Characterization of Cyclopropane Fatty Acid Synthase from *Sterculia foetida*

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Running Title: Cyclopropane Fatty Acid Synthase from *Sterculia foetida*

Summary

Cyclopropane synthase from *Sterculia foetida* developing seeds catalyzes the addition of a methylene group from *S*-adenosylmethionine to the cis-double bond of oleic acid (Bao, et. al. (2002) Proc. Natl. Acad. Sci. 99: 7172-7177. 1). To better understand this enzyme, differential expression in leaf and seed tissues, protein properties and substrate preference of plant cyclopropane synthase were investigated. Immunoblot
analysis with antibodies raised to recombinant *S. foetida* cyclopropane synthase (SfCPA-FAS) revealed that SfCPA-FAS is expressed in *S. foetida* seeds, but not in leaves, and is a membrane protein localized to microsomal fractions. Transformed tobacco cells expressing SfCPA-FAS were labeled *in vivo* with L-[methyl-\(^{14}\)C]methionine and assayed *in vitro* with \( S \)-adenosyl-L-methionine [methyl-\(^{14}\)C]. These kinetic experiments demonstrated that dihydrosterculate was synthesized from oleic acid esterified at the \( sn-1 \) position of phosphatidylcholine (PC). Furthermore analysis of acyl chains at \( sn-1 \) and \( sn-2 \) positions that accumulated in PC from *S. foetida* developing seeds and from tobacco cells expressing SfCPA-FAS also demonstrated that greater than 90% of dihydrosterculate was esterified to the \( sn-1 \) position. Thus, we conclude that SfCPA-FAS is a microsomal localized membrane protein that catalyzes the addition of methylene groups derived from \( S \)-adenosyl-L-methionine across the double bond of oleic acid esterified to \( sn-1 \) position of PC. A survey of plant and bacterial genomes for sequences related to SfCPA-FAS indicated that a peptide domain with a putative flavin binding site is either fused to the methyltransferase domain of the plant protein or is often found encoded by a gene adjacent to a bacterial cyclopropane synthase gene.

**Introduction**

Cyclopropane fatty acids (CPA-FAs) and cyclopropene fatty acids (CPE-FAs) contain three-member carbocyclic rings derived by methyl group addition across a double bond. In the plant kingdom, sterculic and malvalic acid, containing 19 and 18 carbons respectively, are the most commonly found CPE-FAs in seed oils, whereas dihydrosterculic acid is the major carbocyclic fatty acid in the seed oil of *Litchi chinensis* and *Euphoria longana* (2, 3, 4). Long-chain CPA- and CPE-FAs are also found in root, leaf, stem, and callus tissue in plants of the *Malvaceae* family (5, 6). Although the biological role of these fatty acids in plants is uncertain, CPE-FAs might function as anti-fungal agents (7). In tropical areas, CPE-FAs-containing seeds are still often
consumed by humans (8, 9). Among the CPE-FAs-containing oilseeds, cottonseeds are the most prevalent, ranking third in world oilseed crushing (10). Cotton oil contains approximately 1% CPE-FAs, which is the cause of many physiological disorders in animals fed cottonseed meal, such as pink white in eggs, depression of egg production, delay of sexual maturity in hens, and higher levels of hard fats in cows (11, 12). These disorders all reduce product quality or quantity and therefore can be a source of economic loss. The underlying cause of these disorders is due to the fact that CPE-FAs are strong inhibitors of fatty acid desaturases in animals (13,14). Therefore, vegetable oils containing CPE-FAs, particularly cottonseed oil, are generally treated with high temperature or hydrogenation before consumption to remove these CPE-FAs (15). Because of the added processing costs and the creation of trans fatty acids during hydrogenation, a more suitable approach to eliminate CPE-FAs from oilseeds is through genetic engineering. This approach might also be useful to increase carbocyclic fatty acid production for specialty oil applications. Although CPE-FAs are anti-nutritional factors in animal diets, the high chemical reactivity of the cyclopropene ring can potentially be utilized for many oleochemical applications (8). Therefore, methods of both decreasing CPE-FA occurrence in edible oils and increasing their production in industrial oils are desirable. The isolation of a plant cyclopropane synthase cDNA provides an initial step toward achieving these goals (1).

CPA-FAs are also constituents of bacterial and parasitic protozoa lipids (16, 17), but never accompanied by CPE-FAs. Formation of the cyclopropane ring has been extensively studied in bacteria, and the enzymatic reaction has been defined as the
transfer of a methylene group from S-adenosyl-L-methionine (S-AdoMet) to the double bond of an unsaturated acyl chain (18). The position requirement for the cis double bonds is rather loose for the E. coli CPA-FA synthase (19, 20), but more stringent for cyclopropane synthases from Mycobacterium (21). E. coli and other bacterial cyclopropane synthases prefer the monoenoic fatty acids esterified to the sn-2 position of phospholipids, most prominently phosphatidylethanolamine (PE), although the Clostridium butyricum enzyme is selective for the acyl chain located on the sn-1 position of PE (22). Recent analysis of the crystal structures of three cyclopropane synthases from Mycobacterium suggests that acyl-ACP might be a substrate for these enzymes (21).

Interestingly, alignment of the Sf CPA-FAS with bacterial homologues indicates similarity only to the C-terminal half of the plant protein. The N-terminal half (amino acids 1-438) is unique to plant cyclopropane synthase in that known bacterial cyclopropane synthases lack this portion. The cloning of cyclopropane synthase cDNA from developing seeds of S. foetida (1) has provided an opportunity to examine the Sf CPA-FAS with regard to membrane-association, sub-cellular localization, substrate, and positional specificity. We demonstrate that S. foetida cyclopropane synthase catalyzes the addition of a methylene group, derived from S-AdoMet, across the double bond position of oleic acid esterified to PC. In contrast to the E. coli homolog and most other fatty acid modification enzymes of plants, Sf CPA-FAS acts on the sn-1 rather than sn-2 position of phospholipid.

Experimental Procedures

Plant Material and Chemicals

Developing seeds of Sterculia foetida L. were obtained from Montgomery Botanical Center (Miami, FL). Frozen developing seeds were used for lipid analysis and immunoblotting. Tobacco suspension cells (Nicotiana tabacum L.
cv. Bright yellow 2) containing the vector pE1776 or SfCPA-FAS were maintained as described by Bao et. al. (1). L-[methyl-\textsuperscript{14}C]methionine (55 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and S-adenosyl-L-methionine, [methyl-\textsuperscript{14}C] (59 mCi/mmol) from Moravek Biochemicals (Brea, CA). Phospholipase A2 (from Crotalus atrox Venom) was purchased from Sigma (St. Louis, MO).

Production of antibodies to SfCPA-FAS (SfCPA-FAS is 864 amino acid long, and only the carboxyl-half shares significant similarity with known cyclopropane synthases from \textit{E. coli} and \textit{Mycobacteria}. To be able to detect homologues of SfCPA-FAS in other species, the C-terminal half of SfCPA-FAS (470~864 aa) was used as antigen. The corresponding fragment was obtained through PCR with upstream primer JO873 (CCGGAATTCTGTCTAATCCACTGCTCGAAATGTC) and downstream primer JO885 (CCCTCTAGAGCTCGGATAATGAAAACTATTTACG). The resulting PCR fragment was subcloned into the protein expression vector pET28a(+) (Novagen, inc., Madison, WI) at EcoRI and Sacl sites. The expressed peptide contains 36 amino acids from the vector including the histidine tag at the N-terminus. Protein expression and inclusion body purification were conducted according to manufacturer’s instructions. The purified inclusion bodies were resolved by SDS page, stained with Coomassie Brilliant Blue and the partial SfCPA-FAS protein band was excised and stored at -20°C. Frozen gel slices of partial SfCPA-FAS were sent to Cocalico Biologicals, Inc. (Reamstown, PA) for antibody preparation in New Zealand white
rabbits.

**Total protein extraction, characterization of membrane-association and immunoblot analysis**

Plant tissue (1 g) was homogenized with a mortar and pestle in extraction buffer (5 ml) containing 20 mM Tris-HCl pH 7.6, 0.2 M NaCl, 10 mM 3-mercaptopethanol, and 5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride and filtered through two layers of miracloth into centrifuge tubes on ice. Protein was quantitated using a dye-binding assay (BioRad, Hercules, CA) with BSA as the standard. Total protein extracts from transgenic tobacco cell line expressing SfCPA-FAS and developing seeds of *S. foetida* were centrifuged at 30,000 g for 35 minutes. Supernatants were transferred to new tubes and the pellets were resuspended in extraction buffer. SfCPA-FAS enriched membranes (pellet) from tobacco suspension cells were resuspended to 2 mg protein mL$^{-1}$ in 20 mM HEPES-KOH pH 8.0, 10 % (v/v) glycerol, 1 mM dithiothreitol, 1 mM g-amino-n-caproic acid, 0.5 mM benzamidine, 0.5 mM EDTA. Resuspended membranes were washed by 0.5 M NaCl, or 0.1 % (v/v) Triton X-100. Samples were then vortexed for 30 sec and incubated on ice for 10 min. Washed membranes were collected by centrifugation (10 min at 14,000 g) and supernatant was removed and placed into a fresh tube. Protein samples were denatured in SDS-PAGE sample buffer and loaded onto 10% SDS gel for electrophoresis. Proteins were transferred to nitrocellulose, probed with antibodies against SfCPA-FAS (1:1000 dilution) and detected as described previously by Thelen *et. al.* (23).
Subcellular localization of SfCPA-FAS in transgenic tobacco suspension cells

Transgenic tobacco suspension cells expressing SfCPA-FAS (0.5 g) were harvested and homogenized with a Potter homogenizer in 50 mL of homogenization buffer (30 mM TES-KOH pH 7.6, 0.35 M mannitol, 1 mM EDTA, 2 mM dithiothreitol, 14 mM L-cysteine, 0.6 % (w/v) polyvinyl polypyrrolidone) and filtered through two layers of miracloth into a polycarbonate centrifuge tube on ice. All subsequent steps were performed at 4 °C. Organelles were enriched by rate-zonal sedimentation using a procedure adapted from previous protocols (24, 25). Plastids were sedimented by centrifuging the homogenate at 5,000 g for 2 min. The supernatant was decanted into a fresh tube and centrifuged at 20,000 g for 5 min to sediment intact mitochondria. The 20,000 g supernatant was centrifuged at 100,000 g for 60 min to collect low-density membrane particles. The final 100,000 g supernatant was concentrated on an ultrafiltration membrane (5,000 MW cutoff). Sedimented organelles were gently resuspended in ice-cold resuspension buffer (20 mM TES-KOH, pH 7.6, 0.3 M mannitol, 1 mM EDTA, 1 mM dithiothreitol) and protein was quantified. Purity of organelle fractions was determined by immunoblot analyses using antibodies against plastid and mitochondrion marker proteins. Proteins (100 µg) from each fraction was resolved by SDS-PAGE under standard conditions and subsequently transferred to nitrocellulose membranes for antibody probing. Anti-biotin polyclonal antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD) detected the biotin carboxyl carrier protein subunit of the plastid heteromeric acetyl-CoA carboxylase (23) and were therefore used to determine plastid enrichment. Monoclonal antibodies to the
mitochondrial pyruvate dehydrogenase alpha subunit (26) were used to assay mitochondria enrichment.

**In vivo radiolabeling of transgenic tobacco cells**

The tobacco cell line expressing *S/iCPA-FAS*, was maintained in liquid medium and sub-cultured as described previously (1). After three days of sub-culture, 20 μCi of L-[methyl-14C] methionine was added to 100 ml of the cell culture followed with continuous growth under the same condition. At 5 min intervals, 15 ml of cell culture was removed and cells were harvested by brief centrifugation. Lipids were extracted from cell pellets according to Bligh and Dyer (27). Lipids were transmethylated by vortexing at room temperature for three min with 0.5% sodium methoxide in methanol:heptane (1:1, vol/vol). Lipid classes were separated with K6 TLC using two development systems, the first to 12 cm in chlorophorm: methanol: acetic acid: water (85:15:5:2), drying the plate completely and then followed by second development to the top with hexane: diethyl ether: acetic acid (80:20:1). Radioactivity was quantified using an Instant-Imager. PC from each time point was recovered from the TLC plate for positional analysis. To determine the distribution of dihydrosterculic acid (DHSA) on PC, purified PC was subjected to phospholipase A2 treatment as described by Christie (28). Free fatty acids and lyso-PC were separated by TLC using two different mobile phases. The first development in full in hexane: diethyl ether: acetic acid (60:40:2). Free fatty acids were located by spraying with 0.2% (wt/vol) 2’,7’-dichlorofluorescein in ethanol and viewing under UV light. After completely drying the plate, it was developed in chloroform: methanol: acetic acid: water (50:30:8:4) to
immediately below the free fatty acid bands. The distribution of radioactivity between free fatty acids and lyso-PC was quantified using an Instant-Imager. The radioactive lyso-PC of each time point was recovered and FAMEs were prepared as described before; the FAMEs were separated with C<sub>18</sub> reverse-phase TLC developed in acetonitrile: methanol: water (75:25:0.5) and quantified with an Instant-Imager. In a separate experiment, after the PC from tobacco cells expressing S/CPA-FAS and S. foetida developing seeds were treated with phospholipase A2, FAMEs were prepared from both free fatty acids (from sn-2 of PC) and lyso-PC (from sn-1 of PC), and followed by GC/MS analysis using a Hewlett Packard 5890 gas chromatograph MSD 5972 mass analyzer.

**In vitro assay of cyclopropane fatty acid synthase**

The 30,000 g membrane enriched pellets from tobacco cells expressing S/CPA-FAS, and control containing only empty vector pE1776, were used for CPA-FA synthase assays. The membrane-enriched pellet was resuspended in 0.1 M Na Tricine.NaOH (pH 7.0), 15% (v/v) glycerol and 1 mM 2-mercaptoethanol. After protein concentrations were determined, defatted BSA was added to a final concentration of 1% (w/v). CPA-FA synthase assays were performed at 30°C, 0.05 mM [14C-methyl]S-adenosylmethionine substrate and with or without 0.05 mM oleoyl-CoA in a total volume of 0.2 ml. The assay was terminated by either lipid extraction or the addition of 0.5 ml 10% (w/v) KOH and 1.0 ml ethanol, and allowed to stand overnight to completely saponify lipids. The subsequent lipid and fatty acid analyses were performed as described in the *in vivo* labeling experiments. All
Results

*S. foetida* cyclopropane synthase is a seed-expressed, membrane protein. To determine the level and pattern of SfCPA-FAS expression, total proteins were extracted from *S. foetida* leaves, developing seeds, and transgenic tobacco cells expressing SfCPA-FAS. SfCPA-FAS is highly expressed in *S. foetida* developing seeds, but not detected in leaves (Fig. 1A). In tobacco suspension cells, SfCPA-FAS was detected in cell lines expressing SfCPA-FAS cDNA under a constitutive promoter (1) but not in a control line carrying an empty vector. Based on immunoblot analysis, the expression level of SfCPA-FAS in tobacco cells was estimated at less than one tenth of that found in *S. foetida* developing seeds.

SfCPA-FAS is an enzyme of 864 amino acids with a predicted molecular weight of 98 kDa. Analysis of SfCPA-FAS amino acid sequence does not reveal any predicted trans-membrane domain, however, this polypeptide appeared to be associated with membranes (1). To further characterize the enzyme localization, protein extracts from cells expressing SfCPA-FAS and developing seeds were centrifuged at 30,000 g for 35 minutes. The supernatants contained most of the soluble proteins, and pellets were primarily composed of insoluble membrane proteins. SfCPA-FAS was found almost exclusively in the insoluble pellet from both tobacco suspension cells and *S. foetida* developing seeds (Fig. 1B). Thus SfCPA-FAS is a membrane-associated protein in both its native system and after transgenic expression. To determine the nature of the
membrane-association, the enriched membrane pellet were washed with 0.5 M NaCl. After the salt treatment, the majority of SfCPA-FAS remained in the insoluble membrane fraction indicating that the protein was not likely associated by electrostatic bonds (Fig. 1C). In contrast when Triton X-100 was added to the pellet to a final concentration of 0.1%, the SfCPA-FAS was completely released from the membrane pellet (Fig. 1C). These results indicate that SfCPA-FAS is either an integral membrane protein, or it is strongly associated with membranes by non-electrostatic interactions.

**SfCPA-FAS is a microsomal localized enzyme**

To clarify the subcellular location of the cyclopropane synthase, organelle fractionation was performed with tobacco suspension cells expressing SfCPA-FAS. The filtered homogenate was first centrifuged at 5000 g for 2 min to sediment intact plastids. The biotin carboxyl carrier protein (BCCP) subunit of the heteromeric acetyl-CoA carboxylase is located exclusively in plastids and therefore, an anti-biotin antibody was used to determine the enrichment of plastids (23). Indeed, the majority of BCCP was found in the 5,000 g pellet (Fig. 2). The supernatant was further centrifuged at 20,000 g for 5 min to sediment intact mitochondria and this was confirmed with monoclonal antibodies against the mitochondrial pyruvate dehydrogenase alpha subunit (26) (Fig. 2). The 20,000 g supernatant was centrifuged at 100,000 g for 60 min to collect low-density membrane particles derived from microsomes. The SfCPA-FAS was greatly enriched in this fraction (Fig. 2). These results demonstrate that SfCPA-FAS is a microsomal localized enzyme and suggested that CPA-FAs are synthesized outside of plastids.
Phosphatidylcholine is the direct substrate of SfCPA-FAS. In previous in vitro labeling experiments with cell-free extracts of S. foetida developing seeds, most 14C-dihydrosterculate was found on PC suggesting that oleoyl-PC might be a substrate for SfCPA-FAS in vivo (1). Tobacco suspension cells expressing SfCPA-FAS provide a more tractable system to conduct tracer-labeling studies because the system is available year-round, precursors can rapidly enter the cells, and the tissue is uniform and constant throughout the labeling period. To further examine if PC serves as a direct substrate for SfCPA-FAS in vivo, suspension cells were labeled with L-[methyl-14C]methionine under normal growth conditions. Samples were removed at 3 min intervals from 3 min to 24 min, and total lipids from each time point were divided in half. Half of the sample was used to prepare FAMEs, and the other half was used to isolate PC (Fig. 3A, 9 min time point). FAMEs from both PC and total lipid at each time point were fractionated in parallel by C18 reversed-phase TLC. As shown in Fig. 3B (9 min time point), DHSA was the only radioactive fatty acid esterified to PC, whereas FAME samples from total lipids, revealed other radioactive bands (most likely sterols) in addition to DHSA. The percentage of DHSA found in PC versus that from total lipids was calculated and plotted versus assay time (Fig. 3C). At early time points (3 and 6 min), approximately 99% of DHSA was esterified to PC. At later times some labeled DHSA appeared in other lipids but the amount in PC stabilized at approximately 85% after 20 min labeling. This kinetics of appearance of DHSA strongly suggests that PC is a direct substrate for SfCPA-FAS (Fig. 3C).
SfCPA-FAS could also be readily assayed *in vitro*. Assays were standardized by incubating a range of protein concentrations (from 30,000 g pellet of tobacco cells expressing SfCPA-FAS) with 0.05 mM [14C-methyl]S-adenosylmethionine and 0.05 mM oleoyl-CoA at 30°C for 1 hr. Radioactive DHSA product increased approximately linearly with proteins up to 1 mg/ml (Fig. 4A), whereas higher protein concentrations saturated the assays under the conditions used. As shown in Fig. 4B, after a 10~20 min lag, DHSA product accumulated linearly for at least 105 min at a rate of 0.34 :mol/hr/mg protein. Based on these experiments, subsequent *in vitro* assays were conducted with 100 µg of protein from the 30,000 g pellet at 30°C for 1 hr, unless otherwise specified.

To further confirm PC as a direct substrate for SfCPA-FAS by an alternative method, *in vitro* assays were performed with the 30,000g pellet using [14C-methyl]S-adenosylmethionine as the methylene donor. Upon the termination of the reaction, the samples were split equally between two tubes. The reaction in one tube was terminated by adding 0.5 ml of 10% KOH and 1 ml of ethanol. After saponification, fatty acids were extracted and FAMEs were prepared. In the other tube, the reaction was stopped by lipid extraction, and lipid classes were separated by TLC (Fig 5, top panel). PC and other radioactive lipid bands were recovered and used for FAME preparation. Along with the FAMEs from the other half of the reaction, FAMEs from PC and other radioactive bands were separated by C18 reverse-phase TLC (Fig. 5, bottom panel). As shown in Fig. 5, DHSA was only detected in PC, and not in any other lipid. In addition, the amount of radioactive DHSA in PC was approximately the same as that found in the total FAMEs prepared through KOH saponification. This result was consistent with in vivo labeling and further supports the hypothesis that PC is a direct substrate of SfCPA-FAS.

We also considered 18:1-CoA as a potential substrate for SfCPA-FAS. To test this possibility, the 30,000 g pellet was washed three times with assay buffer to remove acyl-CoAs, and then assayed with or without 50 :M oleoyl-CoA. Proteins from control cells (transformed with empty vector) did not possess any cyclopropane...
synthase activity with no DHSA synthesized as shown in the control lane (Fig. 6A). In contrast, DHSA was produced by the 30,000 g pellet from tobacco cells expressing SfCPA-FAS (Fig. 6A). However, the biosynthesis of DHSA from assays with oleoyl-CoA and that without oleoyl-CoA was almost identical based on three independent experiments (Fig. 6B), indicating that the addition of exogenous oleoyl-CoA had no effect on the activity of SfCPA-FAS and therefore, oleoyl-CoA is not likely a substrate of SfCPA-FAS.

**The addition of methylene group takes place on the sn-1 position of PC**

Although Fig. 3 and 5 indicate that PC is the direct substrate for SfCPA-FAS, the modification could take place on oleic acid esterified to the sn-1, sn-2, or both positions. To further clarify the reaction mechanism, *in vivo* labeling was conducted with tobacco suspension cells with SfCPA-FAS using L-[methyl-14C]methionine as radioactive tracer. Samples were taken at 5, 10, 15, 20, and 25 min, and PC was purified and treated with position specific phospholipase A2 to cleave the fatty acids at the sn-2 positions. The resultant free fatty acids and lyso-PC were separated by TLC. As shown in Fig. 7A top panel, radioactivity was only associated with lyso-PC, whereas no radioactivity could be detected in the free fatty acid fraction released form sn-2. Since the head-group of lyso-PC can also be labeled by L-[methyl-14C]methionine, FAMEs were prepared from the labeled lyso-PCs and fractioned by C18 reverse-phase TLC. Only one radioactive spot corresponding to DHSA could be found on the reverse TLC (Fig. 7A.
Radioactivity from total lipids, PC, Lyso-PC, and DHSA were plotted versus labeling time (Fig. 7B). The accumulation of radioactivity in total lipids increased linearly during the 25 min labeling period, however, there was a 10 min lag for the accumulation of radioactivity in PC, Lyso-PC, and DHSA.

As shown in Fig. 7, the in vivo experiment suggested that, in contrast to most acyl modification reactions of PC in plants, the synthesis of DHSA in tobacco cells occurred preferentially on sn-1, instead of the sn-2 position. This conclusion was also supported by analysis of non-radioactive acyl groups from the sn-1 and sn-2 positions of PC from tobacco cells expressing StCPA-FAS and from S. foetida developing. As shown in Fig. 8, approximately 90% of DHSA found on PC (from tobacco cells expressing StCPA-FAS) was located on the sn-1 position, indicating relatively little acyl migration. A similar distribution pattern of DHSA was obtained from PC of S. foetida developing seeds (data not shown). Thus, both in short term labeling and during steady-state accumulation, DHSA is predominantly associated with sn-1 of PC.

To further confirm this observation, PC was purified from the in vitro assay after using [14C-methyl]S-adenosylmethionine as methylene donor. The purified PC sample was split equally into two parts; one part was treated with phospholipase A2, and the resulting free fatty acids and lyso-PC were separated by TLC along with the other half of untreated PC sample. No radioactivity was detected from the free fatty acids derived from the sn-2 position of PC (Fig. 9A). The radioactivity, equal to the untreated PC was exclusively associated with lyso-PC (Fig. 9A). FAMEs were prepared from radioactive lyso-PC and PC and fractioned with C18 reverse-phase
TLC. The amount of radioactivity in DHSA derived from lyso-PC was the same as that derived from PC as measured in Fig. 9B. These in vitro assay data further demonstrated that the addition of the methylene group to oleic acid to form DHSA occurred at the sn-1 position of PC.

Discussion

Cyclopropane synthases have been extensively studied in two systems, namely Escherichia coli and Mycobacterium tuberculosis. E. coli CFA-FA synthase is a soluble enzyme found in the cell cytoplasm loosely associated with the inner membrane (29). The substrates of E. coli CFA synthase are phospholipids (most likely PE) containing unsaturated fatty acids whose double bond must be positioned 9-11 carbon units from the ester linking to the glycerol backbone. It is still unresolved whether this soluble enzyme gains access to the double bond deep within the hydrophobic core of the bilayer structures of the membranes or if acyl groups must be removed from the membrane prior to cyclopropanation (18). In Mycobacteria, data related to the enzymology of cyclopropane synthases are not available due to the unavailability of substrate acyl chains, and it is still not clear whether cyclopropanation occurs during chain elongation or after (30). But Mycobacterial cyclopropane synthases are believed to be soluble proteins because they were purified in buffers without any detergent (21). In plants, S. feotida cylopropane synthase is the only plant enzyme studied by in vitro assays. Western blots (Fig. 1A) indicate the enzyme is relatively abundant in S. feotida developing seeds, consistent with the 0.4% abundance of ESTs found in the seed cDNA
library (1). One of the differences between StCPA-FAS and bacterial cyclopropane synthases is that StCPA-FAS is a more strongly associated membrane protein than its bacterial counterparts (Fig. 1B and C). StCPA-FAS contains a 400 amino acid N-terminal extension when compared to bacterial counterparts that could be responsible for membrane anchorage. The finding that StCPA-FAS is enriched in microsomal fraction is expected because most fatty acid modification enzymes, which yield unusual fatty acids, are located within the ER (31).

In several bacteria, phosphatidylethanolamine (PE) is the likely direct substrate for cyclopropane synthases \textit{in vivo}. In the protozoan \textit{Crithidia fasciculate}, CPA-FAs are found only in PE (32). From experiments with crude enzyme extracts from \textit{S. marcescens} and \textit{C. butyricum}, Zalkin et. al. (33) observed that CPA-FAs were synthesized only with the presence of phospholipids, particular PE. This observation was later further confirmed by Chung and Law (34) with purified enzymes. Most importantly, a synthetic diether analogue of PE was found to be an effective substrate for bacterial cyclopropane synthases, which ruled out the possibility of removal of the acyl group from PE before synthesis of CPA-FAs (35). Other phospholipids might be used as substrate as well, especially worthy of mentioning is PC for \textit{A. tumefaciens} (22).

In the case of \textit{Mycobacterium}, the crystal structures of three cyclopropane synthases revealed a hydrophobic patch very similar to known acyl-ACP utilization enzymes, and believed to be an ACP binding site. This observation lead to the suggestion that acyl-ACP might be the substrate of cyclopropane synthase from \textit{Mycobacterium} (21). In plants, because StCPA-FAS has no plastid targeting sequence, it can be assumed that
acyl-ACP does not serve as a potential substrate for SfCPA-FAS. *In vivo* labeling results (Fig. 3C) indicated that at early time points the newly synthesized radioactive DHSA is exclusively associated with PC. *In vitro* assays with the membrane fraction of tobacco cells with SfCPA-FAS also confirmed that all the radioactive DHSA was esterified to PC. In addition, oleoyl-CoA failed to enhance the rate of reaction. Based upon these observations, we conclude that PC is the direct substrate of plant cyclopropane synthase, although transfer of the acyl group to another carrier for cyclopropanation cannot be completely ruled out.

In PE of *E. coli* and *S. marcescens* and PE and PC of *A. tumefaciens*, CPA-FAs are predominantly in the *sn*-2 position (22), which is also the predominant location of unsaturated fatty acids in most non-photosynthetic bacteria. One exception is the PE of *C. butyricum* in which unsaturated and CPA-FAs are found in greater abundance at the *sn*-1 position (22). Craven and Jeffrey (36) showed through X-ray studies that CPA-FAs have a similar shape and crystal structure to the corresponding monoenoic acids. In addition, phospholipids containing either unsaturated or CPA-FAs are similar in their solubility characteristics in polar solvents and in their ability to form stable micellar dispersions (37, 38). Therefore, in many respects CPA-FAs have both similar physical properties and positional distribution as monounsaturated fatty acids in phospholipids. In plant phospholipids (except PG), saturated fatty acids in general show preferential esterification at *sn*-1 and unsaturated fatty acids are more abundant at *sn*-2. Furthermore, the most common substrates for microsomal fatty acid modification enzymes are acyl groups on the *sn*-2 position of PC. In particular,
although both sn-1 and sn-2 bound acyl groups can serve as substrates, for most desatuases (39,40,42) and for the biosynthesis of ricinoleic acid in endosperm of castor (41) modifications occur predominantly on the sn-2 position of PC. To date, we are not aware of any report of plant fatty acid modification enzymes that specifically act on the acyl group esterified to the sn-1 of PC. The SfCPA-FAS clearly has strong selectivity to the oleic acid on sn-1 position of PC. This feature of sn-1 specificity of SfCPA-FAS not only makes it unprecedented as a plant microsomal fatty acid modification enzyme, but also presents interesting questions for the downstream desaturation. In particular it will be of interest to determine whether the cyclopropane desaturase also acts on the sn-1 position of PC. In the PC fraction of S. foetida developing seeds, 5% of total fatty acids was DHSA of which 91% was located on sn-1 and 9% on sn-2 position (data not shown). In contrast, approximately 37% of total fatty acids of PC was sterculic acid which was equally distributed between sn-1 and sn-2 position. This data implies that the DHSA synthesized on sn-1 of PC may be removed from the sn-1 before desaturation, and subsequently re-incorporated into PC. The positional distribution of CPE-FAs in triacylglycerols of S. foetida seeds indicates that for triacylglycerols containing only one CPE-FA, CPE-FAs are more abundant at sn-1 or 3 positions, but for triacylglycerols containing two CPE-FAs, CPE-FAs are enriched at the sn-2 position (43). These data suggest that the metabolism of cyclopropene fatty acids in seeds oils may involve several acylation steps.

A number of plant DNA sequences are related to the SfCPA-FAS. In Arabidopsis, there are two genes that locate in tandem on chromosome 3 which have
approximately 60% amino acid identity with *Sterculia* cyclopropane synthase and 95% with each other. (Due to annotation errors, At3g23500 and At3g23510 are actually one gene with 854 aa, and At3g23520 and At3g23530 is a second gene with 867 aa). Although CPA-FAs and CPE-FAs have not been reported in *Arabidopsis* so far, these structures might be further modified or used to synthesize complex lipids that are not easily analyzed or extracted. Alternatively, the *Arabidopsis* enzymes might catalyze the formation of related products that originated from the common intermediate as CPA-FAs. Therefore, it will be beneficial to understand the catalytic property of the gene family, which is related to *Sf* CPA-FAS. When *Sf* CPA-FAS is compared with its bacterial counterparts, it possesses a >400 aa long N-terminal extension. This N-terminal portion has some features of oxidases and contains a conserved FAD binding motif. At present, the function(s) of the N-terminal extension are not clear. Interestingly, homologues of the N-terminal portion exist in several cyclopropane-producing bacteria and they locate at very close proximity to cyclopropane synthase genes. In *Mycobacteria*, the hypothetical protein Rv0449c is homologous to the N-terminal portion of *Sf* CPA-FAS and is separated from the cyclopropane synthase (ufaA1) by only 200 bp. In *Agrobacteria*, AGR-C-3599p (N-terminal homolog to *Sf* CPA-FAS) and AGR-C-3601p (C-terminal homolog to *Sf* CPA-FAS) are distinct genes separated by only 802 bp. This striking coincidence between two proteins with adjacent genomic locations in two bacteria and their fusion to form one protein in plants suggests that the N-terminal domain in plants and its homologues in bacteria may play a role(s) related to cyclopropanation. From evolution point of view, it will be interesting to know
how the cyclopropane synthase evolved from *E. coli* (homologous to C-terminal portion) to *Agrobacteria* and *Mycobacteria* (two separated proteins, one homologous to N-terminal and the other to C-terminal portion), and eventually to plants (one fused protein). In *Mycobacteria*, there are at least three genes that encode cyclopropane synthases, but there is only one gene that shares homology with the N-terminal half of *Sterculia* enzyme. If the hypothetical protein (Rv0449c homologous to N-terminal half) is indeed involved in cyclopropanation, drugs that inhibit this protein may provide new strategies for treatment for tuberculosis.

In conclusion, the SfCPA-FAS is a microsomal localized membrane enzyme, which catalyzes the addition of a methylene group derived from *S*-adenosyl-*L*-methionine across the double bond of oleic acid esterified to the *sn*-1 position of PC. Further studies will focus on the function(s) of the N-terminal domain, such as its membrane association, substrate recognition, or whether it is an authentic FAD-containing protein.

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**References**


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Footnotes:

FAME, fatty acid methyl ester; CPA-FA, cyclopropane fatty acid; CPE-FA, cyclopropene fatty acid; SfCPA-FAS, Sterculia foetida cyclopropane fatty acid synthase; DHSA, dihydrosterculic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine.
Figure Legends

**Fig. 1. Western blot analysis of SfCPA-FAS.** A, total proteins (100 µg) were loaded to each lane (lane 1, leaves of *S. foetida*; lane 2, developing seeds of *S. foetida*; lane 3, tobacco with empty vector pE1776; lane 4, tobacco expressing SfCPA-FAS). B, the total protein extracts from transgenic tobacco cell line expressing SfCPA-FAS and developing seeds of *S. foetida* were centrifuged at 30,000 g for 35 minutes. 200 µg of proteins from tobacco cells (lane 1, pellet; lane 2, supernatant) and 100 µg from seeds (lane 3, pellet; lane 4, supernatant) were loaded to each lane. C, the 30,000g pellet from tobacco cells with SfCPA-FAS was washed by 0.5 M NaCl, or 0.1 % (v/v) Triton X-100, then vortexed for 30 sec and incubated on ice for 10 min. After centrifugation (10 min at 14 k g), proteins from supernatant (S) and pellet (P) were resolved in SDS-PAGE. Lane 1, pellet of NaCl wash; lane 2, supernatant of NaCl wash; lane 3, pellet of Triton X-100 wash; and lane 4, supernatant of Triton X-100 Triton X-100 wash.

**Fig. 2. Sub-cellular localization of SfCPA-FAS.** Organelles of tobacco cells expressing SfCPA-FAS were sequentially fractionated. Plastids were sedimented by centrifuging at 5,000 g for 2 min, mitochondria at 20,000 g for 5 min, and the microsomal membranes at 100,000 g for 60 min. Enrichment of organelle fractions was estimated by immunoblot analyses using antibodies against plastid and mitochondrial marker proteins. Anti-biotin antibodies detected the BCCP subunit of plastid acetyl-CoA carboxylase and monoclonal antibodies to the mitochondrial pyruvate dehydrogenase alpha subunit (PDH-) estimated mitochondrial enrichment. The distribution of SfCPA-FAS was determined by antibodies against SfCPA-FAS.
Fig. 3. Appearance of DHSA in PC versus total lipids after \textit{in vivo} labeling. Cell suspension cultures (100 ml) of tobacco expressing SfCPA-FAS, were labeled with 20 µCi of L-[methyl-\textsuperscript{14}C] methionine. Samples were collected at 3 min intervals followed by lipid extraction. For each time point, total lipids were divided equally in two parts. A, One part was used for lipid separation (9 min time point shown). B, FAMEs were prepared from purified PC (lane 1) and the other half of total lipids (lane 2), and fractionated with C\textsubscript{18} reverse-phase TLC (9 min time point). C, Lastly, the percentages of radioactive DHSA found in PC to that in total lipids were plotted with time based on three independent experiments.

Fig. 4. Linear range of SfCPA-FAS in \textit{in vitro} assays. A, Radioactive DHSA formed versus total protein extracted from SfCPA-FAS tobacco cells in 200 µl reaction volume for 1 hour. B, Time course of SfCPA-FAS from 100 µg total protein in 200 µl reaction volume for 1 hour. Each time point is calculated from three determinations.

Fig. 5. Association of DHSA with radioactive lipids synthesized in \textit{in vitro} assays. Standard \textit{in vitro} assays as described in experimental procedures. After the assay, half the reaction was used to prepare FAMEs directly; the other half was used for lipid separation shown on the top. FAMEs were prepared from each radioactive spot and fractionated along with that from the other half on C\textsubscript{18} reverse-phase TLC on the bottom. DHSA was only recovered from the PC band. Absence of radioactive recovery from other bands occurred if the radioactive product is water-soluble after FAME preparation.
Fig. 6. Influence of oleoyl-CoA on SfCPA-FAS in *in vitro* assays. A, FAMEs separated on TLC after *in vitro* assays. pE1776, control (lane 1); SfCPA-FAS assay with addition of 0.05 mM oleoyl-CoA (lane 2); SfCPA-FAS assay without addition of oleoyl-CoA (lane 3). B, SfCPA-FAS activities from three determinates.

Fig. 7. Position analysis of DHSA on PC after *in vivo* labeling of L-[methyl-\(^{14}\)C] methionine. A, PC was purified from each time point, treated with phospholipase A2, and free fatty acids and lyso-PC were separated by TLC (left top). FAMEs were prepared from lyso-PC and fractionated with C\(_{18}\) reverse-phase TLC (bottom). B, The accumulation of radioactivity in different classes is plotted versus time. Radioactivity from lyso-PC was slightly lower than that from PC at given time point, which was due to losses during recovery from TLC and phospholipase A2 treatment.

Fig. 8. Fatty acid composition of \(sn-1\) and \(sn-2\) positions of PC from tobacco cells expressing SfCPA-FAS. PC was isolated from the total lipids of tobacco cells expressing SfCPA-FAS, and then treated with phospholipase A2. FAMEs were prepared from both free fatty acids (from \(sn-2\) of PC) and lyso-PC (from \(sn-1\) of PC), and followed by GC/MS analysis.

Fig. 9. Position analysis of DHSA in PC from *in vitro* assays. A: lane 1, PC purified from *in vitro* assay and treated with phospholipase A2, and lane 2, equal amount of PC without phospholipase A2 treatment. B, DHSA from corresponding lyso-PC (lane 1) and PC (lane 2) in the upper panel.
Fig. 2

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Fig. 5
Characterization of cyclopropane fatty acid synthase from Sterculia foetida
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