ACP desaturase. The Type II coriander cDNA was also expressed to high levels in *E. coli*, but the resulting protein was detected almost entirely as an inclusion body (data not shown). Western blots of urea-solubilized extracts from *E. coli* expressing the Type II clone revealed a 36 kDa immunoreactive peptide indistinguishable from the lower band of coriander seed extracts (data not shown). Because the Type II peptide was insoluble, we were unable to assay for activity directly, and preliminary attempts to obtain *in vitro* activity of the Type II-encoded protein after urea solubilization of the inclusion body were unsuccessful.

Expression of the Coriander Type II cDNA in Tobacco

As a direct means to test the hypothesis that the 36 kDa polypeptide is an acyl-ACP desaturase that results in petroselinic acid accumulation,

the Type II cDNA was cloned into the plant expression vector pBI121 (Jefferson *et al.*, 1987) and transformed into tobacco, a plant which contains no detectable petroselinic acid. Western blot analysis of the transgenic tobacco callus using anti-$\Delta^9$18:0-ACP desaturase antibodies revealed an additional peptide of 36 kDa in extracts of callus containing the Type II cDNA that was absent in callus transformed with only the pBI121 vector (Figure 2.3). The lack of detectable higher molecular weight precursors on western blots suggests that tobacco calli are capable of properly processing the transit peptide of the expressed product of the coriander Type II cDNA.

To determine whether the peptide encoded by the Type II cDNA possessed *in vivo* activity, the fatty acid composition of the transgenic callus was examined by gas chromatography (Figure 2.4A,B). Callus transformed with the Type II cDNA contained two fatty acids not present in callus transformed with the pBI121 vector alone (Figure 2.4B, Peaks 2 & 5). The retention times of methyl ester derivatives of these fatty acids coincided with those of methyl hexadecenoic acid (16:1) and methyl petroselinic acid. In ten callus samples analyzed, levels of each of these fatty acids ranged from 1 to 4 wt% of the total. To determine the double bond position of hexadecenoic acid and confirm the double bond position of petroselinic acid, monounsaturated fatty acid methyl esters of the transgenic calli were derivatized with dimethyl disulfide (Yamamoto *et al.*, 1991), and analyzed by GC-MS (Figure 2.5A,B). The mass spectrum of the derivatized petroselinic acid contained the same diagnostic ions as a petroselinic acid standard. In addition, the mass spectrum of the derivatized hexadecenoic acid of transgenic calli contained ions diagnostic for a Δ^4 isomer (Francis, 1981).

Discussion

The synthesis of petroselinic acid in Umbelliferae species is an apparently tissue-specific event. Seeds of these plants are rich sources

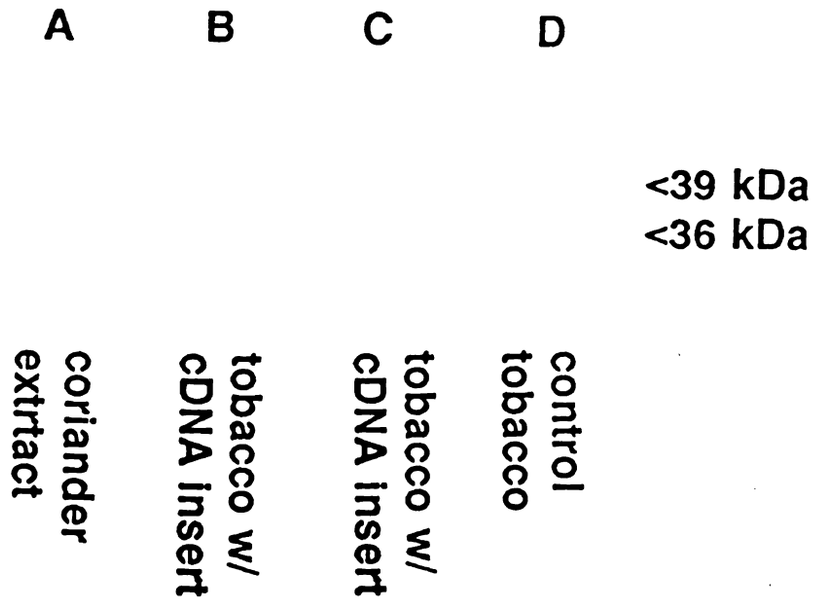

Figure 2.3. Western blot analysis of protein extracts from callus of transgenic tobacco. Proteins of crude extracts were separated by 11% SDS/PAGE, transferred to nitrocellulose, and probed with polyclonal antibodies against the $\Delta^9$18:0-ACP desaturase antibody of avocado. Lanes: A, coriander seed extract; B, C, tobacco callus transformed with pBI121 containing the coriander Type II cDNA; D, tobacco callus transformed with the pBI121 expression vector without insert. Lanes B and C represent extracts from two independent samples of calli transformed with the Type II cDNA.

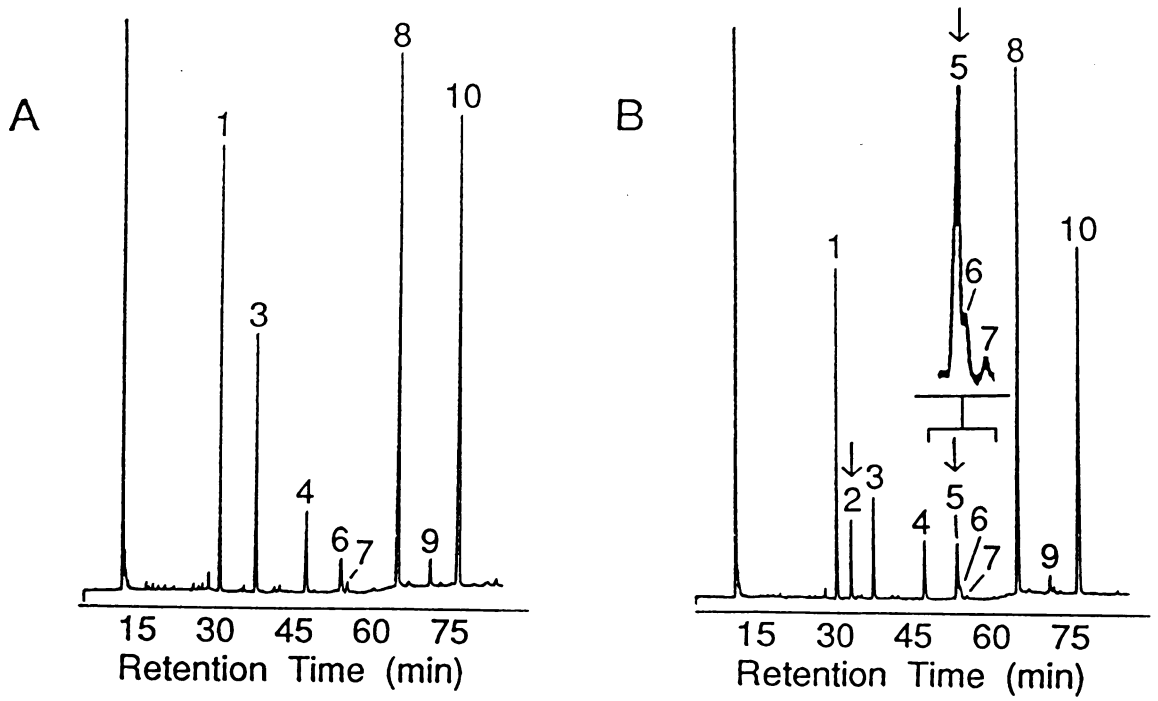

Figure 2.4. Gas chromatograms of fatty acid methyl esters prepared from tobacco callus transformed with the pBI121 vector (*A*) and pBI121 containing the coriander Type II cDNA insert (*B*). Arrows indicate fatty acids present only in callus transformed with the Type II insert. The double bond positions of Δ^4hexadecenoic acid (16:1Δ^4) (Peak 2) and petroselinic acid (18:1Δ^6) (Peak 5) were determined by GC-MS (see Figure 5). Inset in *B* is an enlargement of peaks (Peaks 5, 6 & 7) corresponding to the closely eluting isomers of 18:1. Peak identification: 1, 16:0; 2, 16:1Δ^4; 3, 17:0 (internal standard); 4, 18:0; 5, 18:1Δ^6; 6, 18:1Δ^9; 7, 18:1Δ^{11}; 8, 18:2; 9, 20:0; 10, 18:3.

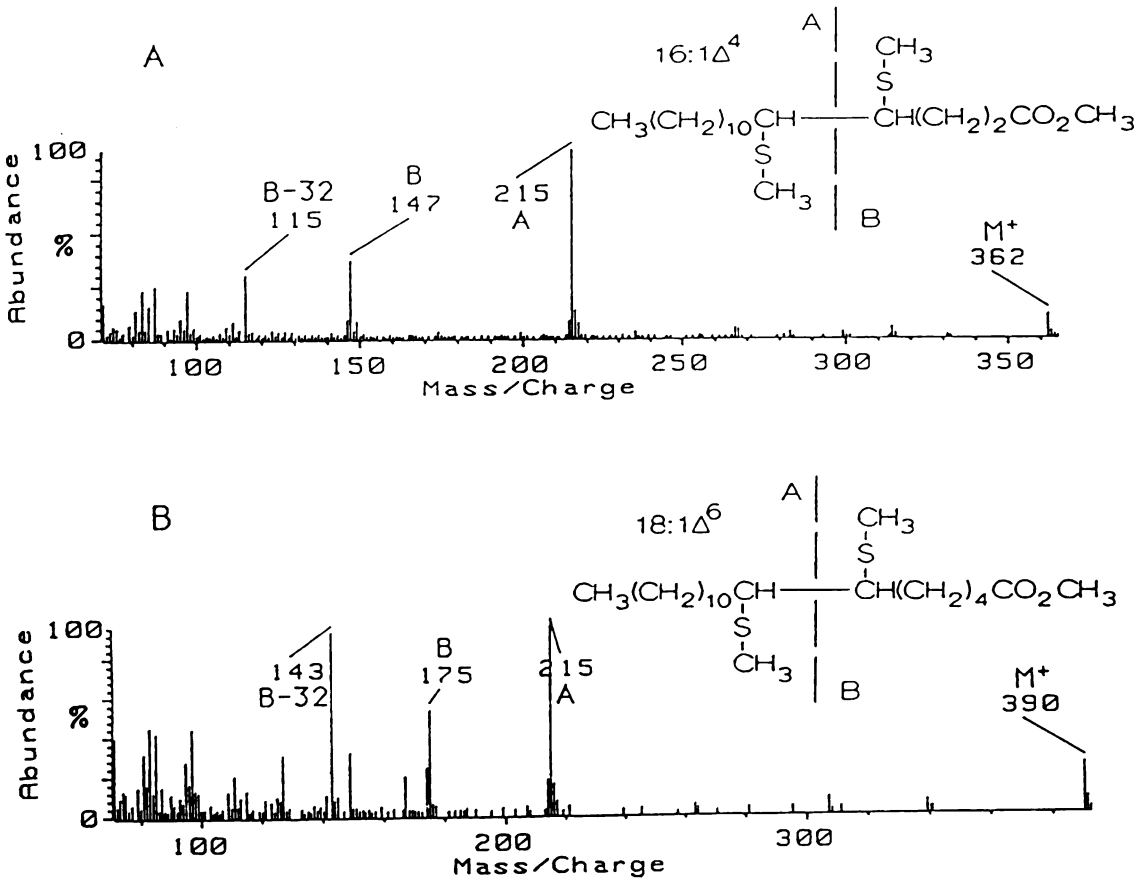

Figure 2.5. Mass spectra of thiomethyl adducts of fatty acid methyl esters detected exclusively in tobacco callus transformed with the Type II coriander cDNA insert in pBI121. Shown are mass spectra of Δ^4hexadecenoic acid (16:1Δ^4) (Peak 2, Figure 4B) (*A*) and petroselinic acid (18:1Δ^6) (Peak 5, Figure 4B) (*B*) derivatives prepared from fatty acid methyl esters of transgenic tobacco callus as described in the text.

of this fatty acid (Ellenbracht *et al.*, 1980; Kleiman and Spencer, 1982; Dutta and Appelqvist, 1991), but other tissues including leaves and roots contain little or no petroselinic acid (Ellenbracht *et al.*, 1980; Dutta and Appelqvist, 1991). Results of preliminary metabolic studies have suggested that petroselinic acid in Umbelliferae seed is derived from the activity of an acyl-ACP desaturase (see Chapter 3). In the current study, using a polyclonal antibody against the $\Delta^9$18:0-ACP desaturase of avocado, proteins of 39 and 36 kDa were detected on western blots of seed extracts of coriander and various other Umbelliferae species. Consistent with a role in petroselinic acid synthesis, the 36 kDa peptide, in contrast to the 39 kDa peptide, was identified only in tissues that produce this fatty acid. A cDNA clone (Type II) encoding a mature peptide of similar mobility on SDS/PAGE as the 36 kDa protein was isolated from a coriander endosperm cDNA library using anti-$\Delta^9$18:0-ACP desaturase antibodies (Shanklin and Somerville, 1991). *Agrobacterium*-mediated transformation of tobacco, a species which lacks petroselinic acid, with a full-length Type II clone resulted in the expression of the 36 kDa peptide and the appearance of petroselinic acid in the transgenic callus. These results demonstrate that the biosynthetic pathway of petroselinic acid involves a desaturation step catalyzed by a 36 kDa peptide with antigenic relation to the $\Delta^9$18:0-ACP desaturase. The high degree of amino acid sequence identity with the $\Delta^9$18:0-ACP desaturase, the presence of a plastid transit peptide in its native form, and results of previous metabolic studies (see Chapter 3) strongly suggest that the 36 kDa desaturase uses an acyl-ACP as its *in vivo* substrate. Previously, oleic acid was the only plant fatty acid known to be synthesized via an acyl-ACP desaturase (Nagai and Bloch, 1968; Jaworski and Stumpf, 1974; McKeon and Stumpf, 1982).

An unexpected finding from this work was the appearance of nearly equal levels of Δ^4hexadecenoic acid and petroselinic acid in tobacco callus expressing the 36 kDa desaturase. Several hexadecenoic acid (16:1) isomers are detectable in lipid extracts of coriander seed. One of these isomers has been identified by mass spectrometry as Δ^4hexadecenoic acid

(unpublished data). However, amounts of this fatty acid are typically ≤0.2 wt% of the total fatty acid of coriander seed extracts. In contrast, petroselinic acid accounts for ≥70 wt% of the total fatty acid of coriander seed (Kleiman and Spencer, 1982).

Despite our demonstration that the synthesis of petroselinic acid involves a unique 36 kDa putative acyl-ACP desaturase, the immediate precursor of this fatty acid cannot be definitively identified from these experiments. The detection of Δ^4hexadecenoic acid in addition to petroselinic acid in transgenic tobacco callus, however, does suggest possible biosynthetic origins of the Δ^6 double bond of petroselinic acid. If the location of double bond placement is determined by the position of carbon atoms from the methyl (or ω) end of acyl chains, then it is conceivable that the 36 kDa peptide is an ω^{12} desaturase. As such, this enzyme could catalyze the ω^{12} desaturation of both palmitoyl (16:0)- and stearoyl (18:0)-ACP resulting in Δ^4hexadecenoyl- and petroselinoyl-ACP, respectively. Alternatively, if double bond placement is dictated by the position of carbon atoms from the carboxyl (or Δ) end of acyl chains, then it is unlikely that the 36 kDa peptide catalyzes both the Δ^4 and Δ^6 desaturation of palmitoyl- and stearoyl-ACP, respectively. Instead, petroselinic acid might result from the desaturation of a shorter chain acyl-ACP by the 36 kDa peptide followed by elongation to petroselinoyl-ACP. For example, a possible biosynthetic pathway might consist of the Δ^4 desaturation of palmitoyl-ACP to Δ^4hexadecenoyl-ACP with subsequent two-carbon elongation to petroselinoyl-ACP.

The synthesis of petroselinic acid and Δ^4hexadecenoic acid in transgenic tobacco demonstrates the ability to produce new unsaturated fatty acids via gene transfer technology. This finding suggests the potential for the development of a new plant oil (*i.e.*, a high petroselinate oil) in an existing oilseed crop. However, levels of petroselinic acid in the transgenic tobacco callus were 1 to 4 wt% of the total fatty acid. This rather low amount of petroselinic acid may suggest that other factors are required for high levels of accumulation. Such

factors may include, for example, an acyl-ACP thioesterase specific for petroselinoyl-ACP. It is also possible that petroselinic acid may not be a preferred substrate for membrane lipid metabolism. As such, higher levels of this fatty acid might be expected to accumulate in the seed oil of transgenic plants.

Finally, the $\Delta^9$18:0-ACP desaturase (Shanklin and Somerville, 1991; Thompson *et al.*, 1991) and the 36 kDa desaturase of coriander share a high degree of amino acid identity. The primary difference between these amino acid sequences occurs near their amino-termini. The divergence in this region includes the absence of 15 amino acids in the 36 kDa desaturase relative to the $\Delta^9$18:0-ACP desaturase. It is intriguing to speculate that the alteration in double bond placement relates to this difference in the primary structures of these enzymes. Regardless, comparative biochemical studies of the $\Delta^9$18:0-ACP desaturase and the 36 kDa desaturase of coriander may allow for a better understanding of the relationship between the catalytic mechanism and the active sites of fatty acid desaturases.

Literature Cited

Browse J, Somerville C (1991) Glycerolipid synthesis: Biochemistry and regulation. Annu Rev Plant Physiol Plant Mol Biol **42**: 467-506

Cheesbrough TM, Cho SH (1990) Purification and characterisation of soyabean stearoyl-ACP desaturase. *In* PJ Quinn, JL Harwood, eds, Plant Lipid Biochemistry, Structure and Utilization. Portland Press, London, p 129-130

Dutta PC, Appelqvist L-A (1991) Lipids and fatty acid patterns in developing seed, leaf, root, and in tissue culture initiated from embryos of *Daucus carota* L. Plant Sci **75**: 177-183

Ellenbracht F, Barz W, Mangold HK (1980) Unusual fatty acids in the lipids from organs and cell cultures of *Petroselinum crispum*. Planta **150**: 114-119

Francis GW (1981) Alkylthiolation for the determination of double-bond position in unsaturated fatty acid esters. Chem Phys Lipids **29**: 369-374

Hall TC, Ma Y, Buchbinder BU, Pyne JW, Sun SM, Bliss FA (1978) Messenger RNA for G1 protein of French bean seeds: Cell-free translation and product characterization. Proc Natl Acad Sci USA **75**: 3196-3200

Jabben M, Shanklin J, Vierstra RD (1989) Ubiquitin-phytochrome conjugates: Pool dynamics during *in vivo* phytochrome degradation. J Biol Chem **264**: 4998-5005

Jaworski JG (1987) Biosynthesis of monoenoic and polyenoic fatty acids. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants: A Comprehensive Treatise, Vol 9, Lipids: Structure and Function. Academic Press, New York, p 159-174

Jaworski JG, Stumpf PK (1974) Fat metabolism in higher plants: Properties of a soluble stearoyl-acyl carrier protein desaturase from maturing *Carthamus tintorius*. Arch Biochem Biophys **162**: 158-165

Jefferson RA, Kavanaugh TA, Bevan MW (1987) GUS fusions: β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6**: 3901-3907

Keegstra K, Olsen LJ, Theg SM (1989) Chloroplastidic precursors and their transport across the envelope membranes. Annu Rev Plant Physiol Plant Mol Biol **40**: 471-501

Kinney AJ, Hitz WD, Yadav NS (1990) Stearoyl-ACP desaturase and a β-ketoacyl-ACP synthetase from developing soybean seeds. *In* PJ Quinn, JL Harwood, eds, Plant Lipid Biochemistry, Structure and Utilization. Portland Press, London, p 126-128

Kleiman R, Spencer GF (1982) Search for new industrial oils: XVI. Umbelliflorae-seed oils rich in petroselinic acid. J Am Oil Chem Soc **59**: 29-38

Laemli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-685

Lütcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA (1987) Selection of AUG initiation codon differs in plants and animals. EMBO J **6**:43-48

McKeon T, Stumpf PK (1981) Stearoyl-acyl carrier protein desaturase from safflower seeds. Methods Enzymol **71**: 275-281

McKeon TA, Stumpf PK (1982) Purification and characterization of stearoyl-acyl carrier protein desaturase and the acyl-acyl carrier protein thioesterase from maturing seeds of safflower. J Biol Chem **257**: 12141-12147

Mersereau M, Pazour GJ, Das A (1990) Efficient transformation of *Agrobacterium tumefaciens* by electroporation. Gene **90**: 149-151

Morris LJ, Wharry DM, Hammond EW (1967) Chromatographic behaviour of isomeric long-chain aliphatic compounds. II. Argentation thin-layer chromatography of isomeric octadecenoates. J Chromatog **31**: 69-76

Murphy DJ (1992) Modifying oilseed crops for non-edible products. Trends Biotechnol **10**: 84-87

Nagai J, Bloch K (1968) Enzymatic desaturation of stearyl acyl carrier protein. J Biol Chem **243**: 4626-4633

Post-Beittenmiller MA, Schmid KM, Ohlrogge JB (1989) Expression of holo and apo forms of spinach acyl carrier protein-I in leaves of transgenic tobacco plants. Plant Cell **1**: 889-899

Rogers SG, Horsch RB, Fraley RT (1986) Gene transfer in plants: Production of transformed plants using Ti plasmid vectors. Methods Enzymol **118**: 627-648

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab, Cold Spring Harbor, NY

Shanklin J, Mullins C, Somerville C (1991) Sequence of a complementary DNA from *Cucumis sativus* L. encoding the stearoyl-acyl-carrier protein desaturase. Plant Physiol **97**: 467-468

Shanklin J, Somerville C (1991) Stearoyl-acyl carrier protein desaturase from higher plants is structurally unrelated to the animal and fungal homologs. Proc Natl Acad Sci USA **88**: 2510-2514

Short JM, Fernandez JM, Sorge JA, Huse WD (1988) λZAP: A bacteriophage λ expression vector with *in vivo* excision properties. Nucleic Acids Res **16**: 7583-7600

Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1990) Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol **185**: 60-89

Thompson GA, Scherer DE, Foxall-Van Aken S, Kenny JW, Young HL, Shintani DK, Kridl JC, Knauf VC (1991) Primary structures of the precursor and mature forms of stearoyl-acyl carrier protein desaturase from safflower embryos and requirement of ferredoxin for enzyme activity. Proc Natl Acad Sci USA **88**: 2578-2582

Yamamoto K, Shibahara A, Nakayama T, Kajimoto G (1991) Determination of double-bond positions in methylene-interrupted dienoic fatty acids by GC-MS as their dimethyl disulfide adducts. Chem Phys Lipids **60**: 39-50

CHAPTER 3[1]

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

Abstract

We have previously demonstrated that the double bond of petroselinic acid (18:1Δ^{6cis}) in coriander seed results from the activity of a 36 kDa desaturase that is structurally related to the Δ^9stearoyl-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 that express the coriander 36 kDa desaturase and as a result produce petroselinic acid and Δ^4hexadecenoic 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-CoAs. Evidence instead 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

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[1]Material in this chapter has been accepted for publication [Cahoon EB, Ohlrogge JB (1994) Plant Physiology (in press)]

the coriander 36 kDa desaturase revealed significant amounts of petroselinic acid and Δ^4hexadecenoic 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 kDa desaturase positions double bond placement relative to the carboxyl end of acyl-ACP substrates. 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 kDa desaturase followed by two-carbon elongation of the resulting Δ^4hexadecenoyl-ACP.

Introduction

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 ($18:1\Delta^{\underline{cis}6}$)(Kleiman and Spencer, 1982). Prior to a recent report of this lab (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 enzymatic 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 phosphatidylcholine (Gurr *et al.*, 1969; Slack *et al.*, 1978; Stymne and Appelqvist, 1978) and the desaturation of palmitic acid to *trans*Δ^3hexadecenoic acid on phosphatidylglycerol (Bartels *et al.*, 1967). In addition, fatty acid unsaturation may arise from reactions which use saturated acyl chains bound to acyl carrier protein (ACP). The primary example of such a pathway is the desaturation of stearoyl-ACP to oleoyl-ACP through the activity of the Δ^9stearoyl-ACP desaturase (Nagai and Bloch, 1969; Jaworski and Stumpf, 1974). Acyl-coenzyme A (CoA) esters may also serve as substrates for fatty acid desaturases. Though such reactions have yet to be conclusively demonstrated in higher plants, the desaturation of eicosanoyl-CoA is the proposed biosynthetic route of Δ^5eicosenoic acid in seeds of meadowfoam (*Limanthes alba*) (Moreau *et al.*, 1980; Pollard and Stumpf, 1980). Finally, Shibahara *et al.* (1991) have proposed a mechanism

in pulp of kaki (*Diospyros kaki*) in which the double bonds of oleic acid and *cis*-vaccenic acid can be enzymatically shifted between the Δ^9 and Δ^{11} positions.

With regard to the biosynthesis of petroselinic acid, we have previously identified a 36 kDa peptide in Umbelliferae endosperm that displays immunological cross-reactivity with antibodies against the Δ^9stearoyl-ACP desaturase of avocado (Cahoon *et al.*, 1992). This peptide is absent in tissues that do not synthesize petroselinic acid, including leaves and roots of coriander, an Umbelliferae species. In addition, expression of a coriander endosperm cDNA for the 36 kDa peptide in tobacco resulted in the production of petroselinic acid and Δ^4hexadecenoic acid ($16{:}1\Delta^4$) in transgenic callus. Furthermore, translation of the coriander cDNA indicated that the 36 kDa peptide is synthesized with a plastid transit peptide and, in its mature form, possesses 70% amino acid identity with the Δ^9stearoyl-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^9stearoyl-ACP desaturase (Cahoon *et al.*, 1992). However, expression of the 36 kDa desaturase in *E. 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 kDa 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 kDa 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 kDa 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 kDa 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 harvested as described (Chapter 4). Studies were also performed with suspension cells derived from calli of tobacco (*Nicotiana tabacum* L.) transformed with a cDNA for the 36 kDa (or Type II) desaturase of coriander endosperm as previously described (Cahoon *et al.*, 1992). The coriander cDNA was inserted behind the cauliflower mosaic virus (CaMV) 35S promoter in the plant expression vector pBI121 (Clontech, Palo Alto, CA). Transgenic and wild-type suspension cells were maintained in liquid media (Linsmaier and Skoog, 1965) with constant shaking at 100 rpm at 28°C. Media 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 to 2 mm thickness) of coriander endosperm (40-100 mg fresh weight) were incubated in loosely-capped 13 x 100 mm tubes containing 3 μCi of the ammonium salts of either [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, Boston, MA), stearic acid (18:0) (55 mCi/mmol, American Radiolabeled Chemicals, St. Louis, MO), or oleic acid (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 Chapter 4. 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 pre-incubated 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 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 (Chapter 4; Morris *et al.*, 1967). TLC plates were prepared by 10 min incubation of silica gel K6 pre-poured TLC plates (250 μ layer thickness) (Whatman) in 15 wt% silver nitrate in acetonitrile followed by air-drying. 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 μ layer) plates (Whatman) 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 non-aqueous 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, 1mM $MgCl_2$, 1mM KCl, 1 mM isoascorbate, and 0.1% (w/v) bovine serum albumin (Fraction V, Sigma, St. Louis, MO). The homogenate was passed through two layers of miracloth (Calbiochem, La Jolla, CA). The approximate protein concentration of homogenates was 7.5 mg/mL as estimated using the method of Bradford (1976) and a bovine albumin standard. [2-^{14}C]Malonyl-CoA was either purchased (55 mCi/mmol, Amersham, Arlington Heights, IL) or chemically synthesized from [2-^{14}C]malonate (56 mCi/mmol, American Radiolabeled Chemicals, St. Louis, MO) using the method of Rutkowski and Jaworski (1978) but without dilution of specific activity.

Reactions were performed in 13 x 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 U/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 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 nmoles of [2-^{14}C]malonyl-CoA (275,000-350,000 dpm) in a 5 μL volume. This assay was based in part on that described by McKeon and Stumpf (1981) for measurement of Δ^9stearoyl-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 minutes. Fatty acid methyl esters were then analyzed by argentation TLC as described above. To confirm the identity of ^{14}C-methyl petroselinic, 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 oxygen 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 nitrogen prior to and after addition of the radiolabeled substrate. In addition, tubes were completely sealed for the duration of the incubation period. The ferredoxin 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 ferredoxin (Sigma) and 3.1 μL of ferredoxin:NADPH reductase (10 U/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 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 following 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. Following 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 non-polar solvent systems as described (Chapter 4). 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 [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" section) were supplemented with 10 μL of 1 mM spinach ferredoxin (Sigma) and 8 μL of ferredoxin: NADPH reductase (Sigma) (10 U/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 pmoles) 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 to 3.1 nmole) as the substrate were conducted in parallel.

Radiolabeled acyl-ACPs were prepared from *E. coli* ACP and [1-^{14}C]myristic acid, palmitic acid, stearic acid according to the method of Rock and Garwin (1979). [1-^{14}C]Stearoyl-CoA was synthesized enzymatically 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 kDa 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/mmole, New England Nuclear, Boston, MA) in 13 mL polypropylene screw cap tubes. (Suspension cells had been subcultured in fresh media four days prior to use in these experiments.) Following 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 thawing on ice, labeled cells were homogenized with a Polytron PT10/35 (Brinkman), and the homogenate was centrifuged at 10,000 x *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 x *g* for 20 min. The supernatant was discarded, and the pellet was centrifuged at 10,000 x *g* for an additional 5 min in order 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 10,000x*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 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 were used per 25 to 30 mg of nitrocellulose. In preliminary studies, approximately 80% of [1-^{14}C]palmitoyl-ACP blotted onto nitrocellulose was recovered as fatty acid methyl esters using this procedure.

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 cm, 14 cm, 18 cm, and 20 cm in toluene at -20°C. Radioactivity in separated fatty acid methyl esters was detected by phosphorimaging of TLC plates and mobilities compared to 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, Boston, MA) for use in radiolabeling studies described above. [U-^{14}C]Oleic acid (a gift from Dr. Edward Emken, USDA 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 of this lab demonstrated that the double bond of petroselinic acid of Umbelliferae endosperm arises from the activity of a 36 kDa peptide that is structurally related to the Δ^9stearoyl-ACP desaturase (Cahoon *et al.*, 1992). The identity of the substrate of the 36 kDa desaturase, however, was not determined in this 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 (1) a glycerolipid, (2) coenzyme A (CoA), or (3) acyl carrier

protein (ACP). In this regard, kinetics of [1-^{14}C]acetate radiolabeling of carrot and coriander endosperm shown in the companion paper gave no indication that petroselinic acid is synthesized via the desaturation of saturated acyl chains esterified to glycerolipids, including phosphatidylcholine (PC) (Chapter 4). 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 desaturases 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 3.1). These results thus 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 presumed that fatty acids are incorporated into glycerolipids as coenzyme A esters (Roughan and Slack, 1982). As such, the inability to synthesize petroselinic acid from exogenous stearic acid would suggest the absence of a Δ^6stearoyl-CoA desaturation pathway in Umbelliferae endosperm.

As with [1-^{14}C]stearic acid, radiolabeled palmitic acid was not metabolized to petroselinic acid when provided exogenously to slices of coriander endosperm (Table 3.1). The lack of palmitic acid incorporation into petroselinic acid would suggest, for example, 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.* (1991) have provided evidence

Table 3.1. Metabolism of exogenous [1-^{14}C]medium- and long-chain fatty acids by slices of coriander endosperm

Three μCi (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) for 6 h, and stearic acid (18:0) for 10 h.

[1-^{14}C]Fatty Acid	Endosperm Fresh Weight	^{14}C Incorporated into Glycerolipids	% of Incorporated Label Elongated/ Desaturated	% 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 x 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	<1.0
16:0	66	61.4	0	-	-	n.d.[c]					
18:0	92	10.4	0	-	-	-	-	n.d.			
18:1Δ^9	60	133	30.2	-	-	-	-	n.d.	-	100	n.d.

[a]Includes a 14:1 isomer which co-migrated with petroselinic acid on argentation TLC plates and tentatively identified as 14:1Δ^6. Also includes a 16:1 isomer which co-migrated with oleic acid on argentation TLC plates. [b]Total radioactivity in fatty acid methyl esters co-migrating 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.

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 3.1). 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 length 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 length 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 3.1). 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 length 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 re-incorporated 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 composed approximately 10 to 15% of the elongation products of [1-^{14}C]lauric acid and myristic acid but ≤2.5% of the [^{14}C] fatty acids resulting [1-^{14}C]acetate labeling of coriander endosperm for a similar time period (Chapter 4) The basis for this difference is not known. However, because 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 presumed that the Δ^6 double bond of petroselinic acid and Δ^6hexadecenoic acid result from the same enzymatic 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 coenzyme A. In contrast to the metabolic fate of long-chain fatty acids, [1-^{14}C]medium-chain length 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 co-factors, including NADPH, NADH, ACP and catalase. Depending upon the experiment, assays were carried out for 7 or 20 minutes. The major radiolabeled acyl products of both 7 and 20 minute reactions were palmitic acid and stearic acid, which typically accounted for 55 to 70% of the radioactivity recovered in fatty acids (Figure 3.1A). 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 to 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^6hexadecenoic acid) that might potentially co-migrate 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 pre-formed, non-radioactive fatty acid (data not shown). Finally, the conversion of [2-^{14}C]malonyl-CoA to petroselinic acid appeared to be dependent upon the developmental stage of coriander endosperm as well as upon 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 three-fold when the assay was conducted under oxygen-limiting conditions (Table 3.2). The apparent dependence of petroselinic acid synthesis on molecular oxygen is consistent with the known catalytic

Table 3.2. *Oxygen- and ferredoxin-dependence of petroselinic acid and oleic acid synthesis from [2-^{14}C]malonyl-CoA by homogenates of coriander endosperm*

Control (or complete) assays contained ferredoxin and ferredoxin:NADPH reductase and were performed as described in "Materials and Methods". Oxygen-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 nitrogen prior to and after addition of substrate ([2-^{14}C]malonyl-CoA). Ferredoxin-dependence (-Fd/FNR) of petroselinic acid and oleic acid synthesis from [2-^{14}C]malonyl-CoA was studied by omitting spinach ferredoxin (Fd) and ferredoxin: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	%Distribution of ^{14}C in Products
Complete	SatFA[a]	56 (5,300)[b]
	18:1Δ^6	29 (2,740)
	18:1Δ^9	15 (1,460)
-Fd/FNR	SatFA	65 (5,430)
	18:1Δ^6	28 (2,340)
	18:1Δ^9	7 (620)
$+N_2$	SatFA	85 (9,790)
	18:1Δ^6	8 (960)
	18:1Δ^9	7 (830)

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

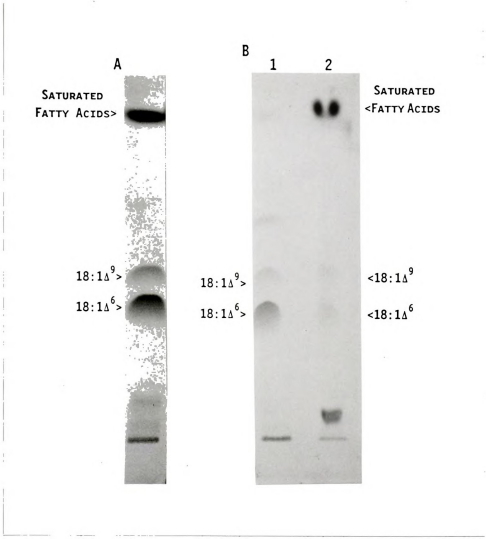


Figure 3.1. Products of the metabolism of [2-^{14}C]malonyl-CoA by crude homogenates of developing coriander endosperm. Shown in (A) is an autoradiogram of fatty acid methyl esters prepared from the total products of a 20 min assay performed as described in "Materials and Methods". Shown in (B) is an autoradiogram of products of a 7 min assay extracted according to the method of Mancha *et al.* (1978). In Lane 1 are fatty acid methyl esters prepared from the isopropanol saturated-petroleum ether extract consisting primarily of free fatty acids. In lane 2 are fatty acid methyl esters derived by transesterification of precipitated acyl-ACPs. Fatty acid methyl esters in (A) and (B) were separated by argentation TLC (see "Materials and Methods"). Radiolabeled material near the origin in lane 2 of panel (B) was not identified.

properties of plant fatty acid desaturases (Jaworski, 1987). Unexpectedly, the addition of ferredoxin and ferredoxin:NADPH reductase had only a small effect upon petroselinic acid synthesis from [2-^{14}C]malonyl-CoA. In contrast, the presence of these co-factors in assays resulted in a two-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 3.3). More than 70% of the radioactivity in free fatty acids of a 7 minute 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 following methanol:chloroform precipitation. Analysis of acyl-ACPs of a 7 min reaction in this manner, revealed a preponderance of radioactivity associated with saturated fatty acids (Figure 3.1B, Table 3.4). 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

Table 3.3. *Glycerolipid and fatty acid products of [2-^{14}C]malonyl-CoA metabolism by crude homogenates of coriander endosperm*

Reactions products of seven and twenty minute 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[d]	140	210
DAG[e]	80	<50
TAG[f]	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]PC, Phosphatidylcholine. [e]DAG, Diacylglycerol. [f]TAG, Triacylglycerol.

Table 3.4. *Products of the metabolism of [2-^{14}C]malonyl-CoA by homogenates of coriander endosperm*

Reaction products of seven minute 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]% of total dpm of ^{14}C recovered in saturated fatty acids. [c]% of total dpm of ^{14}C recovered in petroselinic acid. [d]% of total dpm of ^{14}C recovered in oleic acid.

ether wash, most likely in the form of free fatty acid as indicated by results of 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**

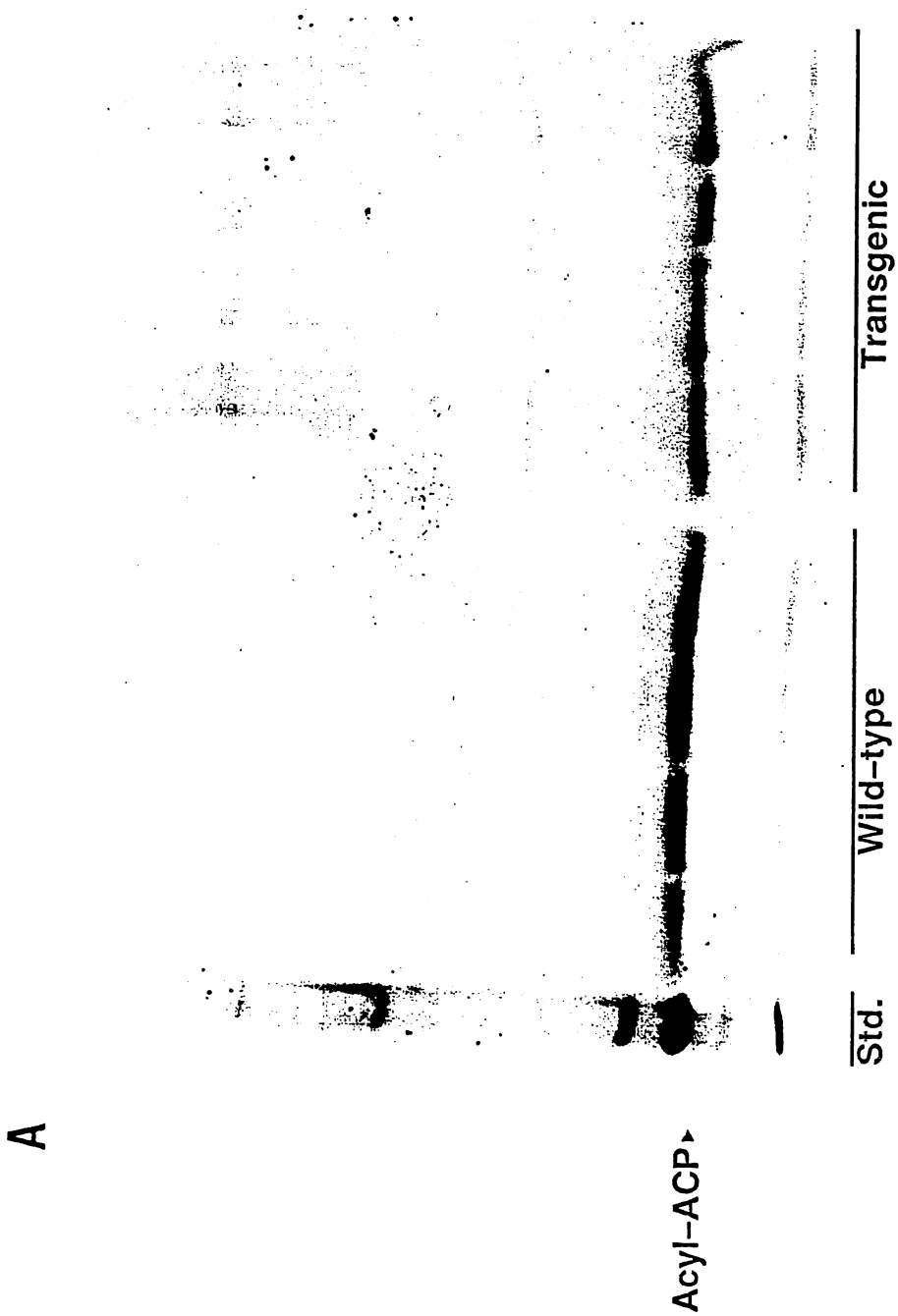
The metabolic evidence presented above and previously reported data demonstrating that the double bond of petroselinic acid arises from the activity of a Δ^9stearoyl-ACP desaturase-like peptide (Cahoon *et al.*, 1992) are consistent with an acyl-ACP route of petroselinic acid biosynthesis. To test this 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 experiments above 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- 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 ferredoxin and ferredoxin: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

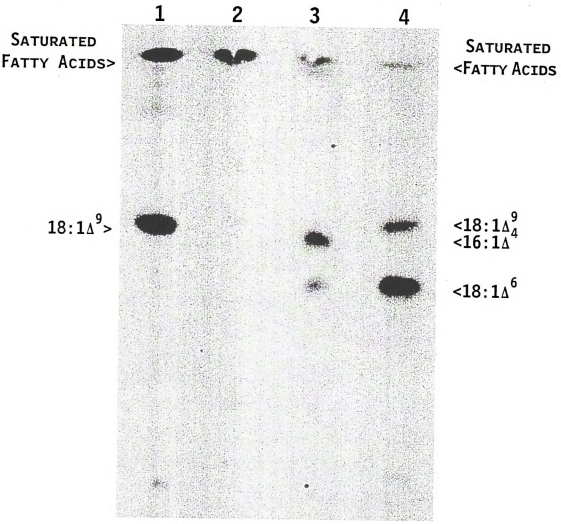
To provide further evidence for the involvement of acyl-ACP-

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 kDa 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^4hexadecenoic acid (16:1Δ^4) (neither fatty acid is detectable in control callus). Because tobacco likely does not contain a 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]mal-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 co-migrated with a tobacco long/medium-chain acyl-ACP standard (Figure 3.2A). Examination of fatty acid methyl esters derived from acyl-ACPs of the transgenic tobacco revealed the presence of a radiolabeled band that co-migrated with a methyl petroselinic acid standard on argentation TLC plates (Figure 3.2B). The major monounsaturated moiety in these samples, however, was a fatty acid methyl ester identified as methyl Δ^4hexadecenoic acid based on its mobility on argentation and reverse-phase TLC and the chain lengths of its permanganate-periodate oxidation products (data not shown). Neither methyl petroselinic acid nor Δ^4hexadecenoic acid waspresent in fatty acid methyl esters prepared from acyl-ACPs of wild-type tobacco cells.

Figure 3.2. Analysis of long/medium chain acyl-ACPs of suspension cultures of wild-type tobacco and transgenic tobacco expressing a cDNA for the coriander 36 kDa 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 of (A) is a mixture of tobacco ACP isoforms esterified to [1-^{14}C]myristic acid. Regions of blots co-migrating 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 of (B) 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.


A
Acyl-ACP
Std.
Wild-type
Transgenic

B


1
2
3
4
SATURATED
FATTY ACIDS>
18:1Δ9>
SATURATED
<FATTY ACIDS
<18:1Δ9
<16:1Δ4
<18:1Δ6

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^4hexadecenoic 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 kDa desaturase contain Δ^4hexadecenoic acid in their long/medium-chain acyl-ACP pool, it is unlikely that, for example, this fatty acid is a partial β-oxidation product of petroselinic acid. As discussed below, a more likely explanation is that Δ^4hexadecenoyl-ACP is the direct precursor of petroselinic acid.

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

We have previously reported that the expression of a cDNA for the 36 kDa 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 kDa desaturase may position the placement of double bonds with respect to the methyl end of acyl-ACPs and is therefore functionally an ω^{12} (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^4hexadecenoic acid. Alternatively, the position of carbon atoms from the carboxyl (or Δ) end of acyl-ACP substrates may dictate where the 36 kDa 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 Δ^6 desaturase. Therefore, the co-occurrence of petroselinic acid and Δ^4hexadecenoic acid in transgenic tobacco would suggest that petroselinic acid is an elongation product of Δ^4hexadecenoic acid.

To determine whether the 36 kDa desaturase positions the placement of 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 length 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 kDa 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 3.5, 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 kDa desaturase does not function as an ω^{12}

Table 3.5. *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	% 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$\Delta^{4/9}$[a]	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 ≤1.3% each of 11:0, 13:0, 15:1$\Delta^{4/9}$, 19:0, and 19:1$\Delta^{4/9}$.

desaturase but instead positions double bond insertion from the carboxyl end of acyl chains. In this regard, the 36 kDa desaturase of coriander endosperm is similar to the Δ^9stearoyl-CoA desaturase of rat liver (Holloway *et al.*, 1963) and yeast (Bloomfield and Bloch, 1960) and the Δ^9stearoyl-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.

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^4hexadecenoyl-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 3.1). 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 → 14:1Δ^2-ACP → 16:1Δ^4-ACP → 18:1Δ^6-ACP

(b) 16:0-ACP → 16:1Δ^4-ACP → 18:1Δ^6-ACP

(c) 18:0-ACP → 18:1Δ^6-ACP

However, [1-^{14}C]myristic acid can also be converted to Δ^6hexadecenoic acid by coriander endosperm (Table 3.1). 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 $^4\Delta$hexadecenoyl-ACP rather than Δ^6hexadecenoyl-ACP). Assuming that the same desaturase is involved in the insertion of the double bond of both petroselinic acid and Δ^6hexadecenoic acid, pathway (a) would therefore be an unlikely route of petroselinic acid formation. In addition, transgenic tobacco calli expressing the coriander 36 kDa desaturase produce not only petroselinic acid but also Δ^4hexadecenoic acid (Cahoon *et al.*, 1992), both of which are

detectable in the acyl-ACP pool of these cells. Δ^4Hexadecenoic acid and petroselinic acid, however, likely cannot arise from both pathways (b) and (c), respectively. Results of nonanoic acid labeling, for example, demonstrate that the 36 kDa desaturase does not function as an ω^{12}desaturase. Such a catalytic property would be necessary for this enzyme to synthesize both Δ^4hexadecenoic acid and petroselinic acid directly. Furthermore, desaturases typically introduce double bonds into specific positions of fatty acid substrates regardless of chain length (*e.g.*, Bloomfield and Bloch, 1960; Cheesebrough and Cho, 1990; Halloway *et al.*, 1963; Higashi and Murata, 1993). As such, it is unlikely that the 36 kDa desaturase acts as both a Δ^4palmitoyl-ACP desaturase (pathway b) and as a Δ^6stearoyl-ACP desaturase (pathway c). More consistent with the double bond-positioning properties of the 36 kDa 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 kDa 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 medium chain-length 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 when supplied exogenously to coriander endosperm (Tables 3.1 and 3.5) suggests that the 36 kDa desaturase does not have an absolute specificity for palmitoyl-ACP.

If petroselinic acid is synthesized from Δ^4hexadecenoyl-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^4hexadecenoic acid in the total lipid extract is on the order of 500:1 (72 wt% petroselinic acid: 0.1 to 0.2 wt% Δ^4hexadecenoic acid) (Chapter 4). This ratio in transgenic tobacco callus, however, is approximately 0.8:1 (4 wt% petroselinic acid: 5 wt% Δ^4hexadecenoic acid) (Cahoon *et al.*,

1992). This substantial difference might indicate that some component(s) of Δ^4hexadecenoyl-ACP elongation in coriander endosperm is absent in transgenic tobacco callus. This component may, for example, be a β-keto-acyl-ACP synthetase that is specialized for the metabolism of Δ^4hexadecenoyl-ACP. Whatever its nature, the enzyme(s) involved in Δ^4hexadecenoyl-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 kDa desaturase and the Δ^9stearoyl-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, ferredoxin, and ferredoxin:NADPH reductase. It cannot be ruled out that the acyl-ACP desaturase assay used in our studies lacked an essential co-factor(s). For example, ferredoxin may not be the preferred electron donor for the 36 kDa desaturase. However, this is not consistent with the plastid localization of this enzyme and its structural similarity to the Δ^9stearoyl-ACP desaturase [*i.e.*, the *in vitro* activities of all plastid desaturases characterized to date, including the Δ^9stearoyl-ACP desaturase, are stimulated by ferredoxin (Jaworski, 1987; Schmidt and Heinz, 1990)]. It is also possible that the 36 kDa desaturase may be inactive with fatty acids esterified to *E. coli* ACP. This enzyme instead might only be capable of using 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 kDa desaturase is functional *in vivo* in tobacco, a "non-Umbelliferae" species. Another possibility is that the 36 kDa desaturase operates in

close association with one or more fatty acid biosynthetic enzymes. In such a scenario, growing acyl chains may be channelled through an elongation/desaturation pathway that is inaccessible *in vitro* to exogenous acyl-ACPs. The existence of metabolic channels or "metabolons" have 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 coenzyme A 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^6hexadecenoic acid.

(3) Crude homogenates of coriander endosperm were capable of incorporating [2-^{14}C]malonyl-CoA into petroselinic acid in an oxygen-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 kDa desaturase following [1-^{14}C]acetate labeling revealed significant amounts of petroselinic acid and Δ^4hexadecenoic 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 kDa desaturase positions double bond placement 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^4hexadecenoyl-ACP. Relative to coriander endosperm, elongation of Δ^4hexadecenoyl-ACP appears to be a limiting reaction in the synthesis of petroselinic acid by transgenic tobacco.

Literature Cited

Bartels CT, James AT, Nichols BW (1967) Metabolism of *trans*-3-hexadecenoic acid by *Chlorella vulgaris* and by lettuce leaf. Eur J Biochem **3**: 7-10

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol **37**: 911-917

Bloomfield DK, Bloch K (1960) The formation of Δ^9-unsaturated fatty acids. J Biol Chem **235**: 337-345

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 **72**: 248-254

Browse J and Slack CR (1985) Fatty-acid synthesis in plastids from maturing safflower and linseed cotyledons. Planta **166**: 74-80

Cahoon EB, Shanklin J, Ohlrogge JB (1992) Expression of a coriander desaturase results in petroselinic acid production in transgenic tobacco. Proc Natl Acad Sci USA **89**: 11184-11188

Cheesbrough TM, Cho SH (1990) Purification and characterisation of soyabean stearoyl-ACP desaturase. *In* PJ Quinn, JL Harwood, eds, Plant Lipid Biochemistry, Structure and Utilization. Portland Press, London, p 129-130

Christie WW (1982) Lipid Analysis, 2nd Edition. Pergamon Press, Oxford

Dörmann P, Frentzen M, Ohlrogge JB (1994) Specificities of acyl-acyl carrier protein (ACP) thioesterase and glycerol-3-phosphate

acyltransferase for octadecenoyl-ACP isomers: Identification of a petroselinoyl-ACP thioesterase in Umbelliferae. Plant Physiol (submitted)

Guerra DJ, Ohlrogge JB, Frentzen M (1986) Activity of acyl carrier protein isoforms in reactions of plant fatty acid metabolism. Plant Physiol **82**: 448-453

Gurr MI, Robinson MP, James AT (1969) The mechanism of formation of polyunsaturated fatty acids by photosynthetic tissue. The tight coupling of oleate desaturation with phospholipid biosynthesis in *Chlorella vulgaris*. Eur J Biochem **9**: 70-78

Higashi S, Murata N (1993) An in vivo study of substrate specificies of acyl-lipid desaturases and acyltransferases in lipid synthesis in *Synechocystis* PCC6803. Plant Physiol **102**: 1275-1278

Holloway PW, Peluffo R, Wakil SJ (1963) On the biosynthesis of dienoic fatty acid by animal tissues. Biochem Biophys Res Comm **12**: 300-304

Jaworski JG (1987) Biosynthesis of monoenoic and polyenoic fatty acids. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants: A Comprehensive Treatise, Vol 9, Lipids: Structure and Function. Academic Press, New York, p 159-174

Jaworski JG, Stumpf PK (1974) Fat metabolism in higher plants: Properties of a soluble stearoyl-acyl carrier protein desaturase from maturing *Carthamus tintorius*. Arch Biochem Biophys **162**: 158-165

Kates M (1972) Techniques of lipidology. *In* TS Work, E Work, eds, Laboratory Techniques in Biochemistry and Molecular Biology, Vol 3, Part 2. Elsevier/North-Holland, Amsterdam, p 444-445

Kannangara CG, Jacobson BS, Stumpf PK (1973) *In vivo* biosynthesis of α-linolenic acid in plants. Biochem Biophys Res Comm **52**: 648-655

Kleiman R, Spencer GF (1982) Search for new industrial oils: XVI. Umbelliflorae-seed oils rich in petroselinic acid. J Am Oil Chem Soc **59**: 29-38

Linsmaier EM, Skoog F (1965) Organic growth factor requirement of tobacco tissue cultures. Physiol Plant **18**: 100-127

Mancha M, Stokes GB, Stumpf PK (1975) Fat metabolism in plants. The determination of acyl-acyl carrier protein and acyl coenzyme A in a complex lipid mixture. Anal Biochem **68**: 600-608

McKeon T, Stumpf PK (1981) Stearoyl-acyl carrier protein desaturase from safflower seeds. Methods Enzymol **71**: 275-281

Moreau RA, Pollard MR, Stumpf PK (1981) Properties of a Δ5-fatty acyl-CoA desaturase in the cotyledons of developing *Limanthes alba*. Arch Biochem Biophys **209**: 376-384

Morris LJ, Wharry DM, Hammond EW (1967) Chromatographic behaviour of isomeric long-chain aliphatic compounds. II. Argentation thin-layer chromatography of isomeric octadecenoates. J Chromatog **31**: 69-76

Murphy DJ (1992) Modifying oilseed crops for non-edible products. Trends Biotechnol **10**: 84-87

Nagai J and Bloch K (1968) Enzymatic desaturation of stearyl acyl carrier protein. J Biol Chem **243**: 4626-4633

Norman HA, St. John JB (1986) Metabolism of unsaturated monogalactosyldiacylglycerol molecular species in *Arabidopsis thaliana* reveals different sites and substrates for linolenic acid synthesis. Plant Physiol **81**: 731-736

Norman HA, Smith LA, Lynch DV, Thompson JR GA (1985) Low-temperature-induced changes in intracellular fatty acid fluxes in *Dunaliella salina*. Arch Biochem Biophys **242**: 157-167

Pollard MR, Stumpf PK (1980) Biosynthesis of C_{20} and C_{22} fatty acids by developing seeds of *Limanthes alba*. Chain elongation and Δ5 desaturation. Plant Physiol **66**: 649-655

Post-Beittenmiller D, Jaworski JG, Ohlrogge JB (1991) *In vivo* pools of free and acylated acyl carrier proteins in spinach: Evidence for sites of regulation of fatty acid biosynthesis. J Biol Chem **266**: 1858-1865

Rock CO, Garwin JL (1979) Preparative enzymatic synthesis and hydrophobic chromatography of acyl-acyl carrier protein. J Biol Chem **254**: 7123-7128

Roughan G, Nishida I (1990) Concentrations of long-chain acyl-acyl carrier proteins during fatty acid synthesis by chloroplasts isolated from pea (*Pisum sativum*), safflower (*Carthamus tinctoris*), and amarathus (*Amaranthus lividus*) leaves. Arch Biochem Biophys **276**: 38-46

Roughan PG, Slack CR (1982) Cellular organization of glycerolipid metabolism. Annu Rev Plant Physiol **33**: 97-123

Roughan PG, Thompson Jr GA, Cho SH (1987) Metabolism of exogenous long-chain fatty acids by spinach leaves. Arch Biochem Biophys **259**: 481-496

Rutkoski A, Jaworski J (1978) An improved synthesis of malonyl-coenzyme A. Anal Biochem **91**: 370-373

Schmidt H, Heinz E (1990) Involvement of ferredoxin in desaturation of lipid-bound oleate in chloroplasts. Plant Physiol **94**: 214-220

Srere PA (1987) Complexes of sequential metabolic enzymes. Annu Rev Biochem **56**: 89-124

Shibahara A, Yamamoto K, Takeoka M, Kinoshita A, Kajimoto G, Nakayama T, Noda M (1990) Novel pathways of oleic and *cis*-vaccenic acid biosynthesis by an enzymatic double-bond shifting reaction in higher plants. FEBS Lett **264**: 228-230

Slack CR, Roughan PG, Balasingham N (1978) Labeling of glycerolipids in the cotyledons of develpoing oilseeds by [1-^{14}C]acetate and [2-^{3}H]glycerol. Biochem J **179**: 421-433

Stymne S, Appelqvist L-A (1978) The biosynthesis of linoleate from oleoyl-CoA via oleoyl-phosphatidylcholine in microsomes of developing safflower seeds. Eur J Biochem **90**: 223-229

Stymne S, Stobart AK (1986) Biosynthesis of γ-linolenic acid in cotyledons and microsomal preparations of the developing seeds of common borage (*Borago officinalis*) Biochem J **240**: 385-393

Taylor DC, Weber N, Hogge LR, Underhill EW (1989) A simple enzymatic method for the preparation of radiolabeled erucoyl-CoA and other long-chain fatty acyl-CoAs and their characterization by mass spectrometry. Anal Biochem **184**: 311-316

CHAPTER 4[1]

Apparent Role of Phosphatidylcholine in the Metabolism of Petroselinic Acid in Developing Umbelliferae Endosperm

Abstract

Studies were conducted to characterize the metabolism of the unusual fatty acid petroselinic acid (18:1*cis*Δ^6) in developing endosperm of the Umbelliferae species coriander and carrot. Analyses of fatty acid compositions of glycerolipids of these tissues revealed a dissimilar distribution of petroselinic acid in triacylglycerols (TAG) and the major polar lipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Petroselinic acid composed 70 to 75 mol% of the fatty acids of TAG but only 9 to 20 mol% of the fatty acids of PC and PE. Though such data appeared to suggest that petroselinic acid is at least partially excluded from polar lipids, results of [1-^{14}C]acetate radiolabeling experiments gave a much different picture of the metabolism of this fatty acid. In time-course labeling of carrot endosperm, [1-^{14}C]acetate was rapidly incorporated into PC in high levels. Through 30 min, radiolabel was most concentrated in PC, and of this, 80 to 85% was in the form of petroselinic acid. One explanation for the large disparity in amounts of petroselinic acid in PC as determined by fatty acid mass analyses and ^{14}C radiolabeling is that turnover of these lipids or the fatty acids of these lipids results in relatively low accumulation of petroselinic acid mass. Consistent with this, the kinetics of [1-^{14}C]acetate time-course labeling of carrot endosperm and "pulse-chase" labeling of coriander endosperm suggested a possible flux of fatty acids from PC into TAG. In time-course experiments, radiolabel initially entered PC at the highest rates but accumulated in TAG at later time points. Similarly, in pulse-chase studies, losses in absolute amounts of radioactivity from PC were

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accompanied by significant increases of radiolabel in TAG. In addition, stereospecific analyses of unlabeled and [1-^{14}C]acetate labeled PC of coriander endosperm indicated that petroselinic acid can be readily incorporated into both the *sn*-1 and *sn*-2 positions of this lipid. Because petroselinic acid is neither synthesized nor further modified on polar lipids, the apparent metabolism of this fatty acid through PC (and possibly other polar lipids) may define a function of PC in TAG assembly apart from its involvement in fatty acid modification reactions.

Introduction

Petroselinic acid, the Δ^{6cis} isomer of octadecenoic acid (18:1), is the major component of the seed oil of most Umbelliferae (or Apiaceae), Araliaceae, and Garryaceae species where it may compose up to 85 wt% of the total fatty acid (Kleiman and Spencer, 1982). Because of its limited natural occurrence, petroselinic acid is considered to be an unusual fatty acid. Many unusual fatty acids of seed oils possess chemical structures that differ significantly from those of the common C_{16} and C_{18} plant fatty acids. Examples of such acyl moieties include erucic acid ($22:1\Delta^{13cis}$) of Brassicaceae species and the medium-chain length (C_8-C_{14}) fatty acids of coconut, palm, and *Cuphea* species. In contrast, the structure of petroselinic acid differs from that of oleic acid ($18:1\Delta^{9cis}$), a common plant fatty acid, by only the position of its double bond.

Because of its structural similarity to oleic acid, petroselinic acid offers an interesting case study of the metabolism of an unusual fatty acid in a developing oilseed. Unusual fatty acids of seeds, especially acyl groups of atypical chemical structures, are often absent or found in only small amounts in membrane (or polar) glycerolipids including phosphatidylcholine (PC), the primary membrane lipid of seeds. These fatty acids are instead concentrated in storage form as triacylglycerol (TAG) (as reviewed by Ohlrogge, 1988; Battey *et al.*, 1989). In theory, the partitioning of unusual fatty acids away from polar glycerolipids insures that "proper" membrane function is not disrupted by the aberrant structures of these acyl moieties (Stymne *et al.*, 1990). It has been proposed that this selective partitioning of unusual fatty acids may result from substrate specificities of enzymes such as diacylglycerol acyltransferase and CDP-choline:diacylglycerol cholinephosphotransferase (Bafor *et al.*, 1990; Browse and Somerville, 1991). Together these two enzymes serve as primary determinants of fatty acid flux via diacylglycerol into storage (TAG) and membrane glycerolipids. It has also been suggested that specialized acyltransferases may contribute to the channeling of unusual fatty acids into TAG (Oo and Huang, 1989). Examples

of such enzymes are lysophosphatidic acid acyltransferases characterized in extracts of palm endosperm (Oo and Huang, 1989) and *Cuphea lanceolata* embryo (Bafor *et al.*, 1990) which display a marked preference for CoA esters of medium-chain length fatty acids. Recent evidence has also suggested that phospholipases may contribute to the exclusion of unusual fatty acids from membrane lipids (Banas *et al.*, 1992).

In contrast, oleic acid and its polyunsaturated derivatives (*e.g.*, linoleic acid and α-linolenic acid) are not excluded from PC in developing oilseeds such as those of soybean, safflower, and linseed (Slack *et al.*, 1978). In these tissues, PC appears to readily participate in the flux of C_{18} polyunsaturated fatty acids into TAG. In addition, the unusual fatty acids γ-linolenic acid and ricinoleic acid may also be metabolized through PC in seeds of borage (Stymne and Stobart, 1986; Griffiths *et al.*, 1988) and castor (Bafor *et al.*, 1991), respectively. In each of these cases, however, the metabolic flow of fatty acids through PC can be attributed to the role of this lipid as a substrate for fatty acid modification reactions including desaturation and hydroxylation.

In this communication, we describe the metabolism of the unusual fatty acid petroselinic acid in the endosperm of developing seeds of the Umbelliferae species coriander and carrot. Reported below are results that are consistent with an apparent movement of petroselinic acid through both stereospecific positions of PC (and perhaps other polar lipids). This route of metabolism occurs despite the fact that petroselinic acid is neither synthesized nor further modified on this glycerolipid in coriander or carrot endosperm (Cahoon *et al.*, 1992; Chapter 3).

Materials and Methods

Plant Material

Developing mericarps (fruits) of coriander (*Coriandrum sativum* L.) were obtained from plants grown under greenhouse conditions of at least 12 h illumination provided by natural or supplemental lighting.

Developing mericarps of wild carrot or Queen Anne's lace (*Daucus carota* L.) were collected from natural stands on the Michigan State University campus. All experiments and lipid analyzes were performed using endosperm dissected from the seed coat and pericarp of coriander and carrot mericarps. The endosperm contained small amounts of an embedded embryo that was not readily separable from endosperm. Collected plant tissue was placed on ice and used immediately after dissection (30 to 45 min after collection). Endosperm was placed in isopropanol for use in lipid analyses or in cold water or 50 mM Mes, pH 5.0 until use in radiolabeling experiments described below.

Total Lipid Extraction and Analyses

Developing endosperm of coriander (approx. 150 mg fresh weight) and carrot (approx. 75 mg fresh weight) was heated at 80°C in 2 mL of isopropanol for 10 minutes. Upon cooling, total lipids were extracted by homogenization of endosperm with a Polytron PT 10/35 (Brinkman, Westbury, NY) in 10 mL of hexane:isopropanol (3:2 v/v) (Hara and Radin, 1978; Post-Beittenmiller *et al.*, 1989). Following 4-6 h of incubation, debris was removed by centrifugation, and the total lipid extract in hexane:isopropanol was dried under N_2 and resuspended in 2 mL of chloroform:acetic acid (100:1 v/v). Lipids were subsequently separated into neutral lipid, glycolipid, and phospholipid fractions by column chromatography using a silica Sep-Pak cartridge (Millipore, Milford, MA) essentially as described by Lynch and Steponkus (1987) except that neutral lipids were eluted with 10 mL of chloroform:acetic acid (100:1 v/v) followed by 5 mL of chloroform:acetone (80:20 v/v). Fractionated lipids were dried under N_2 and stored at -20°C in chloroform:methanol (6:1 v/v) until further use.

The fatty acid composition of glycerolipid classes was determined by GLC following TLC separation and fatty acid transesterification. Neutral lipids, primarily triacylglycerol (TAG) and diacylglycerol (DAG), were separated on silica K6 (0.25 mm thickness) TLC plates (Whatman, Maidstone, England) using a mobile phase of hexane:ethyl ether:acetic acid

(60:40:1 v/v). Glycolipid and phospholipid classes were separated by silica TLC using a solvent system of chloroform:methanol:acetic acid (75:25:8 v/v). Following development, TLC plates were dried in a N_2 atmosphere and separated lipid classes were lightly stained with I_2 and identified by co-chromatography with lipid standards. Lipid bands were scraped from the TLC plate into 1-2 mL of boron trichloride/methanol (10% w/v) (Alltech, Deerfield, IL) containing 17:0 (internal standard) and transesterified at 90°C for 30 min. In the case of neutral lipids, the boron trichloride/methanol was supplemented with 25% (v/v) toluene to increase the solubility of these lipids, and transesterification was carried out for 45 min. Following heating, fatty acid methyl esters were extracted as described (Morrison and Smith, 1964).

The composition of fatty acid methyl esters of individual lipid classes was determined by capillary GLC with a Hewlett Packard 5890 gas chromatograph interfaced to a Spectra-Physics SP4290 integrator. Separation of fatty acid methyl esters was achieved using a 50 m x 0.25 mm ID CP-Sil 88 capillary column (Chrompack, Middelburg, Netherlands) with the oven temperature programmed from 155°C (70 min hold) to 170°C at 2.5°C/min. Injection port and FID detector temperatures were 215°C, and the column head pressure was 7.5 psi He. Under these conditions, the 18:1 isomers methyl petroselinic acid (18:1$\Delta^{6\underline{cis}}$), oleic acid (18:1$\Delta^{9\underline{cis}}$), and *cis*-vaccenic acid (18:1$\Delta^{11\underline{cis}}$) were sufficiently resolved for separate integration of each. The CP-Sil 88 column, however, was not capable of resolving 18:1 and 16:3 in the case of monogalactosyldiacylglycerol (MGDG). Instead, this separation was achieved with a 30 m x 0.25 mm ID DB23 (J&W Scientific, Folsom, CA) column using the same chromatographic conditions as described above.

Several isomers of hexadecenoic acid (16:1) of unknown double bond position were identified by GC-MS of dimethyl disulfide derivatives prepared from monounsaturated fatty acid methyl esters of coriander phospholipids as described (Cahoon *et al.*, 1992; Yamamoto *et al.*, 1991).

Lipid Stereospecific Analyses

Phosphatidylcholine (PC) of coriander endosperm was purified by TLC from the total phospholipid fraction isolated as described above. Phospholipids were separated by silica TLC with chloroform:methanol:acetic acid (75:25:8 v/v). The PC band was visualized with light iodine staining, scraped from the TLC plate, and eluted from the silica with 3 mL of methanol:chloroform:water (100:50:40 v/v). Following centrifugation, the supernatant was recovered, and the silica scrapings were washed with an additional 2 mL of chloroform:methanol:water. Supernatants from the two washes were pooled, and two phases were formed with the addition of 1.8 mL of water and 1.5 mL of chloroform. After centrifugation, PC was recovered in the resulting chloroform layer. The stereospecific fatty acid composition of PC was determined using phospholipase A_2 from *Naja naja* venom (Sigma) dissolved in 100 mM Tris-HCl pH 7.6 and 4 mM $CaCl_2$ using the reaction conditions and extraction procedure described by Griffiths *et al.* (1985). This method produced nearly complete hydrolysis of PC as judged by TLC analysis of the products. The lysoPC and free fatty acid (FFA) products were separated by silica TLC with development to a height of 10 cm with a mobile phase of chloroform:methanol: water (65:25:4 v/v). After drying in a N_2 atmosphere, TLC plates were developed to their full length (20 cm) in hexane:ethyl ether:acetic acid (60:40:1 v/v). The separated lysoPC and FFA were transesterified and analyzed by gas chromatography as described above. The fatty acid composition of the *sn*-2 position of PC was calculated by subtraction of the fatty acid composition of lysoPC from that of total PC. This number agreed closely with the acyl composition of FFA released upon hydrolysis.

The *sn*-2 composition of TAG was determined by Grignard hydrolysis of coriander TAG using the method of Myher and Kuksis (1979). This method was used instead of pancreatic lipase treatment because TAGs rich in petroselinic acid are resistant to lipase digestion (Heimermann *et al.*, 1973). TAG of coriander endosperm was purified by TLC. TAG was

hydrolyzed with ethylmagnesium bromide (1.0 M in *tert*-butyl methyl ether, Aldrich), and reaction products were recovered as described by Myher and Kuksis (1979). Hydrolysates were separated using borate impregnated-silica TLC plates with development in 50:50 hexane:ethyl ether (v/v) (Christie, 1982). TLC plates were prepared by incubation of silica K6 TLC plates for 10 minutes in a solution of 5% (w/v) boric acid in acetonitrile:methanol (60:40 v/v) followed by air-drying. The TLC-purified 1,2;2,3 DAG was transesterified and analyzed by gas chromatography as described above. To minimize isomerization (acyl-migration) of 1,2;2,3 and 1,3 DAG products, all steps were performed in rapid succession. The *sn*-2 acyl composition of TAG was calculated using the formula [(4 x fatty acid mol% of 1,2;2,3 DAG)-(3 x fatty acid mol% of TAG)] for a given fatty acid as described by Christie (1982) and Lawson and Hughes (1988). As a check of this procedure, the erucic acid-rich TAG of *Crambe abyssinica* was analyzed as described above. This TAG, which is composed of approximately 55 mol% erucic acid, is known to contain virtually no erucic acid at its *sn*-2 position (Gurr *et al.*, 1972). In close agreement, only 1.3 mol% of the *sn*-2 fatty acids were determined to be erucic acid using the above calculation.

[1-^{14}C]Acetate Time-Course Labeling of Carrot and Coriander Endosperm

Approximately 15-20 mg fresh weight of freshly harvested carrot endosperm was incubated in 250 μL of 50 mM Mes-NaOH, pH 5.0, containing 0.54 mM sodium [1-^{14}C]acetate (56 mCi/mmol, Amersham, Arlington Heights, IL) in five loosely capped 13 mm x 100 mm test tubes (Slack *et al.*, 1978). Incubations were performed at 25°C with shaking at 100 rpm. At time points of 2, 5, 15, 30, and 60 min, the incubation buffer was quickly removed from each tube, and endosperm was washed two times with 1 mL of ice-cold water. The endosperm was then heated at 80°C in 1 mL of isopropanol for 10 min. After cooling, 1.5 mL of hexane was added, and the endosperm was homogenized in the original incubation test tube with the pestle of a Elvehjem homogenizer. To enhance abrasion of the

endosperm, fine glass beads were added to the tube prior to grinding. Following homogenization of the tissue, an additional 1.5 mL of hexane and 1 mL of isopropanol were added, and lipids were extracted for 4 h. Lipids were recovered following a wash of the hexane:isopropanol extract with 2.5 mL of 6.7% (w/v) sodium sulfate (Hara and Radin, 1978). The aqueous phase was re-extracted three additional times with 2 mL of hexane:isopropanol (7:2 v/v).

A similar procedure was used for ^{14}C-acetate labeling of coriander endosperm slices. In this experiment, transverse slices (approximately 1 mm thick) of coriander endosperm were incubated in 0.23 mM of [1-^{14}C]acetate in 50 mM Mes, pH 5.0. After 3.5 h, label was removed, and the endosperm slices were washed and lipids extracted as described above.

Lipid classes were separated by silica TLC with sequential development of TLC plates to heights of 4 cm and 12 cm in chloroform: methanol:acetic acid (75:25:8 v/v) with drying in a N_2 atmosphere between developments. TLC plates were subsequently developed to their full lengths (20 cm) in hexane:ethyl ether:acetic acid (60:40:1 v/v). This method allowed analysis of polar and neutral lipids on the same TLC plate with full resolution of all major glycerolipid classes. Some cross-contamination of DAG with radiolabeled free sterols was detected, particularly in the 3.5 h labeling experiment with coriander endosperm slices. However, free sterols represented only a small fraction of labeled lipids and could be distinguished upon argentation TLC analysis of fatty acid methyl esters as described below. In addition, identification of radiolabeled PC was confirmed by two-dimensional TLC analysis (Christie, 1982). Radiation in lipid classes was determined by autoradiography and quantified by liquid scintillation counting of lipid bands of TLC plates scraped into 3a20 (Research Products International, Mt. Prospect, IL) scintillation cocktail. Alternatively, the distribution of radiation in fatty acids of specific lipid classes was determined following TLC separation and transesterification of lipids as described above. The resulting fatty acid methyl esters were separated by

argentation TLC (Morris *et al.*, 1967). TLC plates were prepared by immersion of silica plates in a solution of 15% (w/v) $AgNO_3$ in acetonitrile for 10 minutes followed by air-drying. Argentation TLC plates (20 cm in length) were developed sequentially at -20°C to heights of 10 cm, 15 cm, and 20 cm in toluene. TLC plates were air-dried between developments. This procedure resulted in full resolution of saturated fatty acid, di-unsaturated fatty acid, and, in particular, petroselinic acid and oleic acid methyl esters. Separated fatty acid methyl esters were visualized by autoradiography as well as by light spraying with 0.1 wt% 2,7 dichlorofluorescein in methanol (Kates, 1972). Radioactivity in fatty acid methyl ester bands was determined as described above.

[1-^{14}C]Acetate Pulse-Chase Labeling of Coriander Endosperm

Transverse slices (approx. 1 mm thickness) of freshly harvested coriander endosperm (300 mg fresh weight) were incubated in 1 mL of 0.14 mM sodium [1-^{14}C]acetate in 50 mM Mes-NaOH, pH 5.0, for 15 min (Slack *et al.*, 1978) in a loosely-capped 13 mm x 100 mm test tube. The incubation tube was shaken at 100 rpm and maintained at 25°C. After 15 min, the labeling buffer was removed, and endosperm slices were washed three times with 2 mL of ice-cold water and once with 2 mM unlabeled sodium acetate in 50 mM Mes, pH 5.0. The endosperm slices were quickly partitioned among five pre-weighed test tubes. One test tube contained 1 mL isopropanol which was subsequently heated at 80°C for 10 minutes to stop further metabolism of the label. The remaining four test tubes which constituted the chase contained 1 mL of 2 mM unlabeled sodium acetate in 50 mM Mes pH 5.0. At times of 30, 90, 180, and 360 min following the 15 min pulse, buffer was removed from one of the four chase tubes, and the endosperm was washed with 1 mL of ice-cold water. One mL of isopropanol was added to the tube which was subsequently heated at 80°C for 10 min. Lipids were extracted from endosperm slices as described above for [1-^{14}C]acetate time-course labeling experiments. During the chase period, the incubation buffer was removed from each of the four tubes and replaced with fresh buffer containing 2 mM sodium acetate after 15 minutes and again at 30 min

intervals during the first two hours. This was done in order to minimize incorporation of residual [1-^{14}C]acetate.

The distribution of label in lipid classes and fatty acid methyl esters of specific lipid classes was determined as described above. In addition, stereospecific analysis of labeled fatty acids of PC following the initial 15 min pulse was performed as described above. The distribution of label in fatty acid methyl esters derived from lysoPC and FFA was determined by liquid scintillation counting following separation of acyl derivatives by argentation TLC as described above.

Results

Distribution of Petroselinic Acid in Glycerolipids of Carrot and Coriander Endosperm

Petroselinic acid has been previously identified in phosphatidylcholine (Dutta *et al.*, 1992) and the total polar lipid fraction of carrot seed (Dutta and Appelqvist, 1991) as well as the total phospholipids of carrot and coriander seed (Prasad *et al.*, 1987). A more detailed analysis indicated that petroselinic acid is a component of all detectable glycerolipids of developing carrot and coriander endosperm (Table 4.1). Relative amounts of this fatty acid in individual lipid classes, however, were quite different. As is the case with most unusual fatty acids, the highest levels of petroselinic acid were detected in TAG. In extracts of both carrot and coriander endosperm, petroselinic acid accounted for about 70 to 75 mol% of the total fatty acid of TAG. Petroselinic acid was also the major fatty acid of DAG of both coriander and carrot endosperm, which is consistent with the primary role of this lipid as a precursor of TAG in oil-accumulating seeds. In marked contrast, the major phospholipids PC, phosphatidylethanolamine (PE), and phosphatidic acid (PA), which together accounted for nearly 75 mol% of the total polar lipids of carrot and coriander endosperm, contained about one-fourth to one-eighth as much petroselinic acid as TAG. PC of carrot and coriander endosperm, for example, contained 15 and 20 mol% petroselinic

Table 4.1. Glycerolipid content and fatty acid composition of glycerolipids of developing coriander[a] (A) and carrot (B) endosperm

Fatty acid compositions were determined by gas chromatography of fatty acid methyl esters derived from glycerolipids purified by silica column chromatography and TLC. Lipids were quantified by fatty acid mass measured relative to heptadecanoic acid (17:0) (internal standard). Fatty acid compositions are expressed as mol% ± SE (n=3-5), except in the case of fatty acids coriander MGDG and DGDG which are expressed as the average mol% of two determinations.

(A)

	Mol% of Total Lipid	16:0	16:1[b]	18:0	18:1Δ[6]	18:1Δ[9]	18:2	other[c]
					mol%			
TAG	92.9	3.8 ± 0.5	0.4 ± 0.1	0.9 ± 0.2	75.0 ± 1.2	6.3 ± 0.8	12.7 ± 0.4	≤1.0
DAG	2.3	6.3 ± 0.8	0.8 ± 0.3	1.1 ± 0.1	63.0 ± 1.3	8.2 ± 0.3	19.4 ± 1.9	≤1.3
PC	2.1	15.1 ± 0.7	1.4 ± 0.1	1.6 ± 0.3	20.0 ± 1.1	15.7 ± 1.4	44.8 ± 2.7	≤1.7
PE	0.9	26.9 ± 0.5	1.2 ± 0.1	1.6 ± 0.7	9.1 ± 0.1	6.4 ± 0.7	54.4 ± 1.0	≤1.6
PA	0.8	17.4 ± 1.0	0.8 ± 0.2	1.2 ± 0.1	12.0 ± 0.4	14.1 ± 2.3	52.6 ± 2.2	≤2.0
PI	0.5	25.1 ± 0.8	1.1 ± 0.1	2.0 ± 0.3	26.1 ± 1.4	9.0 ± 0.8	35.5 ± 1.8	≤2.2
MGDG	0.1	9.2	1.0	3.1	11.3	3.0	29.1	≤42.5[d]
DGDG	>0.1	13.3	0.6	1.8	23.1	3.3	35.6	≤22.3[e]

(B)

Mol% of Total Lipid		16:0	16:1[b]	18:0	18:1Δ[6]	18:1Δ[9]	18:2	other[c]
				mol%				
TAG	95.2	3.6 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	70.6 ± 0.7	9.4 ± 0.2	14.8 ± 0.5	≤0.7
DAG	1.3	11.8 ± 0.8	0.4 ± 0.1	2.3 ± 0.7	51.2 ± 1.6	14.3 ± 0.6	18.6 ± 2.0	≤1.4
PC	1.6	13.3 ± 0.4	0.9 ± 0.1	1.0 ± 0.2	15.5 ± 1.0	19.4 ± 1.9	48.3 ± 2.5	≤1.6
PE	0.8	20.2 ± 0.7	0.8 ± 0.1	1.2 ± 0.2	7.3 ± 0.3	6.9 ± 0.9	62.3 ± 1.3	≤2.0
PA	1.2	15.5 ± 1.1	0.5 ± 0.1	0.9 ± 0.1	9.3 ± 0.8	15.3 ± 2.4	56.6 ± 3.7	≤2.0
PI	0.5	23.4 ± 1.3	1.5 ± 0.3	2.3 ± 0.3	25.7 ± 1.5	10.5 ± 0.8	34.2 ± 2.0	≤1.5
MGDG	0.2	5.1 ± 0.8	1.2 ± 0.1	1.6 ± 0.3	26.4 ± 1.6	4.8 ± 0.4	18.4 ± 1.2	≤42.7[f]
DGDG	0.2	8.6 ± 0.8	0.7 ± 0.2	1.7 ± 0.1	41.2 ± 1.3	4.5 ± 0.4	23.3 ± 1.2	≤20.2[g]

[a]22-26 DAF. [b]Sum of three isomers (16:1Δ[4], 16:1Δ[6], and 16:1Δ[9]). [c]Includes primarily 14:0, 18:1Δ[11], and 18:3, unless otherwise indicated. [d]Includes 16:2, 6.3%; 16:3, 3.6%; 18:1Δ[11], 1.1%; and 18:3, 30.8%. [e]Includes 18:1Δ[11], 1.3% and 18:3, 20.2%. [f]Includes 16:2, 2.9 ± 0.4%; 16:3, 4.1 ± 0.7%; 18:1Δ[11], 1.0 ± 0.1%; and 18:3, 32.1 ± 2.8%. [g]Includes 18:1Δ[11], 1.3 ± 0.1% and 18:3, 18.5 ± 1.6%.

acid, respectively. In addition, petroselinic acid composed <10 mol% of PE of the endosperm of both species. In contrast to TAG, the major fatty acids of these polar lipids were oleic acid and/or its derivative linoleic acid. Minor glycerolipids, particularly phosphatidylinositol (PI) as well as the plastid galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) of carrot endosperm, contained somewhat higher levels of petroselinic acid than did PC and PE. Still, relative amounts of petroselinic acid in these lipids were roughly one-half to one-third as much as in TAG.

In addition to petroselinic acid, small amounts (≤1.5 mol%) of other unusual fatty acids were detected in glycerolipids of carrot and coriander endosperm. These included *cis*-vaccenic acid ($18:1\Delta^{11\underline{cis}}$) and three hexadecenoic acid (16:1) isomers. Mass spectral analyses of thiomethyl derivatives of hexadecenoic acid methyl esters indicated that these fatty acids were Δ^4, Δ^6, and Δ^9 isomers (Appendix 1).

It is notable that PA levels in developing carrot and coriander endosperm were higher than might be expected for a plant tissue. Slack *et al.* (1978), for example, reported that PA composed between 4 to 13 mol% of the phospholipids of developing cotyledons of safflower, soybean, and linseed. In our analyses, PA accounted for approximately 16 and 24 mol% of the polar lipids of the developing endosperm of coriander and carrot, respectively. It is difficult to attribute this PA to the degradation of PC and PE during or prior to lipid extraction. In order to inactivate potential lipolytic enzymes, endosperm was incubated in hot isopropanol before extraction of lipids (Kates and Eberhardt, 1957). Also, in radioisotope labeling experiments described below, PC was heavily labeled with [1-^{14}C]acetate, but no significant breakdown of this lipid to PA was detected in either time-course or pulse-chase labeling experiments.

Lipid Synthesis During the Development of Coriander Endosperm

The nearly four-fold higher relative amounts of petroselinic acid in TAG versus PC in coriander endosperm described above might reflect

temporal differences in TAG and PC synthesis. For example, the majority of PC might be synthesized during early stages of endosperm development prior to the rapid synthesis and accumulation of petroselinic acid in TAG. To examine this possibility, changes in absolute amounts of total fatty acid and petroselinic acid in PC and TAG were examined over a period of coriander endosperm development ranging from early to mid-maturity. Depending upon greenhouse conditions, a distinct endosperm (*i.e.*, endosperm readily separable from pericarp) could be detected by approximately 12 to 16 DAF and mid-maturity was reached by 18 to 22 DAF. In the example detailed in Table 4.2, the fresh weight of coriander endosperm more than doubled between 16 and 20 DAF. During this period, TAG and PC were both actively synthesized as the fatty acid content of these lipids increased by more than six-fold and five-fold, respectively. Despite this, relative levels of petroselinic acid deposition into TAG and PC were significantly different. Of the increase in fatty acid content of TAG, about 75% could be accounted for by increases in amounts of petroselinic acid [(Δ nmol petroselinic acid/Δ nmol total fatty acid) x 100%], whereas changes in petroselinic acid content constituted about 19% of the increases in fatty acids of PC. These data therefore suggest that differences in relative amounts of petroselinic acid in PC and TAG are not the result of temporal differences in the synthesis of these lipids.

[1-^{14}C]Acetate Time-Course Labeling of Carrot and Coriander Endosperm

Analyses of fatty acid compositions of lipids and developmental changes in these compositions as described above give only an indication of the net products of a myriad of metabolic reactions. To gain a better understanding of how these fatty acid compositions arise, [1-^{14}C]acetate labeling studies of carrot and coriander endosperm were performed. In time-course labeling of carrot endosperm (Figure 4.1A), at least 85% of the radioactivity recovered in glycerolipids was detected in PC, TAG, DAG, and PE at time points through 1 h. Of these lipids, PC and TAG were the most heavily labeled.

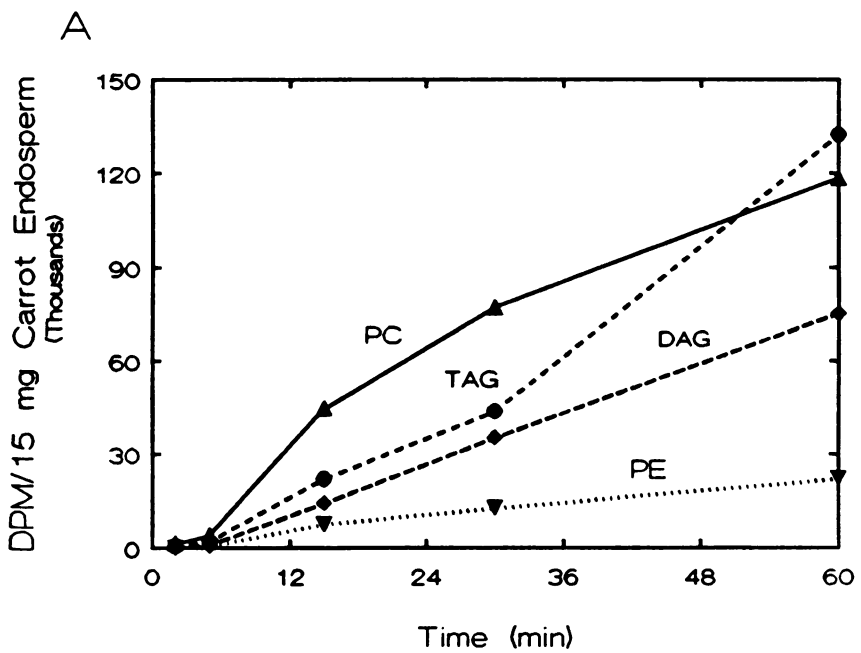
Table 4.2. *Changes in total fatty acid and petroselinic acid content of phosphatidylcholine (PC) and triacylglycerol (TAG) from early (16 DAF) to mid (20 DAF)-development of coriander endosperm*

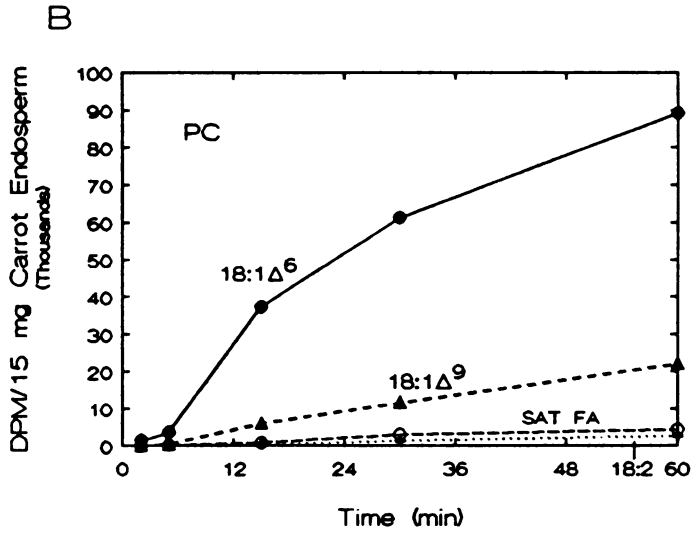
Fatty acids were analyzed by gas chromatography as methyl ester derivatives and amounts determined relative to methyl heptadecanoic acid (17:0) (internal standard).

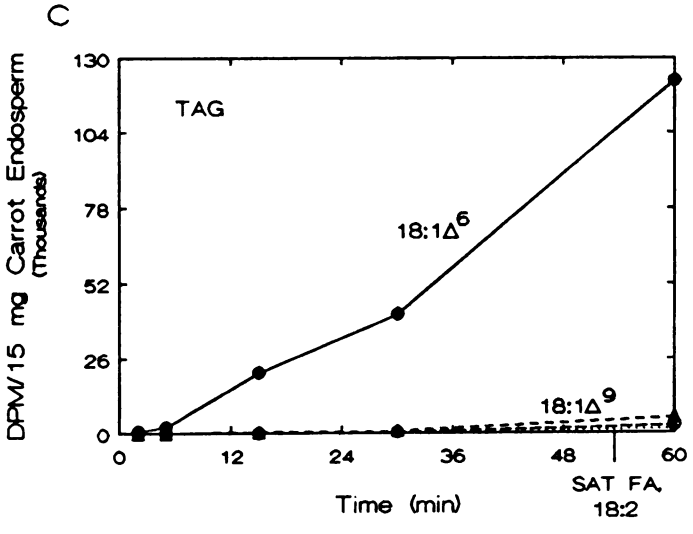
		TAG	PC
DAF[a]	Fresh Weight	Total FA[b]/18:1Δ^6	Total FA/18:1Δ^6
	mg/endosperm	*nmol fatty acid/endosperm*	
16	1.7	199/127	7.2/0.8
18	2.7	719/534	25.3/4.7
20	3.9	1,260/930	38.3/6.7

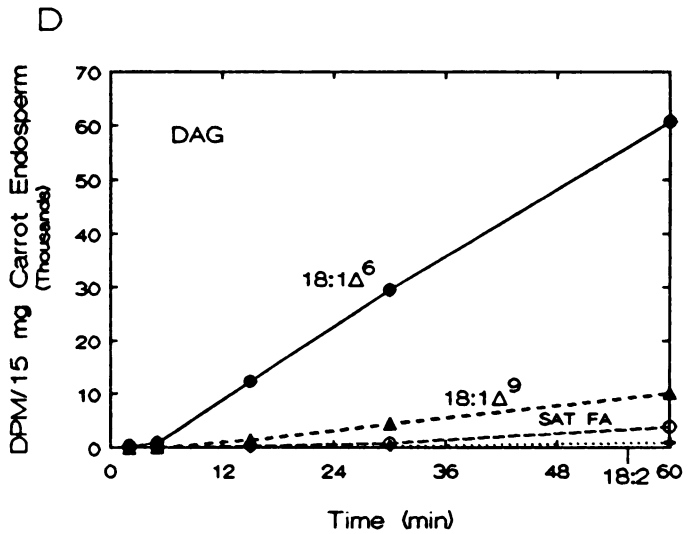
[a]DAF, Days after flowering. [b]FA, Fatty acids.

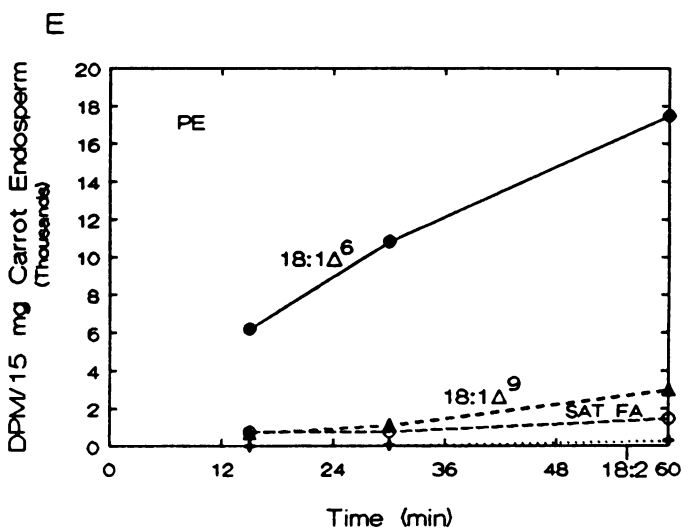
Figure 4.1. Products of [1-^{14}C]acetate time-course labeling of developing carrot endosperm. Shown is the distribution of radioactivity recovered in the major glycerolipid classes (A) and fatty acids of phosphatidylcholine (PC) (B), triacylglycerol (TAG) (C), diacylglycerol (DAG) (D), and phosphatidylethanolamine (PE) (E) at time points through 1 h.


A
DPM/15 mg Carrot Endosperm
(Thousands)
150
120
90
60
30
0
0
12
24
36
48
60
Time (min)
PC
TAG
DAG
PE


B
PC
18:1Δ6
18:1Δ9
SAT FA
DPM/15 mg Carrot Endosperm (Thousands)
Time (min)
18:2
0
12
24
36
48
60
10
20
30
40
50
70
80
90
100


C
TAG
18:1Δ6
18:1Δ9
DPM/15 mg Carrot Endosperm (Thousands)
Time (min)
SAT FA, 18:2
0
12
24
36
48
60
26
52
78
104
130


D
DAG
DPM/15 mg Carrot Endosperm (Thousands)
70
60
50
40
30
20
10
0
18:1Δ6
18:1Δ9
SAT FA
0
12
24
36
48
18:2
60
Time (min)


E
PE
DPM/15 mg Carrot Endosperm (Thousands)
20
18
16
14
12
10
8
6
4
2
0
18:1Δ6
18:1Δ9
SAT FA
0
12
24
36
48
18:2
60
Time (min)

The most striking result was the rapid incorporation of high levels of [1-^{14}C]acetate into PC as petroselinic acid. At time points through 30 min, PC was the most heavily labeled glycerolipid. Of the radioactivity detected in PC during this period, nearly 85% was in the form of petroselinic acid, and even after 1 h, 75% of the label in PC was associated with this fatty acid (Figure 4.1B). Relative to PC, the radiolabel appeared to initially enter TAG at a slower rate. However, by 1 h, the highest amounts of radioactivity were detected in TAG. At this time point, TAG contained approximately 1.1 times as much ^{14}C as PC. In addition, at least 90% of the radiolabel in TAG was present as petroselinic acid throughout the 1 h labeling period (Figure 4.1C). The incorporation of high amounts of [1-^{14}C]acetate initially into PC followed by accumulation into TAG at later time points was suggestive of the metabolic movement of fatty acids from PC into TAG. Such precursor-product labeling kinetics, in fact, were similar to those previously described for the metabolism of oleic acid-derived C_{18} polyunsaturated fatty acids in seeds of soybean and linseed (Slack *et al.*, 1978), in which acyl chains were shown to move from PC into TAG.

In addition to PC and TAG, significant amounts of radioactivity were detected in DAG as well as the polar lipid PE (Figure 4.1D and E). Throughout the 1 h time-course, >80% of the radiolabel in these lipids was detected as petroselinic acid. With regard to PE, however, the total amount of label in this lipid was five- to seven-times less than that in PC.

Similar to carrot endosperm, in pulse-chase labeling experiments of coriander endosperm described below, [1-^{14}C]acetate was also incorporated into PC in high levels as petroselinic acid during an initial 15 min "pulse" labeling period. In addition, in coriander endosperm that had been incubated in [1-^{14}C]acetate for an extended period of time (3.5 h), PC persisted as a major radiolabeled glycerolipid, accounting for nearly 30% of the total radioactivity recovered in the total lipid extract (Table 4.3). Of the label in PC, about 55% was detected as petroselinic acid.

Table 4.3. *Distribution of radiolabel incorporation into glycerolipids of coriander endosperm following 3.5 h incubation in [1-^{14}C]acetate*

Lipid Class	% of Total Incorporated ^{14}C	% Distribution of Radiolabel in Fatty Acids			
		SatFA[a]	18:1Δ^{6}[b]	18:1Δ^{9}	18:2
Total	100	8.1	72.6	18.3	2.6
TAG	39.4	2.0	90.0	5.6	2.3
PC	28.7	6.4	56.4	31.6	5.5
DAG	18.9	4.4	78.3	15.0	2.3
PE	4.0	16.2	63.3	18.6	1.9
PI	3.1	---ND[c]--			
PA	2.8	---ND---			
Other	3.1	---ND---			
	Total Incorporation	25,800 DPM/mg endosperm			

[a]Total saturated fatty acid. [b]May include small amounts of 16:1Δ^{6}. This acyl moiety composes 2.6% of the total radiolabeled fatty acid. [c]Not determined.

In comparison, 39% of the label in the total lipid extract was recovered as TAG, of which 90% was present in the form of petroselinic acid. DAG also contained significantly high levels of recovered label (19% of the total label), and the relative proportion of radiolabeled petroselinic acid in this lipid (78%) was roughly similar to that in the total extract. Although the total amount of label in PE was more than seven-fold less than that in PC, this polar lipid also contained high relative amounts of label in the form of petroselinic acid (63%).

[1-^{14}C]Acetate Pulse-Chase Labeling of Coriander Endosperm

[1-^{14}C]Acetate labeling of carrot and coriander endosperm described above suggested a possible flux of fatty acids from PC into TAG. To determine whether ^{14}C-petroselinic acid does move from PC to TAG, [1-^{14}C]acetate pulse-chase labeling of tissue slices of developing coriander endosperm was conducted. Following a 15 min incubation in labeled acetate, endosperm slices were placed in unlabeled acetate for periods of up to 6 h. After the initial 15 min labeling ("pulse"), more than 85% of the total label was detected in PC, TAG, and DAG (Table 4.4).As with time-course labeling of carrot endosperm (see above), PC was the most heavily labeled lipid after 15 min of incubation of coriander endosperm in [1-^{14}C]acetate. At the end of the 15 min pulse, 42% of the incorporated [1-^{14}C]acetate recovered in the lipid fraction was detected in PC, and 85% of the label in PC was present as petroselinic acid. Some difficulty was encountered in completely removing unincorporated [1-^{14}C]acetate from the endosperm slices at the end of the pulse period. As a result, a 60% increase in levels of incorporated label was detected during the subsequent chase period. Despite this, the absolute amount of radioactivity in PC had declined by close to 2.5-fold at the end of the 6 h chase. This change in amounts of radiolabel in PC included proportional losses in ^{14}C-petroselinic acid. Accompanying this change was a marked increase in the amount of label in TAG. At the end of the 6 h chase, radioactivity in TAG had increased nearly 7-fold, and this change included increases in amounts of radiolabeled petroselinic acid.

Table 4.4. *Redistribution of radioactivity in phosphatidylcholine (PC), triacylglycerol (TAG), and diacylglycerol (DAG) following transfer of coriander endosperm slices from [1-^{14}C]acetate to media containing unlabeled acetate*

Slices of coriander endosperm at mid-development were incubated in 0.14 mM [1-^{14}C]acetate for 15 min, washed, and transferred to media containing 2.0 mM unlabeled acetate (see Materials and Methods). Results below indicate recovery of radioactivity in PC, TAG, and DAG at time points following transfer to unlabeled media.

Time in unlabeled media	Total ^{14}C Incorporated	PC		TAG		DAG
		Tot.[a]	Δ^{6b}	Tot.	Δ^{6}	Tot.
min	*dpm (x10^{-2})/mg endosperm*	*dpm (x10^{-2})/mg endosperm*				
0	10.4	4.3	3.7	2.7	2.5	2.2
30	11.0	3.6	2.9	4.3	3.8	2.1
90	12.9	3.4	2.9	5.3	4.8	2.2
180	15.1	2.9	2.5	7.5	6.6	3.2
360	16.7	1.7	1.4	9.1	8.1	3.8

[a]Tot., Total radioactivity in fatty acids of given lipid class. [b]Δ^{6}, Radioactivity in petroselinic acid of given lipid class.

Because of the increase in total recovered label during the chase period, it was not possible to definitively assign label lost from PC to that gained by TAG. However, losses in absolute amounts of radioactivity in PC accompanied by increases in radiolabel in TAG during the chase was consistent with a precursor-product relationship between these lipids.

Stereospecific Patterns of Petroselinic Acid Metabolism in Glycerolipids of Coriander Endosperm

In many seeds, the *sn*-2 position of glycerolipids contains almost exclusively C_{18} fatty acids with Δ^9 unsaturation, *e.g.*, oleic acid and linoleic acid (Stymne and Stobart, 1987). The biochemical basis for this acylation pattern is believed to reside in the substrate specificity of lysoPA acyltransferase, the enzyme which catalyzes the esterification of acyl-CoA moieties to the *sn*-2 position of the glycerol backbone (Ichihara *et al.*, 1987). However, in seeds which synthesize certain unusual fatty acids, specialized lysoPA acyltransferases appear to exist with substratespecificities that permit the accumulation of unusual acyl moieties at the *sn*-2 position of TAG (Oo and Huang, 1989; Bafor *et al.*, 1990; Cao *et al.*, 1990).

With regard to petroselinic acid, this fatty acid, in terms of mass, was found to be most concentrated in the *sn*-1 position of PC isolated from coriander endosperm (Table 4.5). However, significant amounts of petroselinic acid were also present in the *sn*-2 position of this lipid. In fact, approximately 40% of the total petroselinic acid in PC was detected in this stereospecific position. Similarly, petroselinic acid accounted for nearly 50 mol% of the fatty acid mass of the *sn*-2 position of coriander TAG (Table 4.6). This value closely agreed with that determined by Gunstone (1991) using ^{13}C NMR. Furthermore, in PC from coriander endosperm labeled with [1-^{14}C]acetate for 15 min, nearly 85% of the total radiolabeled fatty acids were composed of ^{14}C-petroselinic acid. Of the labeled fatty acids of the *sn*-2 position of PC, slightly more than 80% was detected as petroselinic acid (Table 4.7). Thus, fatty acid mass and radiolabeling data demonstrate the ability of coriander endosperm to

Table 4.5. *Stereospecific fatty acid composition of phosphatidylcholine of developing coriander endosperm*

PC was purified from phospholipid extracts of developing coriander endosperm (22-26 DAF). Fatty acid compositions are expressed as mol% of the total fatty of PC or the total fatty acid of the *sn*-1 or *sn*-2 stereospecific positions of PC. Values are the average of two determinations.

Fatty Acid	Total FA[a]	*sn*-1	*sn*-2
		mol%	
16:0	15.3	30.0	0.5
16:1[b]	1.4	2.0	0.9
18:0	2.2	3.9	0.5
18:1Δ^6	21.8	26.7	16.9
18:1Δ^9	14.4	6.4	22.5
18:1Δ^{11}	1.4	2.0	0.7
18:2	43.6	29.3	58.0

[a]FA, Fatty acids. [b]Sum of three detectable 16:1 isomers.

Table 4.6. *Stereospecific fatty acid composition of triacylglycerol of developing coriander endosperm (22 to 26 DAF)*

Fatty acid compositions are expressed as mol% ± SE (n=3) of the total FA of TAG, 1,2;2,3-DAG (Grignard hydrolysis of TAG), or the calculated *sn*-2 position of TAG. The *sn*-2 position of TAG was calculated using the formula [(4 x 1,2;2,3-DAG fatty acid mol%)-(3xTAG fatty acid mol%)] (see Materials and Methods).

Fatty Acid	Total FA[a]	1,2;2,3-DAG	*sn*-2
		mol%	
16:0	3.9 ± 0.7	3.3 ± 0.7	1.5 ± 0.7
16:1[b]	0.4 ± 0.1	0.4 ± 0.2	0.8 ± 0.1
18:0	1.0 ± 0.2	0.7 ± 0.1	0
$18:1\Delta^{6}$	74.0 ± 0.9	67.6 ± 0.9	48.5 ± 0.7
$18:1\Delta^{9}$	7.2 ± 0.8	10.7 ± 1.0	21.2 ± 2.1
$18:1\Delta^{11}$	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
18:2	12.7 ± 0.6	16.4 ± 1.0	27.4 ± 2.1

[a]FA, Fatty acids. [b]Sum of three detectable 16:1 isomers.

Table 4.7. *Stereospecific distribution of radioactivity in PC following 15 min incubation of coriander endosperm slices in [1-^{14}C] acetate*

Fatty Acid	% of Total ^{14}C in PC	% of Total ^{14}C in *sn*-1	% of Total ^{14}C in *sn*-2
Saturated FA[a]	2	3	2
18:1Δ^6	85	90	81
18:1Δ^9	13	7	18
Ratio of ^{14}C *sn*-1/^{14}C *sn*-2		42/58	

[a]FA, Fatty acids.

readily use both the *sn*-1 and *sn*-2 positions of the glycerol backbone of lipids in the metabolism of petroselinic acid.

Discussion

Like many other unusual fatty acids of seeds, petroselinic acid was most concentrated in TAG of developing carrot and coriander endosperm. In contrast, relative amounts of petroselinic acid in the major polar lipids PC and PE were much reduced. In coriander and carrot endosperm, for example, petroselinic acid composed 70 to 75 mol% of the fatty acids of TAG but only 15 to 20 mol% and <10 mol% of the fatty acids of PC and PE, respectively (Table 4.1). One explanation for such differences is that metabolic mechanisms exist in cells of these tissues which maintain active flux of petroselinic acid into TAG but limit or partially exclude the accumulation of this fatty acid in polar lipids. In seeds of *Cuphea lanceolota*, for example, it has been proposed that medium-chain length fatty acids are excluded from PC by the high activity of DAG acyltransferase for DAG molecules rich in these fatty acids (Bafor *et al.*, 1991). In such a scenario, unusual fatty acids are rapidly shunted into TAG synthesis and effectively precluded from incorporation into PC and other polar lipids. Our results from [1-^{14}C]acetate labeling studies of carrot and coriander endosperm, however, give a much different view of petroselinic acid metabolism. In this regard, incubation of carrot and coriander endosperm in [1-^{14}C]acetate for short periods (15 to 30 minutes) was accompanied by the rapid incorporation of label into PC in high levels, and >80% of the radioactivity in PC and PE was associated with petroselinic acid. Relative to PE, however, incorporation of radiolabel into PC was more significant as this lipid typically contained about seven times more radioactivity than PE in these experiments. Thus, results of [1-^{14}C]acetate radiolabeling studies indicated that there is virtually no exclusion of petroselinic acid incorporation into polar lipids, especially PC, despite the fact that this fatty acid does not accumulate to high levels in these lipids in terms of mass.

If little or no exclusion of petroselinic acid from PC and perhaps other polar glycerolipids occurs, what prevents high levels of accumulation of petroselinic acid in these lipids? One possibility is that turnover of polar lipids, particularly PC, results in reductions in amounts of petroselinic acid. Such turnover could involve either removal of fatty acids from the glycerol backbone or metabolic movement of the complete DAG moiety of PC into TAG. Turnover of PC in coriander and carrot endosperm is suggested by at least three observations. First, in time-course labeling of carrot endosperm with [1-^{14}C]acetate, radiolabel most rapidly entered PC at early time points (15 to 30 minutes), and the radioactivity detected in PC was primarily in the form of petroselinic acid (Figure 4.1). However, as the rate of incorporation of [1-^{14}C]acetate into PC declined at later time points, radioactivity accumulated in TAG at an accelerating rate and after 1 h, amounts of radiolabel in this lipid exceeded that in PC. Of the label accumulated in TAG, >90% was detected in petroselinic acid. Such labeling kinetics suggest that petroselinic acid first enters PC prior to its accumulation in TAG.

A second indication of fatty acid turnover in PC was obtained from pulse-chase labeling of coriander endosperm with [1-^{14}C]acetate (Table 4.4). In this study, significant amounts of label which were incorporated into PC during a 15 min incubation in [1-^{14}C]acetate were lost in a subsequent "chase" period. Accompanying this loss were significant increases in radioactivity in TAG, primarily in the form of petroselinic acid. The results of this experiment are thus consistent with a turnover of fatty acids in PC and, more specifically, suggest the possible movement of petroselinic acid, in route to TAG, through PC.

A third indication of fatty acid turnover in PC is the large disparity in the rates of fatty acid accumulation in PC and TAG as determined by radiolabeling studies and fatty acid mass analyses. In time-course labeling studies with carrot endosperm, amounts of radiolabeled fatty acids in TAG and PC were essentially equal after 1 h

incubation in [1-^{14}C]acetate (Figure 4.1). Similarly, after 3.5 h of incubation of coriander endosperm in [1-^{14}C]acetate, levels of radioactivity in fatty acids of TAG only 1.6-fold greater than in PC (Table 4.3). Based on these results, if no turnover of the fatty acids of PC occurs, one would expect to find high, perhaps nearly equal levels of fatty acid mass in PC and TAG. However, in developmental studies of coriander endosperm, the rate of fatty acid mass accumulation into TAG was at least 35 times greater than into PC (Table 4.2). One way to explain this large difference is that fatty acids (primarily petroselinic acid) are metabolized through PC such that they are readily detectable in this lipid in radiolabeling experiments. However, movement of petroselinic acid in the form of acyl or DAG moieties from PC results in relatively low levels of fatty acid mass accumulation in this lipid.

The involvement of PC in TAG biosynthesis in developing oilseeds has been previously documented for fatty acid flux in seeds which accumulate oleic acid derived-C_{18} polyunsaturated fatty acids (*e.g.*, linoleic acid and α-linolenic acid) (Slack *et al.*, 1978). In seeds such as those of safflower, linseed, and soybean, C_{18} polyunsaturated fatty acids synthesized from ^{14}C-acetate appear to move from PC into TAG in both time course and pulse-chase labeling experiments (Slack *et al.*, 1978) in a manner similar to that described here for petroselinic acid. However, with regard to C_{18} polyunsaturated fatty acids, PC acts as a substrate for the Δ^{12} desaturation of oleic acid and the Δ^{15} desaturation of linoleic acid in the endoplasmic reticulum (Browse and Somerville, 1991). It is generally believed therefore that the movement of polyunsaturated fatty acids through PC in developing oilseeds is related to the role of this lipid in fatty acid desaturation. In other words, in order for the cell to maintain a pool of unsaturated fatty acids for membrane lipid synthesis, oleic acid must be directed first through PC prior to incorporation into TAG. The unusual fatty acids γ-linolenic acid and ricinoleic acid are also metabolized through PC in seeds of borage (Stymne and Stobart, 1986; Griffiths *et al.*, 1988) and castor (Bafor *et al.*,

1991), respectively. Like the C_{18} polyunsaturated fatty acids, though, these unusual fatty acids are synthesized on PC via reactions involving oleic acid or linoleic acid. The Δ^6 double bond of petroselinic acid, however, does not derive from fatty acid desaturation on PC (Cahoon *et al.*, 1992; Chapter 3), and no further modification of petroselinic acid occurs on this lipid. Therefore, the apparent flux of petroselinic acid through PC suggests that the role of PC in TAG metabolism is not limited to its involvement in fatty acid desaturation or other modification reactions. PC, for example, could participate in the metabolic or physical movement of fatty acids to intracellular or intramembrane sites of TAG synthesis. In this regard, the radiolabeled PC detected in our studies might correspond to a pool of PC dedicated to TAG assembly rather than to membrane biogenesis. Alternatively, because of the similarity in structures of oleic acid and petroselinic acid, phospholipid biosynthetic enzymes such as CDP-choline:DAG cholinephosphotransferase may not be able to effectively distinguish DAG moieties rich in petroselinic acid. As such, the movement of petroselinic acid out of PC and perhaps other polar lipids might represent an editing or retailoring activity. Such activity has recently been demonstrated in studies by Banas *et al.* (1992) in which microsomal extracts of several oilseeds were able to remove unusual oxygenated fatty acids from PC via endogenous phospholipase activity.

It is interesting to speculate why petroselinic acid does not accumulate to high levels in polar lipids. It has been proposed that certain unusual fatty acids of oil seeds may be disruptive to "proper" membrane structure and function (Stymne *et al.*, 1990). In this regard, the melting point of petroselinic acid is twice as high as that of oleic acid (30°C vs. 14°C). In addition, plants cells apparently lack the ability to further desaturate petroselinic acid (*i.e.*, the occurrence of petroselinic acid-derived 18:2 or 18:3 has yet to be detected in plants). As a result, PC molecules rich in petroselinic acid would have significantly higher T_ms than those rich in oleic acid, and cells containing large amounts of petroselinic acid in PC would be less capable

of increasing levels of membrane unsaturation in response to environmental changes. However, coriander and carrot endosperm do contain significant amounts of petroselinic acid in polar lipids compared to seeds rich in other unusual fatty acids including medium-chain length fatty acids, erucic acid, and ricinoleic acid. Because the structure of petroselinic acid does not differ greatly from that of the common fatty acid oleic acid, it is likely that membranes of plant cells can tolerate somewhat higher levels of petroselinic acid than other unusual fatty acids of more divergent structures.

Another finding of this study was the ability of coriander endosperm to incorporate high levels of petroselinic acid in the *sn*-2 position of glycerolipids, particularly PC and TAG. In this regard, Dutta *et al.* (1992) have demonstrated that microsomes of carrot endosperm are capable of incorporating petroselinoyl-CoA at the *sn*-2 position of lysoPA. However, oleoyl-CoA was a much preferred substrate for this reaction. It was also reported that, in the presence of lysoPC, microsomes of carrot endosperm incorporate petroselinoyl-CoA more readily than oleoyl-CoA at the *sn*-2 carbon (Dutta *et al.*, 1992). Therefore, our results may indicate the activity of a lysoPA and/or lysoPC acyltransferase specialized for the metabolism of petroselinic acid. The detection of significant amounts of ^{14}C-petroselinic acid in the *sn*-2 position of PC may also reflect the composition of the acyl-CoA pool available for esterification at the *sn*-2 carbon. That is, the acyl-CoA pool may be enriched in ^{14}C-petroselinoyl-CoA relative to oleoyl-CoA following acetate radiolabeling of coriander endosperm.

The results of labeling experiments presented here provide a somewhat unique perspective of the metabolism of an unusual fatty acid in a developing seed. In this regard, in a proceedings report by Grobois and Mazliak (1979), it was noted that high levels of radiolabeled petroselinic acid were detected in the total phospholipid fraction of seed of English ivy (an Araliaceae species) that had been incubated in ^{14}C-acetate. This finding provides at least a partial, independent confirmation of our

results and suggests that the metabolism of petroselinic acid may be similar in seeds of families other than the Umbelliferae which accumulate high levels of this fatty acid.

An unanswered questions of this study is the mechanism through which the apparent flux of petroselinic acid from PC (and perhaps other polar lipids) to TAG occurs in developing coriander and carrot endosperm. [^{14}C]Acetate labelling as used in our experiments gives only an indication of the metabolism of fatty acids. To confirm the results presented above and to determine whether the intact glycerol backbone moves from PC to TAG, [1,3-^{14}C]glycerol labelling of coriander endosperm was attempted (results not shown). However, in these preliminary studies, only low levels of radiolabeled glycerol were incorporated into lipids, and a clear interpretation of the movement of petroselinic acid among lipid classes was not possible. The majority of the glycerol incorporated in these experiments was associated with DAG. Lesser amounts were detected in TAG, and only a small portion of the label was present in PC (data not shown). With time, levels of ^{14}C-glycerol in TAG increased. This distribution of radioactivity in glycerolipids is similar to that recently reported for ^{3}H-glycerol labeling of *Brassica napus* embryos (Perry and Harwood, 1993). The detection of low amounts of ^{14}C-glycerol in PC was also observed by Dutta *et al.* (1992) in studies of ^{14}C-glycerol-3-phosphate metabolism by carrot seed microsomes.

Another unanswered question is the relevance of our results to the metabolism of other unusual fatty acids, especially those of more atypical chemical structures. In particular, are these fatty acids metabolized through PC during periods of rapid TAG synthesis? Results of radiolabeling studies with seeds that accumulate medium-chain fatty acids have suggested a more limited involvement of PC in the flux of these fatty acids into TAG (Slabas *et al.*, 1982; Bafor *et al.*, 1990). In studies with seeds of plants (*e.g.*, Brassicaceae sp., *Limanthes* sp., and *Tropaeolum majus*) that synthesize high levels of very long-chain fatty acids ($\geq C_{20}$), radiolabel associated with these acyl moieties is often detected in PC and

other polar lipids (Gurr *et al.*, 1974; Pollard and Stumpf, 1980a,b; Fehling *et al.*, 1990; Löhden and Frentzen, 1992; Taylor *et al.*, 1992). The relative amounts of radiolabeled very long-chain fatty acids in PC and other polar lipids are typically in excess of that determined by measurement of the fatty acid mass composition of these lipids. Very long-chain fatty acids, however, are generally believed to enter TAG directly (through reactions of the Kennedy pathway) (Fehling *et al.*, 1990; Löhden and Frentzen, 1992; Taylor *et al.*, 1992). Still, it is interesting to speculate that a small, rapidly metabolized pool of PC might participate in the movement of other unusual fatty acids, including very long chain fatty acids, into TAG.

Literature Cited

Bafor M, Jonsson L, Stobart AK, Stymne S (1990) Regulation of triacylglycerol biosynthesis in embryos and microsomal preparations from the developing seeds of *Cuphea lanceolata*. Biochem J **272**: 31-38

Bafor M, Smith MA, Jonsson L, Stobart K, Stymne S (1991) Ricinoleic acid biosynthesis and triacylglycerol assembly in microsomal preparations from developing castor-bean (*Ricinus communis*) endosperm. Biochem J **280**: 507-514

Banas A, Johansson I, Stymne S (1992) Plant microsomal phospholipases exhibit preference for phosphatidylcholine with oxygenated acyl groups. Plant Sci **84**: 137-144

Battey JF, Schmid KM, Ohlrogge JB (1989) Genetic engineering for plant oils: Potential and limitations. Trends Biotech **7**: 122-125

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol **37**: 911-917

Browse J, Somerville C (1991) Glycerolipid synthesis: Biochemistry and regulation. Annu Rev Plant Physiol Plant Mol Biol **42**: 467-506

Cahoon EB, Shanklin J, Ohlrogge JB (1992) Expression of a coriander desaturase results in petroselinic acid production in transgenic tobacco. Proc Natl Acad Sci USA **89**: 11184-11188

Cao YZ, Oo K-C, Huang AHC (1990) Lysophosphatidate acyltransferase in the microsomes from maturing seeds of meadowfoam (*Limanthes alba*). Plant Physiol **94**: 1199-1206

Christie WW (1982) Lipid Analysis, 2nd Edition. Pergammon Press, Oxford

Dutta PC, Appelqvist L-A (1991) Lipids and fatty acid patterns in developing seed, leaf, root, and in tissue culture initiated from embryos of *Daucus carota* L. Plant Sci **75**: 177-183

Dutta PC, Appelqvist L-A, Stymne S (1992) Utilization of petroselinate (C $18:1^{\Delta 6}$) by glycerol acylation enzymes in microsomal preparations of developing embryos of carrot (*Daucus carota* L.), safflower (*Carthamus tinctorius* L.) and oil rape (*Brassica napus* L.). Plant Sci **81**: 57-64

Fehling E, Murphy DJ, Mukherjee KD (1990) Biosynthesis of triacylglycerols containing very long chain monounsaturated acyl moieties in developing seeds. Plant Physiol **94**: 492-498

Griffiths G, Stobart AK, Stymne S (1985) The acylation of *sn*-glycerol 3-phosphate and the metabolism of phosphatidate in microsomal preparations from the developing cotyledons of safflower (*Carthamus tinctorius* L.) seed. Biochem J. **230**: 379-388

Griffiths G, Stobart AK, Stymne S (1988) Δ^6- and Δ^{12}-dependent activities and phosphatidic acid formation in microsomal preparations from the developing cotyledons of common borage (*Borago officinalis*). Biochem J **252**: 641-647

Grobois M, Mazliak P (1979) Ultrastructural sites involved in petroselinic acid (C18:1Δ^6) biosynthesis during ivy seed (*Hedera helix*) development. *In* L-A Appelqvist, C Lijenberg, eds, Advances in the Biochemistry and Physiology of Plant Lipids. Elsevier/North-Holland Biomedical Press, Amsterdam, p 409-414

Gunstone FD (1991) The ^{13}C-NMR spectra of six oils containing petroselinic acid and of aquilegia and meadowfoam oil which contain Δ5 acids. Chem Phys Lipids **58**: 159-167

Gurr MI, Blades J, Appleby RS (1972) Studies on seed-oil triglycerides. The composition of *Crambe abyssinica* triglycerides during seed maturation. Eur J Biochem **29**: 362-368

Gurr MI, Blades J, Appleby RS, Smith CG, Robinson MP, Nichols BW (1974) Studies on seed-oil triglycerides. Triglyceride biosynthesis and storage in whole seeds and oil bodies of *Crambe abyssinica*. Eur J Biochem **43**: 281-290

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

Heimermann WH, Holman RT, Gordon DT, Kowalyshyn DE, Jensen RG (1973) Effect of double bond position in octadecanoates upon hydrolysis by pancreatic lipase. Lipids **8**: 45-47

Ichihara K, Asahi T, Fujii S (1987) 1-Acyl-*sn*-glycerol-3-phosphate acyltransferase in maturing safflower seeds and its contribution to the non-random fatty acid distirbution of triacylglycerol. Eur J Biochem **167**:339-347

Kates M (1972) Techniques of lipidology. *In* TS Work, E Work, eds, Laboratory Techniques in Biochemistry and Molecular Biology, Vol 3, Part 2. Elsevier/North-Holland, Amsterdam

Kates M, Ebehardt FM (1957) Isolation and fractionation of leaf phosphatides. Can J Bot **35**: 895-905

Kleiman R, Spencer GF (1982) Search for new industrial oils: XVI. Umbelliflorae-seed oils rich in petroselinic acid. J Am Oil Chem Soc **59**: 29-38

Lawson LD, Hughes BG (1988) Triacylglycerol structure of plant and fungal oils containing γ-linolenic acid. Lipids **23**: 313-317

Löhden I, Frentzen M (1992) Triacylglycerol biosynthesis in developing seeds of *Tropaeolum majus* L. and *Limanthes douglasii* R.Br. Planta **188**: 215-224

Lynch DV, Steponkus PL (1987) Plasma membrane lipid alterations associated with cold acclimation of winter rye seedlings (*Secale cereale* L. cv Puma). Plant Physiol **83**: 761-767

Morris LJ, Wharry DM, Hammond EW (1967) Chromatographic behaviour of isomeric long-chain aliphatic compounds. II. Argentation thin-layer chromatography of isomeric octadecenoates. J Chromatog **31**: 69-76

Morrison WR, Smith LM (1964) Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron trifluoride methanol. J Lipid Res **5**: 600-608

Myher JJ, Kuksis A (1979) Stereospecific analysis of triacylglycerols via racemic phosphatidylcholines and phospholipase C. Can J Biochem **57**: 117-124

Ohlrogge JB (1988) Molecular approaches to the study and modification of oilseed fatty acid synthesis. *In* TH Applewhite, ed, Proceedings World Conference on Biotechnology for the Fats and Oils Industry. American Oil Chemists Society, Champaign, IL, p 87-92

Oo K-C, Huang AHC (1989) Lysophosphatidate acyltransferase activities in the microsomes from palm endosperm, maize scutellum, and rapeseed cotyledon of maturing seeds. Plant Physiol **91**: 1288-1295

Perry HJ, Harwood JL (1993) Use of [2-^{3}H]glycerol precursor in radiolabelling studies of acyl lipids in developing seeds of *Brassica napus*. Phytochemistry **34**: 69-73

Pollard MR, Stumpf PK (1980a) Long chain (C_{20} and C_{22}) fatty acid biosynthesis in developing seeds of *Tropaeolum majus*. An *in vivo* study. Plant Physiol **66**: 641-648

Pollard MR, Stumpf PK (1980b) Biosynthesis of C_{20} and C_{22} fatty acids by developing seeds of *Limanthes alba*. Chain elongation and Δ5 desaturation. Plant Physiol **66**: 649-655

Post-Beittenmiller MA, Schmid KM, Ohlrogge JB (1989) Expression of holo and apo forms of spinach acyl carrier protein-I in leaves of transgenic tobacco plants. Plant Cell **1**: 889-899

Prasad RBN, Rao YN, Rao SV (1987) Phospholipids of palash (*Butea monosperma*), papaya (*Carica papaya*), jangli badam (*Sterculia foetida*), coriander (*Coriandrum sativum*) and carrot (*Daucus carota*) seeds. J Am Oil Chem Soc **64**: 1424-1427

Slabas AR, Roberts PA, Ormesher J, Hammond EW (1982) *Cuphea procumbens*. A model system for studying the mechanism of medium-chain fatty acid biosynthesis in plants. Biochem Biophys Acta **711**:411-420

Slack CR, Roughan PG, Balasingham N (1978) Labeling of glycerolipids in the cotyledons of developing oilseeds by [1-^{14}C]acetate and [2-^{3}H]glycerol. Biochem J **179**: 421-433

Stymne S, Stobart AK (1986) Biosynthesis of γ-linolenic acid in cotyledons and microsomal preparations of the developing seeds of common borage (*Borago officinalis*). Biochem J **240**: 385-393

Stymne S, Stobart AK (1987) Triacylglycerol biosynthesis. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants: A Comprehensive Treatise, Vol 9, Lipids: Structure and Function. Academic Press, New York, p 175-211

Stymne S, Bafor M, Jonsson L, Wiberg E, Stobart K (1990) Triacylglycerol assembly. *In* PJ Quinn, JL Harwood, eds, Plant Lipid Biochemistry, Structure and Utilization. Portland Press, London, p 191-197

Taylor DC, Barton DL, Rioux KP, MacKenzie SL, Reed DW, Underhill EW, Pomeroy MK, Weber N (1992) Biosynthesis of acyl lipids containing very-long chain fatty acids in microspore derived and zygotic embryos of *Brassica napus* L. cv Reston. Plant Physiol (1992) **99**: 1609-1618

Yamamoto K, Shibahara A, Nakayama T, Kajimoto G (1991) Determination of double-bond positions in methylene-interrupted dienoic fatty acids by GC-MS as their dimethyl disulfide adducts. Chem Phys Lipids **60**: 39-50

CHAPTER 5

Δ^6Hexadecenoic Acid is Synthesized by the Activity of a Soluble Δ^6Palmitoyl-Acyl Carrier Protein Desaturase in *Thunbergia alata* Endosperm

Abstract

The seed oil of *Thunbergia alata* Bojer ex Sims is composed of more than 80 wt% of the unusual fatty acid Δ^6hexadecenoic acid. Studies were conducted to determine the biosynthetic origin of the double bond of this fatty acid. Assays of soluble fractions of developing *T. alata* seed endosperm with [1-^{14}C]palmitoyl (16:0)-acyl carrier protein (ACP) revealed the presence of significant amounts of Δ^6 desaturase activity. This activity was greatest when 16:0-ACP was provided as a substrate, whereas no desaturation of the coenzyme A ester of this fatty acid was detected. In addition, $\Delta^6$16:0-ACP desaturase activity in *T. alata* endosperm extracts was dependent on the presence of ferredoxin and molecular oxygen and was stimulated by catalase. Most of the desaturase activity was lost by the addition of 1 mM potassium cyanide or hydrogen peroxide to assays. Using PCR with degenerate oligonucleotides corresponding to conserved regions of Δ^9stearoyl (18:0)- and $\Delta^4$16:0-ACP desaturases, a nucleotide probe encoding a portion of a diverged acyl-ACP desaturase was amplified from a *T. alata* endosperm cDNA library. A full-length cDNA corresponding to this probe was isolated. The mature peptide encoded by this cDNA shared 66% amino acid identity to the mature castor $\Delta^9$18:0-ACP desaturase and 57% amino acid identity to the mature coriander $\Delta^4$16:0-ACP desaturase. Extracts of *Escherichia coli* that express the *T. alata* cDNA catalyzed the Δ^6 desaturation of 16:0-ACP. These results demonstrate that Δ^6hexadecenoic acid in *T. alata* endosperm is formed by the activity of a soluble $\Delta^6$16:0-ACP desaturase that is structurally related to the $\Delta^9$18:0- and $\Delta^4$16:0-ACP desaturases. Implications of this work to an understanding of active site structures of acyl-ACP desaturases are discussed.

Introduction

Fatty acid desaturases of plants have received considerable attention because of their contributions to the physiology and economic value of plants. The activity of fatty acid desaturases, for example, may be a component of the ability of certain species to adjust levels of membrane unsaturation in response to stresses such as chilling (Cheesbrough, 1990; Wada *et al.*, 1990; Miquel *et al.*, 1993). In addition, the degree of fatty acid unsaturation resulting from desaturase activity is often a major determinant of the nutritional and industrial quality of plant seed oils (Battey *et al.*, 1989).

Plants typically contain a variety of fatty acid desaturases. The most common are membrane-associated desaturases that use fatty acids bound to glycerolipids as substrates (Browse and Somerville, 1991). In addition, the synthesis of oleic acid (18:1Δ^9) in plants and certain other organisms such as *Euglena* is catalyzed by a desaturase that functions on fatty acids esterified to acyl carrier protein (ACP) (Nagai and Bloch, 1965; Nagai and Bloch, 1968; Jaworski and Stumpf, 1974). In contrast to all previously characterized desaturases, this enzyme, the Δ^9stearoyl (18:0)- ACP desaturase, displays soluble activity (Nagai and Bloch, 1965). In the presence of radiolabeled 18:0-ACP and cofactors including NADPH, ferredoxin, and ferredoxin-NADPH reductase, the activity of the $\Delta^9$18:0-ACP desaturase is readily detectable in extracts of most plant tissues (McKeon and Stumpf, 1981). Due in part to its soluble nature and relative ease of assay, the $\Delta^9$18:0-ACP desaturase has been purified from several plant sources (McKeon and Stumpf, 1982; Cheesbrough and Cho, 1990; Kinney *et al.*, 1990; Shanklin and Somerville, 1991; Thompson *et al.*, 1991), and a number of cDNAs encoding this enzyme have been isolated (Kinney *et al.*, 1990; Shanklin and Somerville, 1991; Shanklin *et al.*, 1991; Thompson *et al.*, 1991; Nishida *et al.*, 1992; Sato *et al.*, 1992; Slocombe *et al.*, 1992; Taylor *et al.*, 1992). In addition to the $\Delta^9$18:0-ACP desaturase, a $\Delta^4$16:0-ACP desaturase has recently been identified in plants (Cahoon *et al.*, 1992; Chapter 3). This enzyme is a component of the petroselinic acid

(18:1Δ^6) biosynthetic pathway in endosperm of coriander (*Coriandrum sativum* L.) and other Umbelliferae species. Translation of a cDNA for the $\Delta^4$16:0-ACP desaturase has revealed that this enzyme shares extensive amino acid identity with the $\Delta^9$18:0-ACP desaturase (Cahoon *et al.*, 1992).

The existence of structurally-related acyl-ACP desaturases with different substrate recognition and double bond-positioning properties offers the opportunity to characterize the active site structure of this family of enzymes using techniques such as site-directed mutagenesis. These studies would be aided by the isolation of cDNAs for other functionally variant acyl-ACP desaturases in addition to those for the $\Delta^9$18:0- and $\Delta^4$16:0-ACP desaturases. A potential source of such a desaturase is seed of *Thunbergia alata* (a member of the Acanthaceae family). The oil of this tissue consists of nearly 85 wt% of the unusual fatty acid Δ^6hexadecenoic acid (16:1Δ^6) (Spencer *et al.*, 1971). In this report, we have used biochemical and molecular biological approaches to examine whether 16:1Δ^6 in *T. alata* seed endosperm is synthesized via an acyl-ACP desaturase that is related to the $\Delta^9$18:0- and $\Delta^4$16:0-ACP desaturases.

Materials and Methods

Plant Material

Studies were conducted using developing endosperm dissected from fruits of *Thunbergia alata* Bojer ex Sims (black-eyed susan vine) (Northrup King, Minneapolis, MN). Fruits were collected from plants grown either outdoors in pots during summers in East Lansing, Michigan or under greenhouse conditions with natural illumination. In the latter case, flowers required extensive hand-pollination for adequate fruit set. Endosperm was frozen in liquid nitrogen following dissection and stored at -70°C until use in enzyme assays or RNA extraction.

Acyl-ACP Desaturation Assays

Approximately 200 mg of developing *T. alata* endosperm was homogenized in 3 mL of buffer consisting of 100 mM Tris-HCl (pH 7.5), 2.5 mM DTT, 1 mM isoascorbate, 10% (v/v) glycerol, and 1.5% (w/w)

polyvinylpolypyrrolidone (PVPP) using an Elvehjem tissue grinder. The extract was subsequently centrifuged for 5 min at 14,000x*g* in order to remove debris and PVPP. The supernatant was then passed through two layers of miracloth (Calbiochem) and spun for an additional 10 min at 30,000x*g*. The soluble phase was removed while attempting to avoid recovery of the floating fat layer. A portion of contaminating fat was extracted by passing the supernatant through glass wool loosely packed in a Pasteur pipet. The 30,000x*g* supernatant was then centrifuged at 100,000x*g* for 60 min. All centrifugation steps were performed at 5°C. The resulting supernatant was used immediately for desaturation assays described below or frozen in aliquots in liquid N_2 and stored at -70°C until further use. Of note, extensive phenolic oxidation occurred in extracts developed a brown color, presumably due to extensive phenolic oxidation, when maintained at -20°C for longer than one to two weeks.

Acyl-ACP desaturation assays were based on those previously described by Jaworski and Stumpf (1974) and McKeon and Stumpf (1981). Assays were performed in a total volume of 150 μL in loosely-capped 13 x 100 mm tubes and consisted of 1.25 mM NADPH (from a freshly prepared stock in 100 mM Tricine, pH 8.2), 3.3 mM ascorbate, 0.7 mM DTT, 8000 U bovine liver catalase (Sigma), 5 μg bovine serum albumin (Fraction V) (Sigma), 20 μg spinach ferredoxin (Sigma), 80 mU spinach ferredoxin:NADPH reductase (Sigma), 33 mM Pipes (pH 6.0) and 118 pmoles of [1-^{14}C]acyl-ACP or -CoA. Reactions were started with the addition of the 100,000x*g* supernatant of homogenized *T. alata* endosperm (typically 20 to 25 μg total protein) and were conducted at room temperature (approx. 22°C) with shaking (100 rpm). Assays were terminated with the addition of 850 μL of 2.35 M NaOH and carrier fatty acids (30 μg of palmitic acid and petroselinic acid). The stopped reactions were then heated at 85°C for 1 h. Following acidification with 350 μL of 4 M H_2SO_4, the resulting free fatty acids were recovered by three extractions with 2.5 mL of hexane. Fatty acids were converted to methyl ester derivatives with 10% (w/v) boron trichloride (Alltech) using the method of Morrison and Smith (1964).

Reaction products were then analyzed on 15% $AgNO_3$ TLC plates developed sequentially to heights of 10 cm and 20 cm in toluene at -20°C. Radioactivity was detected by autoradiography and quantified by liquid scintillation counting of TLC scrapings in 3a20 complete cocktail (Research Products International).

To confirm the identity of 16:1Δ^6 produced from palmitoyl-ACP, assays were conducted as described above using [U-^{14}C]palmitoyl-ACP as the substrate. The methyl ester derivative of the monounsaturated product was purified by argentation TLC as described above and eluted from TLC scrapings with hexane:ethyl ether (2:1 v/v). The monounsaturated methyl ester was then cleaved at its double bond using permanganate-periodate oxidation (Christie, 1982). Chain lengths of oxidation products were determined relative to ^{14}C-fatty acid standards by reverse-phase TLC using a mobile phase of acetonitrile:methanol:water (75:25:0.5).

Inhibition of desaturase activity was examined by supplementing assays with 1 mM KCN (neutralized) or 1 mM H_2O_2. In the latter case, catalase was omitted from reactions. Oxygen-dependence of desaturase activity was characterized by purging assay tubes completely with nitrogen prior to and after addition of plant extract, and the reaction tube was tightly capped for the duration of the assay.

Radiolabeled acyl-ACPs were synthesized enzymatically according to the method of Rock and Garwin (1979) using *E. coli* ACP. The following fatty acids were used in the synthesis of acyl-ACPs: [1-^{14}C]myristic acid (American Radiolabeled Chemicals, St. Louis, MO) (sp. act. 55 mCi/mmol), [1-^{14}C]palmitic acid (New England Nuclear, Boston, MA) (sp. act. 58 mCi/mmol), [U-^{14}C]palmitic acid (New England Nuclear) (sp. act. 800 mCi/mmol), and [1-^{14}C]stearic acid (American Radiolabeled Chemicals) (sp. act. 55 mCi/mmol). [1-^{14}C]Palmitoyl-CoA (sp. act. 52 mCi/mmol) was purchased from Amersham.

***Thunbergia alata* Endosperm cDNA Library Construction**

Total RNA was isolated from *T. alata* endosperm using the method of Hall *et al.* (1978) with minor modifications. The ratio of buffer to

endosperm used in tissue homogenization was 3.5 mL/g, and the buffer was heated to 80°C prior to adding to the ground endosperm. In addition, the homogenization buffer contained freshly added 10 mM DTT rather than 5 mM DTT as previously described (Hall *et al.*, 1978) and precipitated RNA was washed three times with ice-cold 2 mM LiCl. Furthermore, the LiCl-washed RNA was dissolved in 10 mM Tris-HCl (pH 7.5) and centrifuged at 12,000x*g* for 20 min to remove any insoluble material. RNA was subsequently precipitated with the addition of potassium acetate (pH 5.5) to a final concentration of 200 mM and 2.5 volumes of ethanol.

RNA was subsequently passed through a column of Sigma Cell 50 (Sigma) in order to reduce amounts of polysaccharides potentially recovered along with the RNA. The column consisted of 0.25 g of Sigma Cell 50 placed in a Pasteur pipet. The Sigma Cell 50 was then sequentially washed three times with 1 mL aliquots of water; three times with 1 mL aliquots of 100 mM sodium hydroxide, 5 mM EDTA; three times with water; and five times with 1 mL aliquots of 20 mM Tris (pH 7.5), 0.5 M sodium chloride, 1 mM EDTA, 0.1% (w/v) SDS. RNA resuspended in 1 mL of the final column wash buffer was loaded onto the column and eluted with 5 mL of the final column wash buffer. The purified RNA was recovered following ethanol precipitation.

Poly A^+ RNA was enriched by passing total RNA once through a column of oligo-dT cellulose (Pharmacia) and subsequently used in the construction of a Uni-ZAP XR (Stratagene) cDNA expression library according to the instructions of the manufacturer. A portion of the total amplified library packaged in phage was *in vivo* excised as described by Hay and Short (1992) yielding pBluescript II SK (-) harboring cDNA inserts. The recovered plasmid DNA was used for cDNA isolation by colony hybridization and PCR amplification as described below.

PCR Amplification of Nucleotide Sequences Encoding Acyl-ACP Desaturases

Fully-degenerate sense and antisense oligonucleotides were prepared which corresponded respectively to the conserved amino acid sequences GlyAspMetIleThrGluGlu and GluLysThrIleGlnTyrLeu present in Δ^9stearoyl-

(Shanklin and Somerville, 1991; Shanklin *et al.*, 1991; Nishida *et al.*, 1992; Sato *et al.*, 1992; Slocombe *et al.*, 1992; Taylor *et al.*, 1992) and Δ^4palmitoyl-ACP desaturases (Cahoon *et al.*, 1992). The sequence of the resulting sense and antisense oligonucleotides were 5'GG(A/C/G/T)GA(C/T)ATGAT(A/C/T)AC(A/C/G/T)GA(A/G)GA3' and 5'A(A/G)(A/G)TATTG(A/G/T)AT(A/C/G/T)GT(C/T)TT(C/T)TC3', respectively. Included on the 5' terminus of each oligonucleotide was sequence (5'CAUCAUCAUCAU3' or 5'CUACUACUACUA3') that allowed for insertion of PCR products into the pAMP1 vector (GibcoBRL). Template for PCR amplification was generated by transformation of the SOLR strain (Stratagene) of *E. coli* with an aliquot of the *in vivo* excised *T. alata* endosperm cDNA library. Following growth of transformed *E. coli* to stationary phase in a 3 mL liquid culture, plasmid DNA was purified for use as template in PCR amplification. Reactions were performed in a 50 μL volume and consisted of 10 μM sense and antisense oligonucleotides, 150 to 300 ng plasmid DNA derived from the *T. alata* cDNA library, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1x *Taq* reaction buffer (Gibco BRL), and 5 units *Taq* polymerase (Gibco BRL). Temperature conditions for PCR amplification were 5 min at 95°C and 25 cycles of 1 min at 95°C, 1.5 min at 55°C, and 1.5 min at 72°C. This was followed by an additional 10 min extension at 72°C. PCR fragments of approximately 215 bp were gel-purified, ligated into the pAMP1 vector using the CloneAmp system (GibcoBRL) according to the manufacturer's protocol, and introduced into *E. coli* DH5α. The resulting colonies were screened using colony hybridization as described by Sambrook *et al.* (1989). A "negative" screening protocol was used to reduce the chances of re-isolating cDNAs (pTAD1, 2, and 3) encoding $\Delta^9$18:0-ACP desaturases that were previously obtained by antibody screening of the *T. alata* endosperm cDNA library (see "Results"). DNA probes for library screening were formed by PCR amplification of portions of pTAD 1, 2, and 3. Primers and PCR reaction conditions were the same as those described above. An equimolar mixture of the PCR products derived from pTAD1, 2, and 3 was used as template for the synthesis of [α-^{32}P]dCTP random-primed labeled

probes. Hybridization of plasmids of lysed colonies with radiolabeled probes was carried out in 6x SSC and 0.05x BLOTTO with shaking for 4 h at 53°C as described by Sambrook *et al.* (1989). Filters were washed three times in 1x SSC and 0.1% SDS at 60°C for 45 min and exposed to autoradiography. Plasmid DNA was subsequently isolated from ten of these colonies, and nucleotide sequence was obtained by dideoxy chain termination using Sequenase 2.0 (US Biochemical) according to the manufacturer's instructions. Two classes of plasmids were identified (designated pEC6 and 7), both of which contained inserts encoding for portions of apparent acyl-ACP desaturases (based on amino acid identity with known Δ^9stearoyl- and Δ^4palmitoyl-ACP desaturases).

Screening of the *T.alata* Endosperm cDNA Library

Aliquots of the *in vivo* excised-*T. alata* endosperm cDNA library were used to transform *E. coli* SolR cells. Approximately 50,000 of the resulting colonies were screened using colony hybridization as previously described (Sambrook *et al.*, 1989). Nucleotide probes for screening were generated by [α-^{32}P]dCTP random-primed hexamer labeling of inserts of pEC6 and 7. Hybridization and washing conditions were the same as those described above. Colonies containing plasmid DNA that strongly hybridized to the probe derived from pEC6 were isolated, and nucleotide sequence was obtained for the longest of these cDNAs (the corresponding plasmid was designated pTAD4) using Sequenase 2.0. Because of a relative lack of abundance, colonies containing plasmid hybridizing to pEC7 were not further characterized.

***E. coli* Expression of a Putative cDNA for Δ^6Palmitoyl-ACP Desaturase**

To determine the activity of the desaturase encoded by pTAD4, the portion of the clone corresponding to the mature peptide (total protein minus plastid transit peptide) was expressed in *E. coli*. This region of the cDNA insert of pTAD4 was first amplified by PCR using Vent DNA polymerase (New England Biolabs). The nucleotide sequence of the sense primer was 5'GCTTCGACTATTACTCAC3'. M13 (-20) primer was used as the antisense oligonucleotide. The PCR product was blunt-end ligated into the

Nco I site of the *E. coli* expression vector pET3d (Novagen) as described (Sambrook *et al.*, 1989). The *Nco*I-digested vector had been previously treated with Klenow to fill-in 5′ protruding ends. The junction between the vector and the 5′ terminus of the insert was sequenced to confirm that the PCR product was ligated into pET3d in the proper reading frame. This construct was subsequently introduced into the *E. coli* strain BL21 pLysS and grown in LB media with carbenicillin (125 μg/mL) and chloroamphenicol (30 μg/mL) selection. At a cell density of $O.D._{600} \approx 0.8$, cultures were induced with the addition of IPTG to a final concentration of 0.5 mM and grown for an additional 4 h. Cells were then washed in 50 mM Tris-HCl (pH 7.5), lysed by two freeze-thaw cycles (using a liquid nitrogen bath for freezing and a 22°C water bath for thawing) and incubated with bovine pancreas DNase I (Boehringer Mannheim Biochemical) (20 μg/mL) for 15 min at 22°C. The extract was subsequently centrifuged at 14,000x *g* for 5 min. The resulting supernatant was used for 16:0-ACP desaturation assays as described above. Reactions were performed using 230 μg of protein from the *E. coli* extract. Radiolabel in the TLC-analyzed reaction products was detected using a Bioscan System 200 image scanner.

The double bond position of the monounsaturated product was determined by GC-MS analysis of its dimethyl disulfide derivative (Francis, 1981). In these studies, desaturation assays described above were scaled up six-fold, and reactions were conducted with 2.6 nmole of [1-^{14}C]16:0-ACP and 1 mg of *E. coli* protein. Assays were conducted for 4 h. High protein concentrations and long incubation periods were used to insure the synthesis of sufficient amounts of monounsaturated fatty acid for mass spectral analyses. Reaction products were converted to fatty acid methyl esters as described above and subsequently reacted with 100 μL of an iodine solution (60 mg/mL ethyl ether) and 350 μL of dimethyl disulfide (Aldrich). After 2 to 3 h incubation with shaking (250 rpm at 37°C), dimethyl disulfide derivatives of unsaturated fatty acid methyl esters were extracted as previously described (Yamamoto *et al.*, 1991). Dimethyl disulfide derivatives dissolved in hexane were analyzed by GC-MS

using a Hewlett Packard HP5890II gas chromatograph interfaced to a HP5971 mass selective detector. Separation of analytes was achieved using a DB23 (30m x 0.25 mm I.D.) column (J&W Scientific) with the oven temperature programmed from 185°C (3 min hold) to 230°C at rate of 2.5°C/min.

Results

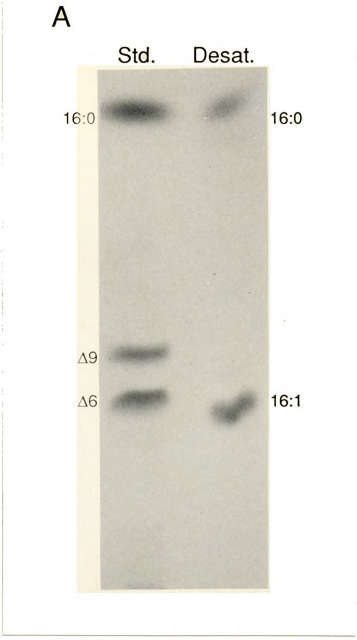
Demonstration of a Soluble Δ^6Palmitoyl-ACP Desaturase in *Thunbergia alata* Endosperm Extracts

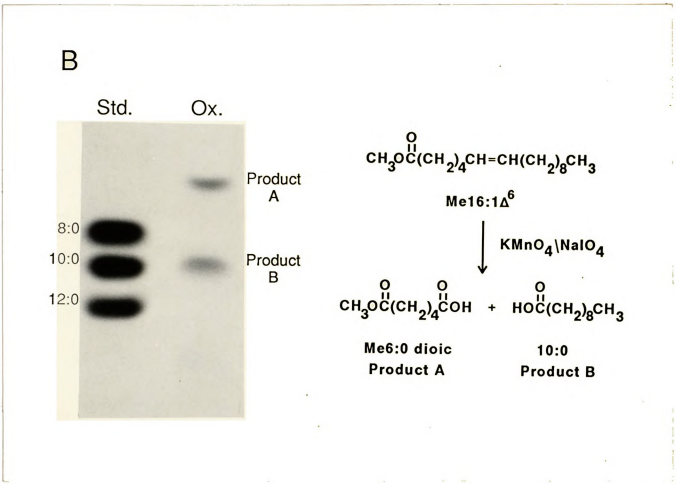
The seed oil of *Thunbergia alata* is composed of nearly 85 wt% of the unusual monounsaturated fatty acid Δ^6hexadecenoic acid (16:1Δ^6). To examine whether this acyl moiety is the product of a Δ^6palmitoyl (16:0)-ACP desaturase, the 100,000x*g* supernatant of a homogenate of developing *T. alata* seed endosperm was incubated with ^{14}C-16:0-ACP and potential co-factors including ferredoxin, NADPH, and ferredoxin-NADPH reductase. Significant amounts of 16:0-ACP desaturase activity were detected in the soluble endosperm extract. The 16:1 moiety produced as a result of this desaturase activity displayed mobility on argentation TLC plates similar to that of the Δ^6 monounsaturated fatty acid petroselinic acid, when analyzed as methyl ester derivatives (Figure 5.1A). In addition, the chain lengths of the permanganate-periodate oxidation products of this 16:1 moiety were consistent with the insertion of the double bond at the Δ^6 carbon (Figure 5.1B).

Substrate Properties of the Δ^6Acyl-ACP Desaturase

To confirm that the Δ^6desaturase identified above is most active with 16:0-ACP, assays were conducted using ^{14}C-saturated acyl-ACP substrates containing 14, 16, and 18 carbons. Under the assay conditions used, $\Delta^6$16:0-ACP desaturase activity in the 100,000x*g* supernatant of *T. alata* endosperm homogenate was essentially linear over ten minutes (Figure 5.2). When assays were conducted over this time period with either [1-^{14}C]14:0- or 18:0-ACP provided as a substrate, low but detectable levels

Figure 5.1. Autoradiograms of intact (*A*) or oxidized (*B*) products of [^{14}C]16:0-ACP desaturation assays conducted with a 100,000x*g* supernatant of *Thunbergia alata* endosperm homogenates. Shown (*A*, Desat.) is the methyl ester derivative of 16:1 produced by desaturation activity in *T. alata* endosperm extracts. Also shown (*A*, Desat.) is the methyl ester of 16:0 derived from unreacted [1-^{14}C]16:0-ACP. Fatty acid methyl esters were resolved by argentation TLC as shown. In the standard (Std.) lane of panel *A* are methyl 16:0, 18:1Δ^9 (Δ^9), and 18:1Δ^6 (Δ^6). Radiolabeled 18:1Δ^6 was produced by the incubation of coriander endosperm in [1-^{14}C]acetate (see Chapter 4). In panel *B* are the permanganate-periodate oxidation products of the methyl ester derivative of 16:1 formed by acyl-ACP desaturation activity in *T. alata* endosperm homogenates. Oxidation products were separated by reverse-phase TLC as shown.


A
Std.
Desat.
16:0
16:0
Δ9
Δ6
16:1


B
Std.
Ox.
Product A
Product B
8:0
10:0
12:0
Me16:1Δ6
KMnO4\NaIO4
Me6:0 dioic
Product A
10:0
Product B

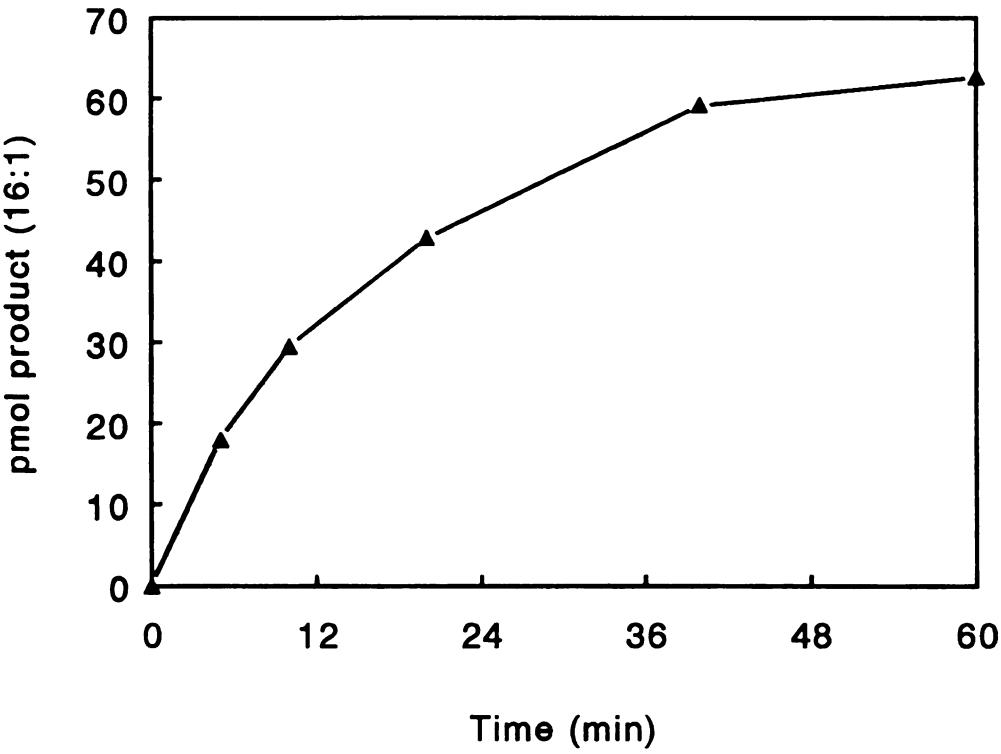


Figure 5.2. Time course of [1-^{14}C]16:0-ACP desaturation activity in a 100,000x*g* supernatant of a *T. alata* endosperm homogenate. Assays were conducted with 118 pmoles of [1-^{14}C]16:0-ACP and 23 μg protein.

of Δ^6 desaturase activity were measured (Table 5.1). Values obtained with the latter substrate, however, were obscured because of the presence of competing $\Delta^9$18:0-ACP desaturase activity in the endosperm extract. In contrast to results obtained with 14:0- and 18:0-ACP, approximately 7-fold higher levels of activity were detected when the 100,000x*g* supernatant was assayed with 16:0-ACP. Because double bonds were inserted at the Δ^6 carbon of 14:0-, 16:0-, and 18:0-ACP, it can be concluded that the Δ^6 desaturase of *T. alata* endosperm positions the placement of unsaturation with regard to the carboxyl- rather than the methyl-terminus of acyl-ACP substrates. This double bond positioning property has been previously observed with the $\Delta^9$18:0- and $\Delta^4$16:0-ACP desaturases (Cheesbrough and Cho, 1990; Chapter 3). Finally, no desaturase activity was detected when [1-^{14}C]16:0-CoA was presented as a potential substrate. Overall, these results indicated that Δ^6 desaturase is most active *in vitro* with palmitic acid (16:0) esterified to ACP.

Catalytic Properties of Δ^6Palmitoyl-ACP Desaturase Activity

Virtually no $\Delta^6$16:0-ACP desaturase activity was detected in the 100,000x*g* supernatant of *T. alata* endosperm homogenates when assays were conducted in the absence of ferredoxin and molecular oxygen (Figure 5.3). $\Delta^6$16:0-ACP desaturase activity was also reduced when catalase was omitted from assays. Furthermore, the inclusion of 1 mM KCN or H_2O_2 in reactions resulted in the loss of most of the desaturase activity. Such catalytic properties of the *T. alata* $\Delta^6$16:0-ACP desaturase are similar to those previously described for the $\Delta^9$18:0-ACP desaturase (Nagai and Bloch, 1968; Jaworski and Stumpf, 1974; McKeon and Stumpf, 1982).

Isolation of a cDNA Encoding a Diverged Acyl-ACP Desaturase from *T. alata* Endosperm

In the studies described above, the $\Delta^6$16:0-ACP desaturase appeared to possess catalytic properties similar to those previously reported for the $\Delta^9$18:0-ACP desaturase. These included the ability to catalyze the soluble desaturation of acyl-ACPs and the requirement of reduced ferredoxin for detectable activity. Based on these functional

Table 5.1. *In vitro substrate specificities of acyl-ACP or -CoA desaturases of T. alata endosperm*

Desaturase assays were conducted for 10 min using 118 pmoles of [1-^{14}C]acyl-ACP or -CoA substrate and 23 μg total protein from a 100,000x*g* supernatant of a *T. alata* endosperm homogenate.

Substrate	Monounsaturated Products[1]	
	Δ^6	Δ^9
	pmol	
14:0-ACP	3.0	n.d.[2]
16:0-ACP	22.7	n.d.
16:0-CoA	n.d.	n.d.
18:0-ACP	2.8	39.8

[1] Δ^6 and Δ^9 isomers were identified by their mobilities on argentation TLC plates.

[2] Not detected.

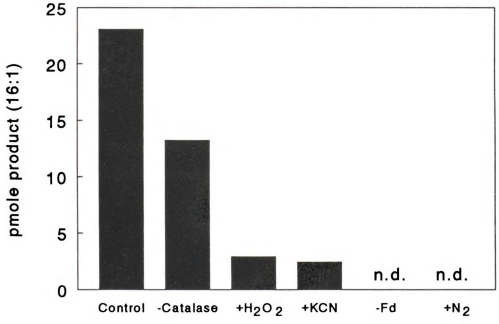


Figure 5.3. Effect of catalase, ferredoxin (Fd), nitrogen (N_2), potassium cyanide (KCN), or hydrogen peroxide (H_2O_2) on $\Delta^6$16:0-ACP desaturation activity in *T. alata* endosperm extracts. Assays were conducted for 10 min using 118 pmoles of [1-^{14}C]16:0-ACP and 23 μg protein from a 100,000x*g* supernatant of endosperm homogenate. Assays with potassium cyanide and hydrogen peroxide contained 1 mM of each compound. Catalase was omitted from assays containing hydrogen peroxide. n.d., not detected.

similarities, we examined whether the $\Delta^6$16:0-ACP desaturase is also structurally related to the $\Delta^9$18:0-ACP desaturase. To address this question, attempts were made to isolate a cDNA for the $\Delta^6$16:0-ACP desaturase using $\Delta^9$18:0-ACP desaturase-derived probes. As a first approach, a cDNA expression library prepared from polyA(+) RNA of *T. alata* endosperm was screened using antibodies against the $\Delta^9$18:0-ACP desaturase of avocado (Shanklin and Somerville, 1991). This method was previously used to obtain a cDNA for the $\Delta^4$16:0-ACP desaturase of coriander endosperm (Cahoon *et al.*, 1992). In the present study, however, antibody screening of the *T. alata* endosperm expression library yielded only cDNAs for three apparent isoforms of the $\Delta^9$18:0-ACP desaturase, which were designated pTAD1, 2, and 3 (see Appendix 2).

As an alternative approach, PCR amplification of a $\Delta^6$16:0-ACP desaturase-specific nucleotide probe was attempted using degenerate sense and anti-sense oligonucleotides prepared against two conserved regions of amino acid sequences of $\Delta^9$18:0- and $\Delta^4$16:0-ACP desaturases. One of the regions GlyAspMetIleThrGluGlu is encoded by the cDNA for the $\Delta^4$16:0-ACP desaturase and all known cDNAs for the $\Delta^9$18:0-ACP desaturase. The second region IleGluGlnThrIleTyrLeu is also encoded by the $\Delta^4$16:0-ACP desaturase cDNA and all known $\Delta^9$18:0-ACP desaturase cDNAs except that of safflower (Thompson *et al.*, 1991). Products of approximately 200 bp obtained following one round of PCR amplification of the total *T. alata* cDNA library (in plasmid form) were screened after subcloning into the pAMP1 vector. To delineate products of the previously isolated cDNAs pTAD1, 2, and 3, colonies containing PCR-derived clones were screened in a "negative" manner with random-labeled probes for pTAD1, 2, and 3 and conditions of moderate to high stringency. One of the resulting clones (pEC6) which displayed weak or no hybridization to the probes encoded an amino acid sequence that was somewhat diverged from those of known $\Delta^9$18:0-ACP desaturases.

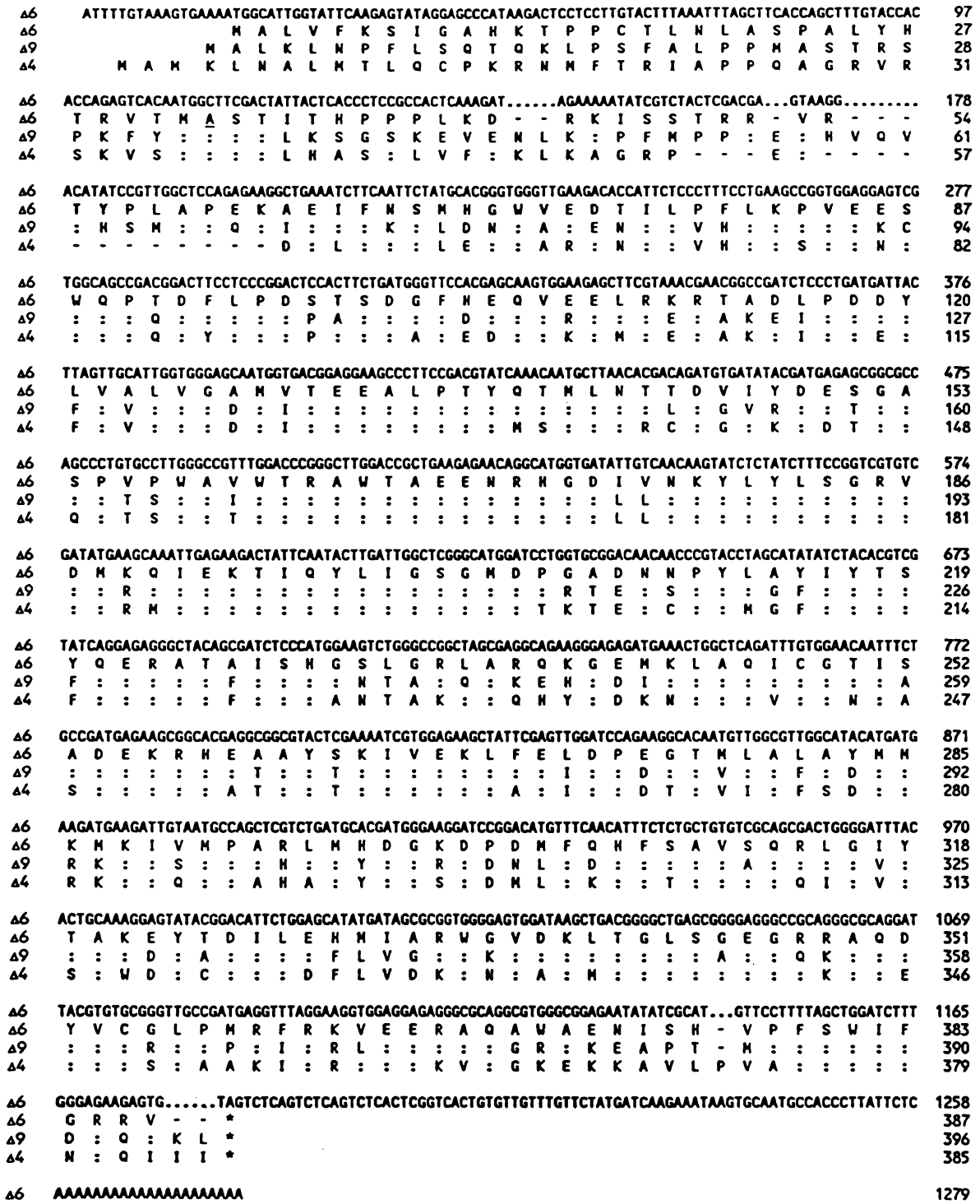
When the *T. alata* endosperm library was screened with a random-labeled probe prepared from pEC6, >0.1% of the total cDNAs examined

strongly hybridized to this probe. The longest of a selected portion of these cDNAs (designated pTAD4) contained 1279 bp and had an open-reading frame encoding a 387-amino acid peptide with considerable identity to known $\Delta^4$16:0- and $\Delta^9$18:0-ACP desaturases (Figure 5.4). Based on similarity of flanking bases to the consensus sequence proposed by Lütcke *et al.* (1987), the translational start site of the cDNA insert of pTAD4 likely occurs at nucleotide 17. In addition, from homology with $\Delta^4$16:0- and $\Delta^9$18:0-ACP desaturases, the mature peptide encoded by pTAD4 likely begins at amino acid 33. As such, the 32 amino acids preceding this residue correspond to a putative plastid transit peptide as is present in all acyl-ACP desaturases characterized to date. Interestingly, the cDNA insert of pTAD4 lacks nucleotide sequence for six to seven amino acids found near the amino-terminus of all previously characterized $\Delta^9$18:0-ACP desaturases. This region is also altered in the cDNA for the coriander $\Delta^4$16:0-ACP desaturase (Cahoon *et al.*, 1992) as compared to cDNAs for $\Delta^9$18:0-ACP desaturases. In this case, the encoding sequence for 15 amino acids is absent in the ^{4}16:0-ACP desaturase cDNA relative to the castor $^9\Delta$18:0-ACP desaturase cDNA (Shanklin and Somerville, 1991). The pTAD4-encoded peptide also contains two less amino acids at its carboxyl-terminus than both the $\Delta^4$16:0- and $\Delta^9$18:0-ACP desaturases. Despite these differences, the interior regions of the putative desaturase encoded by pTAD4 share significant identity with portions of the primary structures of $\Delta^4$16:0- and $\Delta^9$18:0-ACP desaturases, and the spacing between conserved regions of amino acids is the same in all three desaturase types. Overall, the mature peptide encoded by the cDNA insert of pTAD4 has 67% identity to the castor $\Delta^9$18:0-ACP desaturase and 57% identity to the coriander $\Delta^4$16:0-ACP desaturase, disregarding any missing amino acids.

Activity of an *E. coli*-Expressed cDNA for a Diverged Acyl-ACP Desaturase of *T. alata* Endosperm

To determine the activity of the desaturase corresponding to the cDNA insert of pTAD4, the mature peptide-encoding region of this clone was placed behind the T7 polymerase promoter of the pET3d vector (Novagen) and

Figure 5.4. Nucleotide sequence of the cDNA insert of pTAD4 (Δ^6), and a comparison of the deduced amino acid sequences of pTAD4 (Δ^6) and cDNAs for the coriander $\Delta^4$16:0-ACP desaturase (Δ^4) (Cahoon *et al.*, 1992) and the castor $\Delta^9$18:0-ACP desaturase (Δ^9) (Shanklin and Somerville, 1991). Identical amino acids are indicated by colons. Amino acids that are absent relative to the castor $\Delta^9$18:0-ACP desaturase are indicated by dashed lines. Alignment of the nucleotide sequence of the cDNA insert of pTAD4 is maintained with a dotted line. The underlined alanine at amino acid 33 is the likely start of the mature-peptide encoded by pTAD4.



expressed in *E. coli*. When assayed with [1-^{14}C]16:0-ACP, crude extracts of IPTG-induced recombinant *E. coli* catalyzed the synthesis of ^{14}C-16:1 (Figure 5.5). In addition, the methyl ester of the 16:1 product displayed mobility on argentation TLC plates similar to that of a methyl petroselinic acid standard, suggesting that this monounsaturated product is a Δ^6isomer. Furthermore, like the activity found in *T. alata* endosperm extracts, the desaturase expressed in *E. coli* displayed a substrate preference in *in vitro* assays for 16:0-ACP rather than 14:0- or 18:0-ACP (data not shown). No acyl-ACP desaturase activity was detected in extracts of *E. coli* harboring the pET3d vector without cDNA insert or in uninduced recombinant *E. coli* (Figure 5.5).

The ^{14}C-16:1 moiety produced *in vitro* from the *E. coli*-expressed desaturase was conclusively identified as a Δ^6 isomer through GC-MS analysis of its dimethyl disulfide derivative (Figure 5.6). Significant amounts of non-radiolabeled or ^{12}C-16:1Δ^6 were also detected among the desaturase assay products. This was indicated by the presence of the additional ions 143, 175 and 362 *m/z* in the mass spectrum of ^{14}C-16:1Δ^6 as well as by an enrichment in the abundance of ion 187 *m/z* in this spectrum. The unlabeled 16:1Δ^6 likely did not result from *in vivo* synthesis by *E.coli*. In this regard, *E. coli* does not normally produce 16:1Δ^6 (Cronan and Rock, 1987). Furthermore, gas chromatographic analysis of fatty acids of *E. coli* expressing the pTAD4-encoded desaturase failed to detect any 16:1Δ^6 in the bacterial lipids (data not shown). However, given the relatively high concentrations of *E. coli* protein used in these assays, unlabeled 16:1Δ^6 likely arose from the *in vitro* desaturation of endogenous *E. coli* 16:0-ACP present in crude extracts.

Of note, expression levels of the *T. alata* cDNA in *E. coli* appeared to be low relative to expression levels often obtained with cDNAs placed behind the T7 polymerase promoter (Studier *et al.*, 1990). The expressed protein, for example, could not be distinguished on Coomassie-stained SDS-polyacrylamide gels of either the total soluble or insoluble protein fractions of the lysed *E. coli* (data not shown).

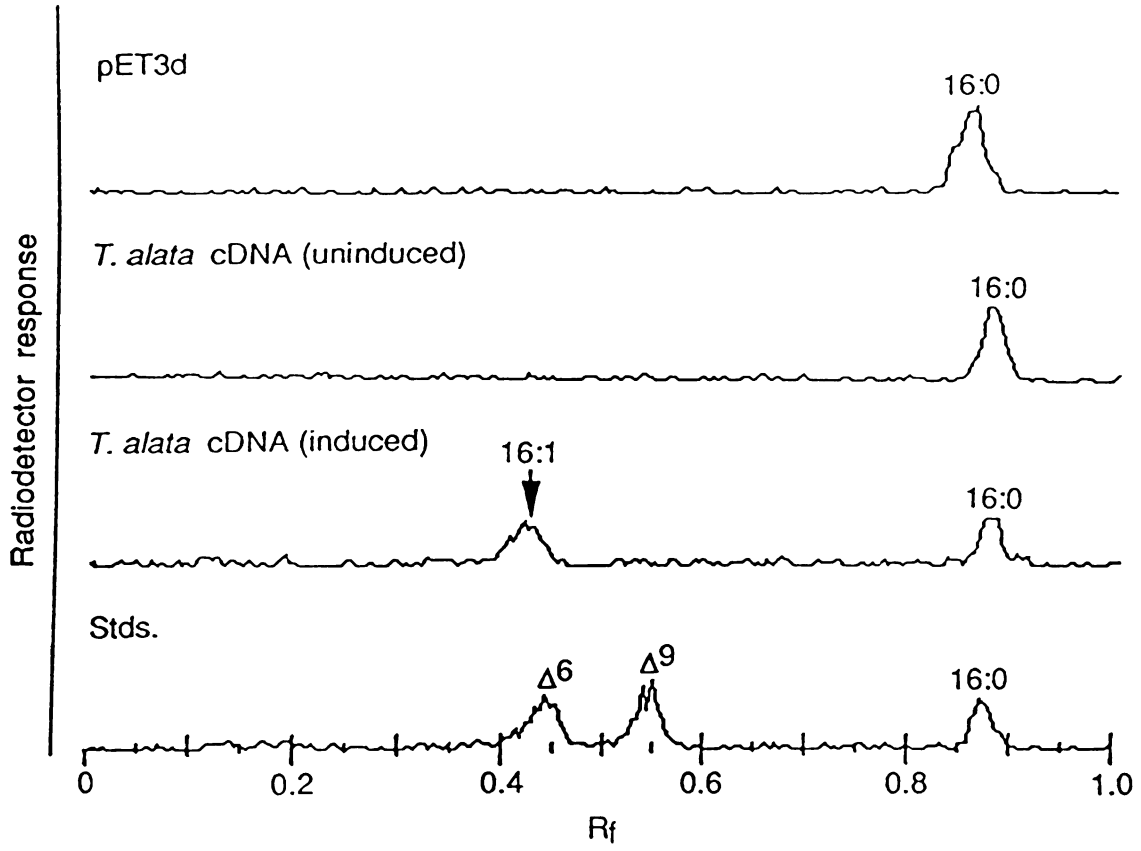


Figure 5.5. Comparison of the $\Delta^6$16:0-ACP desaturase activity of extracts of *E. coli* BL21 pLysS containing only the vector pET3d or pET3d with insert derived from the *T. alata* cDNA of pTAD4 (with or without IPTG induction). The methyl ester of 16:1 formed by the *E. coli* expressed desaturase was separated from methyl 16:0 of the unreacted substrate by argentation TLC as shown. Radiation on TLC plates was detected using a Bioscan 200 image scanner. Assays were conducted for 60 min using 230 μg of *E. coli* protein and 118 pmoles of [1-^{14}C]16:0-ACP. Shown in the standard chromatogram (Std.) are methyl ^{14}C-16:0, -18:1Δ^9, and -18:1Δ^6.

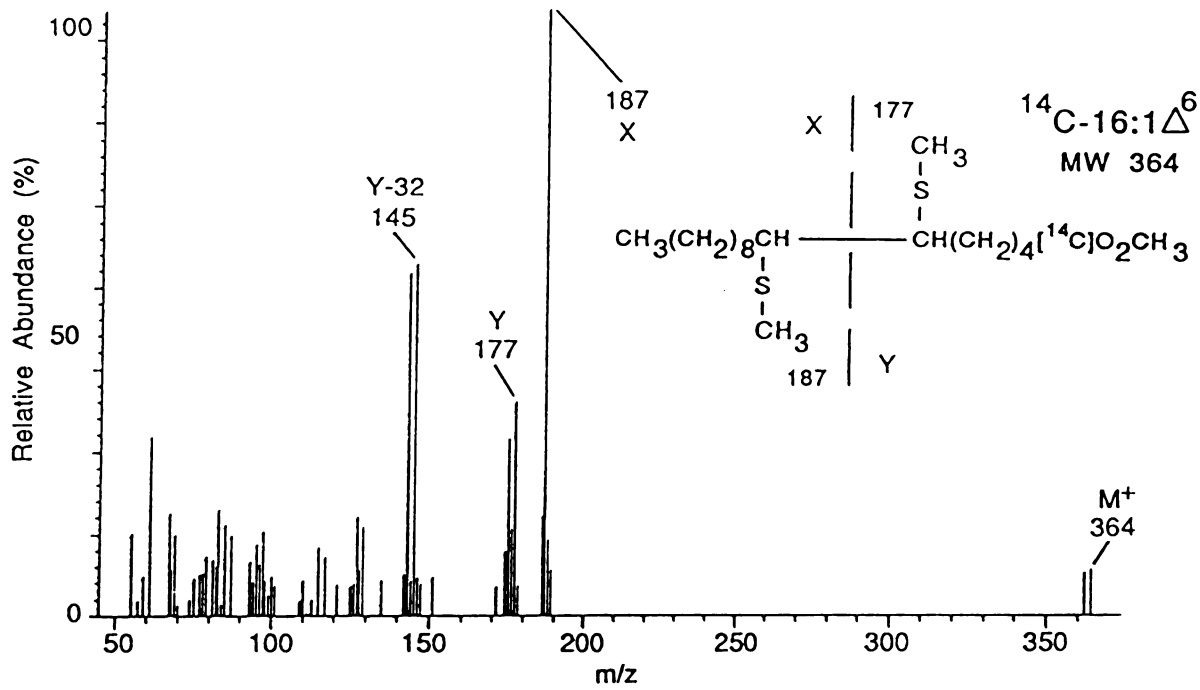


Figure 5.6. Mass spectrum of derivatives of [1-^{14}C]16:1 formed by 16:0-ACP desaturase activity in extracts of *E. coli* expressing the mature peptide-encoding region of pTAD4. The [1-^{14}C]16:1 desaturation product was converted to a methyl ester derivative and reacted with dimethyl disulfide prior to mass spectral analysis.

Discussion

The results presented here demonstrate the involvement of a soluble $\Delta^6$16:0-ACP desaturase in the synthesis of Δ^6hexadecenoic acid in the endosperm of *Thunbergia alata*. The activity of this enzyme has several properties similar to those previously described for the $\Delta^9$18:0-ACP desaturase. These include the requirement of reduced ferredoxin for detectable *in vitro* activity, the stimulation of activity by catalase, and the inhibition of activity by potassium cyanide and hydrogen peroxide. In addition, a cDNA encoding an acyl-ACP desaturase that shares considerable amino acid identity with the $\Delta^9$18:0-ACP desaturase was isolated from a cDNA library prepared from polyA(+) RNA of *T. alata* endosperm. Extracts of *E. coli* that express this cDNA catalyzed the Δ^6 desaturation of 16:0-ACP. These data thus indicate that the $\Delta^6$16:0-ACP desaturase is a functional and structural analog of the $\Delta^9$18:0-ACP desaturase.

The major difference between the primary structures of the mature $\Delta^6$16:0-, $\Delta^4$16:0-, and $\Delta^9$18:0-ACP desaturases occurs in a region near their amino termini. In this region, the *T. alata* $\Delta^6$16:0-ACP desaturase contains six less amino acids than the castor $\Delta^9$18:0-ACP desaturase. Similarly, this portion of the coriander $\Delta^4$16:0-ACP desaturase lacks 15 less amino acids relative to the castor $\Delta^9$18:0-ACP desaturase. One possibility is that differences in substrate recognition (16:0-ACP vs. 18:0-ACP) and double bond positioning properties of these desaturases are associated with this divergence in their primary structures. For example, as a result of amino acids that are missing relative to $\Delta^9$18:0-ACP desaturases, the binding pocket of the Δ^4 and $\Delta^6$16:0-ACP desaturases may be smaller and active site residues involved in double bond insertion may be positioned closer to the carboxyl end of acyl chains. Alternatively, this amino-terminal region of the $\Delta^9$18:0-ACP desaturase may not contribute significantly to the catalytic properties of this enzyme. As such, if the Δ^4 and $\Delta^6$16:0-ACP desaturases evolved from the $\Delta^9$18:0-ACP desaturase,

then there may have been little selective pressure to maintain this region in the variant 16:0-ACP desaturases.

Ultimately, an understanding of how differences in the amino acid sequences of $\Delta^4$16:0-, $\Delta^6$16:0-, and $\Delta^9$18:0-ACP desaturases contribute to variations in their functional properties will require comparisons of the three-dimensional structures of these enzymes. In this regard, elucidation of the crystal structure of the castor $\Delta^9$18:0-ACP desaturase is currently in progress (Schneider *et al.*, 1992). With such information, it may be possible to overlap amino acid sequences of the Δ^4 and $\Delta^6$16:0-ACP desaturases onto the three-dimensional structure of $\Delta^9$18:0-ACP desaturase to more precisely identify residues associated with the different substrate recognition and double bond positioning properties of these enzymes. This could eventually lead to the design of "tailor-made" desaturases that are capable of inserting double bonds into a variety of positions of acyl moieties of a range of carbon chain lengths.

An interesting observation from the studies described above was the lack of detectable amounts of 16:1Δ^6 in lipids of *E. coli* expressing the *T. alata* cDNA. Similarly, Thompson *et al.* (1991) reported that expression of the safflower $\Delta^9$18:0-ACP desaturase cDNA did not lead to the *in vivo* production of oleic acid in recombinant *E. coli*. The latter result can be explained by the fact that *E. coli* contains little 18:0-ACP (Rock and Jackowski, 1982; Ohlrogge, unpublished data). However, 16:0-ACP is a major component of the acyl-ACP pool of *E. coli* (Ohlrogge, unpublished data). Therefore, the lack of 16:1Δ^6 synthesis in *E. coli* expressing the *T. alata* cDNA is not due to the presence of insufficient substrate for the desaturase. In addition, *E. coli* has been reported to contain ferredoxin (Knoell and Knappe, 1974), the apparent electron donor for the $\Delta^6$16:0-ACP desaturase. However, as proposed by Thompson *et al.* (1991), *E. coli* ferredoxin may not be functional with plant acyl-ACP desaturases. Alternatively, *E. coli* may not contain adequate amounts of ferredoxin in a reduced form as required for $\Delta^6$16:0-ACP desaturase activity.

In addition to 16:1Δ^6, *T. alata* seed contains the unusual fatty acid 18:1Δ^8, which composes about 2 wt% of the oil of this tissue (Spencer *et al*, 1971). We have previously shown that petroselinic acid (18:1Δ^6) is formed by elongation of 16:1Δ^4-ACP in Umbelliferae endosperm (Chapter 3). In an analogous manner, we would predict that 18:1Δ^8 arises from the elongation of 16:1Δ^6-ACP rather than from the Δ^8 desaturation of 18:0-ACP. Unlike the synthesis of petroselinic acid, though, elongation of 16:1-ACP in *T. alata* endosperm would likely not be a major pathway as the ratio of amounts of 16:1Δ^6:18:1Δ^8 in this tissue is approximately 40:1. In contrast, the ratio of amounts of 16:1Δ^4:18:1Δ^6 in endosperm of the Umbelliferae coriander is more than 1:500 (Chapter 3).

Finally, significant efforts have been directed toward the development of transgenic crops that produce high-value specialty oils (Murphy, 1992). In this regard, the physical and industrial properties of 16:1Δ^6 have received little study. However, like petroselinic acid, 16:1Δ^6 can be oxidatively cleaved at its double bond to yield adipic acid, which is used in the synthesis of nylon 6,6. In addition, high palmitic acid (16:0) mutants of crop plants including soybean (Bubeck and Fehr, 1989) and *Brassica campesteris* (Perrsson, 1985) exist that may serve as appropriate backgrounds for the transgenic expression of the cDNA for the *T. alata* $\Delta^6$16:0-ACP desaturase. Still, the success of such research would likely require additional studies to determine whether enzymes other than $\Delta^6$16:0-ACP desaturase are specialized for the synthesis and metabolism of 16:1Δ^6 in *T. alata* endosperm. For example, a petroselinoyl-ACP specific thioesterase has been identified in Umbelliferae endosperm extracts that efficiently releases this fatty acid following its synthesis on ACP (Dörmann *et al.*, 1994). A related-enzyme may also be required for high levels of Δ^6hexadecenoic acid production in transgenic plants.

Literature Cited

Battey JF, Schmid KM, Ohlrogge JB (1989) Genetic engineering for plant oils: Potential and limitations. Trends Biotech **7**: 122-125

Browse J, Somerville C (1991) Glycerolipid synthesis: Biochemistry and regulation. Annu Rev Plant Physiol Plant Mol Biol **42**: 467-506

Bubeck DM, Fehr WR, Hammond EG (1989) Inheritance of palmitic and stearic acid mutants of soybean. Crop Sci **29**: 652-656

Cahoon EB, Shanklin J, Ohlrogge JB (1992) Expression of a coriander desaturase results in petroselinic acid production in transgenic tobacco. Proc Natl Acad Sci USA **89**: 11184-11188

Cheesbrough TM (1990) Decreased growth temperature increases soybean stearoyl-acyl carrier protein desaturase activity. Plant Physiol **93**: 555-559

Cheesbrough TM, Cho SH (1990) Purification and characterisation of soyabean stearoyl-ACP desaturase. *In* PJ Quinn, JL Harwood, eds, Plant Lipid Biochemistry, Structure and Utilization. Portland Press, London, p 129-130

Christie WW (1982) Lipid Analysis, 2nd Edition. Pergammon Press, Oxford

Cronan Jr JE, Rock CO (1987) Biosynthesis of membrane lipids. *In* FC Neihardt, ed, *Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology. American Society of Microbiology, Washington, DC, p 474-497

Dörmann P, Frentzen M, Ohlrogge JB (1994) Specificities of acyl-acyl carrier protein (ACP) thioesterase and glycerol-3-phosphate acyltransferase for octadecenoyl-ACP isomers: Identification of a petroselinoyl-ACP thioesterase in Umbelliferae. Plant Physiol (in press)

Francis GW (1981) Alkylthiolation for the determination of double-bond position in unsaturated fatty acid esters. Chem Phys Lipids **29**: 369-374

Hall TC, Ma Y, Buchbinder BU, Pyne JW, Sun SM, Bliss FA (1978) Messenger RNA for G1 protein of French bean seeds: Cell-free translation and product characterization. Proc Natl Acad Sci USA **75**: 3196-3200

Hay B, Short JM (1992) ExAssist helper phage and SOLR cells for Lambda Zap II excisions. Strategies **5**: 16-18 (Stratagene publication)

Rock CO, Jackowski S (1982) Regulation of phospholipid synthesis in *Escherichia coli*. Composition of the acyl-acyl carrier protein pool *in vivo*. J Biol Chem **257**: 10579-10765

Jaworski JG (1987) Biosynthesis of monoenoic and polyenoic fatty acids. *In* PK Stumpf, EE Conn, eds,The Biochemistry of Plants: A Comprehensive Treatise, Vol 9, Lipids: Structure and Function. Academic Press, New York, p 159-174

Jaworski JG, Stumpf PK (1974) Fat metabolism in higher plants: Properties of a soluble stearoyl-acyl carrier protein desaturase from maturing *Carthamus tinctorius*. Arch Biochem Biophys **162**: 158-165

Kinney AJ, Hitz WD, Yadav NS (1990) Stearoyl-ACP desaturase and a β-ketoacyl-ACP synthetase from developing soybean seeds. *In* PJ Quinn, JL Harwood, eds, Plant Lipid Biochemistry, Structure and Utilization. Portland Press, London, p 126-128

Knoell H-E, Knappe J (1974) *Escherichia coli* ferredoxin, an iron-sulfur protein of the adrenodoxin type. Eur J Biochem **50**: 245-252

Lütcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA (1987) Selection of AUG initiation codon differs in plants and animals. EMBO J **6**: 43-48

McKeon T, Stumpf PK (1981) Stearoyl-acyl carrier protein desaturase from safflower seeds. Methods Enzymol **71**: 275-281

McKeon TA, Stumpf PK (1982) Purification and characterization of stearoyl-acyl carrier protein desaturase and the acyl-acyl carrier protein thioesterase from maturing seeds of safflower. J Biol Chem **257**: 12141-12147

Miquel M, James Jr D, Dooner H, Browse J (1993) *Arabidopsis* requires polyunsaturated lipids for low temperature survival. Proc Natl Acad Sci USA **90**: 6208-6212

Murphy DJ (1992) Modifying oilseed crops for non-edible products. Trends Biotechnol **10**: 84-87

Nagai J and Bloch K (1965) Synthesis of oleic acid by *Euglena gracilis*. J Biol Chem **240**: PC3702-PC3703

Nagai J and Bloch K (1968) Enzymatic desaturation of stearyl acyl carrier protein. J Biol Chem **243**: 4626-4633

Nishida I, Beppu T, Matsuo T, Murata N (1992) Nucleotide sequence of a cDNA encoding a precursor to stearoyl-(acyl-carrier-protein) desaturase from spinach, *Spinacia oleracea*. Plant Mol Biol **19**: 711-713

Perrson C (1985) High palmitic acid content in summer turnip rape (*Brassica campestris* var. annua L.). Cruciferae Newslett 10, Scottish Crop Research Institute, Invergowerie, Dundee, p 137

Rock CO, Garwin JL (1979) Preparative enzymatic synthesis and hydrophobic chromatography of acyl-acyl carrier protein. J Biol Chem **254**: 7123-7128

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sato A, Becker CK, Knauf VC (1992) Nucleotide sequence of a complimentary DNA clone encoding stearoyl-acyl carrier protein desaturase from *Simmondsia chinensis*. Plant Physiol **99**: 362-363

Schneider G, Lindqvist Y, Shanklin J, Somerville C (1992) Preliminary crystallographic data for stearoyl-acyl carrier protein desaturase from castor seed. J Mol Biol **225**: 561-564

Shanklin J, Somerville C (1991) Stearoyl-acyl carrier protein desaturase from higher plants is structurally unrelated to the animal and fungal homologs. Proc Natl Acad Sci USA **88**: 2510-2514

Shanklin J, Mullins C, Somerville C (1991) Sequence of a complementary DNA from *Cucumis sativus* L. encoding the stearoyl-acyl-carrier protein desaturase. Plant Physiol **97**: 467-468

Slocombe SP, Cummins I, Jarvis RP, Murphy DJ (1992) Nucleotide sequence and temporal regulation of a seed-specific *Brassica napus* cDNA encoding a stearoyl-acyl carrier protein (ACP) desaturase. Plant Mol Biol **20**: 151-155

Spencer GF, Kleiman R, Miller RW, Earle FR (1971) Occurrence of *cis*-6-hexadecenoic acid as the major component of *Thunbergia alata* seed oil. Lipids **6**: 712-714

Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1990) Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol **185**: 60-89

Taylor MA, Smith SB, Davies HV, Burch LR (1992) The primary structure of a cDNA clone of the stearoyl-acyl carrier protein desaturase gene from potato (*Solanum tuberosum* L.). Plant Physiol **100**: 533-534

Thompson GA, Scherer DE, Foxall-Van Aken S, Kenny JW, Young HL, Shintani DK, Kridl JC, Knauf VC (1991) Primary structures of the precursor and mature forms of stearoyl-acyl carrier protein desaturase from safflower embryos and requirement of ferredoxin for enzyme activity. Proc Natl Acad Sci USA **88**: 2578-2582

Wada H, Gombos Z, Murata N (1990) Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation. Nature **347**: 200-203

Yamamoto K, Shibahara A, Nakayama T, Kajimoto G (1991) Determination of double-bond positions in methylene-interrupted dienoic fatty acids by GC-MS as their dimethyl disulfide adducts. Chem Phys Lipids **60**: 39-50

CHAPTER 6

Conclusions and Future Research Perspectives

Conclusions

Studies presented in this thesis have resulted in the identification of two "new" acyl-acyl carrier protein (ACP) desaturases in plants: (1) a Δ^4palmitoyl (16:0)-ACP desaturase that is associated with the synthesis of petroselinic acid in Umbelliferae endosperm and (2) a $\Delta^6$16:0-ACP desaturase that is involved in the synthesis of Δ^6hexadecenoic acid (16:1) in *Thunbergia alata* endosperm. cDNAs encoding both desaturases were also isolated. Translation of these cDNAs revealed that the primary structures of the Δ^4 and $\Delta^6$16:0-ACP desaturases are related to that of the Δ^9stearoyl-ACP desaturase, the enzyme that catalyses the synthesis of oleic acid in plants. This finding indicates that it may be possible to manipulate the amino acid sequences of acyl-ACP desaturases to produce functional enzymes with altered substrate recognition and double bond positioning properties.

Work described in this thesis has also led to the proposal of a biosynthetic pathway for petroselinic acid in Umbelliferae endosperm as shown in Figure 6.1. This pathway involves the conversion of 16:0-ACP to 16:1Δ^4-ACP via the activity of the $\Delta^4$16:0-ACP desaturase followed by two carbon elongation to form petroselinoyl-ACP. Essential to an understanding of the biosynthesis of petroselinic acid were results obtained from the transformation of tobacco with a cDNA for the coriander $\Delta^4$16:0-ACP desaturase. Though the resulting transgenic callus expressed the desaturase in an active form, these cells apparently lacked other enzymes specialized for the synthesis of petroselinic acid. As a result, the transgenic tobacco callus, in contrast to Umbelliferae endosperm, accumulated significant amounts of 16:1Δ^4 as well as petroselinic acid in glycerolipids. These fatty acids were also found to be major components of acyl-ACP pools of transgenic tobacco cells. This detection of 16:1Δ^4

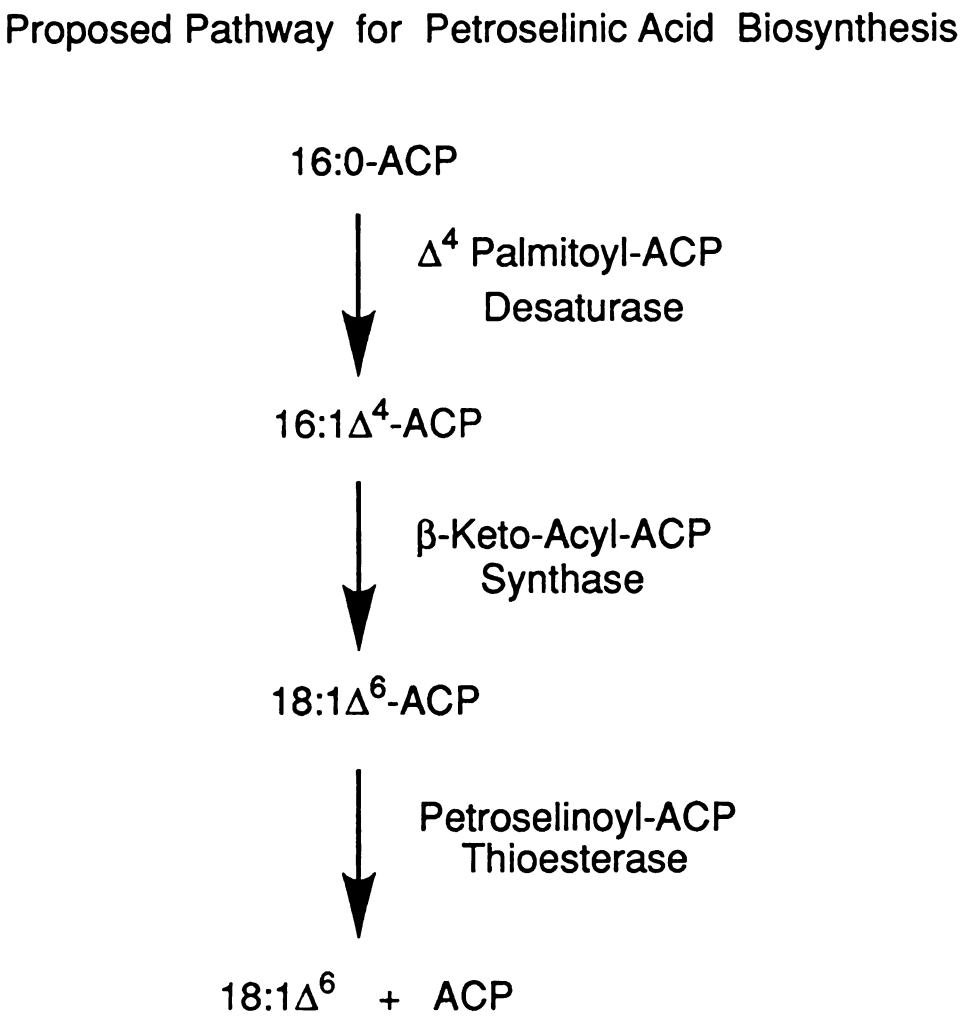


Figure 6.1. Proposed pathway for petroselinic acid biosynthesis. Studies described in Chapters 2 and 3 have provided substantial evidence for the involvement of a $\Delta^4$16:0-ACP desaturase in the synthesis of petroselinic acid in Umbelliferae endosperm. Dörmann *et al.* (1994) have also demonstrated that Umbelliferae endosperm contain a petroselinoyl-ACP-specific thioesterase. Furthermore, results of preliminary research indicate that the elongation of 16:1Δ^4-ACP is a specialized process in the synthesis of petroselinic acid in Umbelliferae endosperm (Cahoon, unpublished data).

in transgenic tobacco provided a perspective of petroselinic acid synthesis that could not have been obtained from studies of Umbelliferae endosperm. These results indicate that it is possible to produce fatty acids of unusual structure in transgenic plant cells. Moreover, these results demonstrate the potential usefulness of transgenic plant material in the elucidation of biochemical pathways associated with unusual fatty acid synthesis.

Finally, through the use of [1-^{14}C]acetate radiolabeling, it was shown that petroselinic acid readily enters phosphatidylcholine (PC) of carrot and coriander endosperm yet does not accumulate to high levels in this lipid relative to triacylglycerol (TAG). This result indicates that an unusual fatty acid can be metabolized through PC in a developing seed. As petroselinic acid is neither synthesized nor further modified on PC, this finding also suggests that flux of fatty acids into PC in seeds is not necessarily related to the role of this lipid in fatty acid modification reactions such as desaturation.

Future Research Perspectives

Acyl-ACP Desaturase Structural Studies

One area in which the work described in this thesis can be continued is the characterization of active site structures of acyl-ACP desaturases. Now available are cDNAs for three acyl-ACP desaturases with related primary structures but with different substrate binding and double bond positioning properties. Also available are amino acid sequences derived from at least 10 cDNAs encoding $\Delta^9$18:0-ACP desaturases of a wide range of plant species (Kinney *et al.*, 1990; Shanklin and Somerville, 1991; Shanklin *et al.*, 1991; Thompson *et al.*, 1991; Nishida *et al.*, 1992; Sato *et al.*, 1992; Slocombe *et al.*, 1992; Taylor *et al.*, 1992; Appendix 2). In addition, efforts to determine the three-dimensional crystal structure of the castor $\Delta^9$18:0-ACP desaturase are at an advanced stage (Schnieder *et al.*, 1992). With this cumulative information, it is (or will be) possible to make predictions about specific amino acids or regions of amino acids that may contribute to the catalytic and substrate binding

properties of acyl-ACP desaturases. Based on such predictions, it may be possible to change the primary structure of a given acyl-ACP desaturase through techniques such as site-directed mutagenesis to produce a "new" desaturase that inserts a double bond at a different position or recognizes a substrate of a different chain length. A similar approach has been successful in the modification of enzymes including trypsin (Craik *et al.*, 1985), substilisin (Wells *et al.*, 1987), lactate dehydrogenase (Wilks *et al.*, 1988), and lipoxygenase (Sloane *et al.*, 1991). Not only would protein engineering studies of acyl-ACP desaturases provide new information regarding structure-function properties of these enzymes but might also result in new renewable sources of industrially valuable chemicals. For example, if an acyl-ACP desaturase were designed that could introduce a double bond at a position beyond the Δ^9 carbon, the resulting fatty acid might be useful in long-chain nylon production. As a result, the introduction of such a desaturase into an existing oilseed crop could potentially lead to the development of a new high-value industrial oil.

From the amino acid sequences of the Δ^4 and $\Delta^6$16:0-ACP desaturase presented in this thesis, the most obvious starting point for mutagenesis is the region near the N-termini of these enzymes. Both desaturases contain deletions of amino acids in this region relative to the $\Delta^9$18:0-ACP desaturase. One possibility is that these deletions reduce the size of the binding pocket of 16:0-ACP desaturases so that they more effectively accommodate C_{16} rather than C_{18} acyl chains. These deletions might also position catalytic residues involved in double bond insertion closer to the carboxyl end of acyl chains. As a result, unsaturation may be introduced at the Δ^4 or Δ^6 carbon rather than at the more distant Δ^9 carbon.

Of the desaturases identified in this thesis, the *Thunbergia alata* $\Delta^6$16:0-ACP desaturase represents a useful tool in mutagenesis studies. Like the $\Delta^9$18:0-ACP desaturase, this enzyme displays soluble activity and can be expressed in *E. coli* in an active form. Therefore, the nucleotide

sequence of the cDNA for the $\Delta^6$16:0-ACP desaturase could be altered and then introduced into *E. coli*. Subsequent assay of extracts of the recombinant *E. coli* could then be performed to determine whether manipulations of the primary structure of the desaturase lead to alterations in its activity. In addition, large amounts of the $\Delta^6$16:0-ACP desaturase could potentially be generated through expression in *E. coli* or other systems and used for X-ray crystallographic determination of its three-dimensional structure. Comparisons of the crystal structure of this enzyme to that of the $\Delta^9$18:0-ACP desaturase would allow a more direct assessment of the basis for the different substrate recognition and double bond positioning properties of these enzymes. In addition, the parameters that have been devised for crystallization of the castor $\Delta^9$18:0-ACP desaturase (Schneider *et al.*, 1992) might also be useful for similar studies with the $\Delta^6$16:0-ACP desaturase.

Development of Transgenic Crops that Produce Petroselinic Acid and Δ^6Hexadecenoic Acid

As described in Chapter 1, oils that contain petroselinic acid and Δ^6hexadecenoic acid are of economic value. In this regard, with cDNAs for the Δ^4 and $\Delta^6$16:0-ACP desaturases, it should be possible, in theory, to develop transgenic crops that accumulate petroselinic acid or Δ^6hexadecenoic acid in their seed oil. Results presented in Chapter 2 partially support such an assertion. In these studies, tobacco callus expressing the coriander $\Delta^4$16:0-ACP desaturase behind the CaMV 35S promoter synthesized petroselinic acid as well as 16:1Δ^4 to levels of 4 wt% each

of the total fatty acid. However, not reported in this thesis, amounts of petroselinic acid in tissues, including seeds, of tobacco plants derived from this callus were generally $\leq$1 wt% of the total fatty acid. Based on further biochemical studies, it is believed that other enzymes in addition to the desaturase are specialized for the synthesis of petroselinic acid in Umbelliferae endosperm. The absence of these enzymes in tobacco likely limits the transgenic production of petroselinic acid.

For example, a petroselinoyl-ACP-specific thioesterase has been identified in Umbelliferae endosperm that is required for the efficient release of petroselinic acid from the reactions of *de novo* fatty acid synthesis (Dörmann *et al.*, 1994). Furthermore, preliminary investigations indicate that the elongation of 16:1Δ^4-ACP is also a specialized process in Umbelliferae endosperm (unpublished data). Therefore, it may be necessary to express genes encoding such enzymes along with the gene for the $\Delta^4$16:0-ACP desaturase in transgenic crops to produce high levels of this fatty acid. Furthermore, acyltransferases, acyl-CoA synthetases, and lipases may also be specialized for the metabolism of petroselinic acid in Umbelliferae endosperm.

No work has yet been initiated with regard to the expression of *Thunbergia alata* $\Delta^6$16:0-ACP desaturase cDNA in transgenic plants. As with the synthesis of petroselinic acid, specialized enzymes other than the desaturase may be needed for high levels of Δ^6hexadecenoic acid production in a transgenic oilseed. One such enzyme, for example, might be a Δ^6hexadecenoyl-ACP-specific thioesterase. However, high palmitic acid mutants of crop plants and *Arabidopsis* exist that may provide good genetic backgrounds for initial studies involving the transgenic expression of the *T. alata* $\Delta^6$16:0-ACP desaturase cDNA (Perrson, 1985; Bubeck, 1989; James and Dooner, 1990).

An additional consideration is the possibility that naturally occurring plants that produce unusual monounsaturated fatty acids, particularly petroselinic acid, could be developed into oilseed crops. Many Umbelliferae species are grown commercially as vegetable and spice crops and for essential oils. In addition, significant efforts have been initiated to establish coriander as an agronomic oilseed crop for the production of petroselinic acid (Anonymous, 1991). The major limitation is the relatively low oil content of its seed (18 wt%). It is possible that Umbelliferae species such as coriander could be developed as agronomic sources of petroselinic acid-rich oils through conventional plant breeding (Anonymous, 1991). Such efforts represent an alternative

to duplicating entire pathways for petroselinic acid synthesis and metabolism in transgenic plants. Regardless, one must also consider the large amount of basic knowledge that could be gained by characterizing enzymes and corresponding genes specialized for these pathways. Like the acyl-ACP desaturases, variant acyl-ACP thioesterases, β-keto-acyl-ACP synthases, and acyltransferases may also be present in Umbelliferae and *Thunbergia alata* endosperm. These enzymes could serve as comparative biochemical tools in understanding active-site structures of fatty acid and lipid biosynthetic enzymes. Such information may be important for precisely modifying various enzymes as attempts are made to produce fatty acids of more divergent structure in transgenic plants.

Further Characterization of Petroselinic Acid Metabolism

Finally, radiolabeling studies presented in Chapter 4 indicated that petroselinic acid may be metabolized through PC during periods of rapid triacylglycerol (TAG) production in Umbelliferae endosperm. However, no details of the mechanism involved in this process were presented. Therefore, additional radiolabeling studies are required to determine whether, for instance, the intact glycerol backbone moves from PC into triacylglycerol or whether PC is edited or retailored by the activity of phospholipases such as those recently described by Banas *et al.* (1992). More general questions that need to be addressed are what role, if any, does PC have in the metabolic or physical movement of fatty acids into TAG in developing seeds, and are the results obtained regarding the metabolism of petroselinic acid applicable to other unusual fatty acids?

Literature Cited

Anonymous (1991) European researchers develop oil sources. INFORM **2**: 690-691

Banas A, Johansson I, Stymne S (1992) Plant microsomal phospholipases exhibit preference for phosphatidylcholine with oxygenated acyl groups. Plant Sci **84**: 137-144

Bubeck DM, Fehr WR, Hammond EG (1989) Inheritance of palmitic and stearic acid mutants of soybean. Crop Sci **29**: 652-656

Craik CS, Largman C, Fletcher T, Roczniak S, Barr PJ, Fletterick R, Rutter WJ (1985) Redesigning trypsin: Alteration of substrate specificity. Science **228**: 291-297

Dörmann P, Frentzen M, Ohlrogge JB (1994) Specificities of acyl-acyl carrier protein (ACP) thioesterase and glycerol-3-phosphate acyltransferase for octadecenoyl-ACP isomers: Identification of a petroselinoyl-ACP thioesterase in Umbelliferae. Plant Physiol (in press)

James Jr DW, Dooner HK (1990) Isolation of EMS-induced mutants in *Arabidopsis* altered in seed fatty acid composition. Theor Appl Genet **80**: 241-245

Kinney AJ, Hitz WD, Yadav NS (1990) Stearoyl-ACP desaturase and a β-ketoacyl-ACP synthetase from developing soybean seeds. *In* PJ Quinn, JL Harwood, eds, Plant Lipid Biochemistry, Structure and Utilization. Portland Press, London, p 126-128

Nishida I, Beppu T, Matsuo T, Murata N (1992) Nucleotide sequence of a cDNA encoding a precursor to stearoyl-(acyl-carrier-protein) desaturase from spinach, *Spinacia oleracea*. Plant Mol Biol **19**: 711-713

Perrson C (1985) High palmitic acid content in summer turnip rape (*Brassica campestris* var. annua L.). Cruciferae Newslett 10, Scottish Crop Research Institute, Invergowerie, Dundee, p 137

Sato A, Becker CK, Knauf VC (1992) Nucleotide sequence of a complimentary DNA clone encoding stearoyl-acyl carrier protein desaturase from *Simmondsia chinensis*. Plant Physiol **99**: 362-363

Schneider G, Lindqvist Y, Shanklin J, Somerville C (1992) Preliminary crystallographic data for stearoyl-acyl carrier protein desaturase from castor seed. J Mol Biol **225**: 561-564

Shanklin J, Somerville C (1991) Stearoyl-acyl carrier protein desaturase from higher plants is structurally unrelated to the animal and fungal homologs. Proc Natl Acad Sci USA **88**: 2510-2514

Shanklin J, Mullins C, Somerville C (1991) Sequence of a complementary DNA from *Cucumis sativus* L. encoding the stearoyl-acyl-carrier protein desaturase. Plant Physiol **97**: 467-468

Sloane DL, Leung R, Craik CS, Sigal E (1991) A primary determinant for lipoxygenase positional specificity. Nature **354**: 149-152

Slocombe SP, Cummins I, Jarvis RP, Murphy DJ (1992) Nucleotide sequence and temporal regulation of a seed-specific *Brassica napus* cDNA encoding a stearoyl-acyl carrier protein (ACP) desaturase. Plant Mol Biol **20**: 151-155

Taylor MA, Smith SB, Davies HV, Burch LR (1992) The primary structure of a cDNA clone of the stearoyl-acyl carrier protein desaturase gene from potato (*Solanum tuberosum* L.). Plant Physiol **100**: 533-534

Thompson GA, Scherer DE, Foxall-Van Aken S, Kenny JW, Young HL, Shintani DK, Kridl JC, Knauf VC (1991) Primary structures of the precursor and mature forms of stearoyl-acyl carrier protein desaturase from safflower embryos and requirement of ferredoxin for enzyme activity. Proc Natl Acad Sci USA **88**: 2578-2582

Wells JA, Cunningham BC, Graycar TP, Estell DA (1987) Recruitment of substrate-specificity properties from one enzyme into a related one by protein engineering. Proc Natl Acad Sci **84**: 5167-5171

Wilks HM, Hart KW, Feeney R, Dunn CR, Muirhead H, Chia WN, Barstow DA, Atkinson T, Clarke AR, Holbrook JJ (1988) A specific, highly active malate dehydrogenase by redesign of a lactate dehydrogenase framework. Science **242**: 1541-1544

Appendix 1

GC-MS Identification of C_{16} and C_{18} Monounsaturated Fatty Acids of Coriander and *Thunbergia alata* Endosperm

Seed oils of coriander and *Thunbergia alata* contain a number of other monounsaturated fatty acids in addition to petroselinic acid or Δ^6 hexadecenoic acid. As shown below, GC-MS analysis of derivatives of fatty acid methyl esters was used to conclusively identify the C_{16} and C_{18} monounsaturated fatty acids of endosperm of these plants.

Derivatives were prepared by reaction of fatty acid methyl esters with dimethyl disulfide in the presence of iodine. This is a relatively new technique that is superior to many of the older methods for the preparation of monounsaturated fatty acids for GC-MS analysis (Francis, 1981). In this regard, the synthesis of dimethyl disulfide (or thiomethyl) derivatives is not technically difficult and can be performed in one step. In addition, dimethyl disulfide reacts directly with double bonds. As a result, the mass spectra obtained from these derivatives contain, in addition to a molecular ion, at least three abundant ions diagnostic for the position of the double bond. These ions include those of fragments corresponding to portions of the molecule on either side of the double bond and an ion formed by the loss of methanol from the fragment containing the carboxyl end of the fatty acid (this ion corresponds to Y-32 in the spectra shown below). Many previous methods of GC-MS analysis of double bond position have used derivatives prepared by reaction of the carboxyl group of fatty acids with reagents such as pyrrolidine (Andersson and Holman, 1974; Christie, 1982). In contrast to mass spectra of dimethyl disulfide adducts, diagnostic ions of these "indirect" derivatives are typically of lower abundance and, as a result, assigning the exact location of a double bond of a fatty acid can be somewhat ambiguous.

Of the monounsaturated fatty acids identified below, Δ^4hexadecenoic

acid ($16:1\Delta^4$) had been not previously detected in lipids of Umbelliferae seeds. As described in Chapters 2 and 3, the presence of this fatty acid in transgenic tobacco expressing the $\Delta^4$16:0-ACP desaturase cDNA provided an essential clue in determining the biosynthetic pathway of petroselinic acid. The detection of $16:1\Delta^4$ in lipids of coriander endosperm provides further support for the pathway proposed in Chapter 3. Amounts of this fatty acid in the total lipid extract of coriander endosperm were ≤0.1 wt%, but somewhat higher levels (≤0.5 wt%) were detected in phosphatidylcholine of this tissue. The other two 16:1 isomers ($16:1\Delta^4$ and Δ^6) identified in extracts of coriander endosperm were present in levels of 0.1 to 0.2 wt% of the total fatty acid. *cis*-Vaccenic acid ($18:1\Delta^{11}$) composed between 0.9 to 1.6 wt% of fatty acids of the detectable glycerolipid classes of coriander endosperm. Amounts of petroselinic acid ($18:1\Delta^6$) and oleic acid ($18:1\Delta^9$) in glycerolipids of coriander endosperm are reported in Chapter 4.

Fatty acid compositions, including C_{16} and C_{18} monounsaturated isomers, of glycerolipids of developing *T. alata* endosperm is provided in Table Appendix 1.1. The unusual monounsaturated isomers Δ^6hexadecenoic acid ($16:1\Delta^6$) and Δ^8octadecenoic acid ($18:1\Delta^8$) were previously identified in *T. alata* seed extracts by Spencer *et al.* (1971) using mass spectrometry of ozonolysis products.

Dimethyl disulfide derivatives were prepared from fatty acid methyl esters of phospholipids or neutral lipids of coriander and *T. alata* endosperm and analyzed by GC-MS using methods and instrumentation described in Chapter 5.

Literature Cited

Andersson BA, Holman RT (1974) Pyrrolidides for mass spectrometric determination of the position of the double bond in monounsaturated fatty acids. Lipids **9**: 185-190

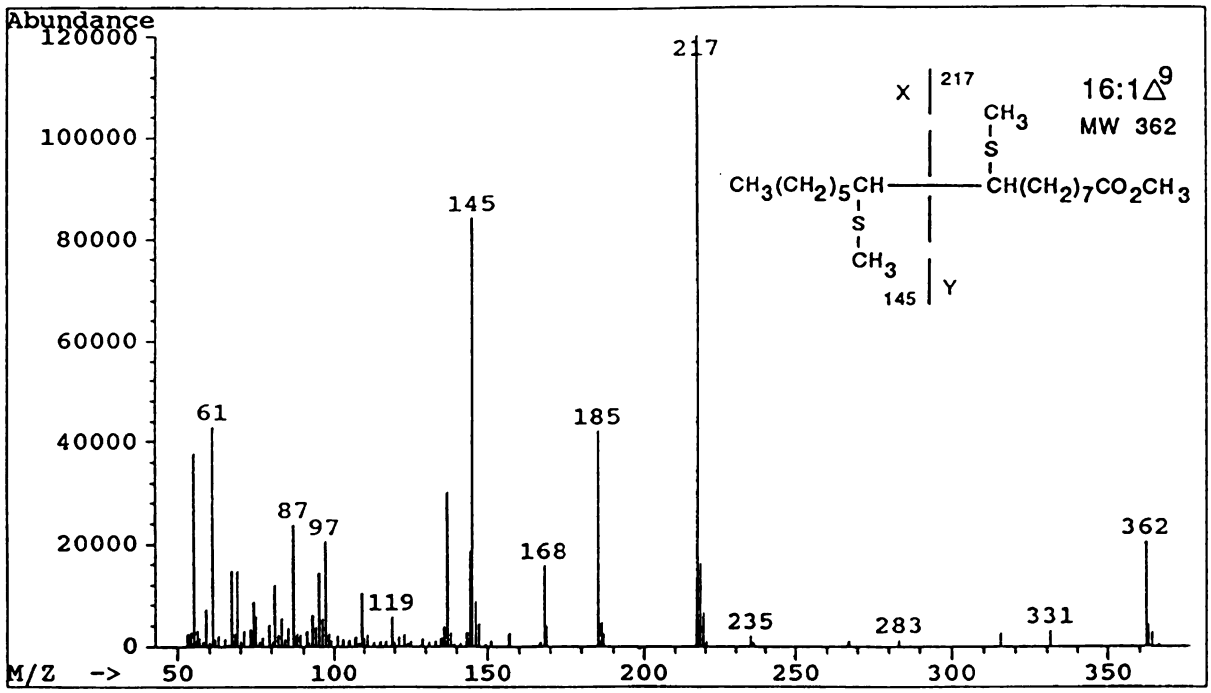
Christie WW (1982) Lipid Analysis, 2nd Edition. Pergammon Press, Oxford

Francis GW (1981) Alkylthiolation for the determination of double-bond position in unsaturated fatty acid esters. Chem Phys Lipids **29**: 369-374

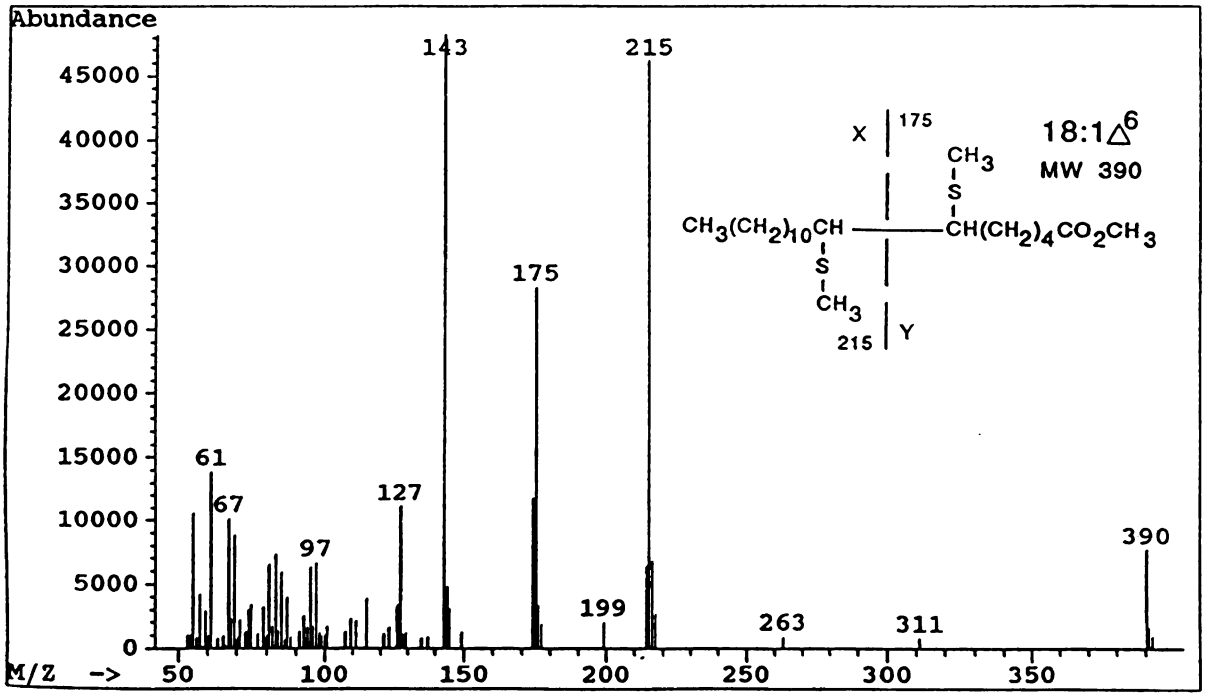
Spencer GF, Kleiman R, Miller RW, Earle FR (1971) Occurrence of *cis*-6-hexadecenoic acid as the major component of *Thunbergia alata* seed oil. Lipids **6**: 712-714

Figure Appendix 1.1. Mass spectra of thiomethyl adducts of C_{16} and C_{18} monounsaturated fatty acid standards (*A* and *B*) and C_{16} and C_{18} monounsaturated fatty acids of coriander (*C-H*) and *Thunbergia alata* (*I-L*) endosperm glycerolipids.

(A) 16:1Δ^9 Standard

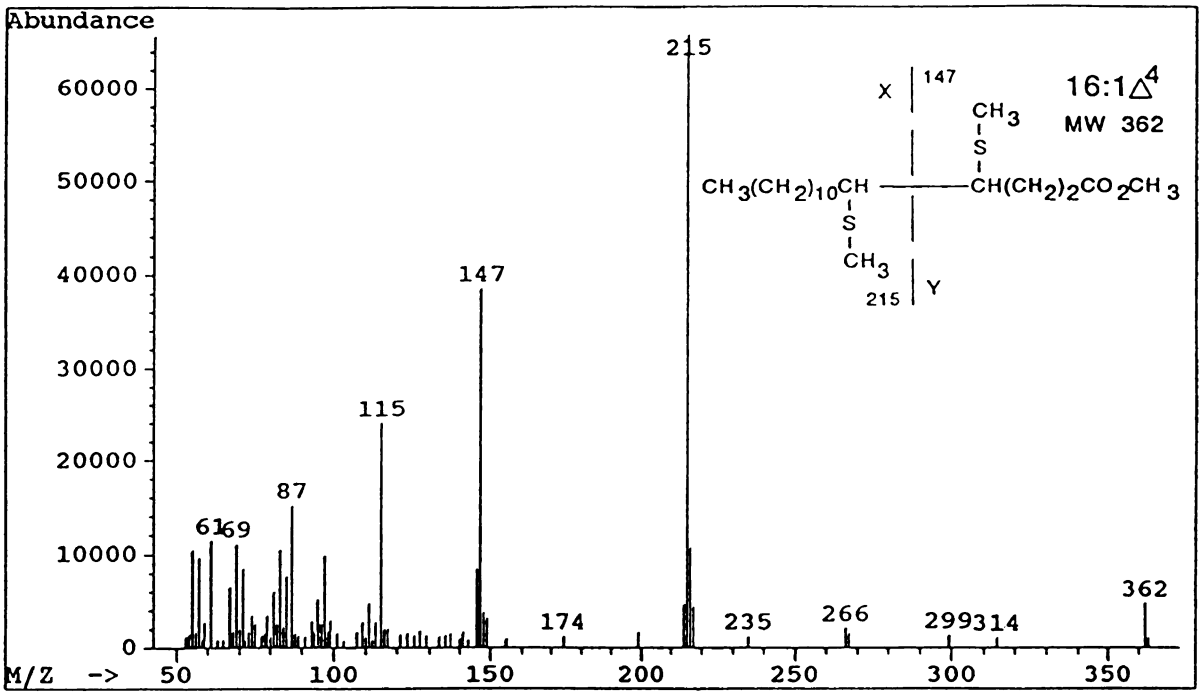


(B) 18:1Δ^6 Standard

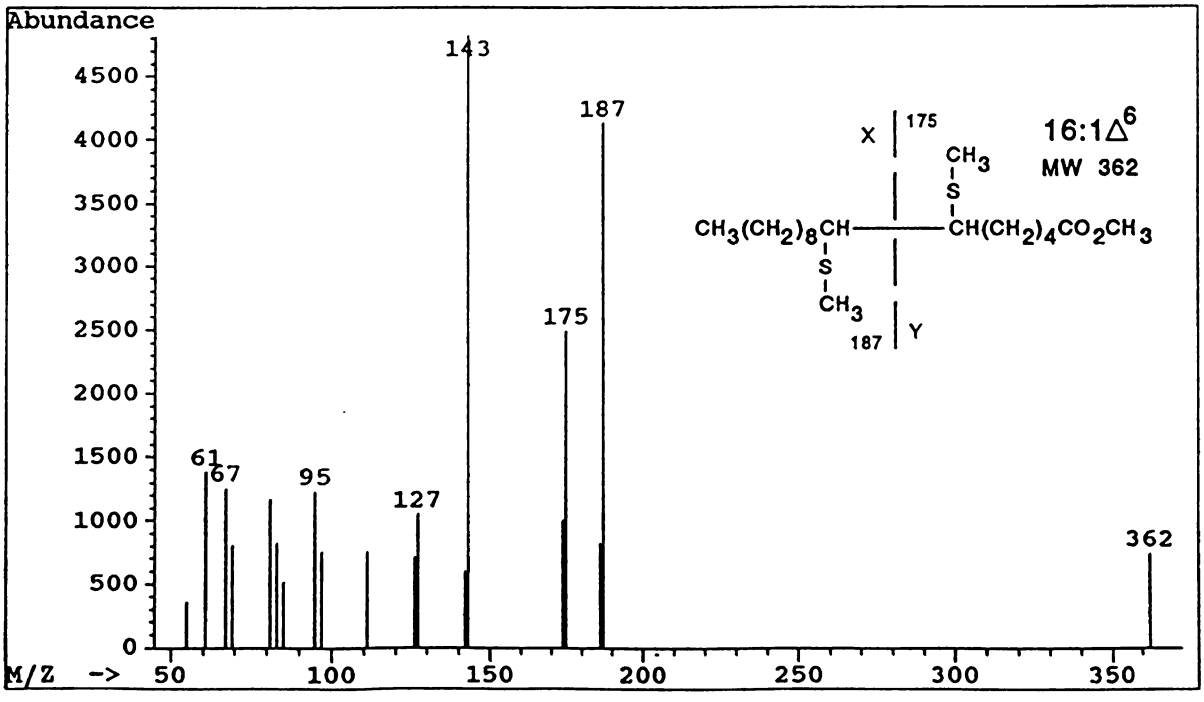


Coriander Endosperm C_{16} and C_{18} Monounsaturated Fatty Acids

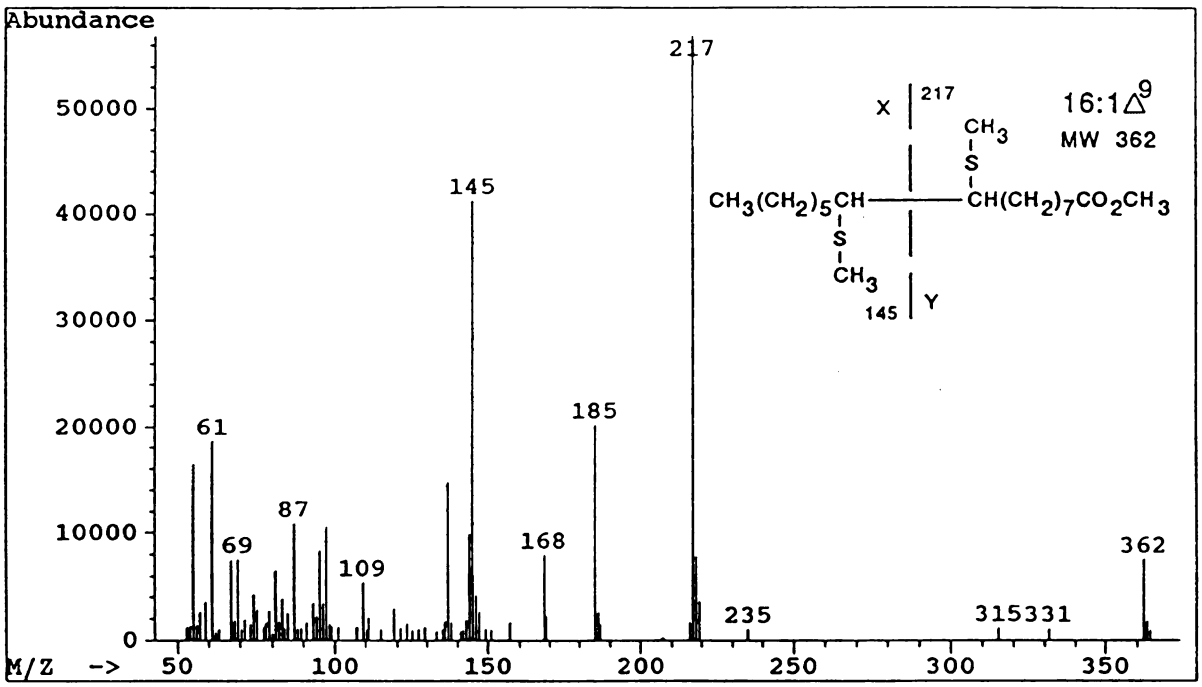
(C) 16:1Δ^4 (Phospholipid fraction)



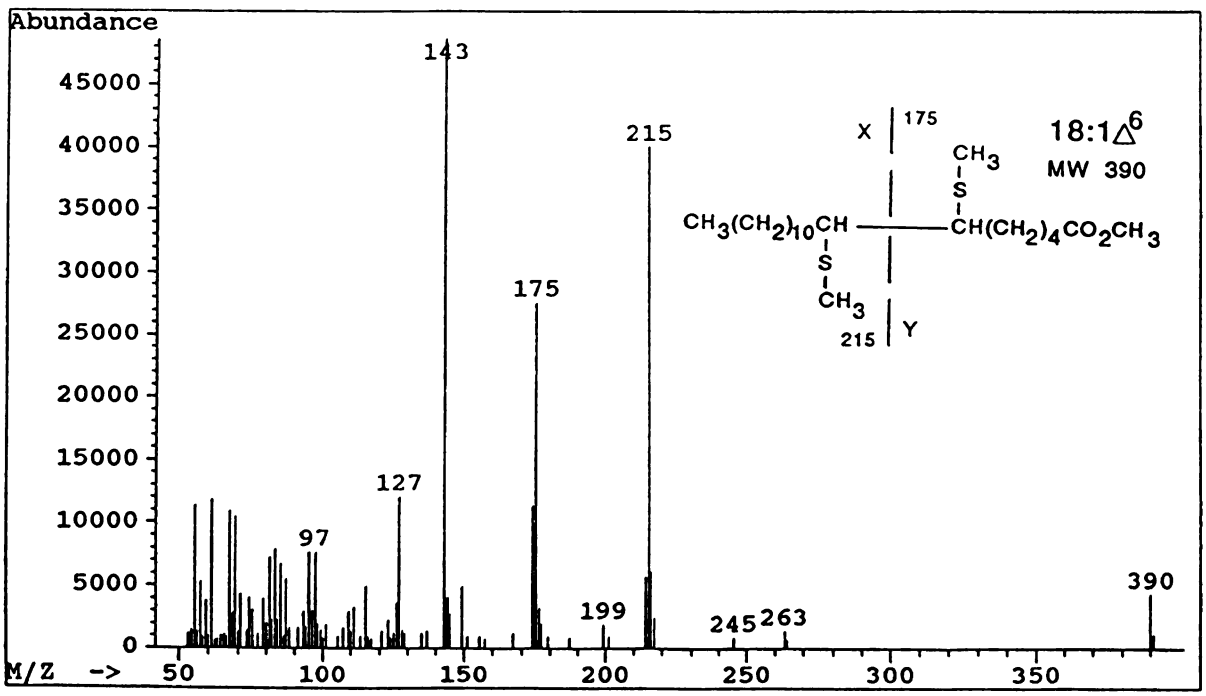
(D) 16:1Δ^6 (Phospholipid fraction)



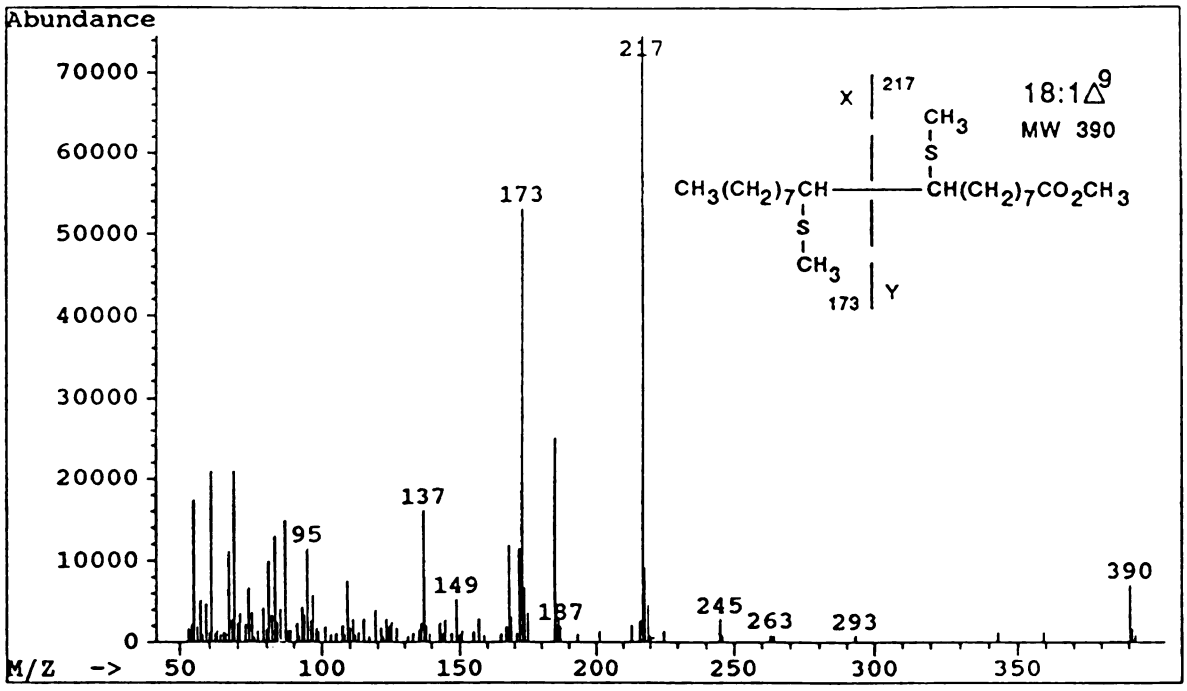
(E) 16:1Δ^9 (Phospholipid fraction)



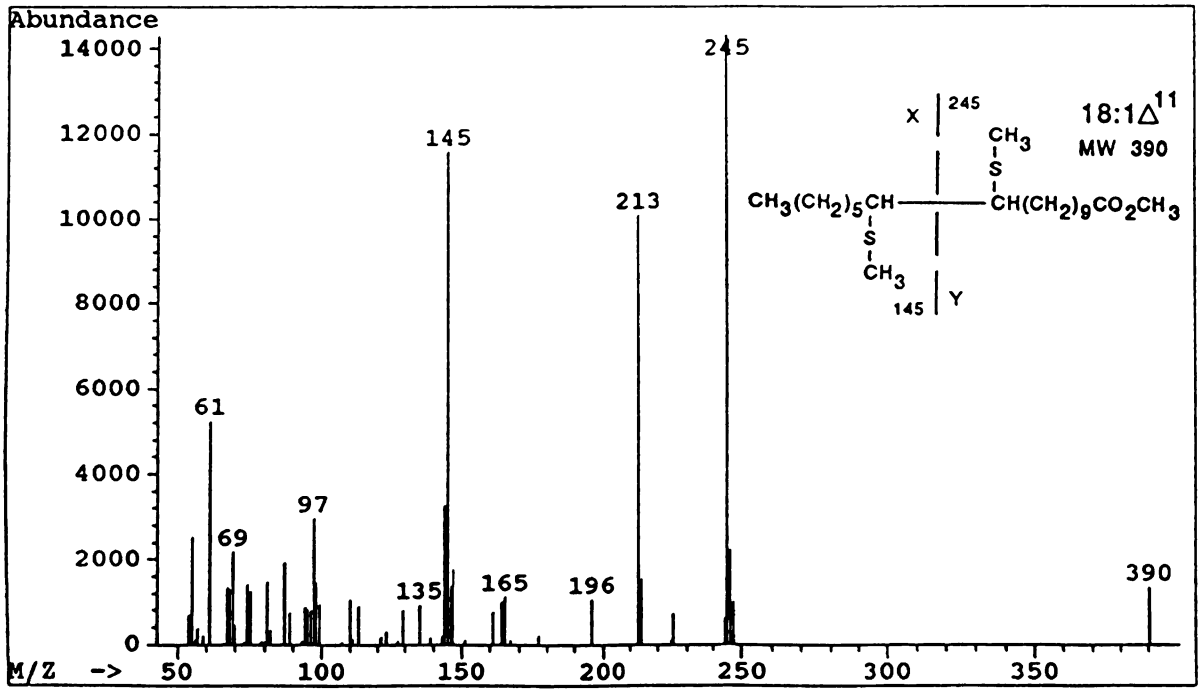
(F) 18:1Δ^6 (Phospholipid fraction)



(G) 18:1Δ^9 (Phospholipid fraction)

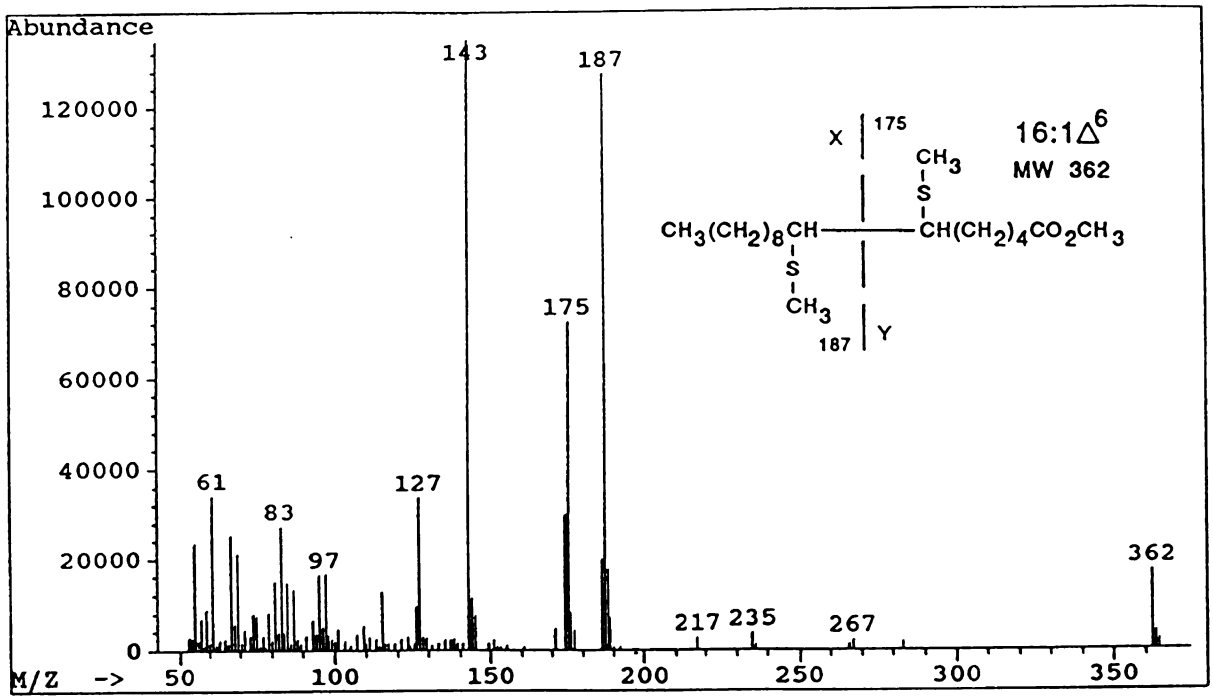


(H) 18:1Δ^{11} (Phospholipid fraction)

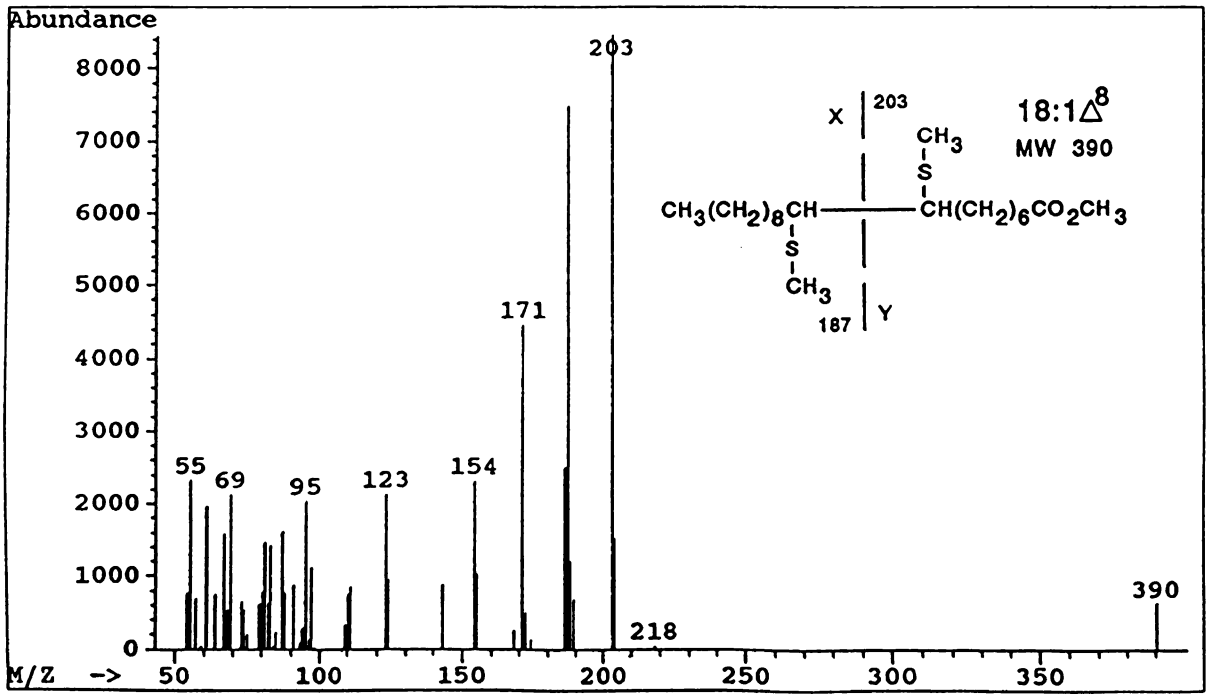


Thunbergia alata Endosperm C_{16} and C_{18} Monounsaturated Fatty Acids

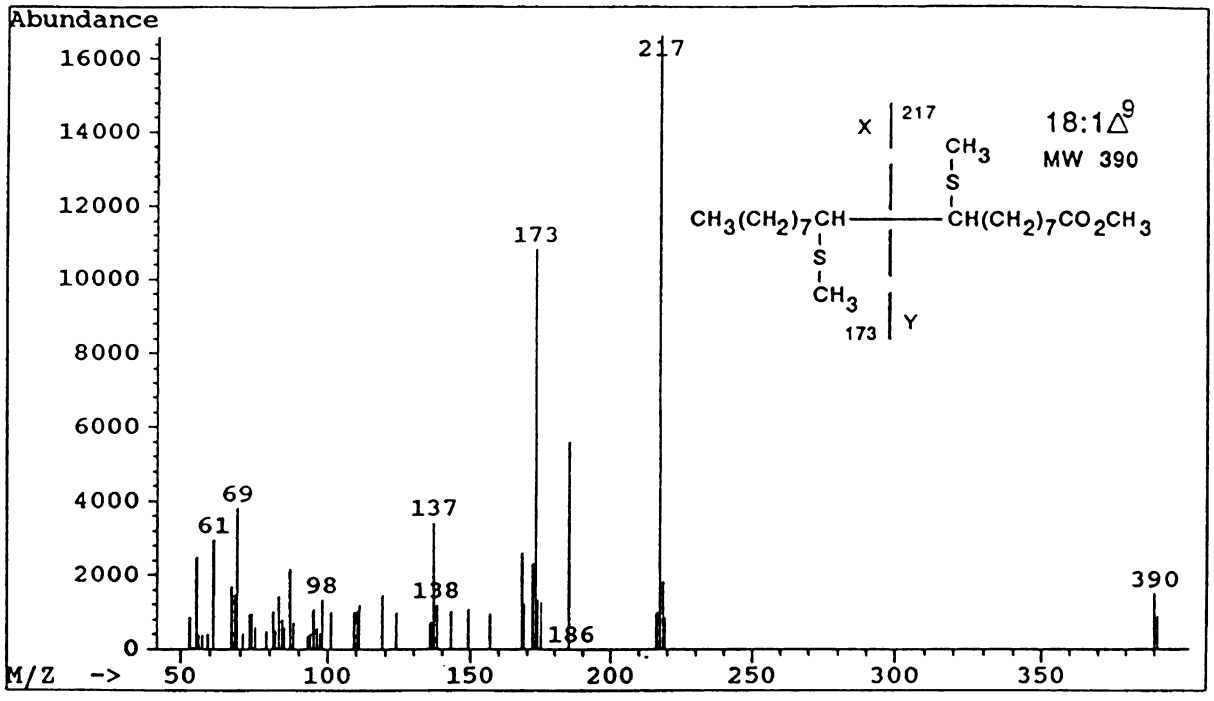
(I) 16:1Δ^6 (Triacylglycerol)



(J) 18:1Δ^8 (Phosphatidylcholine)



(K) 18:1Δ^{9} (Phosphatidylcholine)



(L) 18:1Δ^{11} (Phosphatidylcholine)

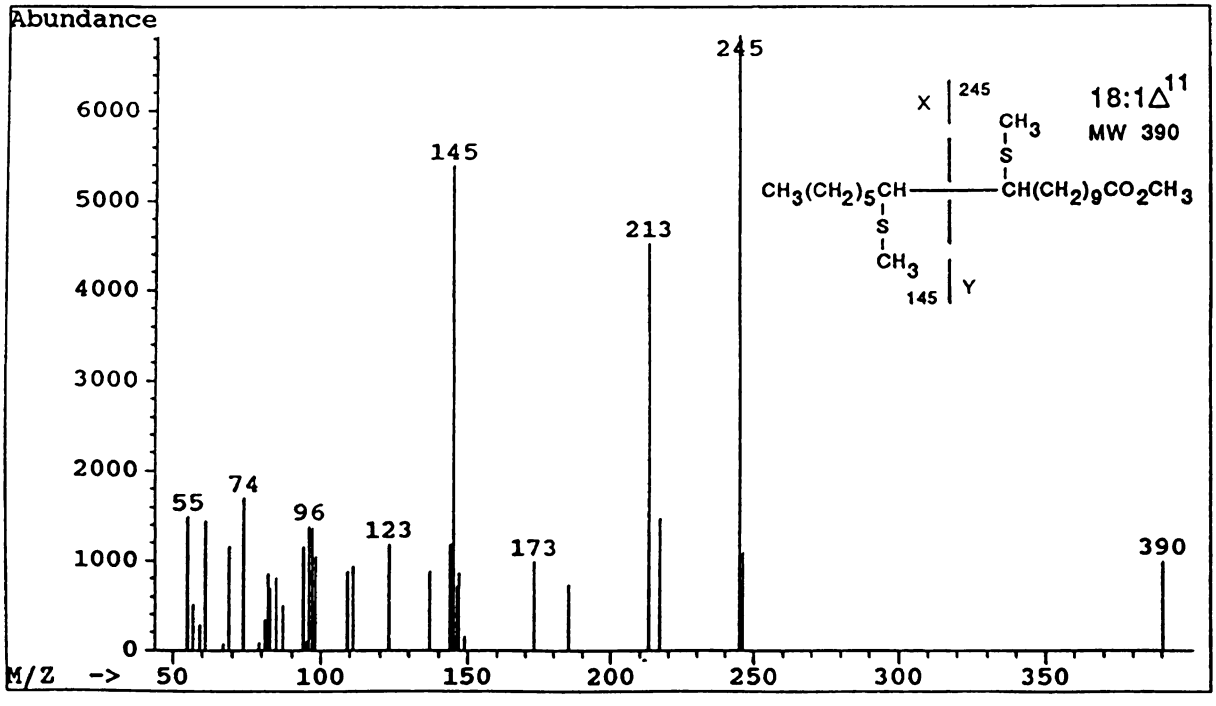


Table Appendix 1.1. Fatty acid compositions of triacylglycerol (TAG), diacylglycerol (DAG), and phosphatidylcholine (PC) of developing Thunbergia alata endosperm

Glycerolipids were purified and fatty acids quantified using chromatographic conditions described in Chapter 4. Fatty acid compositions are presented as mol% ± SE (n=4).

	Fatty Acid						
Lipid Class	16:0	16:1Δ^6	18:0	18:1Δ^8	18:1Δ^9	18:2	other[a]
				mol%			
TAG (740)[b]	9.1 ± 0.7	80.2 ± 1.5	0.8 ± 0.2	2.2 ± 0.2	3.7 ± 0.4	3.7 ± 0.3	≤0.3
DAG (17.3)	15.4 ± 1.8	59.2 ± 5.1	3.7 ± 0.8	3.3 ± 0.9	10.7 ± 1.8	7.7 ± 0.9	≤0.1
PC (56.7)	9.7 ± 0.4	26.1 ± 0.5	1.3 ± 0.2	4.3 ± 0.5	29.9 ± 1.6	27.5 ± 2.7	≤1.2

[a]Includes 14:0, 18:1Δ^{11}, and 18:3

[b]nmoles/endosperm

Appendix 2

cDNAs for Apparent Isoforms of the Δ^9Stearoyl-ACP Desaturase in *Thunbergia alata* Endosperm

As described in Chapter 5, screening of a *Thunbergia alata* endosperm cDNA library with antibodies against the avocado $\Delta^9$18:0-ACP desaturase (Shanklin and Somerville, 1991) lead to the isolation of three cDNAs encoding $\Delta^9$18:0-ACP desaturases (designated pTAD1, pTAD2, and pTAD3). pTAD1 and 2 were initially obtained by antibody screening of the library, and pTAD3 was isolated by screening with a random labeled probe derived from pTAD2. Shown below are the nucleotide sequences of these cDNAs as well as the corresponding amino acid sequences (Figures Appendix 2.1, 2, and 3). From comparisons with previously isolated $\Delta^9$18:0-ACP desaturases cDNAs, pTAD2 and 3 appear to contain open reading frames for complete transit and mature peptides. The cDNA insert of pTAD1, however, lacks coding sequence for the plastid transit peptide and at least two amino acids of the mature peptide. The amino acid sequences encoded by the inserts of pTAD2 and 3 share 87% identity, whereas amino acid sequences corresponding to cDNAs of pTAD1 and 2 share 78% identity (Figure Appendix 2.4).

The double bond positioning properties of desaturases encoded by pTAD1, 2, and 3 were determined by *in vitro* assay of recombinant protein. cDNAs were expressed in *E. coli* using the pET3a vector (Novagen). Inserts were prepared by PCR amplification of the mature peptide-encoding regions of cDNAs. In the case of pTAD1, the sense PCR primer contained additional sequence for an alanine and serine residue. Extracts of *E. coli* expressing each of the cDNAs catalyzed the Δ^9 desaturation of [1-^{14}C]stearoyl (18:0)-ACP (data not shown).

Overall, the isolation of three $\Delta^9$18:0-ACP desaturase cDNAs from a library derived from polyA(+) RNA of *T. alata* endosperm suggests that isoforms of this enzyme can exist in a given species and in a single tissue.

Literature Cited

Shanklin J, Somerville C (1991) Stearoyl-acyl carrier protein desaturase from higher plants is structurally unrelated to the animal and fungal homologs. Proc Natl Acad Sci USA **88**: 2510-2514

```
pTAD1  ACGAGAACTATTCTAAGGGAGGTGGAAACTGCCAATAAACATTTTACTCCCCCCAGCGAGGTTCATGTTCAAGTGACGCACTCA   84
PTAD1   T  R  T  I  L  R  E  V  E  T  A  N  K  H  F  T  P  P  S  E  V  H  V  Q  V  T  H  S    28

pTAD1  ATGCCCCCACAGAAAATTGAGATATTCAAATCCTTGGAAGACTGGGCTGCAGATAATATATTAGTTTACCTTAAGCCTGTTGAA   168
pTAD1   M  P  P  Q  K  I  E  I  F  K  S  L  E  D  W  A  A  D  N  I  L  V  Y  L  K  P  V  E    56

pTAD1  AAATGTTGGCAACCTCAGGATTTCCTTCCAGATCCAGCATCTGATGGTTTTCATGATCAGGTCAAGGAATTGAGAGAAAGAGCC   252
pTAD1   K  C  W  Q  P  Q  D  F  L  P  D  P  A  S  D  G  F  H  D  Q  V  K  E  L  R  E  R  A    84

pTAD1  AGGGAGATACCAGATGATTATTTTGTTGTTCTAGTTGGTGATATGATCACAGAAGAAGCTCTTCCAACATATCAGACAATGTTG   336
pTAD1   R  E  I  P  D  D  Y  F  V  V  L  V  G  D  M  I  T  E  E  A  L  P  T  Y  Q  T  M  L   112

pTAD1  GATACCTTAGATGGTGTGCGGGATGAAACAGGAGCGAGCCCAACTCCTTGGGCAATTTGGACAAGGGCATGGACTGCTGAAGAA   420
pTAD1   D  T  L  D  G  V  R  D  E  T  G  A  S  P  T  P  W  A  I  W  T  R  A  W  T  A  E  E   140

pTAD1  AATAGGCATGGAGACCTTCTTAATAAATATCTTTATCTTTCTGGGAGAGTGGATATGAGACAGATTGAGAAGACTATTCAGTAT   504
pTAD1   N  R  H  G  D  L  L  N  K  Y  L  Y  L  S  G  R  V  D  M  R  Q  I  E  K  T  I  Q  Y   168

pTAD1  CTAATCGGCTCTGGAATGGACCCAAGGACAGAAAACAGTCCATACCTTGGATTTATATACACATCCTTCCAAGAAAGGGCTACT   588
pTAD1   L  I  G  S  G  M  D  P  R  T  E  N  S  P  Y  L  G  F  I  Y  T  S  F  Q  E  R  A  T   196

pTAD1  TTCATCTCCCATGGAAACACTGCCCGGCTCGCCAGACAACATGGGGACAATAAGCTAGCCCAAATCTGTGGTACAATTGCCTCA   672
pTAD1   F  I  S  H  G  N  T  A  R  L  A  R  Q  H  G  D  N  K  L  A  Q  I  C  G  T  I  A  S   224

pTAD1  GATGAGAAGCGTCATGAAACTGCATACACCAAGATAGTGGAGAAGCTATTCGAGATTGACCCTGATGGAACAATGCTATCTTTG   756
pTAD1   D  E  K  R  H  E  T  A  Y  T  K  I  V  E  K  L  F  E  I  D  P  D  G  T  M  L  S  L   252

pTAD1  GCCGACATGATGAAGAAGAAGGTCTCTATGCCAGCCCACTTGATGTATGATGGCCATGACGAAAACCTCTTTGAGAACTTCTCA   840
pTAD1   A  D  M  M  K  K  K  V  S  M  P  A  H  L  M  Y  D  G  H  D  E  N  L  F  E  N  F  S   280

pTAD1  GCTGTTGCTCAGCGACTTGGTGTTTACACAGCCAAAGACTATGCTGACATCCTAGAACATTTGATTGCCAGATGGAAAGTGTCA   924
pTAD1   A  V  A  Q  R  L  G  V  Y  T  A  K  D  Y  A  D  I  L  E  H  L  I  A  R  W  K  V  S   308

pTAD1  GATTTGACCGGGCTATCAGGGGAAGGTCAGAAAGCTCAGGACTATGTGTGTCGGCTGCCACCAAGAATCAGAAAACTGGAGGAG  1008
pTAD1   D  L  T  G  L  S  G  E  G  Q  K  A  Q  D  Y  V  C  R  L  P  P  R  I  R  K  L  E  E   336

pTAD1  AGAGCTCAAGCTCGAATAAAAGAAGGACCAAGAATCCCATTTAGCTGGATATACAATAGAGAGTTGCTACTATAGATTTGATGT  1092
pTAD1   R  A  Q  A  R  I  K  E  G  P  R  I  P  F  S  W  I  Y  N  R  E  L  L  L  *            360

pTAD1  GTTCTCTCTGGGCAGCCGTTGCTCACCTGTAGGTGAAAAGCTCAAGGAAATTTGCATTGTAGGTTGTTTGCAGCTTAAAATTAT  1176

pTAD1  GTTGAGAATGAAGAATGCTGGTGCAAGTGTTACTGTGTCGTGTCATTGTGTTCTAATATTTTTTCGTAGCTCTCAGGACTGTAA  1260

pTAD1  AAACTGTCAGAACGTTGTAAAAGGATTTTGGTGATGATTGCTGTGTTTTGCACTTTGACCAACAAAAAAAAAAAAAAAAAAAA  1343
```

Figure Appendix 2.1. Nucleotide and deduced amino acid sequences of the cDNA insert of pTAD1.

```
pTAD2  CACAAAACCTCATTTTTCTGAAGAAATATAGACGAAATGGCGCTGAAATGTAGCGTAACCCCCCACCAGGTGCCTTCTTTTCCT   84
PTAD2                                    M  A  L  K  C  S  V  T  P  H  Q  V  P  S  F  P    16

pTAD2  GTTAATCAGCTCAGATCTCACCGAGTTTACATGGCTTCAACTCTCGATTCGGCATCCGCTAATGTTGGGAAAGGTAAAAAGGCT  168
pTAD2   V  N  Q  L  R  S  H  R  V  Y  M  A  S  T  L  D  S  A  S  A  N  V  G  K  G  K  K  A   44

pTAD2  TTCACCCCCCCTCGAGAAGTCAAGGTTCAACTGACGCATCCCATGGCTCCAGAAAAGCGCGAGATCTTCCATTCACTGCACGGT  252
pTAD2   F  T  P  P  R  E  V  K  V  Q  L  T  H  P  M  A  P  E  K  R  E  I  F  H  S  L  H  G   72

pTAD2  TGGGCGGAAGAAAACCTTCTGTCTCTCTTGAAGCCTGTTGAGAAGTGTTGGCAGCCCAACGACTTTCTTCCCGACCCTTCTTCA  336
pTAD2   W  A  E  E  N  L  L  S  L  L  K  P  V  E  K  C  W  Q  P  N  D  F  L  P  D  P  S  S  100

pTAD2  GAAGGCTTTGATGAACAGGTCAGGGAGCTTCGGCTAAGAACCAAGGAACTACCCGATGAATACTTTGTTGTGTTGGTTGGCGAC  420
pTAD2   E  G  F  D  E  Q  V  R  E  L  R  L  R  T  K  E  L  P  D  E  Y  F  V  V  L  V  G  D  128

pTAD2  ATGATCACGGAAGAAGCTCTCCCTACTTATCAGACGATGATCAACACTTTAGATGCAGTCCGTGATGAGACCGGTGCAAGCCTT  504
pTAD2   M  I  T  E  E  A  L  P  T  Y  Q  T  M  I  N  T  L  D  A  V  R  D  E  T  G  A  S  L  156

pTAD2  ACTCCTTGGGCTATTTGGACTAGAGCATGGACTGCGGAAGAGAATAGGCACGGTGATCTTCTCAACAAATATCTTTACCTTTCG  588
pTAD2   T  P  W  A  I  W  T  R  A  W  T  A  E  E  N  R  H  G  D  L  L  N  K  Y  L  Y  L  S  184

pTAD2  GGACGCGTGGACATGAGGCAAATTGAGAAGACGATTCAATACCTCATCGGTTCAGGAATGGATCCTCGCACGGATAACAACCCG  672
pTAD2   G  R  V  D  M  R  Q  I  E  K  T  I  Q  Y  L  I  G  S  G  M  D  P  R  T  D  N  N  P  212

pTAD2  TACCTCGGATTCATCTACACCTCGTTCCAGGAGAGGGCGACATTCATTTCTCATGGGAACACGGCTAGGCTTGCCAAGGAACAC  756
pTAD2   Y  L  G  F  I  Y  T  S  F  Q  E  R  A  T  F  I  S  H  G  N  T  A  R  L  A  K  E  H  240

pTAD2  GGGGACCTTAAACTGGCACAGATATGCGGCAGCATAGCTGCAGATGAGAAACGGCACGAAACTGCTTACACCAAAATTATCGAA  840
pTAD2   G  D  L  K  L  A  Q  I  C  G  S  I  A  A  D  E  K  R  H  E  T  A  Y  T  K  I  I  E  268

pTAD2  AAGCTGTTTGAGATCGACCCTGATGGGACAGTACTTGCTCTAGCCGATATGATGAGGAAGAAAGTGTCTATGCCGGCACATTTG  924
pTAD2   K  L  F  E  I  D  P  D  G  T  V  L  A  L  A  D  M  M  R  K  K  V  S  M  P  A  H  L  296

pTAD2  ATGTATGATGGACAAGATGAAAATCTGTTTGAACACTTTGCAGCTGTGGCGCAACGCATTGGAGTGTACACTGCGAAAGACTAT  1008
pTAD2   M  Y  D  G  Q  D  E  N  L  F  E  H  F  A  A  V  A  Q  R  I  G  V  Y  T  A  K  D  Y  324

pTAD2  GCTGATATACTGGAATTTTTAGTTGGGAGATGGGAGGTGGAGAAACTAACAGGACTTTCAGGGGAGGGTCGTAACGCGCAGGAG  1092
pTAD2   A  D  I  L  E  F  L  V  G  R  W  E  V  E  K  L  T  G  L  S  G  E  G  R  N  A  Q  E  352

pTAD2  TACGTGTGCGGGTTGGCTCCTCGGATCAGAAGGTTGGAAGAGAGAGCACAGGCACGGGCAAAGCAGGGGGCACCTGTGCCCTTC  1176
pTAD2   Y  V  C  G  L  A  P  R  I  R  R  L  E  E  R  A  Q  A  R  A  K  Q  G  A  P  V  P  F  380

pTAD2  TCCTGGGTTTATGGTCGAGAAGTTAAAATTTGAACCGTCGGACGGATTCCGAGTTAGTTTGCTTCTGTGAACTCCTCTTTTCAG  1260
pTAD2   S  W  V  Y  G  R  E  V  K  I  *                                                      390

pTAD2  CTTTGCTATTAGCAAGAAAATTTTTATCAAGTTTATGTGTTTTTAAAATATATAATATCAATTGTTTTTGGGTTTTAAAAAAAA  1344

pTAD2  AAAAAAAAAAA                                                                          1355
```

Figure Appendix 2.2. Nucleotide and deduced amino acid sequences of the cDNA inserts of pTAD2. The underlined amino acid corresponds to the likely transit peptide cleavage site.

```
pTAD3   TTTTTCTGGTGTCGATTTGAAGAAATATAGAGGAAATGGCGCTTAAATTTAGCATTACGCCCCACAAGATGCCTTCCTTCCCT   83
PTAD3                                        M  A  L  K  F  S  I  T  P  H  K  M  P  S  F  P    16

pTAD3   GATTTCCAGCTCAGATCTCACCGGGTTTGCATGACTTCAACTCTCTATTCTGCATCCGTTGAGGTCGGCAATGGTAAAAAGCCT   167
pTAD3    D  F  Q  L  R  S  H  R  V  C  M  I  S  T  L  Y  S  A  S  V  E  V  G  N  G  K  K  P    44

pTAD3   TTCAGTCCCCCTCGAGAAGTCAATATTCAAGTGACACATCCCATGCCTCCAGAAAAGCGCGAGATCTTCAACTCATTGCATGGA   251
pTAD3    F  S  P  P  R  E  V  N  I  Q  V  T  H  P  M  P  P  E  K  R  E  I  F  N  S  L  H  G    72

pTAD3   TGGGCGGAAACTAATCTTCTGTCTCTCTTGAAGCCTGTTGACAAGTGTTGGCAGCCCAGTGACTTTCTACCCGACCCTTCCGCA   335
pTAD3    W  A  E  T  N  L  L  S  L  L  L  P  V  D  K  C  W  Q  P  S  D  F  L  P  D  P  S  A   100

pTAD3   GATGGCTTTGACGAGCAGGTCAGAGAGTTACGGAAAAGAACCAAGGAACTACCCGATGAATATTTTATTGTGTTGATTGGTGAC   419
pTAD3    D  G  F  D  E  Q  V  R  E  L  R  K  R  T  K  E  L  P  D  E  Y  F  I  V  L  I  G  D   128

pTAD3   ATGATTACGGAGGAAGCTCTCCCAACTTATCAGACTATGATCAACACGCTTGATGCAGTCCGGGATGAGACTGGTGCAAGCCTT   503
pTAD3    M  I  T  E  E  A  L  P  T  Y  Q  T  M  I  N  T  L  D  A  V  R  D  E  T  G  A  S  L   156

pTAD3   ACTCCTTGGGCTATTTGGAATAGAGCATGGACTGCTGAAGAGAATAGGCATGGTGATCTTCTCAACAAATATCTCTACCTTTCG   587
pTAD3    T  P  W  A  I  W  N  R  A  W  T  A  E  E  N  R  H  G  D  L  L  N  K  Y  L  Y  L  S   184

pTAD3   GGACGTGTGGACATGAAGCAAGTCGAAAAGACAATTCAGTACCTGATTGGTTCAGGAATGGACCCTCATACTGATAACAACCCG   671
pTAD3    G  R  V  D  M  K  Q  V  E  K  T  I  Q  Y  L  I  G  S  G  M  D  P  H  T  D  N  N  P   212

pTAD3   TACCTTGGATTCATCTACACATCGTTTCAGGAGAGAGCAACATTCATTTCTCATGGGAACACGGCTAGGCTTGCCAAGGAACAC   755
pTAD3    Y  L  G  F  I  Y  T  S  F  Q  E  R  A  T  F  I  S  H  G  N  T  A  R  L  A  K  E  H   240

pTAD3   GGTGATATGAAACTGGCACAGATTTGTGGTACCATCGCTGCAGATGAGAAACGTCATGAAACTGCCTACACAAAAATTATTGAA   839
pTAD3    G  D  M  K  L  A  Q  I  C  G  T  I  A  A  D  E  K  R  H  E  T  A  Y  T  K  I  I  E   268

pTAD3   AAGCTGTTTCAACTGGACCCTGATGGGACAATACTCGCTCTAGCTGACATGATGCGGAAGAAAGTGTCGATGCCTGCCCACCTG   923
pTAD3    K  L  F  Q  L  D  P  D  G  T  I  L  A  L  A  D  M  M  R  K  K  V  S  M  P  A  H  L   296

pTAD3   ATGTTTGATGGGAAAGATCAAAATCTATTTGAACACTTCTCTGCTGTGGCGCAACGTATTGGAGTGTACACTGCCAAGGACTAT  1007
pTAD3    M  F  D  G  K  D  Q  N  L  F  E  H  F  S  A  V  A  Q  R  I  G  V  Y  T  A  K  D  Y   324

pTAD3   GCTGATATATTGGAATATCTAGTGGCGAGATGGGAGGTGGAGAAGCTGACAGGGCTTACAGGAGAGGGGCGTAAAGCGCAAGAG  1091
pTAD3    A  D  I  L  E  Y  L  V  A  R  W  E  V  E  K  L  T  G  L  T  G  E  G  R  K  A  Q  E   352

pTAD3   TATGTGTGTGGCTTGGCTCCGAGGATCAGAAGGTTGGATGAGAGAGCACAGGCACGTGCAAAGGAAGCAGCGCCTGTGCCCTTT  1175
pTAD3    Y  V  C  G  L  A  P  R  I  R  R  L  D  E  R  A  Q  A  R  A  K  E  A  A  P  V  P  F   380

pTAD3   ACATGGATTTTTGGCCGAGAAGTTCGTCTCTAGTAATACTATTGAATGGTCAAATAAAACATGCAGCAAGGATGTGGAAACGGG  1259
pTAD3    T  W  I  F  G  R  E  V  R  L  *                                                      390

pTAD3   CTATTAGGTCGATTTTGCCATAAGCCTATTTACTTGAAGAAAACCTTAAATTATTAAAAGGATTGCTGTTGAGGCCTAAAAATT  1343

pTAD3   GCAATACATCAAAGACACATATTCACCAACATTATTAATGTATTGTGTTTTTGTGAGGTTATGAAGTACTCATTTATTAAAAAA  1427

pTAD3   AAAAAAAAAAAA                                                                          1439
```

Figure Appendix 2.3. Nucleotide and deduced amino acid sequences of the cDNA insert of pTAD3. The underlined amino acid corresponds to the likely transit peptide cleavage site.

```
pTAD2   MALKCSVTPHQVPSFPVNQLRSHRVYMASTLDSASANVGKGKKAF   45
pTAD1                                    TRTILRE:ETAN:H:   15
pTAD3   ::::F:I:::KM::::DF:::::::C:T:::Y:::VE::N:::P:   45

pTAD2   TPPREVKVQLTHPMAPEKREIFHSLHGWAEENLLSLLKPVEKCWQ   90
pTAD1   :::S::H::V::S:P:Q:I:::K::ED::AD:I:VY:::::::::   60
pTAD3   S:::::NI:V::::P:::::::N:::::::T:::::::::D::::   90

pTAD2   PNDFLPDPSSEGFDEQVRELRLRTKELPDEYFVVLVGDMITEEAL  135
pTAD1   :Q::::::A:D::HD::K:::E:AR:I::D:::::::::::::::  105
pTAD3   :S::::::::AD::::::::::K::::::::::I::I:::::::::  135

pTAD2   PTYQTMINTLDAVRDETGASLTPWAIWTRAWTAEENRHGDLLNKY  180
pTAD1   ::::::LD:::G::::::::P:::::::::::::::::::::::::  150
pTAD3   ::::::::::::::::::::::::::::N:::::::::::::::::  180

pTAD2   LYLSGRVDMRQIEKTIQYLIGSGMDPRTDNNPYLGFIYTSFQERA  225
pTAD1   ::::::::::::::::::::::::::::E:S::::::::::::::  195
pTAD3   :::::::::K:::::::::::::::::H:::::::::::::::::  225

pTAD2   TFISHGNTARLAKEHGDLKLAQICGSIAADEKRHETAYTKIIEKL  270
pTAD1   ::::::::::::RQ:::N:::::::T::S::::::::::::V:::  240
pTAD3   ::::::::::::::::::::::::::T::::::::::::::::::  270

pTAD2   FEIDPDGTVLALADMMRKKVSMPAHLMYDGQDENLFEHFAAVAQR  315
pTAD1   ::::::::M:S:::::K::::::::::::H::::::N:S:::::  285
pTAD3   :QL:::::I:::::::::::::::::::F::K:Q::::::S:::::  315

pTAD2   IGVYTAKDYADILEFLVGRWEVEKLTGLSGEGRNAQEYVCGLAPR  360
pTAD1   L::::::::::::::H:IA::K:SD::::::::QK::D:::R:P::  330
pTAD3   ::::::::::::::Y::A:::::::::T::::K::::::::::::  360

pTAD2   IRRLEERAQARAKQGAPVPFSWVYGREVKI*                390
pTAD1   ::K::::::::I:E:PRI::::I:N::LLL*                360
pTAD3   ::::D::::::::EA:::::T:IF::::RL*                390
```

Figure Appendix 2.4. Comparison of amino acid sequences encoded by cDNA inserts of pTAD1, 2, and 3. As shown, the primary structures of polypeptides corresponding to pTAD1 and 3 are compared to that of pTAD2. Colons indicate identical amino acids, and the underlined amino acids in the pTAD2 and 3 sequences represent likely transit peptide cleavage sites.