Castor LPCAT and PDAT1A Act in Concert to Promote Transacylation of Hydroxy-Fatty Acid onto Triacylglycerol[1][OPEN]

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Oilseeds produce abundant triacylglycerol (TAG) during seed maturation to fuel the establishment of photoautotrophism in the subsequent generation. Commonly, TAG contains 18-carbon polyunsaturated fatty acids (FA), but plants also produce oils with unique chemical properties highly desirable for industrial processes. Unfortunately, plants that produce such oils are poorly suited to agronomic exploitation, leading to a desire to reconstitute novel oil biosynthesis in crop plants. Here, we studied the production and incorporation of hydroxy-fatty acids (HFA) onto TAG in Arabidopsis (*Arabidopsis thaliana*) plants expressing the castor (*Ricinus communis*) FAH12 hydroxylase. One factor limiting HFA accumulation in these plants is the inefficient removal of HFA from the site of synthesis on phosphatidylcholine (PC). In Arabidopsis, lysophosphatidic acid acyltransferase (LPCAT) cycles FA to and from PC for modification. We reasoned that the castor LPCAT (RcLPCAT) would preferentially remove HFA from PC, resulting in greater incorporation onto TAG. However, expressing RcLPCAT in Arabidopsis expressing FAH12 alone (line CL37) or together with castor acyl:coenzyme A:diacylglycerol acyltransferase2 reduced HFA and total oil yield. Detailed analysis indicated that RcLPCAT reduced the removal of HFA from PC, possibly by competing with the endogenous LPCAT isozymes. Significantly, coexpressing RcLPCAT with castor phospholipid:diacylglycerol acyltransferase increased novel FA and total oil contents by transferring HFA from PC to diacylglycerol. Our results demonstrate that a detailed understanding is required to engineer modified FA production in oilseeds and suggest that phospholipase A2 enzymes rather than LPCAT mediate the highly efficient removal of HFA from PC in castor seeds.

Seeds are the most common form of plant reproduction and supply the necessary reserves to sustain seedlings until they can establish photoautotrophic growth. A critical carbon reserve in seeds of many plant species is oil in the form of triacylglycerol (TAG). Oils from crops like canola (*Brassica napus*) and soybean (*Glycine max*) contain fatty acid (FA) that is a crucial source of human and animal nutrition. Some plant species produce seed oils that contain unique bond structures or side groups that impart unique properties valued in a wide array of industries (Dyer et al., 2008). Unfortunately, the plants that natively accumulate these oils are typically not suited for agronomic exploitation (Ohlrogge et al., 2018). One solution to this problem is to engineer the oil composition of established crop species to produce modified oils for industrial demands (Napier et al., 2014).

Engineering plants to produce desirable industrial oils requires understanding the biosynthesis and incorporation of modified FA onto TAG. We use hydroxy-fatty acid (HFA) production in Arabidopsis (*Arabidopsis thaliana*) as an exemplar of how plants synthesize and incorporate modified FA onto TAG. The fatty acid hydroxylase enzyme (RcFAH12) from castor (*Ricinus communis*) introduces a hydroxy group at the Δ12 position of oleic acid (18:1) attached to phosphatidylcholine (PC; van de Loo et al., 1995). Expressing RcFAH12 in the seed of Arabidopsis did result in some synthesis of the desired HFA product, but in quantities far below castor (Kumar et al., 2006; Lu et al., 2006). Additionally, the accumulation of HFA resulted in lower oil yields (Bates et al., 2014) and reduced storage lipid mobilization during seedling establishment (Lunn et al., 2018b).

Analysis of the acyl fluxes involved in seed TAG synthesis in RcFAH12-expressing Arabidopsis revealed that reduced flux of HFA from PC into TAG synthesis limited the accumulation of HFA (Bates et al., 2012). This bottleneck in the processing of HFA-PC resulted in a feedback mechanism that reduced de novo FA synthesis, consequently lowering the amount of seed oil (Bates et al., 2014). Expressing genes known to elevate oil levels somewhat alleviated this effect (Adhikari

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et al., 2016; Lunn et al., 2018a), implying the need for mechanisms to not only synthesize HFA on PC but to also efficiently transfer the modified FA through the reactions of lipid metabolism into TAG. Overexpressing Arabidopsis enzymes that incorporate acyl groups onto TAG produced no gains in HFA or oil accumulation (Burgal et al., 2008; van Erp et al., 2011; Hu et al., 2012). However, expressing the castor homologs of these enzymes raised HFA yield and partially alleviated low oil levels (Burgal et al., 2008; van Erp et al., 2011). Furthermore, coexpressing multiple TAG assembly enzymes increased novel FA, even producing TAG with three HFA moieties (Lunn et al., 2019). These experiments demonstrate that the expression of castor TAG assembly enzymes overcomes production barriers by preferentially utilizing HFA-containing substrates and lipid intermediates.

In wild-type Arabidopsis, polyunsaturated FA are synthesized on PC by activities of the fatty acid desaturases 2 and 3 (Wallis and Browse, 2010). Three pathways move newly synthesized polyunsaturated FA from PC toward TAG precursors. Most directly, transacylation by phospholipid:diacylglycerol acyltransferase (PDAT) transfers an acyl moiety from PC to diacylglycerol, forming TAG (Dahlqvist et al., 2000). A second pathway removes the choline head group from PC, forming diacylglycerol by either phosphatidylcholine:diacylglycerol cholinephosphotransferase (Lu et al., 2009), phospholipase C, or phospholipase D. An acyl group is then attached to PC-derived diacylglycerol by acyl-coenzyme A:diacylglycerol acyltransferase (DGAT) or PDAT to produce TAG. Lastly, FA may be removed from PC by phospholipase A enzymes or by lysophosphatidylcholine acyltransferase (LPCAT). The phospholipase A reaction releases a free FA for lipid synthesis after conversion to acyl-CoA by long-chain acyl-CoA synthetases (Shockey et al., 2002). LPCAT catalyzes the reversible interconversion of lysophosphatidylcholine and acyl-CoA with PC. The forward reaction utilizes acyl-CoA to acylate the *sn*-2 position of lysophosphatidylcholine forming PC. The reverse reaction removes acyl moieties from the same position of PC, producing lysophosphatidylcholine and acyl-CoA (Bates et al., 2012). By incorporating acyl groups for desaturation on PC and releasing others back to acyl-CoA pool, LPCAT enzymes greatly influence FA composition in the seed oil.

Since the reduced flux of HFA from PC into TAG synthesis limits novel FA yield, research has focused on expressing castor homologs of enzymes involved in these three pathways. Expression of *RcPDAT1A* in hydroxy-accumulating Arabidopsis yielded a 60% increase in the proportion of HFA and a substantial increase in seed oil content (van Erp et al., 2011). However, the use of castor isozymes from the remaining pathways for HFA mobilization from PC has been far less successful. Coexpression of castor phosphatidylcholine:diacylglycerol cholinephosphotransferase or phospholipase C resulted in only modest increases in HFA and seed oil (Hu et al., 2012; Aryal and Lu, 2018), while castor phospholipase D reduced HFA accumulation (Tian et al., 2020). Expression of castor phospholipase A enzymes to liberate HFA from PC also produced mixed results, with significantly higher oil levels but drastically reduced proportions of HFA (Bayon et al., 2015; Lin et al., 2019). One possible reason for these results is that Arabidopsis seeds cannot activate unesterified HFA released from PC by the castor phospholipase A to HFA-CoA. We reasoned that castor LPCAT (RcLPCAT) might be an efficient enzyme for releasing HFA from PC because it produces HFA-CoA directly. The forward reaction of RcLPCAT would incorporate 18:1 onto LPC providing high levels of substrate for hydroxylation. The reverse reaction would then liberate HFA from PC, producing HFA-CoA for incorporation into TAG by acyltransferases of the Kennedy pathway (Lunn et al., 2019). Thereby, the reverse RcLPCAT reaction would simultaneously mitigate the inhibition of de novo FA while increasing HFA yield.

In this report, we demonstrate the surprising effects of RcLPCAT expression on FA flux and TAG synthesis. Coexpression of RcLPCAT and RcFAH12 reduced both HFA accumulation and total oil content. Expression of RcLPCAT together with the acyl-CoA dependent acyltransferase RcDGAT2 in hydroxylase-expressing Arabidopsis also reduced HFA yield and oil levels. However, coexpression of RcLPCAT and RcPDAT1A increased both the proportion of HFA and seed oil content. These results indicate that RcLPCAT and RcPDAT1A act in concert to promote transacylation of HFA from PC onto TAG.

RESULTS

Coexpression of RcLPCAT with *RcFAH12* Reduces HFA Accumulation

We reasoned that RcLPCAT would increase HFA-PC turnover by removing HFA from the site of synthesis at the *sn*-2 position of PC. Using a BLAST search of the castor genome database with the protein sequences of either AtLPCAT1 or AtLPCAT2 returns only a single hit (30190.m011126; our RcLPCAT), with a probability of 1.1e-142. We amplified this sole castor LPCAT, whose predicted protein sequence had close similarity to AtLPCAT2 (AT1G63050), from a cDNA library. We subcloned this coding sequence, under the control of the strong seed-specific β-conglycinin promoter (Sebastiani et al., 1990), into a plant transformation vector with dsRed selection We transformed the well-characterized FAH12-expressing Arabidopsis line, CL37 (*fae1*_RcFAH12; Lu et al., 2006), with this RcLPCAT expression construct. Using dsRed fluorescence, we identified 30 independent T1 transformants and grew them to maturity. To determine seed fatty acid composition, we selected red T2 seeds, generated fatty acid methyl esters (FAME), and analyzed these by gas chromatography (GC). Compared with the parental CL37 line,

transformation with *RcLPCAT* reduced the proportion of HFA in nearly every case (Supplemental Fig. S1A). These CL37:RcLPCAT lines were designated LPT. We cultivated three independent lines (LPT4, LPT15, and LPT29) with low HFA levels and whose marker segregation ratio in T2 seed (approximately 3 red:1 brown) indicated a single genomic insertion site. During the cultivation of homozygous T3 plants, we collected developing seed and confirmed strong *RcLPCAT* expression using reverse transcription quantitative PCR (RT-qPCR; Supplemental Fig. S1B). Consistent with previous reports (Lu et al., 2006), analysis of FAME showed that mature CL37 seed contained 17.2% ± 0.2% HFA, with isogenic segregant seed statistically similar (Supplemental Fig. S1C). However, each homozygous line expressing RcLPCAT had significantly less HFA, averaging only 12.2% ± 0.2% (4), 12.4% ± 0.2% (15), and 12.5% ± 0.4% (29; Fig. 1A). Collectively, these data show that RcLPCAT expression in CL37 substantially reduced HFA accumulation.

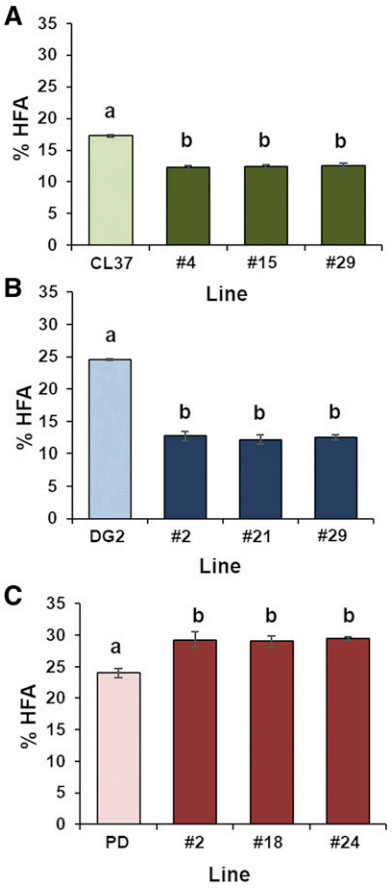

Figure 1. Accumulation of HFA in various *RcLPCAT* expression lines. A, LPT. B, DG2_LPT. C, PD_LPT. Transgenic lines are colored as follows: *fae1*:RcFAH12 (CL37; light green), CL37:RcLPCAT (LPT; dark green), CL7:RcDGAT2 (DG2; light blue), CL7:RcDGAT2_RcLPCAT (DG2_LPT; dark blue), CL37:PDAT1a (PD; light red), and CL37:RcPDAT1a_RcLPCAT (PD_LPT; dark red). Error bars indicate SD with $n = 3$ independent replicates of seed pooled from 25 plants. Statistical analysis was performed using a one-way ANOVA and posthoc Tukey's test ($P < 0.001$), with lowercase letters applied to columns to mark significance.

Coexpression of RcLPCAT and RcDGAT2 Reduces HFA Accumulation

The reduced HFA in the LPT lines might result from poor utilization of HFA-CoA in the absence of an acyltransferase with substrate preference toward HFA. To test this hypothesis, we transformed our RcLPCAT expression construct into the previously characterized hydroxy-accumulating Arabidopsis line coexpressing RcDGAT2 (CL7:RcDGAT2, referred to here as DG2). The RcDGAT2 enzyme preferentially incorporates HFA-CoA onto diacylglycerol to produce TAG (Burgal et al., 2008). We cultivated 30 independent T1 transformants and harvested mature seed for FA analysis via FAME preparation and GC. Surprisingly, while the parental DG2 line averaged 25.5% ± 0.9% HFA, all the RcLPCAT transgenic lines contained significantly lower proportions of HFA (Supplemental Fig. S2A). These transgenics were designated DG2_LPT, and three lines with low HFA and 3:1 segregation of the DsRed marker (lines DG2_LPT21, DG2_LPT22, and DG2_LPT29) were cultivated and developing seeds analyzed to confirm the presence of *RcLPCAT* transcript (Supplemental Fig. S2B). Then, we analyzed the FA composition of samples of mature T3 seed from homozygous T2 plants. Control DG2 seed contained an average of 24.5% ± 0.1% HFA, similar to previously published values (Burgal et al., 2008), with each isogenic segregant similar to the parent (Supplemental Fig. S2C). However, each homozygous DG2_LPT line had significantly lower HFA levels than DG2, at 12.7% ± 0.7% (21), 12.1% ± 0.6% (22), and 12.5% ± 0.3% (29; Fig. 1B), indicating that even in the presence of an acyltransferase with substrate preference for HFA-CoA, RcLPCAT expression still reduces HFA accumulation.

Coexpression of RcLPCAT with RcPDAT1A Increases HFA Accumulation

The reductions observed in both LPT and DG2_LPT led us to question whether RcLPCAT was efficient in producing HFA-CoA as a substrate for incorporation by RcDGAT2 or endogenous Arabidopsis acyltransferases into TAG. We decided to express RcLPCAT in a well-characterized HFA-accumulating Arabidopsis line coexpressing the castor phospholipid:diacylglycerol acyltransferase, RcPDAT1A (line CL37:RcPDAT1A, referred to here as PD), which transacylates HFA from PC onto diacylglycerol to produce TAG (van Erp et al., 2011). We speculated that if RcLPCAT is effective at removing HFA from PC, it will compete with RcPDAT1A for the HFA-PC substrate, leading to lower HFA accumulation. We expressed RcLPCAT using a vector conferring BASTA resistance, since line PD already contained a dsRed marker linked to the RcPDAT1A

transgene. Expression of *RcLPCAT* in the pPhas-BAR:RcLPCAT construct is under the control of the phaseolin promoter (Slightom et al., 1983), with expression levels similar to β-conglycinin (Shockey et al., 2015). We selected 30 independent T1 transformants and collected mature T2 seed from each plant for FA analysis. Compared with the PD controls that averaged 24% ± 0.1% HFA, 21 of the 30 transgenics produced T2 seed with significantly increased HFA in the oil (Supplemental Fig. S3A). We identified and grew homozygous PD_LPT lines for three of these transgenics with high HFA, along with isogenic lines lacking the RcLPCAT transgene. During seed maturation, we harvested developing seeds from the homozygous plants and used RT-qPCR to confirm high levels of *RcLPCAT* expression (Supplemental Fig. S3B). In this experiment, the FA composition of mature seed from PD controls averaged 24% ± 0.7% HFA, similar to previously published values (van Erp et al., 2011), and each isogenic segregant line was statistically similar (Supplemental Fig. S3C). In contrast, homozygous plants of the PD_LPT lines exhibited a substantial 20% increase in HFA. Lines PD_LPT 2, 21, and 24 contained 29% ± 1.2%, 29% ± 0.8%, and 29.4% ± 0.2% HFA, respectively, in the mature seeds (Fig. 1C). These data show that, in contrast to our other transgenics, coexpressing RcLPCAT with RcPDAT1A increases HFA accumulation. To further examine these different outcomes, we chose to investigate three lines, LPT29, DG2_LPT29, and PD_LPT24, in more detail.

Expression of RcLPCAT Alters HFA-TAG Accumulation over Time

We reasoned that RcLPCAT expression in LPT and DG2_LPT limited HFA accumulation by reducing the rate of HFA flux through lipid metabolism into TAG. To test this, we determined the HFA-TAG accumulation over time in developing seeds of the RcLPCAT-expressing lines and the corresponding parental controls during oil filling. We grew the plants and collected developing seeds at 8, 10, 12, and 14 d after flowering (DAF). Using thin-layer chromatography (TLC), we separated each lipid species and collected TAG for FAME preparation and GC analysis. Throughout the time course, both LPT and DG2_LPT seeds accumulated less HFA-containing TAG than their respective parents. The accumulation of HFA-TAG in LPT was 12.1% ± 1.2% at 12 DAF, 19% less than the 15% ± 0.4% in TAG from CL37 (Fig. 2A). More dramatically, the DG2_LPT line had only 10.4% ± 0.2% HFA in TAG at 12 DAF, a 47% reduction compared with the 19.6% ± 0.3% for the DG2 parental line (Fig. 2B). In contrast to these decreases, line PD_LPT showed higher HFA-TAG accumulation at each time point, averaging 25.7% ± 0.3% at 12 DAF, 25% higher than the 20.6% ± 0.6% HFA in TAG found in the seed of PD (Fig. 2C).

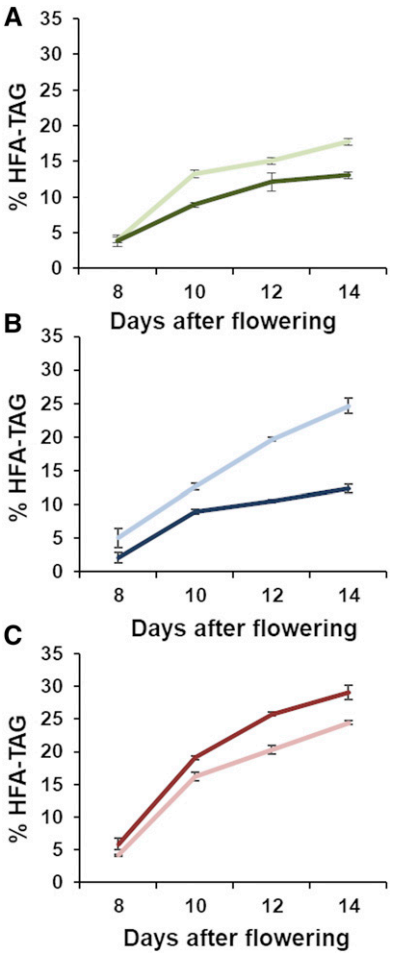

Figure 2. Accumulation of HFA-TAG during oil filling. A, LPT. B, DG2_LPT. C, PD_LPT. Transgenic lines are colored as follows: *fae1*:RcFAH12 (CL37; light green), CL37:RcLPCAT (LPT; dark green), CL7:RcDGAT2 (DG2; light blue), CL7:RcDGAT2_RcLPCAT (DG2_LPT; dark blue), CL37:RcPDAT1a (PD; light red), and CL37:RcPDAT1a_RcLPCAT (PD_LPT; dark red). Error bars indicate SD with $n = 3$ independent replicates of seeds pooled from 30 siliques of 10 plants.

Expression of RcLPCAT Alters Oil Content

Inefficient utilization of HFA-containing lipids during seed maturation results in FAH12-expressing CL37 plants having lower oil content (Bates et al., 2014), but coexpression of some TAG-assembly isozymes from castor alleviates this effect (Lunn et al., 2019). We considered the possibility that RcLPCAT activity in our transgenics might increase oil content if it partially alleviated the bottleneck in HFA flux through lipid metabolism in the seeds. To examine this possibility, we grew LPT, DG2_LPT, and PD_LPT along with the corresponding parental lines and *fae1* as controls. Mature seeds from *fae1* looked round and uniform in size, while many CL37 seeds were shrunken and misshapen (Fig. 3A), consistent with previous reports (Lunn et al., 2019), indicating lower oil content. Despite having a lower proportion of HFA in the seed oil than CL37, LPT

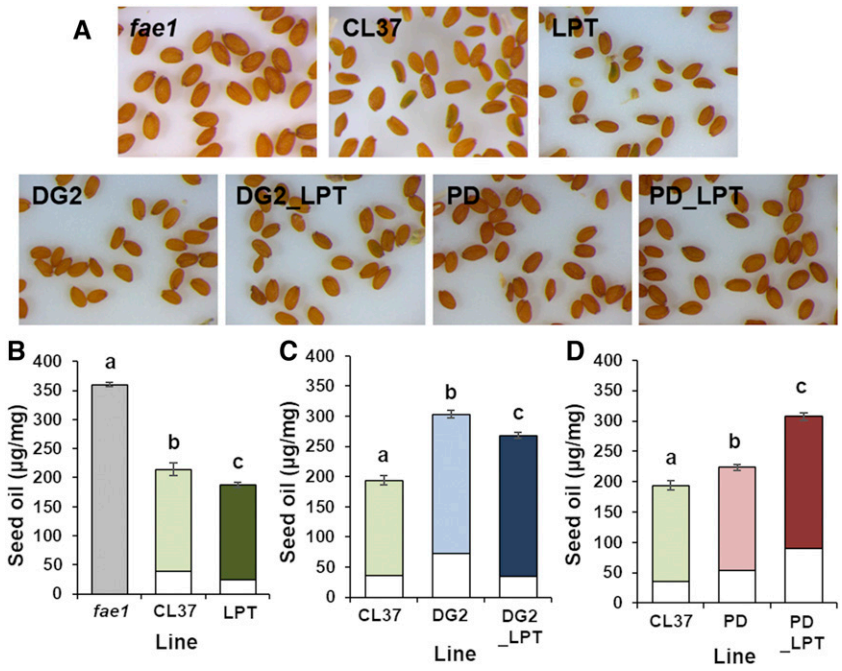

Figure 3. Seed oil content in *RcLPCAT* expression lines. A, Representative images of mature seed in all lines. B to D, Total oil per mg of seed in LPT (B), DG2_LPT (C), and PD_LPT (D). Transgenic lines are colored as follows: *fae1* (gray), *fae1*:RcFAH12 (CL37; light green), CL37:RcLPCAT (LPT; dark green), CL7:RcDGAT2 (DG2; light blue), CL7:DGAT2_LPCAT (DG2_LPT; dark blue), CL37:RcPDAT1a (PD; light red), and CL37:RcPDAT1a_RcLPCAT (PD_LPT; dark red), with white sections indicating HFA content. Error bars indicate SD with $n = 3$ independent replicates of seed pooled from 25 plants. Statistical analysis was performed using a one-way ANOVA ($P < 0.001$) with posthoc Tukey's test, with lowercase letters applied to columns to mark significance.

seeds surprisingly remained shrunken and misshapen. Seeds of DG2 and DG2_LPT appeared misshapen only rarely, with many plump seeds similar in size and appearance to seed of *fae1*. Seeds from PD were often misshapen, while RcLPCAT expression in PD_LPT relieved this phenotype so that PD_LPT seeds were largely indistinguishable from *fae1* (Fig. 3A). To quantify the differences in oil content, we generated FAME from mature seed, including a known quantity of 17:0 TAG as an internal control. The oil content of each parental line was comparable to previously published data (Lunn et al., 2019), with the *fae1* parent containing 359.8 ± 3.6 µg of oil per mg of seed. The RcFAH12-expressing CL37 had 40% less oil, averaging 214.4 ± 10.3 µg mg^{-1}, while LPT was further reduced by 12%, at only 187.7 ± 4.8 µg mg^{-1} (Fig. 3B). Seed from DG2 contained 303.5 ± 6.3 µg mg^{-1}, with DG2_LPT also significantly reduced by 12%, to 268.2 ± 4.7 µg mg^{-1} (Fig. 3C). However, RcLPCAT expression in PD produced a substantial 37% increase in oil, from 223.6 ± 4.7 µg mg^{-1} in PD to 307.2 ± 6.2 µg mg^{-1} in PD_LPT (Fig. 3D). Contrary to expectation, the expression of RcLPCAT in the LPT and DG2_LPT lines resulted in both a lower proportion of HFA and a lower oil content. Only expressing RcLPCAT along with RcPDAT1A in the PD_LPT line resulted in higher HFA and oil content.

We analyzed the molecular species of HFA-TAG from mature seed in each RcLPCAT expression line. We used TLC to separate TAG bands corresponding to 0-, 1-, and 2-HFA moieties for FAME analysis (Supplemental Fig. S4), using an internal standard to determine the percentage contribution of each TAG species. We discounted 3-HFA-TAG from our calculation, as it accounted for less than 1% of the total TAG in each line. The seed of LPT contained 66.7% ± 1.7% 0-HFA-TAG, a 30% increase over the 51.5% ± 2.3% found in CL37 seed. The increased 0-HFA-TAG accompanied a 39% reduction in 1-HFA-TAG, from 40% ± 1.5% in CL37 to 24.6% ± 2.8% in LPT, but no significant change in 2-HFA-TAG (Fig. 4A). Expressing RcLPCAT in DG2 also significantly increased 0-HFA-TAG by 68%, from 35.5% ± 2.7% to 59.5% ± 0.3%. This accompanied a 34% reduction in 1-HFA-TAG, from 39.2% ± 3.6% to 25.7% ± 1.6%, as well as a 39% reduction in 2-HFA-TAG, from 24.6% ± 1.8% to 14.9% ± 1.1% (Fig. 4B). The TAG species in PD and PD_LPT seed revealed a different pattern, with 0-HFA-TAG declining 19%, from 40.8% ± 1.6% to 32.9% ± 2.3%. Paired with this decline in 0-HFA-TAG was a 28% increase in 1-HFA-TAG, from 38.7% ± 0.5% to 53.7% ± 2.1%; there was no significant change in 2-HFA-TAG (Fig. 4C). These data imply that RcLPCAT expression decreases HFA availability for TAG synthesis in the LPT and DG2_LPT lines but increases it in PD_LPT.

Altered Stereochemical Distribution of HFA in 1-HFA-TAG

The altered levels of 1-HFA-TAG in our three RcLPCAT expression lines implied a shift in the balance of HFA incorporation through different TAG biosynthesis pathways. Since acyltransferases incorporate acyl groups at a specific location, the distribution of HFA moieties on 1-HFA-TAG indicates the flux through TAG assembly. To investigate this dynamic, we extracted lipids from mature seeds of our six parental and RcLPCAT-expressing lines and collected 1-HFA-TAG by TLC. We determined the stereochemistry by digesting 1-HFA-TAG with a lipase that specifically

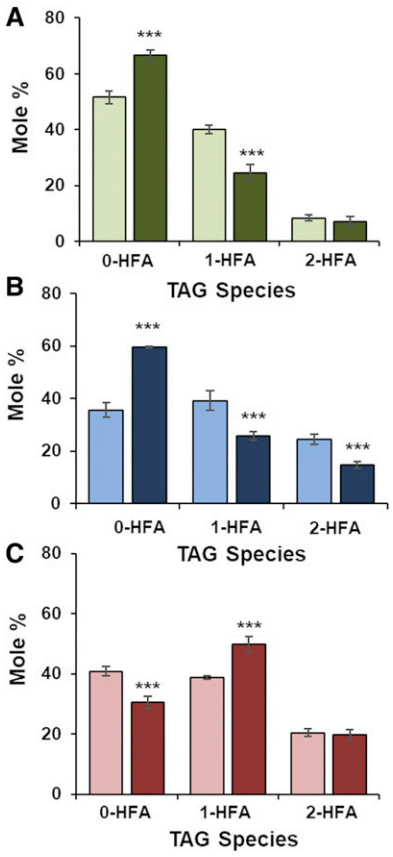

Figure 4. The proportion of each HFA-TAG species in RcLPCAT expression lines. All graphs display the 0-, 1-, and 2-HFA-TAG in LPT (A), DG2_LPT (B), and PD_LPT (C). Transgenic lines are colored as follows: *fae1*:RcFAH12 (CL37; light green), CL37:RcLPCAT (LPT; dark green), CL7:RcDGAT2 (DG2; light blue), CL7:RcDGAT2_RcLPCAT (DG2_LPT; dark blue), CL37:RcPDAT1a (PD; light red), and CL37:RcPDAT1a_RcLPCAT (PD_LPT; dark red). Error bars indicate SD with $n = 3$ independent replicates of seeds pooled from 25 plants. Statistical analysis was performed using Student's *t* test, with asterisks denoting significance (***$P < 0.001$) compared with the parental line.

removes FA from the *sn*-1/3 position of TAG and quantifying the free FA and monoacylglycerol products. In CL37, 1-HFA-TAG contained 70.2% ± 0.8% HFA at the *sn*-2 position, while LPT seeds increased the proportion by 14%, to 80.1% ± 0.8% (Fig. 5A). Consistent with RcDGAT2 biochemical action, HFA in DG2 had lower *sn*-2 and higher *sn*-1/3 levels than CL37, averaging 59.5% ± 0.8% and 40.4% ± 0.8%, respectively. However, RcLPCAT expression in DG2_LPT reversed this shift, with 24.4% ± 1% and 75.5% ± 1% HFA at the *sn*-1/3 and *sn*-2 positions, respectively (Fig. 5B). Coexpression of RcPDAT1A with the FAH12 hydroxylase in PD provided for a substantial increase in HFA at *sn*-1/3, from 29.8% ± 0.8% in CL37 to 43% ± 0.6%. Interestingly, PD_LPT was the only RcLPCAT expression line without increased HFA incorporation at the *sn*-2 of 1-HFA-TAG relative to its parental control line. Instead, it exhibited a 24% increase in HFA at the *sn*-1/3 position, to 53.4% ± 2% (Fig. 5C). Collectively, these data are consistent with RcLPCAT expression causing a decrease in HFA flux from PC into the acyl-CoA pool and a relatively increased flux through PDAT transfer to the *sn*-3 position of TAG.

Altered Levels of HFA Accumulation in PC during Seed Maturation

Hydroxylase-expressing transgenic oilseeds exhibit a negative correlation between oil content and the level of HFA in PC during seed development (van Erp et al., 2011; Hu et al., 2012; Snapp et al., 2014; Aryal and Lu, 2018; Lunn et al., 2019). The results of our stereochemical analysis of 1-HFA-TAG raised the possibility that rather than facilitating HFA transfer from PC to HFA-CoA, RcLPCAT expression reduced this transfer. This would be consistent with the LPT and DG2_LPT lines having reductions in oil content, relative to the

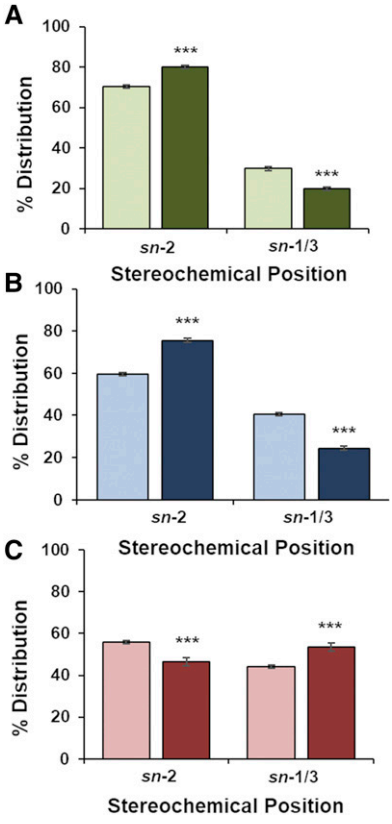

Figure 5. Stereochemical distribution of 1-HFA-TAG in *RcLPCAT*-expression lines. All graphs display the proportion of HFA at the *sn*-2 and *sn*-1/3 positions of 1-HFA-TAG in LPT (A), DG2_LPT (B), and PD_LPT (C). Transgenic lines are colored as follows: *fae1*:RcFAH12 (CL37; light green), CL37:RcLPCAT (LPT; dark green), CL7:RcDGAT2 (DG2; light blue), CL7:RcDGAT2_RcLPCAT (DG2_LPT; dark blue), CL37:RcPDAT1a (PD; light red), and CL37:RcPDAT1a_RcLPCAT (PD_LPT; dark red). Error bars indicate SD with $n = 3$ independent replicates of seeds pooled from 25 plants. Statistical analysis was performed using Student's *t* test, with asterisks denoting significance (***$P < 0.001$) compared with the parental line.

corresponding parental lines, because of increased HFA on PC exacerbating the bottleneck that causes feedback inhibition of de novo fatty acid synthesis (Bates and Browse, 2011). In the PD_LPT line, RcPDAT1A would foster an alternative route for the removal of HFA from PC, potentially allowing for the increased oil content we observed in this line relative to the PD parental line.

Using developing seeds from our RcLPCAT expression lines, we determined the proportion of HFA on PC at 8, 10, 12, and 14 DAF (Fig. 6). We extracted lipids from these seeds and separated the PC fraction using TLC, followed by FAME analysis. Levels of HFA-PC in CL37 rose continually through the time course, reaching 10.4% ± 0.5% at 14 DAF. In LPT, the proportion of HFA-PC was higher throughout the time course, totaling 12.2% ± 0.4% at 14 DAF (Fig. 6A). Similarly, DG2_LPT contained significantly higher HFA in PC than the parental DG line, reaching 12.1% ± 0% at 14 DAF compared with 9.7% ± 0.3% in DG2 (Fig. 6B). However, the results for PD and PD_LPT are similar at

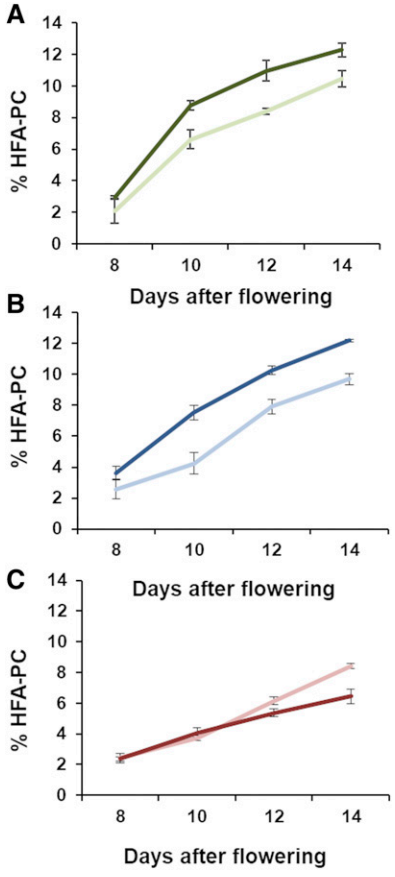

Figure 6. The proportion of HFA in PC during oil filling. All graphs display HFA-PC accumulation in LPT (A), DG2_LPT (B), and PD_LPT (C). Transgenic lines are colored as follows: *fae1*:RcFAH12 (CL37; light green), CL37:RcLPCAT (LPT; dark green), CL7:RcDGAT2 (DG2; light blue), CL7:RcDGAT2_RcLPCAT (DG2_LPT; dark blue), CL37:RcPDAT1a (PD; light red), and CL37:RcPDAT1a_RcLPCAT (PD_LPT; dark red). Error bars indicate SD with $n = 3$ independent replicates of seeds pooled from 30 siliques of 10 plants.

8 and 10 DAF and then show less HFA in the PC of PD_LPT. The HFA in PC at 14 DAF averaged 6.4% ± 0.4% for PD_LPT, a reduction of 24% relative to the 8.4% ± 0.1% for PD seeds (Fig. 6C). Collectively, these results are consistent with RcLPCAT expression reducing rather than increasing the flux of HFA from PC into HFA-CoA.

DISCUSSION

Understanding the biochemistry of TAG assembly and utilization is essential for producing industrially useful oils in crop plants. Research in this area has led to important discoveries about the enzymology, organization, and flux dynamics of lipid metabolism in oilseeds (Bates and Browse, 2011; Bates et al., 2014; Napier et al., 2014; Haslam et al., 2016; Lunn et al., 2019). We have used Arabidopsis expressing the oleoyl-PC hydroxylase from castor (lines CL37 and CL7) as a model to identify strategies to improve the low yields of HFA and other modified FA in transgenic plants. One advance in this area was that castor isozymes are often more effective at producing and processing HFA-containing lipid substrates and intermediates than the endogenous Arabidopsis homologs (Burgal et al., 2008; van Erp et al., 2011; Bayon et al., 2015; Lunn et al., 2019). A second was our discovery that poor metabolism of HFA-containing lipids creates a bottleneck that leads to inhibition of acetyl-CoA carboxylase and FA synthesis, lowering the overall yield of seed oil (Bates et al., 2014). Poor conversion of HFA-containing PC limits both HFA yield and accumulation of seed oil in FAH12-expressing Arabidopsis.

Considering the previous discoveries, we believed that expressing the castor LPCAT homolog in hydroxy-accumulating Arabidopsis seeds might enhance the release HFA from PC into the acyl-CoA pool, making it available for TAG synthesis by enzymes of the Kennedy pathway. Furthermore, the efficient removal of HFA from PC by RcLPCAT would also increase oil production by alleviating the feedback inhibition of FA synthesis. Surprisingly, the expression of RcLPCAT in both CL37 and DG2 backgrounds markedly reduced both HFA content (Fig. 1, A and B) and HFA accumulation in TAG throughout seed development (Fig. 2, A and B). In contrast to these results, RcLPCAT when coexpressed with RcPDAT1A elevated the rate of HFA accumulation and the final proportion of HFA in the seed oil, relative to PD seeds (Figs. 1C and 2C), indicating concerted action between these two enzymes.

Interestingly, the reduced HFA levels in LPT (CL37_RcLPCAT) and DG2_LPT (CL7_RcDGAT2_RcLPCAT) lines were paired with lower total oil content, relative to CL37 and DG2, respectively (Fig. 3). By contrast, PD_LPT (CL37_RcPDAT1A_RcLPCAT) plants had both a higher proportion of HFA and increased total oil content compared with the PD control (Fig. 3C). By comparison, experiments with transgenic expression of several other acyltransferases (including RcDGAT2 and RcPDAT1A),

by us and others, have reported a positive correlation between increases in the proportion of HFA and total oil content (Cahoon et al., 2006; Burgal et al., 2008; Bates et al., 2014; Snapp et al., 2014; van Erp et al., 2014; Yu et al., 2018; Lunn et al., 2019; Shockey et al., 2019). Here, again, the results for RcLPCAT are contrary to expectations.

Transgenic Arabidopsis lines expressing the FAH12 hydroxylase alone contain high levels of HFA in PC during the period of seed oil synthesis (van Erp et al., 2011, 2014; Hu et al., 2012; Lunn et al., 2019). Additionally, many Arabidopsis lines engineered to produce other novel FA also display a correlation between high levels of the desired product in PC and low seed oil (Cahoon et al., 2006; Fahy et al., 2013; Snapp et al., 2014; Yu et al., 2018). The result is a bottleneck in seed lipid metabolism that results in feedback inhibition of acetyl-CoA carboxylase and lowered oil yields (Bates and Browse, 2011; Bates et al., 2014). The coexpression of castor acyltransferase isozymes with FAH12 has typically resulted in lowered HFA-PC levels paired with increased oil (van Erp et al., 2014; Lunn et al., 2019). However, expression of RcLPCAT in both LPT and DG2_LPT resulted in higher proportions of HFA in PC than in the corresponding parental line throughout oil filling (Fig. 6, A and B) and in reduced oil contents (Fig. 3, B and C). In contrast, the coexpression of RcPDAT1A in PD_LPT produced an increase in seed oil content relative to the PD line (Fig. 3D) coupled with some reduction in HFA-PC at 12 and 14 DAF (Fig. 6C). These results indicate that HFA in PC is increased upon RcLPCAT expression, resulting in lower yield and exacerbating the metabolic bottleneck and feedback inhibition of acetyl-CoA carboxylase observed in CL37 (Bates and Browse, 2011; Bates et al., 2014).

In seeking an explanation for these results, it is useful to recognize that RcLPCAT in our transgenic lines is competing with the endogenous Arabidopsis AtLPCAT1 and AtLPCAT2 isozymes (Bates et al., 2012). Additionally, previous work (Lager et al., 2013) on the substrate specificity and selectivity of Arabidopsis and castor LPCAT enzymes expressed in yeast indicated that RcLPCAT is considerably less efficient at removing HFA from *sn*-2 of PC than AtLPCAT2. Based on these observations, our data allow us to present a model (Fig. 7) that explains the changes we observe in each of our RcLPCAT expression lines. Here, newly synthesized 18:1 from the plastid is loaded onto lysophosphatidylcholine to form PC by LPCAT acylation. Hydroxylation of the resulting *sn*-2-18:1 PC occurs via RcFAH12, producing *sn*-2-HFA-PC. We propose that a base flux of HFA from PC to the acyl-CoA pool in CL37 can be somewhat enhanced in DG2 plants by the efficient utilization of HFA-CoA by the RcDGAT2 isozyme (Burgal et al., 2008). However, RcLPCAT expression in LPT and DG2_LPT lowers either or both the selectivity and activity for HFA-PC. The result is increased retention of HFA on PC and reduced availability of HFA-CoA for incorporation into TAG by either endogenous Arabidopsis acyltransferases or RcDGAT2 (Fig. 7A). This competition-based reduction in HFA flux from

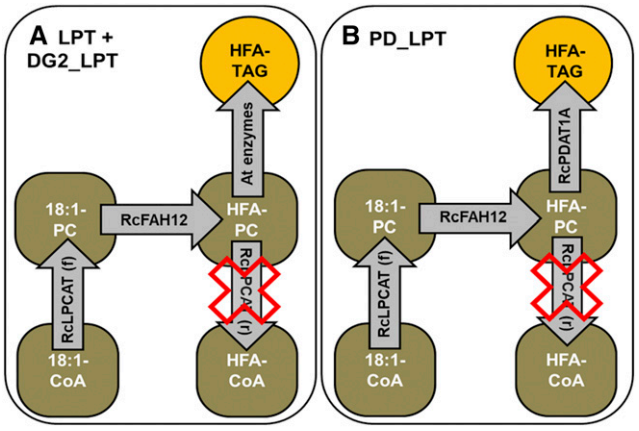

Figure 7. The flux of HFA onto TAG in hydroxy-accumulating Arabidopsis lines expressing RcLPCAT. A, Acyl flux of HFA in CL37:RcLPCAT (LPT) and CL7:RcDGAT2_RcLPCAT (DG2_LPT). B, Acyl flux of HFA in CL37:RcPDAT1a_RcLPCAT (PD_LPT). Gray arrows denote enzyme fluxes and indicate the direction of HFA-containing precursors toward TAG formation. Red crosses show highly reduced activity due to substrate preference of RcLPCAT. 18:1-CoA, Oleoyl-CoA; 18:1-PC, oleoyl PC. Enzymes are as follows: RcLPCAT with the acylation (r) and deacylation (f) reactions, RcFAH12, RcPDAT1A, and endogenous Arabidopsis enzymes (At enzymes).

PC to HFA-CoA will also occur in PD_LPT plants relative to PD. However, the presence of RcPDAT1A efficiently transfers HFA from PC to the *sn*-3 position of diacylglycerol (Fig. 7B). Therefore, in PD_LPT, both enzymes act synergistically to enhance HFA-TAG synthesis and alleviate the feedback inhibition caused by HFA-PC.

The stereochemical analysis of 1-HFA-TAG from our different lines shows this shift in metabolic flux (Fig. 5). Determining the number and location of HFA moieties in TAG reflects the relative fluxes of HFA from PC to TAG. In hydroxylase-expressing Arabidopsis, 1-HFA-TAG with the HFA moiety located at the *sn*-2 position is the predominant TAG molecular species (van Erp et al., 2015; Lunn et al., 2019; Shockey et al., 2019). This distribution indicates that the flux of HFA into TAG occurs through the interconversion of HFA-PC to HFA-diacylglycerol. The increased proportions of HFA at *sn*-2 in LPT and DG2_LPT indicate reduced availability of HFA-CoA for incorporation at the *sn*-1/3 positions by endogenous acyltransferases and RcDGAT2. The reduction of HFA-CoA and increased *sn*-2-HFA-PC (containing HFA at *sn*-2) causes the dominant flux of HFA onto TAG being through the interconversion of *sn*-2-HFA-diacylglycerol by phosphatidylcholine:diacylglycerol cholinephosphotransferase in LPT and DG2_LPT. Comparatively, PD plants have an increased proportion of HFA at *sn*-1/3, reflecting the selectivity for HFA and transacylation action of RcPDAT1A. In PD_LPT, RcLPCAT lowers HFA in the acyl-CoA pool, leading to further HFA-PC substrate for transacylation at the *sn*-3 position of TAG.

Castor seeds have up to 90% HFA in TAG, with most of oil accumulated in the endosperm. Although

synthesized at the *sn*-2 position of PC, the proportion of HFA-PC is under 5%, with no HFA at the *sn*-1 position (Donaldson and Beevers, 1977; van de Loo et al., 1995). One reason for excluding HFA from the PC could be to maintain membrane structure and function. Instead, the rapid turnover of HFA-PC to either HFA-CoA or transacylation onto diacylglycerol occurs by enzymes of the Kennedy pathway (Bafor et al., 1991) and PDAT (van Erp et al., 2011). The reverse reaction of LPCAT is the most direct way to transfer HFA moieties from PC to the acyl-CoA pool. However, our data and in vitro assays (Lager et al., 2013) indicate that this route is unlikely to have a significant role. Although we cannot exclude the possibility that these results do not reflect the RcLPCAT kinetics in castor endosperm, they do suggest an alternative route for removing HFA from PC.

Three possible routes exist for the cycling of HFA into TAG, either releasing the acyl-CoA, interconversion of PC to diacylglycerol, or through direct transfer via PDAT. Since our work indicates that RcLPCAT is not a major contributor and phospholipase A enzymes reduced HFA accumulation, it is possible that the conversion of PC to diacylglycerol is most important. However, while the expression of castor phospholipase C and phosphatidylcholine:diacylglycerol cholinephosphotransferase did increase HFA accumulation (Hu et al., 2012; Aryal and Lu, 2018), the conversion of PC to diacylglycerol would not allow the formation of 3-HFA-TAG. Therefore, it is more likely that, despite lowering HFA in hydroxy-accumulating Arabidopsis, phospholipase A enzymes are the principal mode of FA flux out of PC, followed by the synthesis of HFA-CoA by a long-chain acyl-CoA synthetase. Transcript levels support this hypothesis, with phospholipase A enzymes in castor endosperm being particularly high during oil filling (Troncoso-Ponce et al., 2011; Horn et al., 2016; Sturtevant et al., 2019). The expression of phospholipase A2α and AIIIβ in hydroxy-accumulating Arabidopsis substantially lowered HFA levels in PC but also significantly reduced HFA content of the seed oil (Bayon et al., 2015; Lin et al., 2019). These data suggest that phospholipase A enzymes efficiently remove HFA from PC in both castor and transgenic Arabidopsis. However, Arabidopsis lacks a long-chain acyl-CoA synthetase enzyme capable of activating the unesterified FA product to HFA-CoA for reentry into TAG synthesis. To conclude, these results show how RcLPCAT interacts with other enzymes to shape the FA composition of seed oil and highlight its role in the removal of HFA from PC.

MATERIALS AND METHODS

Plant Growth Conditions

We dispersed Arabidopsis (*Arabidopsis thaliana*) seeds on plates with one-half strength Murashige and Skoog nutrients (Sigma), 0.75% (w/v) agar, and 1% (w/v) Suc at pH 5.7. After growth under 100 μmol m^{-2} s^{-1} continuous light at 22°C for 12 d, we transferred plantlets to the soil and continued cultivation until senescence under 120 μmol m^{-2} s^{-1} continuous white light from broad-spectrum fluorescent lamps at 22°C with 70% humidity.

Statistical Analyses

We analyzed data with GraphPad Prism (GraphPad Software) using either one-way ANOVA with posthoc Tukey's test or Student's *t* test.

Cloning and Transformation of *RcLPCAT*

We amplified the RcLPCAT coding sequence from a castor (*Ricinus communis*) developing seed cDNA library with KOD hot-start polymerase (Novagen) using primers designed to append *Eco*RI and *Xho*I restriction sites. Using the manufacturer's instructions, we ligated isolated amplicons into ZeroBlunt D-TOPO (Invitrogen). We compared the sequence of a single clone against the reference genome available at http://blast.jcvi.org/er-blast/index.cgi?project=rca1 for fidelity. We liberated the *Eco*RI-*RcLPCAT*-*Xho*I fragment and ligated it into the same sites in transformation vector β-conglycinin_dSRed. We constructed the BASTA RcLPCAT expression vector using Gateway cloning. We amplified RcLPCAT using primers to append CACC directly upstream of the coding sequence. We then subcloned the sequence into pENTR-D-TOPO (Invitrogen) and confirmed the fidelity of a single colony. Using the entry vector, we performed the LR reaction per the manufacturer's instructions and confirmed the presence of the construct from a single colony by PCR. We transformed *Agrobacterium tumefaciens* strain GV3101 with the expression vector and isolated a single clone for the transformation of Arabidopsis lines CL37 (Lu et al., 2006), CL7:RcDGAT2 (Burgal et al., 2008), and CL37:RcPDAT1A (van Erp et al., 2011) by floral dip (Clough and Bent, 1998).

RNA Extraction and cDNA Synthesis

We harvested developing Arabidopsis siliques at 10 DAF and flash froze them in liquid nitrogen. Seeds were released from siliques as described (Bates et al., 2013). RNA was extracted from seeds with the RNeasy Plant Mini Kit (Qiagen), and the quality and quantity were determined by nanophotomer (Implen). We normalized RNA from each extraction to 100 ng μL^{-1} and produced cDNA using the SuperScript III First-Strand Synthesis System (Life Technologies). RT-qPCR used Platinum SYBR Green (Invitrogen) per the manufacturer's instructions, with analysis conducted with a RealPlex Mastercycler (Eppendorf).

Determination of Seed Oil

We determined fatty acid proportions, seed oil, and composition of TLC samples by the preparation of FAME followed by GC analysis. Quantification of FAME by GC used flame ionization detection from a wax column (EC Wax; 30 m $\times$ 0.53 internal diameter. $\times$ 1.2 μm; Alltech) with parameters of 210°C for 1 min followed by a ramp to 250°C at 10°C min^{-1}, with a final 9-min temperature hold. The comparative analysis of FA composition used 15 to 20 μg of seed from each line. We measured oil content by coderivatization of a 17:0 TAG standard and seed of known weight. Calculation of total oil used the ratio of oil to 17:0 TAG added before derivation and normalization to the seed sample weight.

Accumulation of HFA-PC and HFA-TAG during Seed Development

We cultivated plant lines as above until the first six siliques emerged. We then scored each subsequent silique at the first visible white tinge as day 1. Counting continued until each independent replicate accounted for 100 siliques at 8, 10, 12, and 14 DAF. We extracted seeds by immersing siliques in liquid nitrogen followed by rapid thawing to break open the wall (Bates et al., 2013). We extracted lipids from the seeds and separated TAG and PC using TLC with a single development in a solvent system of chloroform:methanol:acetic acid (75:25:8, v/v/v). We dried the silica plate under nitrogen and stained with 0.005% (w/v) primulin in 80% (v/v) acetone for visualization under UV light, collected bands from our sample corresponding to standards for TAG and PC, and prepared FAME for analysis by GC.

Lipid Extraction

We heat quenched 15 to 20 μg of mature seed with isopropanol containing 0.01% (w/v) butylated hydroxytoluene (1 mL) for 15 min at 85°C. After incubation, we thoroughly homogenized the heat-quenched seeds with ground glass homogenizers. Rinsing with methanol (3 mL) and chloroform (2 mL) recovered all lipid material. The addition of water (1.6 mL), chloroform (2 mL), and 0.88% (w/v) KCl (2 mL) stimulated phase separation, which we consolidated by centrifugation at 4,383*g* for 2 min. We collected the chloroform layer and then performed two further back-extractions for maximum recovery. We removed the chloroform by drying under nitrogen and resuspended the samples in toluene containing 0.05% (w/v) butylated hydroxytoluene (0.5 mL). We capped the test tubes and stored the extracted lipids at −20°C to prevent oxidation until analysis.

Characterization of TAG Species

To extract lipids, we sampled 20 mg of dried mature seed from each line. Using solvents containing 0.005% (w/v) butylated hydroxytoluene, we separated individual lipid species by TLC (Silica gel 250; 20 × 20 cm; Analtech). Quantification of the number of hydroxy groups in TAG species was determined using two developments (12 and 19 cm) in a solvent system first of chloroform:methanol:acetic acid (93:3:1, v/v/v), then of chloroform:methanol:acetic acid (99:0.5:0.5, v/v/v; Bates and Browse, 2011). Plates were dried for 15 min under vacuum after each development. We visualized lipid bands stained with 0.005% (w/v) primulin in 80% (v/v) acetone under UV light and collected species corresponding to known standards for GC analysis.

Stereochemical Analysis

We separated extracted lipids from 50 mg of dried mature seed and isolated bulk TAG using TLC in a single development, using a solvent system of chloroform:acetone:acetic acid (96:3.5:0.5, v/v/v). Bulk TAGs were collected and eluted from the silica with two washes of chloroform:methanol (4:1, v/v). We induced phase separation with methanol (2 mL) and 0.88% (w/v) KCl (4 mL), followed by a 2-min centrifugation at 4,383*g*. The chloroform layer was collected, and a single back-extraction with chloroform (5 mL) was conducted. We removed chloroform by drying the samples under nitrogen and resuspending in toluene containing 0.05% (w/v) butylated hydroxytoluene (0.5 mL). We dried lipid samples of 50 μL under nitrogen and resuspended in diethyl ether (1 mL) and 0.8 mL of buffer (50 mM sodium bromide, pH 7.6, and 5 mM calcium chloride) in the presence of *Rhizomucor miehei* lipase (Sigma). The reaction proceeded for 40 min under constant vortex mixing and was quenched with 2 mL of 1:1 (v/v) methanol:chloroform. The chloroform layer was collected and lipids separated by TLC with a single development in a solvent system of chloroform:methanol:acetic acid (97.5:2:0.5, v/v/v). We vacuum dried the plate and stained with 0.005% (w/v) primulin in 80% (v/v) acetone and then visualized under UV light, before collecting the TAG and monoacylglycerol bands for analysis by GC.

Accession Numbers

Sequence data for castor LPCAT can be found in the UniProt database under accession number EEF51096.1. Sequence data for castor PDAT1A and DGAT2 can be found in the National Center for Biotechnology Information library under accession numbers XM_002514026 and EU391592, respectively. Sequence data for Arabidopsis LPCAT1 and LPCAT2 can be found in the Araport data library under accession numbers At1g12640 and At1g63050.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Analysis of CL37 expressing RcLPCAT.

Supplemental Figure S2. Analysis of DG2 expressing RcLPCAT.

Supplemental Figure S3. Analysis of PD expressing RcLPCAT.

Supplemental Figure S4. Representative picture of hydroxy-accumulating Arabidopsis lipid extract separation on TLC.

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