Running Head:

Engineering cyclopropane fatty acid accumulation

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Coexpressing *E. coli* cyclopropane synthase with *Sterculia foetida* lysophosphatidic acid acyltransferase enhances cyclopropane fatty acid accumulation

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Summary:

Expression in plant seeds of *E. coli* cyclopropane synthase (EcCPS) which acts on 18:1 primarily at the sn-1 position of PC facilitates higher accumulation of cyclopropane fatty acids (CPA) than higher plant CPSs; coexpression of SfLPAT results in its incorporation at the sn-2 position of LPA by enabling a cycle that enriches for the accumulation of CPA at both sn-1 and sn-2 positions of PC.
Footnotes:

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**Abbreviations footnote**
CPS: cyclopropane fatty acid synthase; CPA, cyclopropane fatty acid; CFA: cyclic fatty acid; LPAT: lysophosphatidic acid acyltransferase; FA, fatty acid(s); FAS, fatty acid synthesis FAME, fatty acid methyl ester; WT, wild type.

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

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 <20% of the desired mFA in transgenic seed (Napier, 2007) compared to levels found in the natural source. For example ricinoleic acid accounts for >90% of the FA of castor seeds and Tung (Aleuities fordii) seeds accumulate >80% α-Eleostearic acid (Drexler et al., 2003; Thelen and Ohlrogge, 2002). 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 FAs (CFA) 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 CFA results in ring opening to produce methyl-branched FA. Branched chain FA are ideally suited for the oleochemical industry as feedstocks for the production of lubricants, plastics, paints, dyes, and coatings (Carlsson et al., 2011). Cyclopropane FAs (CPA) have been found in certain gymnosperms, Malvales, Litchi and other Sapindales. They accumulate to as much as 40% in seeds of Litchi chinensis (Gaydou et al., 1993; Vickery, 1980). Sterculia foetida accumulates the desaturated cyclic FA i.e., cyclopropene FA to >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 CPS enzyme which transfers a methyl group to C9 of the oleoyl-phospholipid followed by cyclization to form the cyclopropane ring (Bao et al., 2002; Bao et al., 2003; Grogan and Cronan, 1997). None of the known natural sources of CPA are suitable for its commercial production. It would therefore be desirable to create an oil seed crop plant that accumulates high levels of CPA by heterologously expressing CPS in seeds. However, to date, heterologous expression of plant cyclopropane synthase genes have led to only ~1.0% CPA in the transgenic seeds (Yu et al., 2011).

Two pathways for the biosynthesis of TAG exist in plants (Bates and Browse, 2012) (Fig. 1). The de novo biosynthesis from glycerol-3-phosphate and acyl-CoA occurs via the Kennedy pathway and includes three acyltransferases: Glycerol-2-phosphate Acyltransferase (GPAT), Acyl-CoA: Lysophosphatidic Acid Acyltransferase (LPAT) and Acyl-CoA: Diacylglycerol Acyltransferase (DGAT) (Kennedy, 1961). Alternatively acyl-CoAs can be redirected from Phosphatidyl Choline (PC) via the action of a PhosphoLipase C (PLC), Choline PhosphoTransferase (CPT), Phosphatidylcholine: Diacylglycerol Cholinephosphotransferase (PDCT) (Hu et al., 2012; Lu et al., 2009) or by Phospholipid: Diacylglycerol Acyltransferase (PDAT) (Dahlqvist et al., 2000). An acyl group can be released from PC to generate lyso-PC by the back reaction of acyl-CoA: lyso-phosphatidylcholine acyltransferase (LPCAT; (Stymne and Stobart, 1984; Wang et al., 2012)) or a phospholipase A/acyl-CoA synthase (ACS) (Chen et al., 2011).

LPAT is a pivotal enzyme controlling the metabolic flow of lysophosphatidic acid into different phosphatidic acids in diverse tissues. Membrane-associated LPAT activities, identified in bacteria, yeast, plant, and animal cells, catalyze the transfer of acyl groups from acyl-CoA to lysophosphatidic acid (lysoPA) to synthesize phosphatidic acid (PA). In plants and other
organisms, LPAT activities have been identified in the endoplasmic reticulum (ER) (Kim et al., 2005), plasma membrane (Bursten et al., 1991) and mitochondria (Zborowski and Wojtczak, 1969). In higher plants, ER-localized LPAT plays an essential role transferring FA from CoA esters to the sn-2 position of lysophosphatidic acid (LPA) in the synthesis of PA, a key intermediate in the biosynthesis of membrane phospholipids and storage lipids in developing seeds (Maisonneuve et al., 2010). LPAT from developing seeds of flax (Linum usitatissimum), rape (Brassica napus) and castor bean (Ricinus communis) preferentially incorporate oleoyl-CoA, weakly incorporate cyclopropane acyl-CoA and were unable to incorporate methyl branched acyl-CoA when presented with an equimolar mix of these potential substrates (Nlandu Mputu et al., 2009). Thus, LPAT activity from agronomic plants constitutes a potential bottleneck for the incorporation of branched chain acyl-CoA into PA. In this work we investigate the utility of an LPAT from a cyclopropanoid-syntheizing plant, S. foetida, with respect to its ability to enhance cyclopropane fatty acid accumulation. In our efforts to enhance CPA accumulation in transgenic plants, we screened CPS genes from diverse sources and identified E. coli CPS (EcCPS) as an effective enzyme for the production of CPA in plants. However, EcCPS is reported to prefer the sn-2 position of E. coli phospholipid (Hildebrand and Law, 1964), the data presented here shows its expression primarily leads to the accumulation of CPA at the sn-1 position. Moreover coexpression of SfLPAT results in the incorporation of CPA at the sn-2 position of LPA. Thus, coexpression of EcCPS and SfLPAT 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. This work underscores the utility of coexpressing an acyltransferase from mFA accumulating species with mFA-synthesizing enzymes to help mitigate bottlenecks in mFA TAG synthesis.
RESULTS

Expression of CPS in yeast

Previously, we expressed four plant CPS genes, three from cotton and one from Sterculia, individually in yeast. Results from this work showed that expression of GhCPS1, a CPS from cotton, led to highest levels of CPA production in both yeast (5.3%) and plants (~ 1.0%) (Yu et al., 2011). To identify a CPS gene that leads to the accumulation of higher levels of cyclopropane fatty acid, CPS from *E. coli*, *Agrobacterium* and five from Arabidopsis were cloned and expressed in yeast. As shown in Fig. 2, the fatty acid composition of yeast expressing EcCPS showed substantial CPA accumulation. In samples from EcCPS-expressing lines (Fig 2A), two peaks corresponding to 17:0 CPA and 19:0 CPA (chemical structure shown in supplementary Fig. 1) were identified (Fig. 2B) based on their mass ions. Expression of EcCPS led to the accumulation of 27% 17:0 CPA and 17% 19:0 CPA yielding a total of 44% CPA accumulation, which is about 8-fold of that observed upon over-expression of the cotton CPS gene GhCPS1 (Fig. 2C). The expression of *Agrobacterium* CPS and 5 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 17C and 19C 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 (phas) promoter and transformed into Arabidopsis *fad2fae1* plants (Meesapyodsuk and Qiu, 2008). This background was chosen because its seed contains more than 80% of 18:1, the CPS FA substrate. T1 seeds expressing EcCPS yielded the highest content of dihydrosterculic acid (DHSA, 19-carbon CPA) (average 5.0 %), no 17-carbon CPA
products were detected. Expression of GhCPS1 and *Sterculia foetida* CPS led to the accumulation of at most 1% CPA (Yu, et al. 2011) whereas expression of 5 Arabidopsis and two cotton orthologs (GhCPS2 and 3) resulted in no detectable accumulation of CPA. T1 *fad2fae1* seeds expressing EcCPS germinated with similar frequency to those of non-transformed seeds, and T2 lines with 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% CFA (Fig.3).

**Isolation of an LPAT from *Sterculia* seed**

RNA from *Sterculia* leaf and young seed were extracted and subject to 454 sequencing. The AtLPAT2 gene sequence encoding the ubiquitous endoplasmic reticulum (ER)–located LPAT (Kim et al., 2005) was used to BLAST the *Sterculia* EST sequences derived from *Sterculia* which contains 23162 seed ESTs and 26083 leaf ESTs. A *Sterculia* homolog was identified and showed preferential expression in seed with 56 occurrences in the cotyledon and embryo of developing seeds vs. 27 occurrences in leaf tissue. Oligonucleotides were designed to amplify the full length cDNA which was cloned and designated SfLPAT (GenBank Accession number KC894726). SfLPAT has an 1164 base pair open reading frame that encodes a 387 amino acid protein with a predicted molecular weight of 43,723 Da and a theoretical pI of 9.63. The predicted amino acid sequence of SfLPAT shows strong homology to Arabidopsis LPAT2 (79.2%), *Brassica* LPAT2 (79.1%), Arabidopsis LPAT3 (61.9%) and 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 (see supplemental Figure 2). *Sterculia* LPAT groups with AtLPAT2 and 3 LPATs from *Brassica*, and forms a clade with corn LPAT and Arabidopsis LPAT3.
Coexpression of EcCPS and SfLPAT in fad2fae1 Arabidopsis seeds

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

CPA accumulation in the progeny of EcCPS-SfLPAT co-expressing transgenic fad2fae1 seeds

Only approximately 30% of the EcCPS-SfLPAT T1 seeds germinated and were able to develop into mature plants, compared to almost 100% for seeds expressing EcCPS alone. The approximately 70% of non-viable seeds showed no radical penetration of the seed coat suggesting a failure of germination rather than 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 SfLPAT 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 co-expressing EcCPS-SfLPAT that contained more than 11.5% CPA exhibited reduced germination rates even with the supplement of 1% (w/v) sucrose in the media. For example, no germination was observed for EcCPS-SfLPAT line 2 which accumulated 11.5% CPA in T2 seeds, and only one of 800 seeds of EcCPS-SfLPAT geminated 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, this
data suggests elevated accumulation of CPA rather than the presence of SfLPAT was responsible for the observed lack of germination.

Six individual T3 plants from EcCPs line 17, 38, 43 and EcCPS-SfLPAT line 8, 37 and 40 each line 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 Fig. 5 and supplemental Table 1, EcCPS T4 homozygous transgenic seeds yielded up to 9.1% CPA, and the progeny of EcCPS-SfLPAT-expressing seeds produced CPA ranging from 10.8-13.3%. SfLPAT expression was detected in lines coexpressing EcCPS and SfLPAT, and the EcCPS expression levels were slightly lower than those observed in several of the lines expressing only EcCPS (Fig. 6), providing further support that increased CPA accumulation results from SfLPAT expression.

**CPA effect on seed weight and oil content**

FAs 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 ± 0.61µg. The three homozygous lines expressing EcCPS showed no significant differences from the parental line, whereas lines coexpressing SfLPAT with EcCPS resulted in a significant (student T-test, p<0.05) 18% decrease in total fatty acid (Fig. 7A). In addition to FA content, seed weights were also determined. As shown in Fig. 7B, parental fad2fae1 seeds weighed 20.0 ± 1.24 µg and T4 seeds of EcCPS expressing lines showed no significant difference, whereas equivalent lines coexpressing EcCPS and SfLPAT showed a significant (student T-test, p<0.05) decrease in seed weight of up to 11%, with the largest decreases occurring in lines that accumulate the most
CFA. Together these data show that CPA accumulation above 9% causes a decrease in seed weight that correlates with reduced accumulation of seed FA (Fig. 7C).

**CPA distribution in transgenic plants**

In Arabidopsis, most mFA in TAG originate from the PC pool (Bates et al., 2009). Consistent with this, the substrate for Sterculia CPS, is 18:1 at the sn-1 position of PC (Bao et al., 2003). We found that CPA accumulates at 25-26% in the polar lipid fraction of EcCPS-expressing Arabidopsis lines that accumulate 5-9% CPAs in the seed oil at maturity (Fig. 5). 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 (Table 1 and 2).

**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* (Bao et al., 2003; Hildebrand and Law, 1964). 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% of 18:1 (Table 3). 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 sn2-positions of PC in Arabidopsis (Table 3); however, significantly higher levels of CPA are present...
at the sn-1 position implying a possible >2-fold preference 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 PC CPA in EcCPS-SfLPAT37.

In contrast to the different sn 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% of 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 4).

**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) co-expression 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 FA in primary transformants, 3) the accumulation of CPA correlates with reduced seed fatty acid content and germination rate.

**The source of CPS enzyme 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, *Sterculia* and Arabidopsis in the *fad2fae1* Arabidopsis background tailored to accumulate the CPS substrate oleate. Of the plant CPS genes tested, expression of only *Sterculia* and a cotton CPS resulted in the accumulation 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 *Agrobacterium*. Expression of the *E. coli* CPS resulted in the accumulation of ~5.0% CPA in primary transformants. These hemizygous T1 seeds were apparently limiting for CPS enzyme because CPS accumulation shows 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 FA, ricinoleic acid in plants. In this case, the expression of an oleate hydroxylase from the fungus *Claviceps purpurea* yielded more hydroxy product than 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% of CPA upon the coexpression of EcCPS with SfLPAT suggests that the substrates supply i.e., 18:1 FA and S-adenosylmethionine (SAM), are 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 $V_{\text{max}}$ or lower $K_m$ for its substrates than the plant CPS enzymes, or that 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 *Sterculia* LPAT with homology to the Arabidopsis LPAT2 was identified. Because sterculic acid preferentially accumulates in seeds relative to vegetative tissues, we hypothesized that SfLPAT may have evolved to accommodate the transfer of cyclic FA. A large increase of up to 35% CFA accumulated in the T1 seeds of Arabidopsis *fad2fae1* when SfLPAT was co-expressed 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 FA and a small increase in 16:0 FA in both TAG and total seed FA fractions. That SfLPAT facilitates the accumulation of CPA is interesting because *S. foetida* accumulates cyclopropene rather than cyclopropane fatty acids. The mechanism and substrate of CPA desaturation to produce cyclopropene fatty acids in *S. foetida* remains elusive it is therefore unresolved whether SfLPAT has preference for CPA, cyclopropene fatty acids or both.

**Decreased fatty acid content, seed weight and poor germination are associated with coexpression of SfLPAT and EcCPS**

When SfLPAT was co-expressed 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 non-transformed control seeds and contained reduced fatty acid content. In transformants expressing only EcCPS that showed the high levels of CPA accumulation, oil content was decreased similarly to that 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 10-20% have been reported for transgenics accumulating hydroxy or conjugated FA (Cahoon et al., 2006; van Erp et al., 2011). Reduction of seed weight and FA content of ricinoleic acid-accumulating transgenics has been attributed to feedback inhibition of fatty acid synthesis (FAS) upon the accumulation of
hydroxy FA lipid intermediates (Bates and Browse, 2011; van Erp et al., 2011). Acyltransferase enzymes facilitate the conversion of membrane lipids to TAG, the sink for FA. 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; Hu et al., 2012; van Erp et al., 2011). We hypothesize that elevated levels of CPA in membrane lipids could result in a wrinkled phenotype by similar feedback inhibition of FAS that was observed upon the accumulation of ricinoleate in membrane lipids, i.e., that the FA released from turnover of CPA-containing lipids reduce the total rate of fatty acid synthesis (Andre et al., 2012; Bates and Browse, 2011; van Erp et al., 2011). RcFAH-expressing seeds exhibit poor germination, but upon coexpression of castor DGAT2 or PDAT1, higher levels of ricinoleoyl TAG accumulates however, germination improves because elevated TAG levels were associated with reduced hydroxy FA 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 germination is more sensitive to CPA accumulation than seed maturation. Upon germination, mobilization of FA from TAG, or FA transport and catabolism could be compromised by the presence of mFA in TAG. That the transgenics germinate poorly on sucrose-containing media, 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 CFA 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 on sn-2 position of E.coli phospholipid (Bao et al., 2003; Hildebrand and Law, 1964). 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 which contains predominantly (79%) saturated FA. In fad2fae1 Arabidopsis seeds, 18:1 is present at ~84% of the total FA, 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 CFA 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 co-expression 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 (see Fig. 1). However, that coexpression of SfLPAT doesn’t affect the CPA distribution in TAG, implies a preference for normal FA 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 slightly elevated at the sn-1 position of PC in the EcCPSSfLAT37 line (Table 3).

Engineering metabolism to further increase CFA 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 results 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 or 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 FA 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; Hu et al., 2012; Kim et al., 2011; Li et al., 2012; van Erp et al., 2011). It is interesting to note that when acyltransferases are expressed in the absence of an mFA-synthesizing enzyme, more rapid movement of FA into TAG generally stimulates higher levels of FA 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 Brassica napus (Maisonneuve et al., 2010). These reports are consistent with the removal of feedback inhibition of FAS by reducing levels of intermediates in the pathway of TAG.

Conclusions

Expression of 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 >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 CFA 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 cDNA of CPSs from E.Coli, Agrobacterium, Sterculia, cotton and Arabidopsis At3g23460, At3g23470, 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 TAIR (http://www.arabidopsis.org/). For expression in plant, E.Coli CPS was amplified from E.Coli strain K-12 (Substr. MG1655) using primers ECPS-5’PacI and ECPS-3’XmaI and cloned into 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 phaseolin promoter (Pidkowich et al., 2007), E.Coli CPS and phaseolin terminator were amplified in separate PCR reactions using appropriate primer pairs (Supplementary Table 4). The PCR products were gel purified and assembled in a PCR reaction primed with terminal primers Phas5’EcoRI and Phas3’EcoRI, and cloned into the pPhasECPS vector with the EcoRI restriction site (New England Biolabs, Ipswich, MA). Further restrictions screen the p2PhasECPS in which the two set of promoters are in the same direction. Sterculia
LPAT was amplified from native plant. Sterculia LPAT was further cloned into p2PhasECPS through restriction of Pac I and XmaI. Primer sequences are listed in supplementary Table 2.

**Plant growth conditions and transgenic analyses**

Developing seeds and leaves of *S. foetida* L. were collected from Montgomery Botanical Center (Miami, FL). 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 for 16 h photoperiod. Binary vectors were introduced into *Agrobacterium 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 a X5 LED flashlight (Inova) in conjunction with a 25A red camera filter (Pidkowich et al., 2007).

**Sterculia EST analysis.**

RNA from *Sterculia* leaf and seeds at different development stages were extracted according to Schultz *et al.* (Schultz et al., 1994). RNA quality and concentration were determined by gel electrophoresis and Nanodrop spectroscopy. EST analysis was performed after reverse transcription, size fractionation of the cDNA: mRNA was purified using Illustra mRNA purification kit (GE Healthcare), 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 ug of mRNA using SUPERSCRIPT II reverse transcriptase (Invitrogen), and Clontech first strand buffer, along with a modified CDS III /3’ cDNA synthesis primer 5’
TAGAGGCGAGGCGGCCGACATGTTTTGTTTTTTTTTTTTTTTTTTTTNTN. 14 thermal cycles were used for cDNA amplification. The cDNA fractions containing sequences of > 500bp were used for subsequent 454 sequencing and assembly by the Research Technology Support Facility at Michigan State University.

**Phylogenetic analysis**

Phylogenetic analysis of the lysophosphatidic acid acyltransferase (LPAT) family was conducted by using full length protein sequences from *Sterculia, rapeseed, Saccharomyces cerevisiae, E. coli*, coconut, *Brassica napus*, *Zea mays*, rice and Arabidopsis. Full-length amino-acid sequences were first aligned by CLUSTALW version 2.0.12 (Thompson et al., 1994) with default parameters ([http://www.ebi.ac.uk/Tools/clustalw/](http://www.ebi.ac.uk/Tools/clustalw/)), 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 (NJ) method (Saitou and Nei, 1987) implemented in MEGA, with the pair-wise deletion option for handling alignment gaps, and the Poisson correction model for computing distance. The final tree graphic was generated using TreeView program (Page, 1996).

**Transgene expression analysis**

Arabidopsis RNA from Arabidopsis seeds from *fad2fae1* and 3 lines of each from EcCPS and EcCPS-SfLPAT transgenic plants were extracted according to Wu et al. (Wu et al., 2002), RNA quality and concentration were determined by gel electrophoresis and Nanodrop spectroscopy. RT and qRT PCR analysis of EcCPS and SfCPS gene expression was carried out as described in (Yu et al., 2011). Primers AtUbiq10-F (5'- TCTTCGCCGGAAAGCAACTTGA -3’) and AtUbiq10-R (5'- TGGCCTTCACGTGTCAATGGT -3’) were used to amplify ubiquitin 10 as internal standard. Gene-specific primers used were qEcCPS-F (5'- GTACCGTATCGCCAACGAATTAC -3’) and qEcCPS-R (5'-
CAATACGCAGCGTGTCTTTGA -3') for EcCPS, and qSfLPAT-F (5'-ACTTCTTGGGCATGCTTTGT -3) and qSfLPAT-R (5'- CTACTGCTTTTGTCGTCCTTG -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 of fresh weight 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 hr and extracted with hexane. Fatty acid methyl esters (FAMEs) from single seeds were prepared by incubating the seed with 30 µL 0.2M 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 (GC/MS) and Agilent J&W DB 23 capillary column (30 m × 0.25 µm × 0.25 µm). The injector was held at 225°C and the oven temperature was varied from 100–160°C at 25°C/min, then to 240°C at 10°C/min. The percentage values were converted to mole percent and presented as a mean 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 vol/vol). The organic solvent was extracted with 250 µL of 1 M KCl, 0.2 M H₃PO₄ twice. The organic phase was dried under N₂ and suspended in hexane. Lipids were separated by TLC with hexane:diethylether:acetic acid (80:20:1, vol/vol). Internal standard heptadecanoic acid was added to each fraction and fatty acid methyl esters (FAMEs) were prepared with 1 mL of methanol:HCl at 90°C for 1 hr and extracted with hexane. FAMEs were quantified by GC-MS, as previously described (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 3 ml Supelco Supel Clean LC-Si SPE column (SIGMA), polar lipid was analyzed with the use of activated ammonium sulfate-impregnated silica gel TLC plates developed with Acetone/Toluene/H2O (91:30:7, v/v/v) and stained with 0.05% primuline to isolate PC. PC was digested with phospholipase A2 from *Naja mossambica mossambica* (SIGMA) in borate buffer (0.5M Boric acid, 0.4 mM CaCl2, pH 8.2) for 25 min at room temperature. Reaction products were separated as previously described (Bates et al., 2007). The stereospecific analysis of the fatty acid composition of TAG was performed as described by Cahoon et al (Cahoon et al., 2006) except TLC mobile phase was replaced with chloroform/methanol/Acetic acid (70:30:1, v/v/v).

Authors' contributions

JS and XHY designed the study. XHY, RR and MS performed the research. JS, XHY and RR prepared the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Dr. M. Patrick Griffith (Director of the Montgomery Botanical Center) for assistance with *Sterculia* tissue collection; Dr. Sean McCorkle (BNL) and Dr. Kevin Carr (Michigan State University) for bioinformatics support; and Dr. Changcheng Xu (BNL), Dr. Phil. Bates, (University of Southern Mississippi) and Xiangjun Li (University of Nebraska) for technical advice on sn positional analysis.
Literature Cited


Figure legends

Figure 1. Schematic representation of the plant triacylglycerol biosynthesis network.

Acyl editing can provide PC-modified FAs for de novo DAG/TAG synthesis. Abbreviations:
DAG, diacylglycerol; G3P, glycerol-3-phosphate; LPA, lyso-phosphatidic acid; LPC, lyso-
phosphatidylcholine; mFA, modified FA; mFAS, modified FA synthase (in this work, mFAS is
cyclopropane synthase (CPS); PA, phosphatidic acid; PC, phosphatidylcholine; TAG,
triacylglycerol; CPT, CDP-choline: DAG choline phosphotransferase; DGAT, acyl-CoA:DAG
acyltransferase; GPAT, acyl-CoA:G3P acyltransferase; PLA, phospholipase A; ACS, Acyl CoA
Synthase; PLC, phospholipase C; LPCAT, acyl-CoA: LPC acyltransferase; PAP, PA
phosphatase; PDAT, phospholipid:DAGacyltransferase.

Figure 2. Analysis of yeast expressing E. coli and various plant CPS enzymes.

GC analysis of FAME from yeast expressing empty vector pYES2 (Panel A) or E.coli CPS
which produced both 17:0 CFA and 19:0 CFA (Panel B), and CFA production in CPS expressed
yeast (Panel C). FAMEs were analyzed by GC/MS, both 17:0 and 19:0 CFA accumulations
were calculated as a percentage of the total FA. The values represent the mean and standard
deviation of at least three replicates.

Figure 3. CPA production in fad2fae1 T2 seeds

FAMEs were extracted from T2 EcCPS fad2fae1 seeds and analyzed by GC/MS.
The values represent the mean and standard deviation of at least three replicates.
Figure 4. Cyclopropane fatty acid accumulation in individual T1 EcCPS-SfLPAT transgenic fad2fae1 seeds. FAMEs were analyzed by GC/MS, cyclopropane fatty acid are expressed as a percentage of the total FA.

Figure 5. Cyclopropane fatty acid production in T3 seeds upon the expression of indicated EcCPS or EcCPS-SfLPAT in fad2/ fae1 plants.
FAMEs were analyzed by GC/MS, CFA are expressed as a percentage of the total FA.
The values represent the mean and standard deviation of at least three replicates.

Figure 6. EcCPS and SfLPAT expression levels in transgenic plants.
qRT-PCR analysis of EcCPS (Panel A) and SfLPAT (Panel B) expression levels in seeds of fad2/ fae1 and 3 transgenic lines harboring EcCPS and EcCPS-SfLPAT as indicated. The relative expression levels are reported relative to the expression of the ubiquitin 10 transcript.
The values represent the mean and standard deviation of at least three replicates.

Figure 7. Effect of expression of EcCPS and EcCPS-SfLPAT on seed fatty acid content and weight.
A. Total fatty acid content in transgenic Arabidopsis. Fatty acid content of seeds was quantified by GC of fatty acid methyl esters.
The values represent the mean and standard deviation of at least three replicates.
.B, Seed weight based on the measurement of batches of 100 transgenic Arabidopsis seeds. The values represent the mean and standard deviation 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 Panels A and B. Significance levels using the student T-test: **, p< 0.01 and *, p<0.05.
Table 1. Fatty acid composition of the polar lipids from transgenic Arabidopsis seeds expressing EcCPS or EcCPS-SfLPAT. The values represent the mean and standard deviation of at least three replicates.

<table>
<thead>
<tr>
<th></th>
<th>16:0%</th>
<th>18:0%</th>
<th>18:1%</th>
<th>18:2%</th>
<th>CPA%</th>
<th>18:3%</th>
</tr>
</thead>
<tbody>
<tr>
<td>fad2fae1</td>
<td>9.6 ± 0.3</td>
<td>3.2 ± 0.2</td>
<td>79.7 ± 0.5</td>
<td>2.8 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>EcCPS17</td>
<td>10.8 ± 1.7</td>
<td>2.8 ± 0.6</td>
<td>53.8 ± 1.0</td>
<td>3.1 ± 0.2</td>
<td>24.6 ± 2.0</td>
<td>4.9 ± 0.8</td>
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<tr>
<td>EcCPS38</td>
<td>8.2 ± 0.1</td>
<td>2.6 ± 0.3</td>
<td>56.6 ± 0.5</td>
<td>3.5 ± 0.1</td>
<td>25.7 ± 0.1</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>EcCPS43</td>
<td>9.5 ± 0.1</td>
<td>2.4 ± 0.2</td>
<td>56.5 ± 0.3</td>
<td>3.1 ± 0.0</td>
<td>24.9 ± 0.2</td>
<td>3.4 ± 0.2</td>
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<tr>
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<td>46.1 ± 0.2</td>
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<td>33.0 ± 0.1</td>
<td>4.9 ± 0.2</td>
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<tr>
<td>EcCPSSfLPAT40</td>
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<td>26.8 ± 0.7</td>
<td>5.7 ± 0.8</td>
</tr>
</tbody>
</table>

Table 2. Fatty acid composition of TAG from transgenic Arabidopsis seeds expressing EcCPS or EcCPS-SfLPAT. The values represent the mean and standard deviation of at least three replicates.

<table>
<thead>
<tr>
<th></th>
<th>16:0%</th>
<th>18:0%</th>
<th>18:1%</th>
<th>18:2%</th>
<th>CPA%</th>
<th>18:3%</th>
<th>20:0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fad2fae1</td>
<td>7.7 ± 0.1</td>
<td>5.8 ± 0.2</td>
<td>82.5 ± 0.0</td>
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<td>EcCPS17</td>
<td>8.0 ± 0.3</td>
<td>5.8 ± 0.3</td>
<td>79.3 ± 0.3</td>
<td>0.5 ± 0.3</td>
<td>3.8 ± 0.0</td>
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<td>EcCPS38</td>
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<td>5.8 ± 0.2</td>
<td>77.9 ± 0.9</td>
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<td>4.6 ± 0.2</td>
<td>1.1 ± 0.1</td>
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<td>71.7 ± 0.5</td>
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<td>EcCPSSfLPAT8</td>
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<td>1.3 ± 0.2</td>
<td>0.7 ± 0.4</td>
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<tr>
<td>EcCPSSfLPAT37</td>
<td>10.5 ± 0.3</td>
<td>6.1 ± 0.3</td>
<td>71.6 ± 0.9</td>
<td>0.2 ± 0.2</td>
<td>8.8 ± 0.2</td>
<td>1.6 ± 0.1</td>
<td>0.9 ± 0.1</td>
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<tr>
<td>EcCPSSfLPAT40</td>
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<td>6.0 ± 0.1</td>
<td>74.7 ± 0.3</td>
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<td>8.5 ± 0.3</td>
<td>1.0 ± 0.1</td>
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</tr>
</tbody>
</table>
Table 3. Fatty acid composition of sn-1 and positions of PC from transgenic Arabidopsis seeds of EcCPS43 and EcCPSSfLPAT37. The values represent the mean and standard deviation of at least three replicates.

<table>
<thead>
<tr>
<th></th>
<th>16:0%</th>
<th>18:0%</th>
<th>18:1%</th>
<th>18:2%</th>
<th>CPA%</th>
<th>18:3%</th>
</tr>
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<tbody>
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<td>sn-1</td>
<td>12.7 ±0.4</td>
<td>5.0 ± 0.6</td>
<td>79.2 ± 0.8</td>
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<td>0.0 ± 0.0</td>
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<tr>
<td>sn-2</td>
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<td>6.3 ± 1.5</td>
<td>88.3 ± 1.5</td>
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<td></td>
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<tr>
<td>sn-1</td>
<td>10.7 ± 0.5</td>
<td>5.4 ± 1.3</td>
<td>37.9 ± 1.0</td>
<td>1.2 ± 0.0</td>
<td>45.8 ± 1.0</td>
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<tr>
<td>sn-2</td>
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<td>80.7 ± 1.2</td>
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<tr>
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<tr>
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<td>25.7 ± 1.3</td>
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</table>

Table 4. Fatty acid composition of sn position of TAG from transgenic Arabidopsis seeds of EcCPS43 and EcCPSSfLPAT37. The values represent the mean and standard deviation of at least three replicates.

<table>
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<tr>
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<th>18:1%</th>
<th>18:2%</th>
<th>CPA%</th>
<th>18:3%</th>
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<td>75.7 ± 1.4</td>
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<td>1.2 ± 0.4</td>
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<td>sn-2</td>
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<td>0.0 ± 0.0</td>
<td>93.2 ± 0.8</td>
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<td>4.9 ± 0.8</td>
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<td>67.3 ± 0.3</td>
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<td>8.7 ± 0.2</td>
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<td>0.0 ± 0.0</td>
<td>90.6 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>3.8 ± 0.0</td>
<td>4.6 ± 0.0</td>
<td>0.0 ± 0.0</td>
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<tr>
<td>EcCPSSfLPAT37</td>
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<td>63.2 ± 0.7</td>
<td>2.4 ± 0.1</td>
<td>10.0 ± 0.1</td>
<td>4.1 ± 0.0</td>
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<tr>
<td>sn-2</td>
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<td>0.4 ± 0.3</td>
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<td>4.1 ± 0.1</td>
<td>3.8 ± 0.0</td>
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</tr>
</tbody>
</table>
A

Detector response

YES2

B

Detector response

EcCPS

C

CPA (percent of total FA)

17:0 CPA

19:0 CPA (DHSA)

CFA

17:0 CFA

19:0 CFA

Total CFA

www.plantphysiol.org
A bar graph shows the CPA (percent of total FA) for different treatments. The graph includes Control, EcCPS, and EcCPSSfLPAT treatments. The values for each group are as follows:

- Control: 17
- EcCPS: 38
- EcCPSSfLPAT: 43

The bars for EcCPS and EcCPSSfLPAT are significantly higher than the control, indicating a higher CPA percent in the treated groups compared to the control.