

Coexpressing *Escherichia coli* Cyclopropane Synthase with *Sterculia foetida* Lysophosphatidic Acid Acyltransferase Enhances Cyclopropane Fatty Acid Accumulation¹[W][OPEN]

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Cyclopropane fatty acids (CPAs) are desirable as renewable chemical feedstocks for the production of paints, plastics, and lubricants. Toward our goal of creating a CPA-accumulating crop, we expressed nine higher plant cyclopropane synthase (CPS) enzymes in the seeds of *fad2fae1* *Arabidopsis* (*Arabidopsis thaliana*) and observed accumulation of less than 1% CPA. Surprisingly, expression of the *Escherichia coli* CPS gene resulted in the accumulation of up to 9.1% CPA in the seed. Coexpression of a *Sterculia foetida* lysophosphatidic acid acyltransferase (SfLPAAT) increases CPA accumulation up to 35% in individual T1 seeds. However, seeds with more than 9% CPA exhibit wrinkled seed morphology and reduced size and oil accumulation. Seeds with more than 11% CPA exhibit strongly decreased seed germination and establishment, and no seeds with CPA more than 15% germinated. That previous reports suggest that plant CPS prefers the stereospecific numbering (sn)-1 position whereas *E. coli* CPS acts on sn-2 of phospholipids prompted us to investigate the preferred positions of CPS on phosphatidylcholine (PC) and triacylglycerol. Unexpectedly, in planta, *E. coli* CPS acts primarily on the sn-1 position of PC; coexpression of SfLPAAT results in the incorporation of CPA at the sn-2 position of lysophosphatidic acid. This enables a cycle that enriches CPA at both sn-1 and sn-2 positions of PC and results in increased accumulation of CPA. These data provide proof of principle that CPA can accumulate to high levels in transgenic seeds and sets the stage for the identification of factors that will facilitate the movement of CPA from PC into triacylglycerol to produce viable seeds with additional CPA accumulation.

Modified fatty acids (mFAs; sometimes referred to as unusual fatty acids) obtained from plants play important roles in industrial applications as lubricants, protective coatings, plastics, inks, cosmetics, etc. The hundreds of potential industrial uses of mFAs have led to considerable interest in exploring their production in transgenic crop plants. mFAs are produced by a limited number of species, and the transfer of genes encoding mFA-producing enzymes from source plants to heterologous hosts has generally resulted in only modest accumulation, usually less than 20% of the desired mFA in transgenic seed (Napier, 2007) compared with levels found in the natural source. For example, ricinoleic acid

accounts for more than 90% of the fatty acid of castor bean (*Ricinus communis*) seeds, and tung (*Aleurites fordii*) seeds accumulate more than 80% α -eleostearic acid (Thelen and Ohlrogge, 2002; Drexler et al., 2003). In order to elevate the content of mFAs in the engineered plants to that found in the native plant, it is necessary to (1) optimize the synthesis of mFA (Mekhedov et al., 2001), (2) minimize its degradation (Eccleston and Ohlrogge, 1998), and (3) optimize its incorporation into triacylglycerol (TAG; Bafor et al., 1990; Bates and Browse, 2011; van Erp et al., 2011).

Cyclic fatty acids (CFAs) are desirable for numerous industrial applications. The strained bond angles of the carbocyclic ring contribute to their unique chemistry and physical properties, and hydrogenation of CFAs results in ring opening to produce methyl-branched fatty acids. Branched chain fatty acids are ideally suited for the oleochemical industry as feedstocks for the production of lubricants, plastics, paints, dyes, and coatings (Carlsson et al., 2011). Cyclopropane fatty acids (CPAs) have been found in certain gymnosperms, Malvales, *Litchi* spp., and other Sapindales species. They accumulate to as much as 40% in seeds of *Litchi chinensis* (Vickery, 1980; Gaydou et al., 1993). *Sterculia foetida* accumulates the desaturated CFA (i.e. cyclopropene fatty acid) to more than 60% of its seed oil (Bohannon and Kleiman, 1978; Pasha and Ahmad, 1992). The first step in its synthesis is the formation of the CPA by the cyclopropane synthase (CPS) enzyme, which transfers a

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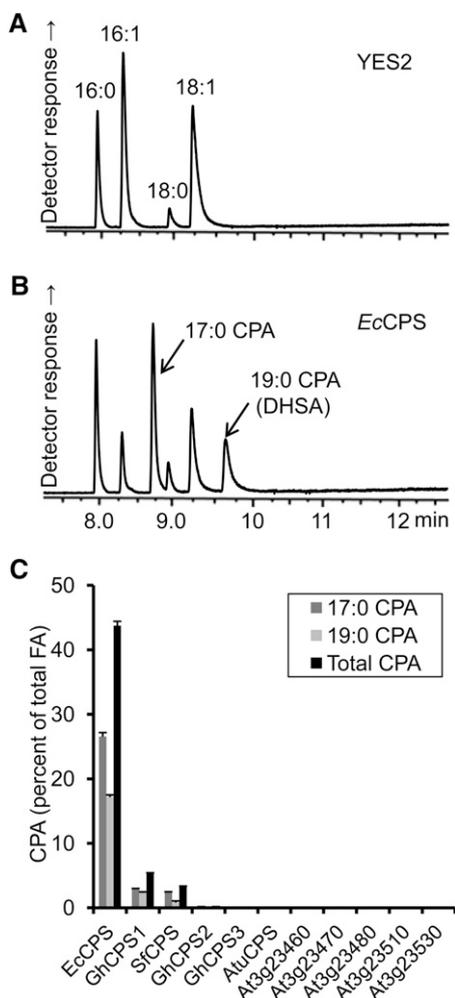


Figure 2. Analysis of yeast expressing *E. coli* and various plant CPS enzymes. GC-MS analysis of FAMES is shown for yeast expressing empty vector pYES2 (A) or *E. coli* CPS, which produced both 17:0 CPA and 19:0 CPA (B), and CPA production in CPS expressed in yeast (C). DHSA, Dihydrostercularic acid. FAMES were analyzed by GC-MS, and both 17:0 and 19:0 CPA accumulations were calculated as a percentage of the total fatty acids (FA). The values represent means and SD of at least three replicates.

of 27% 17:0 CPA and 17% 19:0 CPA, yielding a total of 44% CPA accumulation, which is about 8-fold that observed upon overexpression of the cotton CPS gene GhCPS1 (Fig. 2C). The expression of *A. tumefaciens* CPS and five putative CPS genes from *Arabidopsis* did not yield detectable levels of CPA products. These results demonstrate the efficacy of EcCPS relative to other CPS genes for converting both 16:1 and 18:1 fatty acid substrates to the corresponding 17-carbon and 19-carbon CPA products in yeast.

Expression of CPS in *Arabidopsis fad2fae1*

The CPS open reading frames were transferred into plant expression vectors under the control of the seed-

specific phaseolin promoter and transformed into *Arabidopsis fad2fae1* plants (Meesapyodsuk and Qiu, 2008). This background was chosen because its seed contains more than 80% 18:1, the CPS fatty acid substrate. T1 seeds expressing EcCPS yielded the highest content of dihydrostercularic acid (19-carbon CPA; average 5.0%), and no 17-carbon CPA products were detected. Expression of GhCPS1 and *S. foetida* CPS led to the accumulation of at most 1% CPA (Yu et al., 2011), whereas the expression of five *Arabidopsis* and two cotton orthologs (GhCPS2 and GhCPS3) resulted in no detectable accumulation of CPA. T1 *fad2fae1* seeds expressing EcCPS germinated with similar frequency to those of nontransformed seeds, and T2 lines with a single locus of insertion were identified and screened for CPA production. These T2 seed pools (containing a mixture of heterozygous and homozygous transgenic seeds) accumulated up to 5.8% CPA (Fig. 3).

Isolation of an LPAT from *S. foetida* Seed

RNA from *S. foetida* leaf and developing seed was extracted and subjected to 454 sequencing. The AtLPAT2 gene sequence encoding the ubiquitous endoplasmic reticulum-located LPAT (Kim et al., 2005) was used to BLAST the *S. foetida* EST sequences derived from *S. foetida*, which contains 21,362 seed, and 26,083 leaf EST assemblies. An *S. foetida* homolog was identified that showed preferential expression in seed, with 56 occurrences in the cotyledon and embryo of developing seeds versus 27 occurrences in leaf tissue. Oligonucleotides were designed to amplify the full-length complementary DNA (cDNA), which was cloned and designated SfLPAT (GenBank accession no. KC894726). SfLPAT has a 1,164-bp open reading frame that encodes a 387-amino acid protein with a predicted mass of 43,723 D and a theoretical pI of 9.63. The predicted amino acid sequence of SfLPAT shows strong homology to *Arabidopsis* LPAT2 (79.2%), rapeseed (*Brassica napus*) LPAT2 (79.1%), *Arabidopsis* LPAT3 (61.9%), and

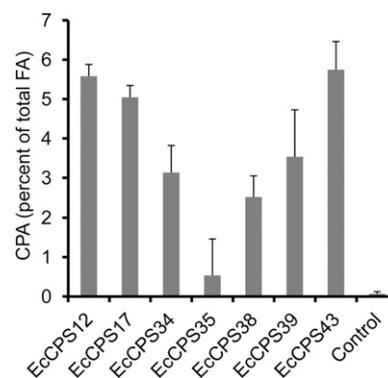


Figure 3. CPA production in *fad2fae1* T2 seeds. FAMES were extracted from T2 EcCPS *fad2fae1* seeds and analyzed by GC-MS. The values represent means and SD of at least three replicates. FA, Fatty acid.

weaker homology to yeast (30.5%) and *E. coli* (23.5%) LPATs. A phylogenetic tree of the amino acid sequences of the various LPAT proteins was constructed by neighbor-joining distance analysis (Supplemental Fig. S2). *S. foetida* LPAT groups with AtLPAT2 and three LPATs from *Brassica* and forms a clade with maize (*Zea mays*) LPAT and Arabidopsis LPAT3.

Coexpression of EcCPS and SflLPAT in *fad2fae1* Arabidopsis Seeds

In order to test if coexpression of the SflLPAT gene along with the EcCPS can enhance CPA accumulation, a single construct containing phaseolin (Phas):EcCPS and Phas:SflLPAT was transformed into the Arabidopsis *fad2fae1* background. T1 seeds were analyzed individually for fatty acid composition. Independent T1 seeds accumulated up to 35% CPA. The 2.9% line appears to be an outlier, with all other lines exceeding 12.7% CPA (Fig. 4). The data clearly demonstrate that coexpression of SflLPAT with EcCPS significantly improves CPA accumulation relative to the expression of EcCPS alone.

CPA Accumulation in the Progeny of EcCPS-SflLPAT-Coexpressing Transgenic *fad2fae1* Seeds

Only approximately 30% of the EcCPS-SflLPAT T1 seeds germinated and were able to develop into mature plants, compared with almost 100% for seeds expressing EcCPS alone. The approximately 70% nonviable seeds showed no radicle penetration of the seed coat, suggesting a failure of germination rather than of establishment. Transgenic lines containing single loci of insertion were identified and allowed to self-fertilize to obtain homozygous individuals. *fad2fae1* T2 seeds containing EcCPS and SflLPAT that accumulated low levels of CPA, along with *fad2fae1* seeds containing only the EcCPS, exhibited close to 100% germination rates, as did the untransformed *fad2fae1* seeds. In contrast, T2 *fad2fae1* seeds coexpressing EcCPS-SflLPAT that contained

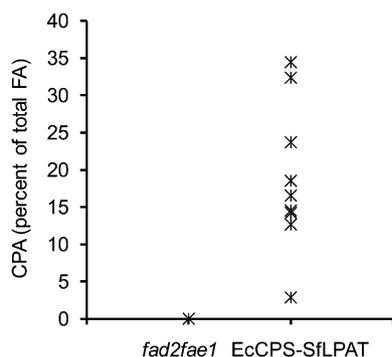


Figure 4. CPA accumulation in individual T1 EcCPS-SflLPAT transgenic *fad2fae1* seeds. FAMES were analyzed by GC-MS, and CPAs were expressed as a percentage of the total fatty acid (FA).

more than 11.5% CPA exhibited reduced germination rates even with the supplement of 1% (w/v) Suc in the medium. For example, no germination was observed for EcCPS-SflLPAT line 2, which accumulated 11.5% CPA in T2 seeds, and only one of 800 seeds of EcCPS-SflLPAT germinated and underwent establishment from line 17 (14.4% CPA) and line 40 (13.3% CPA), and the only T3 progeny identified were found to be heterozygous. Together, these data suggest that elevated accumulation of CPA rather than the presence of SflLPAT was responsible for the observed lack of germination.

Six individual T3 plants from EcCPS lines 17, 38, and 43 and EcCPS-SflLPAT lines 8, 37, and 40 were grown along with parental *fad2fae1* plants under identical conditions. There were no discernible morphological or developmental differences between transformed and non-transformed plants. There were no significant differences in flowering time, seed development, or seed numbers. As shown in Figure 5 and Supplemental Table S1, EcCPS T4 homozygous transgenic seeds yielded up to 9.1% CPA, and the progeny of EcCPS-SflLPAT-expressing seeds produced CPA ranging from 10.8% to 13.3%. SflLPAT expression was detected in lines coexpressing EcCPS and SflLPAT, and the EcCPS expression levels were slightly lower than those observed in two lines expressing only EcCPS (Fig. 6), providing further support that increased CPA accumulation results from SflLPAT expression.

CPA Effect on Seed Weight and Oil Content

Fatty acids from mature seeds were trans-esterified and quantified via gas chromatography with the use of internal standards. Total seed fatty acid content of untransformed *fad2fae1* was $6.45 \pm 0.61 \mu\text{g}$. The three homozygous lines expressing EcCPS showed no significant differences from the parental line, whereas lines coexpressing SflLPAT with EcCPS resulted in a significant (Student's *t* test, $P < 0.05$) 18% decrease in total fatty acid (Fig. 7A). In addition to fatty acid content, seed weights were also determined. As shown in Figure 7B, parental *fad2fae1* seeds weighed $20.0 \pm 1.24 \mu\text{g}$ and T4 seeds of EcCPS-expressing lines showed no significant difference, whereas equivalent lines coexpressing EcCPS and SflLPAT showed a significant (Student's *t* test, $P < 0.05$) decrease in seed weight of up to 11%, with the largest decreases occurring in lines that accumulate the most CPA. Together, these data show that CPA accumulation above 9% causes a decrease in seed weight that correlates with reduced accumulation of seed fatty acid (Fig. 7C).

CPA Distribution in Transgenic Plants

In Arabidopsis, most mFAs in TAG originate from the PC pool (Bates et al., 2009). Consistent with this, the substrate for *S. foetida* CPS is 18:1 at the sn-1 position of PC (Bao et al., 2003). We found that CPA accumulates at 25% to 26% in the polar lipid fraction of

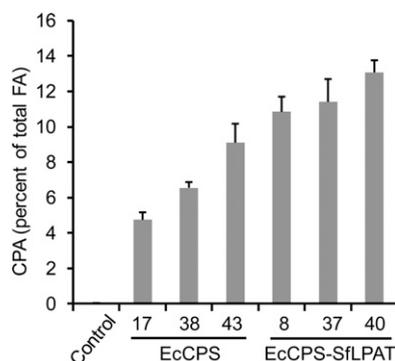


Figure 5. CPA production in T3 seeds upon expression of the indicated EcCPS or EcCPS-SfLPAT in *fad2fae1* plants. FAMES were analyzed by GC-MS, and CPAs are expressed as a percentage of the total fatty acid (FA). The values represent means and SD of at least three replicates.

EcCPS-expressing Arabidopsis lines that accumulate 5% to 9% CPAs in the seed oil at maturity (Fig. 5; Table I). In order to investigate whether the expression of SfLPAT influences the amount of CPA in the polar lipids, we analyzed the CPA content of the polar lipid and TAG fractions of EcCPS- and EcCPS-SfLPAT-expressing seeds. CPA accumulation increased in both polar lipids and TAG when EcCPS was expressed along with SfLPAT (Tables I and II).

sn-Positional Analysis of CPA in PC and TAG

Differences in sn-positional selectivity of plant and bacterial CPSs could potentially explain the increased abundance of CPA in transgenic seeds expressing the *E. coli* CPS relative to the plant enzymes. It is reported that SfCPS prefers the sn-1 position, whereas the EcCPS acts on the sn-2 position in *E. coli* (Hildebrand and Law, 1964; Bao et al., 2003). However, our positional analysis of *fad2fae1* Arabidopsis seeds indicates that 18:1 is present at 85% in PC and that both the sn-1 and sn-2 positions of PC have more than 79% 18:1 (Table III). We selected EcCPS43 and EcCPS-SfLPAT37, which have similar total CFA compositions (approximately 9% and 11%, respectively), for detailed analysis to determine the positions occupied by CPA in the PC pool. To accomplish this, isolated PC was incubated with phospholipase A2. The identification of CPA at both the sn-1 and sn-2 positions implies that EcCPS acts on 18:1 at both sn-1 and sn-2 positions of PC in Arabidopsis (Table III); however, significantly higher levels of CPA are present at the sn-1 position, implying a possible preference of more than 5-fold for sn-1 over sn-2 for EcCPS. This results in the accumulation of 85% of the PC CPA in the sn-1 position in EcCPS43 and 70% of the PC CPA in EcCPS-SfLPAT37.

In contrast to the different sn-positional distributions of CPA in PC for lines expressing EcCPS and coexpression lines expressing EcCPS and SfLPAT, TAG positional analysis shows that the sn-2 position lacks

saturated fatty acids and contains over 90% 18:1. The CPA distribution is similar between the two lines, at approximately 4%, with approximately four times the level of CPA at the sn-1,3 positions relative to the sn-2 position (Table IV).

DISCUSSION

Several findings arise from this work: (1) the expression of EcCPS in transgenic seed leads to the accumulation of higher levels of CPA than the expression of various CPS enzymes from plant sources; (2) the coexpression of EcCPS with SfLPAT results in elevated levels of CPA in the sn-2 position of PC and significantly enhances CPA accumulation to as much as 35% of the total fatty acid in primary transformants; and (3) the accumulation of CPA correlates with reduced seed fatty acid content and germination rate.

The Source of CPS Affects CPA Accumulation in Transgenic Plants

To achieve the goal of accumulating CPA in higher plants, our first approach was to heterologously express CPS genes from other plants, namely cotton, *S. foetida*, and Arabidopsis, in the *fad2fae1* Arabidopsis background tailored to accumulate the CPS substrate oleate. Of the plant CPS genes tested, expression of only *S. foetida* and a cotton CPS resulted in the accumulation

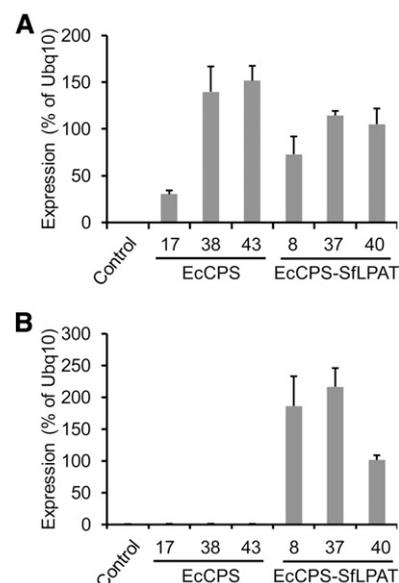


Figure 6. EcCPS and SfLPAT expression levels in transgenic plants. Quantitative reverse transcription-PCR analysis is shown for EcCPS (A) and SfLPAT (B) expression levels in seeds of *fad2fae1* and three transgenic lines harboring EcCPS and EcCPS-SfLPAT as indicated. The expression levels are reported relative to the expression of the UBIQUITIN10 (Ubq10) transcript. The values represent means and SD of at least three replicates.

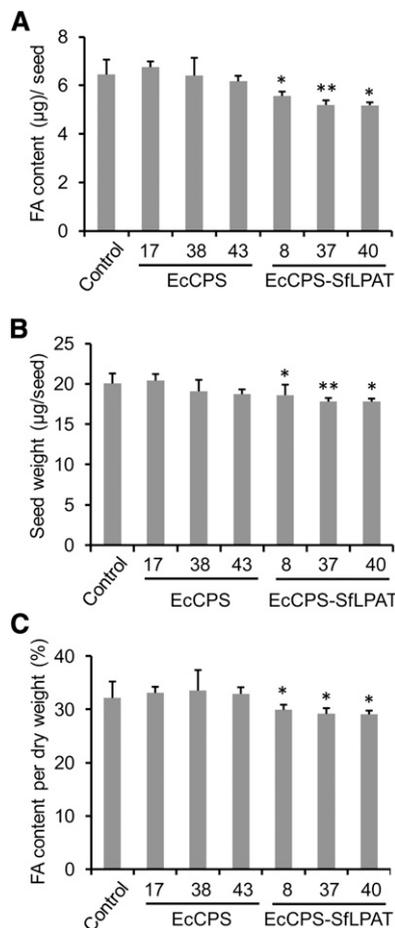


Figure 7. Effects of the expression of EcCPS and EcCPS-SfLPAT on seed fatty acid (FA) content and weight. A, Total fatty acid content in transgenic Arabidopsis. Fatty acid content of seeds was quantified by gas chromatography of FAMES. The values represent means and SD of at least three replicates. B, Seed weight based on the measurement of batches of 100 transgenic Arabidopsis seeds. The values represent means and SD of at least three replicates. C, Total fatty acid content as a proportion of seed weight. The graph is derived from the values used to generate A and B. Significance levels using Student's *t* test are indicated with asterisks: ** $P < 0.01$, * $P < 0.05$.

of detectable CPA (up to approximately 1%; Yu et al., 2011). Expression of the remaining seven plant CPSs yielded no detectable CPA. Our screen was then broadened to include CPS genes from the microbial sources *E. coli* and *A. tumefaciens*. Expression of the *E. coli* CPS resulted in the accumulation of approximately 5.0% CPA in primary transformants. These hemizygous T1 seeds were apparently limiting for the CPS enzyme, because CPS accumulation showed a strong dose dependency (i.e. T3 homozygous seeds accumulated approximately twice as much [9.1%] CPA as hemizygous T1 seeds). It is apparently paradoxical that a microbial CPS gene would outperform higher plant CPSs when heterologously expressed in a higher plant. However, a similar occurrence has been reported for the engineering of the hydroxy fatty acid (HFA) ricinoleic acid in plants.

In this case, the expression of an oleate hydroxylase from the fungus *Claviceps purpurea* yielded more hydroxy product than the expression of a plant homolog (Meesapyodsuk and Qiu, 2008). Reasons for the disparity between the efficacy of plant and bacterial CPSs are currently unknown; however, the accumulation of up to 35% CPA upon the coexpression of EcCPS with SfLPAT suggests that the substrate supply (i.e. 18:1 fatty acid and *S*-adenosyl-Met), is not limiting. Among the possible explanations for the stronger performance of EcCPS relative to the plant CPSs are that the EcCPS may have a higher V_{max} or a lower K_m for its substrates than the plant CPS enzymes, or the bacterial enzyme may be more stable than the plant enzymes. Understanding why EcCPS outperforms plant CPSs when expressed in plants is the subject of ongoing investigation.

Enhanced Cyclopropane Accumulation upon Coexpression of SfLPAT and EcCPS

A preferentially seed-expressed *S. foetida* LPAT with homology to the Arabidopsis LPAT2 was identified. Because stercularic acid preferentially accumulates in seeds relative to vegetative tissues, we hypothesized that SfLPAT may have evolved to accommodate the transfer of cyclic fatty acid. A large increase of up to 35% CPA accumulated in the T1 seeds of Arabidopsis *fad2-fae1* when SfLPAT was coexpressed with EcCPS. We found that *E. coli* CPS acts on 18:1 primarily at the sn-1 position of PC; coexpression of SfLPAT results in the introduction of CPA at the sn-2 position of LPA. This enables a cycle that enriches for the accumulation of CPA at both sn-1 and sn-2 positions of PC and increases the accumulation of CPA. CPA accumulation is also associated with a decrease in 18:1 fatty acid and a small increase in 16:0 fatty acid in both TAG and total seed fatty acid fractions. That SfLPAT facilitates the accumulation of CPA is interesting because *S. foetida* accumulates cyclopropane rather than CPAs. The mechanism and substrate of CPA desaturation to produce cyclopropane fatty acids in *S. foetida* remain elusive; therefore, it is unresolved whether SfLPAT has preference for CPA, cyclopropane fatty acids, or both, relative to normal FA.

Decreased Fatty Acid Content, Seed Weight, and Poor Germination Are Associated with the Coexpression of SfLPAT and EcCPS

When SfLPAT was coexpressed with EcCPS in Arabidopsis, seeds with elevated CPA content exhibited a mildly wrinkled phenotype, similar to that associated with reduced oil content (Focks and Benning, 1998). Quantitative analysis confirmed that wrinkled seeds were lighter than nontransformed control seeds and contained reduced fatty acid content. In transformants expressing only EcCPS that showed high levels of CPA accumulation, oil content was decreased similar to that

Table I. Fatty acid composition of the polar lipids from transgenic *Arabidopsis* seeds expressing *EcCPS* or *EcCPS-SfLPAT*

The values represent means and sd of at least three replicates.

Plant	16:0	18:0	18:1	18:2	CPA	18:3
	%					
<i>fad2fae1</i>	9.6 ± 0.3	3.2 ± 0.2	79.7 ± 0.5	2.8 ± 0.2	0.0 ± 0.0	4.9 ± 0.2
<i>EcCPS17</i>	10.8 ± 1.7	2.8 ± 0.6	53.8 ± 1.0	3.1 ± 0.2	24.6 ± 2.0	4.9 ± 0.8
<i>EcCPS38</i>	8.2 ± 0.1	2.6 ± 0.3	56.6 ± 0.5	3.5 ± 0.1	25.7 ± 0.1	3.5 ± 0.1
<i>EcCPS43</i>	9.5 ± 0.1	2.4 ± 0.2	56.5 ± 0.3	3.1 ± 0.0	24.9 ± 0.2	3.4 ± 0.2
<i>EcCPS-SfLPAT8</i>	11.9 ± 0.1	1.9 ± 0.1	42.1 ± 4.2	2.2 ± 0.0	32.6 ± 0.6	5.0 ± 0.4
<i>EcCPS-SfLPAT37</i>	11.9 ± 0.1	2.0 ± 0.0	46.1 ± 0.2	2.2 ± 0.0	33.0 ± 0.1	4.9 ± 0.2
<i>EcCPS-SfLPAT40</i>	12.5 ± 1.6	3.4 ± 1.8	48.9 ± 1.8	2.7 ± 0.3	26.8 ± 0.7	5.7 ± 0.8

of *EcCPS*- and *SfLPAT*-coexpressing lines, confirming that the developmental deficits arose from elevated *CPS* rather than the expression of *SfLPAT* per se. However, at this time, we are unable to preclude the possibility that the expression of *SfLPAT* results in reduced seed germination. Similar decreases in seed weight and fatty acid content of 10% to 20% have been reported for transgenics accumulating hydroxy or conjugated fatty acid (Cahoon et al., 2006; van Erp et al., 2011). Reduction of seed weight and fatty acid content of ricinoleic acid-accumulating transgenics has been attributed to feedback inhibition of fatty acid synthesis (FAS) upon the accumulation of hydroxy fatty acid lipid intermediates (Bates and Browse, 2011; van Erp et al., 2011). Acyl-transferase enzymes facilitate the conversion of membrane lipids to TAG, the sink for fatty acid. Coexpression of castor seed *DGAT2*, *PDCT*, or *PDAT* along with *RcFAH12* increased ricinoleic acid accumulation in TAG and decreased ricinoleate levels in membrane lipids along with partial reversion of the inhibition of FAS (Burgal et al., 2008; van Erp et al., 2011; Hu et al., 2012). We hypothesize that elevated levels of CPA in membrane lipids could result in a wrinkled phenotype by a similar feedback inhibition of FAS to that observed upon the accumulation of ricinoleate in membrane lipids (i.e. that the fatty acid released from the turnover of CPA-containing lipids reduce the total rate of fatty acid synthesis; Bates and Browse, 2011; van Erp et al., 2011; Andre et al., 2012). *RcFAH*-expressing seeds exhibit poor germination, but upon coexpression of castor *DGAT2* or *PDAT1*, higher levels of ricinoleoyl TAG accumulate; however, germination improves because elevated ricinoleoyl-TAG levels were associated with

reduced hydroxy fatty acid content in membrane lipids. In contrast, coexpression of *EcCPS* with *SfLPAT* resulted in increased CPA in both TAG and membrane lipids, and germination rates decreased as CPA levels increased. In addition to feedback inhibition of FAS, the accumulation of mFA in membrane lipids may result in impaired physiological function of membranes during seed set or desiccation. However, T1 seeds coexpressing *EcCPS* and *SfLPAT* accumulate up to 35% CPA with near-normal development and mildly depressed oil accumulation, suggesting that germination is more sensitive to CPA accumulation than seed maturation. Upon germination, mobilization of fatty acid from TAG, or fatty acid transport and catabolism, could be compromised by the presence of mFA in TAG. That the transgenics germinate poorly on Suc-containing medium, under conditions in which β -oxidation mutants germinate well, is consistent with the view that the buildup of mFA degradation intermediates could underlie the poor germination (Eastmond, 2006).

EcCPS Expression Results in the Accumulation of CPA at the sn-1 Position of PC in Plants

SfCPS prefers the sn-1 position of PC in plants (over 90% of the CPA is found in the sn-1 position), whereas *EcCPS* acts mainly on the monoenoic fatty acid in the sn-2 position of *E. coli* phospholipid (Hildebrand and Law, 1964; Bao et al., 2003). The reported preference of *EcCPS* for the sn-2 position of phospholipid may not reflect its actual preference and likely results from the low occupancy of 18:1 substrate at the sn-1 position,

Table II. Fatty acid composition of TAG from transgenic *Arabidopsis* seeds expressing *EcCPS* or *EcCPS-SfLPAT*

The values represent means and sd of at least three replicates.

Plant	16:0	18:0	18:1	18:2	CPA	18:3	20:0
	%						
<i>fad2fae1</i>	7.7 ± 0.1	5.8 ± 0.2	82.5 ± 0.0	1.2 ± 0.1	0.0 ± 0.0	1.2 ± 0.1	1.2 ± 0.0
<i>EcCPS17</i>	8.0 ± 0.3	5.8 ± 0.3	79.3 ± 0.3	0.5 ± 0.3	3.8 ± 0.0	1.4 ± 0.2	1.2 ± 0.1
<i>EcCPS38</i>	8.1 ± 0.3	5.8 ± 0.2	77.9 ± 0.9	1.5 ± 1.1	4.6 ± 0.2	1.1 ± 0.1	1.1 ± 0.1
<i>EcCPS43</i>	10.4 ± 0.4	6.6 ± 0.0	71.7 ± 0.5	0.8 ± 0.0	7.4 ± 0.2	2.0 ± 0.1	0.9 ± 0.1
<i>EcCPS-SfLPAT8</i>	10.2 ± 0.4	5.9 ± 0.4	72.4 ± 1.1	0.0 ± 0.0	9.8 ± 0.5	1.3 ± 0.2	0.7 ± 0.4
<i>EcCPS-SfLPAT37</i>	10.5 ± 0.3	6.1 ± 0.3	71.6 ± 0.9	0.2 ± 0.2	8.8 ± 0.2	1.6 ± 0.1	0.9 ± 0.1
<i>EcCPS-SfLPAT40</i>	8.7 ± 0.2	6.0 ± 0.1	74.7 ± 0.3	0.0 ± 0.0	8.5 ± 0.3	1.0 ± 0.1	1.1 ± 0.0

Table III. Fatty acid composition of sn-1 and sn-2 positions of PC from transgenic Arabidopsis seeds of EcCPS43 and EcCPS-SfLPAT37

The values represent means and sd of at least three replicates.

Plant	16:0	18:0	18:1	18:2	CPA	18:3
	%					
<i>fad2fae1</i>						
sn-1	12.7 ± 0.4	5.0 ± 0.6	79.2 ± 0.8	1.4 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
sn-2	0.6 ± 0.3	6.3 ± 1.5	88.3 ± 1.5	1.7 ± 0.1	0.0 ± 0.0	3.3 ± 0.4
EcCPS43						
sn-1	10.7 ± 0.5	5.4 ± 1.3	37.9 ± 1.0	1.2 ± 0.0	45.8 ± 1.0	0.0 ± 0.0
sn-2	2.5 ± 0.9	6.4 ± 1.6	80.7 ± 1.2	2.4 ± 0.0	8.0 ± 2.4	0.0 ± 0.0
EcCPS-SfLPAT37						
sn-1	13.2 ± 0.2	5.9 ± 1.1	20.1 ± 0.9	0.7 ± 0.1	60.2 ± 0.1	0.0 ± 0.0
sn-2	7.7 ± 0.0	3.8 ± 1.3	66.2 ± 2.7	2.0 ± 0.2	25.7 ± 1.3	0.0 ± 0.0

which contains predominantly (79%) saturated fatty acid. In *fad2fae1* Arabidopsis seeds, 18:1 is present at approximately 84% of the total fatty acid, occurring at similar levels in both the sn-1 and sn-2 positions of PC. It is interesting that EcCPS results in the accumulation of CPA at the sn-1 position of PC in Arabidopsis when presented with equal levels of 18:1 substrate in both the sn-1 and sn-2 positions. *S. foetida* accumulates sterculic acid equally at all sn-positions, whereas malvic acid is present at only 3% at the sn-2 position (Howarth and Vlahov, 1996). That coexpression of SfLPAT with EcCPS increases the level of CPA in the sn-2 position of PC indicates that CPA from the CoA pool must become esterified to LPA to form PA with CPA at the sn-2 position (Fig. 1). However, that coexpression of SfLPAT does not affect the CPA distribution in TAG implies a preference for normal fatty acid relative to CPA in the sn-2 position by enzymes downstream of PC, such as DGAT, PDCT, and PDAT in the TAG assembly network (Fig. 1). We also note that 16:0 is elevated at the sn-1 and sn-2 positions of PC in the EcCPS-SfLPAT37 line (Table III).

Engineering Metabolism to Further Increase CPA Production

In this work, we have shown that coexpression of SfLPAT along with EcCPS results in the accumulation

of up to 35% CPA in T1 seeds. However, high levels of CPA in membrane lipids result in unwanted decreases in seed weight and TAG accumulation, resulting from a bottleneck in the conversion of CPA to TAG. Heterologous coexpression of two castor acyltransferases, DGAT2 and PDAT1, with the castor 12-hydroxylase resulted in increased HFA accumulation in Arabidopsis (Burgal et al., 2008; Kim et al., 2011; van Erp et al., 2011). Both by analogy with hydroxy fatty acid accumulation and the bias against CPA in the TAG synthetic network observed in this study, it is likely that stacking TAG-synthesizing enzymes such as DGAT, PDCT, or PDAT with preference for PC CPAs into the EcCPS-SfLPAT transgenic lines will result in enhanced CPA accumulation in TAG (Burgal et al., 2008; Kim et al., 2011; van Erp et al., 2011; Hu et al., 2012; Li et al., 2012). It is interesting that when acyltransferases are expressed in the absence of an mFA-synthesizing enzyme, more rapid movement of fatty acid into TAG generally stimulates higher levels of fatty acid accumulation. For example, increased TAG levels have been observed upon the expression of rapeseed microsomal LPAT or yeast LPAT genes (SLC1 and SLC1-1) in Arabidopsis and rape (Maisonneuve et al., 2010). These reports are consistent with the removal of the feedback inhibition of FAS by reducing the levels of intermediates in the pathway of TAG.

Table IV. Fatty acid composition of sn-1, sn-3, and sn-2 positions of TAG from transgenic Arabidopsis seeds of EcCPS43 and EcCPS-SfLPAT37

The values represent means and sd of at least three replicates.

Plant	16:0	18:0	18:1	18:2	CPA	18:3	20:0
	%						
<i>fad2fae1</i>							
sn-1,3	11.1 ± 0.7	5.6 ± 1.0	75.7 ± 1.4	2.8 ± 0.3	0.0 ± 0.0	3.6 ± 0.4	1.2 ± 0.4
sn-2	0.0 ± 0.0	0.0 ± 0.0	93.2 ± 0.8	1.9 ± 0.1	0.0 ± 0.0	4.9 ± 0.8	0.0 ± 0.0
EcCPS43							
sn-1,3	11.3 ± 0.1	6.5 ± 0.0	67.3 ± 0.3	1.3 ± 0.1	8.7 ± 0.2	3.2 ± 0.1	1.6 ± 0.0
sn-2	0.0 ± 0.0	0.0 ± 0.0	90.6 ± 0.0	1.0 ± 0.0	3.8 ± 0.0	4.6 ± 0.0	0.0 ± 0.0
EcCPS-SfLPAT37							
sn-1,3	12.9 ± 0.1	6.2 ± 0.0	63.2 ± 0.7	2.4 ± 0.1	10.0 ± 0.1	4.1 ± 0.0	1.3 ± 0.0
sn-2	0.0 ± 0.0	0.4 ± 0.3	90.3 ± 0.7	1.3 ± 0.0	4.1 ± 0.1	3.8 ± 0.0	0.0 ± 0.0

CONCLUSION

Expression of the *E. coli* CPS gene in *fad2fae1* Arabidopsis results in the accumulation of as much as 9.3% CPA in Arabidopsis seeds. Coexpression of SfLPAT can increase CPA accumulation to as high as 35% and elevate levels of CPA in the sn-2 position of PC. However, the seeds with greater than 7% CPA accumulation appear wrinkled and are reduced in size and seed weight. Seeds with more than 11% CPA exhibit reduced germination rates. *E. coli* CPS acts on 18:1 primarily at the sn-1 position of PC; coexpression of SfLPAT results in the incorporation of CPA at the sn-2 position of LPA. This enables a cycle that enriches the accumulation of CPA at both sn-1 and sn-2 positions of PC and increases the accumulation of CPA. The findings presented here suggest that further optimization of CPA accumulation can potentially be made by stacking additional genes encoding enzymes that will enhance the movement of CPA from membrane lipids into TAG.

MATERIALS AND METHODS

Vector Construction

For expression in yeast, full-length cDNAs of CPSs from *Escherichia coli*, *Agrobacterium tumefaciens*, *Sterculia foetida*, cotton (*Gossypium hirsutum*), and Arabidopsis (*Arabidopsis thaliana*) At3g23460, At3g23470, and At3g23480 were amplified and cloned into yeast expression vector pYES2 by restriction of *SacI* and *EcoRI*. At3g23510 and At3g23530 were amplified from Arabidopsis cDNA and cloned into pYES2 by restriction of *SacI* and *EcoRI*. Primers for Arabidopsis CPS were designed according to their sequences in The Arabidopsis Information Resource (<http://www.arabidopsis.org/>). For expression in plants, *E. coli* CPS was amplified from *E. coli* strain K-12 (Substrain MG1655) using primers ECPS-5'*PacI* and ECPS-3'*XmaI* and cloned into the pDsRed plant expression vector (Pidkowich et al., 2007) to form pPhasECPS. Another expression cassette of *E. coli* CPS was constructed using overlap-extension PCR (Horton et al., 1990). Overlapping fragments of the phaseolin promoter (Pidkowich et al., 2007), *E. coli* CPS, and the phaseolin terminator were amplified in separate PCRs using appropriate primer pairs (Supplemental Table S2). The PCR products were gel purified and assembled in a PCR primed with terminal primers Phas5'*EcoRI* and Phas3'*EcoRI* and then cloned into the pPhasECPS vector with the *EcoRI* restriction site (New England Biolabs). Further restrictions screen p2PhasECPS, in which the two sets of promoters are in the same direction. *S. foetida* LPAT was amplified from a native plant. *S. foetida* LPAT was further cloned into p2PhasECPS through restriction of *PacI* and *XmaI*. Primer sequences are listed in Supplemental Table S2.

Plant Growth Conditions and Transgenic Analyses

Developing seeds and leaves of *S. foetida* were collected from the Montgomery Botanical Center in Florida. The seed coats were removed, and the cotyledons and embryos were frozen with liquid nitrogen and stored at -80°C for RNA extraction and lipid analysis.

Arabidopsis plants were grown in walk-in-growth chambers at 22°C with a 16-h photoperiod. Binary vectors were introduced into *A. tumefaciens* strain GV3101 by electroporation and were used to transform Arabidopsis via the floral dip method (Clough and Bent, 1998). Seeds of transformed plants were screened under fluorescence emitted upon illumination with green light from an X5 light-emitting diode flashlight (Inova) in conjunction with a Quantaray 25A red camera filter (Pidkowich et al., 2007).

S. foetida EST Analysis

RNA from *S. foetida* leaves and seeds at different development stages was extracted according to Schultz et al. (1994). RNA quality and concentration were determined by gel electrophoresis and Nanodrop spectroscopy. EST

analysis was performed after reverse transcription and size fractionation of the cDNA. mRNA was purified using the Illustra mRNA purification kit (GE Healthcare), and cDNA synthesis was carried out with the use of the Creator SMART cDNA Library Construction Kit (Clontech). The first-strand cDNA was synthesized from 2.0 μg of mRNA using SuperScript II reverse transcriptase (Invitrogen) and Clontech first-strand buffer along with a modified CDS III/3' cDNA synthesis primer, 5'-TAGAGGCCGAGCGGCCGCATGTTTTGTTTTTTTCTTTTTTTTTTTVN-3'. Fourteen thermal cycles were used for cDNA amplification. The cDNA fractions containing sequences of more than 500 bp were used for subsequent 454 sequencing and assembly by the Research Technology Support Facility at Michigan State University.

Phylogenetic Analysis

Phylogenetic analysis of the LPAT family was conducted by using full-length protein sequences from *S. foetida*, *Saccharomyces cerevisiae*, *E. coli*, coconut (*Cocos nucifera*), rape (*Brassica napus*), maize (*Zea mays*), rice (*Oryza sativa*), and Arabidopsis. Full-length amino acid sequences were first aligned by ClustalW version 2.0.12 (Thompson et al., 1994) with default parameters and imported into the Molecular Evolutionary Genetics Analysis (MEGA) package version 5.0 (Tamura et al., 2007). Phylogenetic and molecular evolutionary analyses were conducted using the neighbor-joining method (Saitou and Nei, 1987) implemented in MEGA, with the pairwise deletion option for handling alignment gaps and the Poisson correction model for computing distance. The final tree graphic was generated using TreeView (Page, 1996).

Transgene Expression Analysis

Arabidopsis RNA from Arabidopsis *fad2fae1* seeds and three lines each from EcCPS and EcCPS-SfLPAT transgenic plants were extracted according to Wu et al. (2002). RNA quality and concentration were determined by gel electrophoresis and Nanodrop spectroscopy. Reverse transcription-PCR and quantitative reverse transcription-PCR analysis of EcCPS and SfCPS gene expression were carried out as described (Yu et al., 2011). Primers AtUbiq10-F (5'-TCITCGCCGAAAGCAACTTGA-3') and AtUbiq10-R (5'-TGGCCTTCACGTTGTC AATGGT-3') were used to amplify UBQUITIN10 as an internal standard. Gene-specific primers used were qEcCPS-F (5'-GTACCGTATCGCAACGAATTAC-3') and qEcCPS-R (5'-CAATACGCAGCGTCTTTGA-3') for EcCPS and qSfLPAT-F (5'-ACTTCTGGGCATGCTTTGT-3') and qSfLPAT-R (5'-CTACTGCTGTTTGTCTCTTG-3') for SfLPAT.

Fatty Acid Analyses

Yeast culture, expression, and fatty acid analyses were carried out as described (Broadwater et al., 2002). Lipids were extracted in methanol:chloroform (2:1) from 0.1 g fresh weight of cotton tissue, and heptadecanoic acid was added as an internal standard. The isolated lipid was methylated in 1% sodium methoxide at 50°C for 1 h and extracted with hexane. Fatty acid methyl esters (FAMES) from single seeds were prepared by incubating the seed with 30 μL of 0.2 M trimethylsulfonium hydroxide in methanol (Butte et al., 1982). Lipid profiles and acyl group identification were analyzed on a Hewlett-Packard 6890 gas chromatograph equipped with a 5973 mass selective detector for gas chromatography-mass spectrometry (GC-MS) and an Agilent J&W DB 23 capillary column (30 m \times 0.25 mm \times 0.25 μm). The injector was held at 225°C , and the oven temperature was varied from 100°C to 160°C at $25^{\circ}\text{C min}^{-1}$, then to 240°C at $10^{\circ}\text{C min}^{-1}$. The percentage values were converted to mole percent and presented as means of at least three replicates.

CPA Distribution in the TAG

Total lipids were extracted from 20 seeds of each T4 line by homogenizing in 500 μL of methanol:chloroform:formic acid (20:10:1, v/v). The organic solvent was extracted with 250 μL of 1 M KCl and 0.2 M H_3PO_4 twice. The organic phase was dried under N_2 and suspended in hexane. Lipids were separated by thin-layer chromatography (TLC) with hexane:diethylether:acetic acid (80:20:1, v/v). The internal standard heptadecanoic acid was added to each fraction, and FAMES were prepared with 1 mL of methanol:HCl at 90°C for 1 h and extracted with hexane. FAMES were quantified by GC-MS as described previously (Yu et al., 2011).

Stereospecific Analysis of the Fatty Acid Composition of PC and TAG from Transgenic Arabidopsis

Polar lipid and TAG were separated through a 3-mL Supelco Supel Clean LC-Si SPE column (Sigma), and polar lipid was analyzed with the use of activated ammonium sulfate-impregnated silica gel TLC plates developed with acetone:toluene:water (91:30:7, v/v/v) and stained with 0.05% primuline to isolate PC. PC was digested with phospholipase A2 from *Naja mossaibica mossaibica* (Sigma) in borate buffer (0.5 M boric acid, 0.4 mM CaCl₂, pH 8.2) for 20 min at room temperature. Reaction products were separated as described previously (Bates et al., 2007). The stereospecific analysis of the fatty acid composition of TAG was performed as described by Cahoon et al. (2006), except that the TLC mobile phase was replaced with chloroform:methanol:acetic acid (70:30:1, v/v/v).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number KC894726.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Chemical structure of 19:0 cyclopropane fatty acid.

Supplemental Figure S2. Sequence analysis of *Sterculia* cDNAs encoding LPATs.

Supplemental Table S1. Fatty acid composition of transgenic Arabidopsis seeds of T4 lines expressing EcCPS or EcCPS and SFLPAT.

Supplemental Table S2. Oligonucleotide primers.

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