Specialized lysophosphatidic acid acyltransferases contribute to unusual fatty acid accumulation in exotic *Euphorbiaceae* seed oils

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Main conclusion

In vivo and *in vitro* analyses of *Euphorbiaceae* species' triacylglycerol assembly enzymes substrate selectivity is consistent with the co-evolution of seed-specific unusual fatty acid production, and suggests that many of these genes will be useful for biotechnological production of designer oils.

Abstract

Many exotic *Euphorbiaceae* species, including tung tree (*Vernicia fordii*), castor bean (*Ricinus communis*), *Bernardia pulchella*, and *Euphorbia lagascae*, accumulate unusual fatty acids in their seed oils, many of which have valuable properties for the chemical industry. However, various adverse plant characteristics including low seed yields, production of toxic compounds, limited growth range, and poor resistance to abiotic stresses have limited full agronomic exploitation of these plants. Biotechnological production of these unusual fatty acids (UFA) in high yielding non-food oil crops would provide new robust sources for these valuable bio-chemicals. Previous research has shown that expression of the primary UFA biosynthetic gene alone is not enough for high-level accumulation in transgenic seed oils; other genes must be included to drive selective UFA incorporation into oils. Here we use a series of *in planta* molecular genetic studies and *in vitro* biochemical measurements to demonstrate that lysophosphatidic acid acyltransferases from two *Euphorbiaceae* species have high selectivity for incorporation of their respective unusual fatty acids into the phosphatidic acid intermediate of oil biosynthesis. These results are consistent with the hypothesis that unusual fatty acid accumulation arose in part via co-evolution of multiple oil biosynthesis and assembly enzymes that cooperate to enhance selective fatty acid incorporation into seed oils over that of the common fatty acids found in membrane lipids.

Keywords

diacylglycerol acyltransferase

eleostearic acid

ricinoleic acid

lysophosphatidic acid acyltransferase

triacylglycerol

Abbreviations

DGAT	diacylglycerol acyltransferase
ESA	eleostearic acid
FADX	tung tree fatty acid conjugase X
FAH	castor fatty acid hydroxylase
FAME	fatty acid methyl ester
FID	flame ionization detection
GPAT	glycerol-3-phosphate acyltransferase
HFA	hydroxy fatty acids
HPLC	high-performance liquid chromatography
GC	gas chromatography
LPA	lysophosphatidic acid
LPAT	lysophosphatidic acid acyltransferase
PCR	polymerase chain reaction
PDAT	phospholipid:diacylglycerol acyltransferase
PDCT	phosphatidylcholine:diacylglycerol cholinephosphotransferase
TLC	thin layer chromatography
UFA	unusual fatty acid

Introduction

Plant triacylglycerols (TAGs) are a major component of human and animal nutrition. Some plant oils are also useful in the production of various industrial feedstocks and specialized products, including inks, dyes, biodiesel, nylons and plastics. Most edible oil crops have been heavily domesticated, and produce oils containing a limited set of fatty acids that are compatible with cooking and digestion, but of limited utility in the industrial sector. A central goal of green chemistry is to produce industrially-useful fatty acids in microbes or agronomic oilseed crops. Surveys of naturally occurring exotic plant species going back decades have provided hundreds of examples of seed oils containing unusual fatty acids (UFAs) with useful chemical properties, including differing chain-lengths, numbers and positions of carbon-carbon double and triple bonds, and a wide assortment of side-chain functionalities (Gunstone et al. 2007). For our laboratories, key among these are fatty acids produced in the oils of tung tree (*Vernicia fordii*, Hemsl.) and castor bean (*Ricinus communis*), which contain ~80% α-eleostearate (ESA, a conjugated trienoic acid) and ~90% ricinoleate (HFA, a hydroxylated fatty acid), respectively. As of 2013-2014, approximately 645,000 tons of castor oil and ~93,000 tons of tung oil were produced world-wide (McKeon 2016; Shockey et al. 2016). Global production of these two oils is relatively small in relation to that of major commodity oils such as soybean or canola, but given the value of the specialized products that can be produced from them, there is still keen industrial interest to create safe, stable domestic sources of oils containing these functionalities at agronomic scale.

The seminal work from Somerville's laboratory (van de Loo et al. 1995) established that many novel fatty acids in exotic plant species are produced by diverged forms of *fatty acid desaturase-2* (*FAD2*), an otherwise common *FAD* found in all plants. Since that time, proof of concept studies describing the creation and characterization of *Arabidopsis* lines expressing other diverged *FAD2*-like genes, including tung tree linoleate conjugase *FADX* (Dyer et al. 2002; van Erp et al. 2015) or the castor oleate hydroxylase *FAH12* (Lu et al. 2006) have appeared. However, seed lipids in these lines contain only <20% ESA or HFA. An increasingly sophisticated suite of available biotechnological tools have been applied to the analysis of common and exotic oilseeds in recent years. As our collective understanding of the molecular details underlying the processes of fatty acid and triacylglycerol metabolism has gradually advanced, secondary studies have begun to examine the effects of stacking additional genes from the exotic host plants into transgenic lines bearing the primary biosynthetic genes. Co-expression of castor or tung diacylglycerol acyltransferases (DGATs) or phospholipid:diacylglycerol acyltransferases (PDATs, Dahlqvist et al., 2000), two

enzymes which catalyze terminal steps in the TAG biosynthetic pathway, resulted in significant increases in novel fatty acid accumulation (Burgal et al. 2008; van Erp et al. 2011; van Erp et al. 2015), as did expression of phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT), an enzyme that affects TAG precursor pool fatty acid composition via PC:DAG interconversion (Hu et al. 2012). These results and others (Kroon et al. 2006) demonstrated that along with the diverged *FAD2*s, other genes in the exotic species have co-evolved to efficiently utilize substrates containing the UFA.

The secondary studies represented progress, but still only resulted in a maximum of ~30% of the desired product, which is far less than the levels found in native seeds. These results clearly show that much work remains to be done to acquire the full complement of knowledge and genetic tools necessary to achieve predictable metabolic engineering outcomes resulting in marketable new plant products. Our recent efforts have been dedicated to expanding the molecular toolkit even further, with a particular focus on the enzymes that catalyze some of the 'intermediate' steps in these pathways. We describe here the characterization of lysophosphatidic acid acyltransferase (*LPAT*, E.C. 2.3.1.51) genes from tung tree and castor. LPAT catalyzes the transfer of acyl groups from acyl-CoA to the *sn*-2 position of 1-acyl-*sn*-glycerol 3-phosphate (lysophosphatidic acid, LPA). In the ER membrane, the phosphatidic acid (PA) product of the LPAT reaction acts as a precursor for phospholipid biosynthesis (via cytidine diphosphate diacylglycerol or CDP-DAG) or undergoes dephosphorylation by phosphatidic acid phosphatase, yielding DAG, which in turn serves as a substrate for the synthesis of membrane lipids such as phosphatidylcholine and phosphatidylethanolamine, and TAG by way of DGAT or PDAT, reactions (Li-Beisson et al. 2013). As such, the substrate specificities and other biochemical properties of the relevant LPAT isoforms could exert significant influence over the final fatty acid composition of seed oils. Previous studies have shown that plants contain a large, complex family of *LPAT* genes (Bourgis et al. 1999; Kim et al. 2005). The use of acyl-CoA selective *LPAT*s from plants such as coconut and *Cuphea sp*. to accumulate medium chain fatty acids (10-14 carbon chains) in TAG of transgenic plants have demonstrated their value to plant oil engineering (Iskandarov et al. 2017; Kim et al. 2015; Knutzon et al. 1999). Here we have focused on tung and castor *LPAT2* genes. Analysis of the effects of tung and castor LPAT2 enzymes in seed oil metabolism are investigated through expression in both yeast and plants. The relatively fluid degree of functional importance of *LPAT2*, compared to other members of this gene family, is also discussed.

Materials and methods

Gene identification

Tung *DGAT2* and castor *DGAT2* were originally identified as previously described (Shockey et al. 2006; Burgal et al. 2008). Tung *LPAT2* (*VfLPAT2*) was identified from the developing tung seed cDNA libraries described in Pastor et al. (2012), and submitted to Genbank (accession# MH823254). Partial cDNA sequences for *RcLPAT2* and *RcLPAT3A* were originally identified by PCR from a developing castor seed cDNA library using degenerate primers, followed by 5' and 3' rapid amplification of cDNA ends (RACE) to identify the remaining gene fragments, as described previously (Burgal et al. 2008), and have since been identified by Arroyo-Caro et al. (2013).

Plant seed expression plasmid construction

The ORFs for *VfLPAT2* and *RcLPAT2* was amplified by PCR (Phusion polymerase, New England Biolabs, Ipswich, MA, USA), using primers that added *Not*I and *Sac*II sites to the 5'and 3' ends, respectively. All plasmid construction made use of the plant expression vector sets described in Shockey et al. (2015). Purified PCR products for *VfLPAT2* were digested and ligated into either cloning vector pB49 (*At2S-3* promoter, *N*-terminal hemagglutinin (HA) epitope tag) or pK37 (*Phaseolin* promoter, *N*-terminal myc epitope tag) to form shuttle plasmids pB206 and pB318, respectively, promoter:gene:terminator cassettes for which in turn were added into either *FADX* binary plasmid pE29 or *FADX+VfDGAT2* binary plasmid pE259 (built from *FADX* binary plasmid pE116) (Shockey et al. 2015) to produce finished binary plasmids pE188 and pE318. The ORF for *RcLPAT2* was cloned into cloning vector pB35 (beta-conglycinin promoter, no epitope tag) to produce shuttle plasmid pB554. The *Asc*I fragment representing the promoter:gene:terminator cassette from B554 was ligated alone into binary vector pB110 (pE660) or into *RcDGAT2* binary plasmids pE542 and pE565 to generate finished binary plasmids pE659 and pE658, respectively. Finally, castor *GPAT9* (*RcGPAT9*, Genbank accession #EU391594) (Burgal et al. 2008) was cloned into cloning vector pK50 (*glycinin-1* promoter, no epitope tag) to generate shuttle plasmid pB544 then transferred to the *Pac*I site of pE659 to generate finished three-gene binary plasmid pE678. All shuttle plasmids were sequenced to confirm amplification accuracy, and all binary plasmids were mapped with at least two combinations of restriction enzymes to confirm overall structural integrity. The important details for each plasmid described here are summarized in Table 1, and graphical representations of each are shown in Supplemental Figure S1.

Plant growth and transformation

ESA was produced in seeds of the *Arabidopsis fad3fae1* double mutant by overexpression of the tung tree fatty acid conjugase *FADX* (Dyer et al. 2002; Smith et al. 2003; van Erp et al. 2015). The parental line producing hydroxy fatty acids (HFA) for these studies is CL37 (Lu et al. 2006) which expresses the castor hydroxylase *FAH12*, producing ~17-20% HFA of total seed lipids. Finished plant binary plasmids were transformed into *Agrobacterium tumefaciens* strains GV3101 or C58-C1 by electroporation. Colonies were selected on solid media containing kanamycin and gentamycin, grown in liquid culture and transformed into the appropriate *Arabidopsis* lines by floral dip (Clough and Bent 1998).

Screening independent transformant T_2 seeds by gas chromatography

Many independent transformant lines were screened for seed fatty acid composition at the T_2 generation. Fatty acid methyl esters (FAMEs) were prepared from *Arabidopsis* seeds containing HFA by incubation in 5% sulfuric acid in methanol at 85-90 °C for 1-1.5 h in glass tubes sealed with Teflon®-lined caps, followed by quenching with saturated sodium chloride solution and extraction into hexane, approximately as described by Li et al. (2006). FAMEs from ESA-containing seeds were prepared using sodium methoxide at room temperature, as described previously (van Erp et al. 2015). FAMEs were analyzed by gas chromatography (GC) on an Agilent Technologies (Santa Clara, CA, USA) 7890B gas chromatograph with flame ionization detection (FID) using split injection on a 60 m X 0.25 mm inner diameter, 0.2 μm SP-2380 column (Supelco, Sigma-Aldrich, St. Louis, MO, USA). HFA were analyzed with a temperature program of 215-250 ˚C at 5 ˚C/min, followed by 2 min hold at 250 ˚C. Samples containing ESA were analyzed with a temperature program of 170-215 ˚C at 4 ˚C/min, followed by 6 min hold at 215 ˚C. One or two individual lines that produced high levels of the respective unusual fatty acids in segregating T_2 seed samples, and segregated at ~3:1 ratio for presence:absence of the appropriate selectable marker were chosen for further analysis.

HPLC, Regiochemistry, and GC analysis of HFA-containing TAG species

Lipids were extracted as described by Hara and Radin (1978), with some modifications. In brief, 10 mg seed samples were quenched in 1 ml 2-propanol at 87 °C for 10 min, ground with a polytron, and mixed with solvent to achieve a final extract ratio of hexane/2-propanol/H_2O, 6:4:0.5 (v/v/v). The phases were separated by adding 3.5 ml of 6.6% (w/v) aqueous sodium sulphate. The organic phase was collected and the aqueous phase was back-extracted with 3 ml

of hexane:2-propanol (7:2, v/v). Lipid extract was concentrated under N_2 gas and dissolved in toluene for separation by high-performance liquid chromatography (HPLC), which was performed on a Thermo Fisher Ultimate 3000 equipped with: quaternary pump, temperature controlled auto sampler, temperature controlled column compartment, variable wavelength detector, and fraction collector. HPLC analysis was based on previously described methods (Kotapati and Bates 2018). In brief, lipid class separation was carried out on a YMC-Pack PVA-Sil column (250 X 4.6 mm, 5 µm particle size) at 35 ºC in normal phase mode by UV detection at 210 nm. The sampler compartment was maintained at 20 ºC. Crude lipid (500-900 µg) dissolved in toluene was loaded onto the column in 15 µL. The total run time was 30 min, and the column was equilibrated for 15 min prior to each injection. The method gradient was set in such a manner that after all TAGs (hydroxy and non-hydroxy) were separated and collected, any polar lipids present in the sample would be flushed from the column. Standards were used to determine retention times for time-based fraction collection. Solvents (Fisher Scientific Optima Grade) used were: 2-propanol, hexanes, methanol, 2-propanol/water/acetic acid (60/40/0.065, v/v/v). HPLC method gradient parameters and fraction collection parameters are shown in Supplementary Tables S1 and S2, respectively. TAG fractions and polar lipid fractions were collected and solvent removed under a stream of nitrogen, prior to FAME production with 2.5% sulfuric acid in methanol at 85 ºC for 1 h. After addition of 0.2 ml hexane and 1.5 ml 0.9% KCl, the organic phase used directly for GC. FAMEs were analyzed on a Shimadzu GC-2010 Plus with a FID, equipped with a Stabilwax® Crossbond® Carbowax® Polyethylene Glycol column (30.0m long, 0.25mm ID, 0.25 µm film thickness). Samples were injected at a 1:10 split ratio at 250 ºC. The carrier gas was Helium at a constant linear velocity of 35cm/sec. The initial column temperature was held at 175 ºC for 2 min, increased 10 ºC/min to 250 ºC and held for 7 min. The FID was set to 255 ºC. The regiochemical analysis of 1-HFA-TAG was performed as in van Erp et al. (2011).

Yeast expression plasmid construction, transformation, and microsome isolation

The ORFs for *RcLPAT2*, *RcLPAT3A*, and *Arabidopsis LPAT2* were amplified as described above, using primers that added *Not*I and *Pac*I sites to the 5' and 3' ends, respectively. Purified PCR products were digested and ligated into multiple cloning site 1 of the yeast expression vector pESC-URA (Agilent Technologies, Santa Clara, CA, USA). Sequenced plasmids were transformed into the *Saccharomyces cerevisiae ale*Δ mutant (*Mata, his3*Δ*1, leu2Δ0, met15*Δ*0, ura3*Δ*0, ale1*::KANMX) (Ståhl et al. 2008). The empty vector was included as a negative control. Colonies

were selected on solid synthetic uracil drop-out medium containing glucose, then grown overnight at 30°C in liquid synthetic uracil drop-out medium containing 2% glucose. The yeast cells were then transferred to medium supplemented with 2% galactose and grown until OD_{600nm} 3-4 to induce protein expression. Yeast microsomal membranes were prepared as described in Lager et al. (2013).

Substrates for enzyme assays

[1-^{14}C]Oleic acid was purchased from Perkin Elmer and [1-^{14}C]ricinoleic acid was synthesized enzymatically from [1-^{14}C]oleate using microsomal preparations from developing castor bean according to the method described by Bafor et al. (1991). *sn*-1-oleoyl-LPA was synthesized by chemical acylation of oleic acid to glycerol-3-phosphate by the method described by Kanda et al. (1981). Ricinoleoyl-LPA was synthesized from di-ricinoleoyl-PC (a generous gift from ENI/Metapontum Agrobios, Metaponto, Italy) as follows: 1.2 µmol di-ricinoleoyl-PC was dissolved in 200 µl diethylether in a test tube with screw cap with a magnetic flea. One ml of 0.1M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)/10 mM $CaCl_2$ pH 5.6 containing 400 units phospholipase D (from peanut, Sigma Aldrich) was added and the tube was purged with nitrogen, then capped and stirred rapidly at 30 °C. Samples (10 µl) were withdrawn at 1 h intervals and spotted on small (5 x 5 cm) thin layer chromatography (TLC) plates with PC as a standard. The plates were developed in chloroform:methanol:acetic acid:water ($CHCl_3$:MeOH:HAc:H_2O, 85:15:10:3.5, v/v/v/v) and stained with iodine vapor. After 3 h incubation only phosphatidic acid (PA) was seen, with no trace of PC. PA was then extracted into chloroform by adding 3.75 ml MeOH:$CHCl_3$(2:1), 1.25 ml $CHCl_3$ and 1 ml 0.15M HAc and the upper phase was washed once with 2.5 ml $CHCl_3$. The chloroform phases were combined in a test tube with a spin magnet, the chloroform was evaporated with N_2 and the residue dissolved in 200 µl of ether. One ml of 0.1 M Tris-HCl pH 8.9/10 mM $CaCl_2$, containing 400 units of phospholipase A_2 (PLA_2, from *Naja mossambica*, Sigma Aldrich), was added to the ether. The tube was purged with nitrogen, capped and incubated under stirring at 30 °C. Samples (10 µl) were withdrawn at ~12 h intervals and checked on TLC for the conversion of PA into LPA. Four hundred additional units of PLA_2 were added at each check. After about 36 h, no PA remained and the ether was evaporated under a stream of N_2 and 30 µl glacial acetic acid was added to the solution, which was then extracted with 4 x 2 ml of hexane to remove the free fatty acids. After removal of residual hexane from the buffer phase under nitrogen after the last extraction, the LPA was extracted with 3 x 2 ml of water-saturated *n*-butanol. The

butanol phases was combined and evaporated and the residue (ricinoleoyl-LPA) was dissolved in 400 µl of methanol for storage before use in the assays.

Enzyme assays

LPAT activities were assayed by incubating microsomal membranes (corresponding to 8 µg of microsomal protein) prepared from *ale1*Δ yeast transformed with plasmids bearing RcLPAT2, RcLPAT3A, *Arabidopsis* LPAT2, or empty vector with 5 nmol LPA (dissolved in buffer) and 10 nmol of [^{14C}]acyl-CoA in 0.1M phosphate buffer, pH 7.2 in a final volume of 100 µl at 30 °C. The incubations were terminated with addition of 100 µl of 0.15 M HAc and 500 µl of $CHCl_3$:MeOH (1:1, v/v). An aliquot of chloroform phase was analyzed directly by liquid scintillation and the rest was concentrated under nitrogen, spotted on TLC plates (Merck, silica 60) and developed with $CHCl_3$:MeOH:HAc:H_2O (85:15:10:3.5, v/v/v/v). The relative amount of radioactive PA was determined by electronic autoradiography (Instant Imager, Packard Canberra). The total amount of produced radioactive PA was calculated from the relative amounts of PA in the chloroform phase and total amount of radioactivity as determined by liquid scintillation. The reaction was linear between 2 and 4 min using the best substrate combination for each enzyme. Further assays were therefore done for 3 min. All assays were done in triplicate.

Results

Evidence for a positive role for VfLPAT2 in ESA accumulation in transgenic *Arabidopsis*

The positive influence of tung DGAT2 or castor DGAT2 on their respective UFA levels in transgenic *Arabidopsis* seeds has already been established (Burgal et al. 2008; van Erp et al. 2015). We sought to identify additional enzymes that act upstream of the DGAT reaction to further enhance levels of the target fatty acids. We tested this idea first by comparing the levels of ESA produced by plants expressing the tung conjugase *VfFADX* alone (Dyer et al. 2002), to that found in lines containing *VfFADX* and *VfDGAT2*, and to lines containing *VfFADX*, *VfDGAT2*, and *VfLPAT2* (Table 1). Multiple independent transgenic T_1 plants for each of the three constructs were grown to maturity, followed by GC analysis of the segregating T_2 seed samples from each of the independent transformants (Fig. 1). *VfFADX* alone produced just 4.7 weight % ESA on average, while *VfDGAT2* co-expression resulted in a slight increase in average ESA levels to 5.6%. Inclusion of *VfLPAT2*, on the other hand, increased ESA levels significantly from the base *VfFADX* line ($p = 0.0061$), with peak performing lines reaching nearly 13% ESA (Fig. 1).

The influence of *VfLPAT2* was assessed independently by comparing seed ESA levels produced from transgenic lines expressing either *FADX* alone and one containing *FADX* and *VfLPAT2* (Table 1). The difference between the mean ESA values between these two populations was highly significant (Fig. 2, unpaired student's t-test, $p<0.0001$). These two sets of data strongly indicated that tung *LPAT2* is a useful tool for engineering ESA production in *Arabidopsis* seed lipids.

These results encouraged us to extend our analyses to castor *LPAT2*. Detailed biochemical analyses with tung enzymes is hampered by the difficulty of preparing ESA-containing substrates, due to ESA's sensitivity to light, high temperature, and acidic pH. On the other hand the hydroxylated fatty acids (HFA) produced by castor (a related *Euphorbiaceae* species) are significantly easier to work with *in vitro*, due to its enhanced chemical stability relative to ESA. Therefore, study of the castor enzymes allows us to combine both *in vitro* yeast and *in vivo* plant experiments to better understand UFA selectivity.

The effect of various *Ricinus communis* acyltransferases on the production of HFA-containing TAG species in transgenic *Arabidopsis* seeds

The starting point for HFA production in *Arabidopsis* is the previously characterized CL37 line that expresses the castor fatty acid hydroxylase (*RcFAH12*) and produces two HFA, ricinoleic acid and densipolic acid, which together accumulate to ~15-20% of fatty acids in CL37 seeds (Lu et al. 2006). We first compared the ability of castor acyltransferase to influence seed lipid HFA levels in CL37 plants by expressing an empty vector, *RcDGAT2*, or co-expressing *RcLPAT2* and *RcDGAT2* (Fig. 3a). The empty vector line was essentially the same as previous reports for CL37, indicating that the vector has no effect on HFA accumulation. The positive role of *RcDGAT2* in transgenic HFA accumulation has already been established (Burgal et al. 2008); and a new construction of *RcDGAT2* (containing the strong, seed-specific *At2S-3* promoter and a *N*-terminal myc epitope fusion to the protein coding sequence) used here generated very similar results, with a significant increase in total seed HFA levels compared to empty binary vector controls in segregating T_2 seeds from multiple independent T_1 transformants (Fig. 3a). Importantly, expression of *RcLPAT2* alone (Fig. 3b) , or co-expression of *RcLPAT2* with *RcDGAT2* (Fig. 3a) in the CL37 background resulted in additional significant increases in HFA levels. Interestingly, the co-expression of the three acyltransferase enzymes of the Kennedy pathway (GPAT, LPAT, DGAT) from castor in CL37 did not further increase the segregating T_2 seed HFA content (Fig. 3).

In oilseed research, whole seed FAME analysis correlates well to seed oil content (oils compose 90-95% of total seed lipid) but it does include fatty acids found in other seed lipids, such as membrane lipids, diacylglycerols, and, when using some types of catalysts (such as the sulfuric acid/methanol-based approach used for HFA FAME synthesis in this study), free fatty acids as well. Therefore, to learn more about how the expression of these enzymes specifically affected the fatty acid composition of TAG, we quantified how much HFA were found in purified TAG and determined the relative ratios of TAG species containing different numbers of HFA per TAG molecule in each of the transgenic lines. Fig. 4a quantifies the relative amount of the three HFA-containing TAG species (TAGs containing 1-, 2-, or 3-HFA per molecule, no stereochemical location specified) and 0-HFA-TAG in each transgenic line from homozygous T_3 or T_4 seeds. The complete fatty acid composition of each TAG species is shown in Supplementary Fig. S2, and for each TAG species the composition was similar between all transgenic lines. However, the relative amount of each TAG species differed between the control CL37 and the new transgenic lines. When *RcLPAT2* alone is co-expressed with the fatty acid hydroxylase in *Arabidopsis* seeds the amount of 0-HFA-TAG is significantly reduced ($p = 0.0132$), concomitant with a significant increase in the level of 1-HFA-TAG ($p = 0.0449$), and an increase

in total seed TAG HFA content from 20.2% ± 0.3% to 27.4 ± 3.7% (Fig. 4b). This result suggests that the products of the RcLPAT2 reaction are selectively incorporated into TAG in *Arabidopsis* seeds.

Co-expression of *RcDGAT2* alone in the CL37 background also has a similar but more pronounced effect, with the significant increase in total TAG HFA content (Fig. 4b) coming from a large reduction in 0-HFA-TAG (p = 0.0003), and increases in both 2-HFA-TAG (p = 0.0312) and 1-HFA-TAG (p = 0.0993). When the three main acyltransferases of the Kennedy pathway from castor are co-expressed, the least amount of 0-HFA-TAG is observed (9.8% ± 5.2%) and the highest level of 1-HFA-TAG accumulates (68.8% ± 3.9%), but 2-HFA-TAG is not significantly different than expression of *RcDGAT2* alone (Fig. 4a). Total TAG HFA content in the homozygous *RcGPAT9/LPAT2/DGAT2* line was 37.2 ± 2.4%, which is almost double the 20.2 ± 0.3% observed in the CL37 background line (Fig. 4b). Interestingly, very little 3-HFA-TAG accumulated in these lines.

Effect of RcLPAT2 on total seed oil content

Previously, we have demonstrated that production of HFA in line CL37 causes a reduction in seed lipid content from 35-40% of dry weight in wild-type *Arabidopsis* to approximately 20-25% of dry weight in CL37 (van Erp et al. 2011; Bates et al. 2014; Adhikari et al. 2016; Karki and Bates 2018). Metabolic labeling studies have indicated that the reduced seed oil content of CL37 is due to inefficient utilization of HFA by *Arabidopsis* enzymes, which induces the down-regulation of acetyl-CoA carboxylase activity and thus total fatty acid synthesis (Bates et al. 2014; Bates and Browse 2011). In addition, more efficient utilization of HFA by co-expression of castor enzymes for the last step in TAG assembly (RcDGAT2 or RcPDAT1) alleviates the reduced acetyl-coA carboxylase activity and increases oil content (Bates et al. 2014). Here, the negative effect of HFA on seed oil accumulation in CL37 was almost completely alleviated by the addition of *RcLPAT2* alone. The seed lipid content was significantly increased from 24.2±1.2% dry weight in CL37 to 34.2±1.8% (p = 0.0037) (Fig. 5). The average seed lipid content of the *RcGPAT9/LPAT2/DGAT2* line grown at the same time also increased from that of CL37 to 29.6±2.1% (p = 0.0675) (Fig. 5). The overall performance transgenic Arabidopsis lines for oil accumulation is sensitive to environmental conditions as characterized before (Li et al. 2006; Karki and Bates 2018), and to the composition of the transgenes used; plant growth under lower light intensities than described above, resulted in *RcDGAT2* transgenic seed TAG levels similar to parental CL37, while lower light *RcLPAT2/DGAT2* seeds still did contain significantly more seed lipids than CL37 (Supplemental Fig. S3). These results, in conjunction with the increase in total seed HFA content (Fig. 3) and HFA-

TAG accumulation (Fig. 4), suggests RcLPAT2 more efficiently incorporates HFA-containing substrates into TAG precursors than does the endogenous *Arabidopsis* enzyme (AtLPAT2).

Effect of RcLPAT2 on the regiochemical localization of HFA in TAG

The LPAT reaction places an acyl group into the *sn*-2 position of G3P, to form PA. Unless further modified or removed through acyl editing (Bates 2016), this fatty acid will end up predominantly at the *sn*-2 position of TAG in oilseeds. Therefore, to determine the effect of RcLPAT2 on the localization of HFA within TAG we performed lipase-based regiochemical analysis of the major TAG species (1-HFA-TAG) from parental CL37 and each of the lines co-expressing the castor acyltransferases (Fig. 6). Previously, expression of *sn*-3-specfic castor acyltransferases (*RcDGAT2* or *RcPDAT1a*) in CL37 caused a shift in the 1-HFA-TAG regiochemistry, by reducing the amount of HFA at *sn*-2 and increasing the amount at the *sn*-1/3 positions (van Erp et al. 2011). The current CL37 results were consistent with previous measurements that indicated approximately 70% of the HFA in 1-HFA-TAG molecular species resides at the *sn*-2 position (van Erp et al. 2011). The line expressing *RcLPAT2* in CL37 had essentially the same regiochemical localization for HFA in the 1-HFA-TAG molecular species (Fig. 6). This result indicates that the enhanced production of 1-HFA-TAG induced by *RcLPAT2* expression (Fig. 4, Fig. 5) predominantly accumulates HFA at the *sn*-2 position, as expected. Each of the other lines which contain an *sn*-3 acyltransferase did cause a shift in the regiochemistry from s*n*-2 to *sn*-1/3, as expected from previous results (van Erp et al. 2011). Interestingly, the *RcGPAT9/LPAT2/DGAT2* line which contains both *sn*-1 and *sn*-3 acyltransferases had the most HFA in the *sn*-1/3 position.

Comparison of AtLPAT2 and RcLPAT2 biochemical properties *in vitro*

To further confirm our *in vivo* results suggesting RcLPAT2 more efficiently utilizes HFA substrates than AtLPAT2 we compared the biochemical properties of these two enzymes *in vitro*. We first compared the enzymatic properties of AtLPAT2 and RcLPAT2, using combinations of substrates that would be common in untransformed *Arabidopsis*, as well as those that would be found in native castor plants and in HFA-producing CL37 *Arabidopsis*. Enzyme activities were measured in microsomal fractions prepared from the yeast *ale1*Δ strain overexpressing the enzymes. Although *ALE1* is responsible for the major microsomal LPAT activity in yeast, these microsomes still contain substantial residual LPAT activity, likely catalyzed by the SLC1 enzyme (Jasieniecka-Gazarkiewicz et al. 2017). Both

AtLPAT2 and RcLPAT2 efficiently acylated 18:1-LPA with ricinoleoyl-CoA, whereas 18:1-CoA was hardly used above background activity in the acylation of ricinoleoyl-LPA by AtLPAT2 and RcLPAT2 (Fig. 7). Ricinoleoyl-CoA was efficiently acylated to ricinoleoyl-LPA by the castor enzyme whereas the AtLPAT2 did not exhibit any significant activity with the same combination of substrates. As expected, the *Arabidopsis* enzyme was effective in acylating 18:1-CoA to 18:1-LPA while RcLPAT2, interestingly, had no significant activity with this substrate combination (Fig. 7), indicating that castor has another LPAT housekeeping isoform that is responsible for the synthesis of 'normal' non-hydroxylated DAG that can be used for membrane lipid synthesis. Together with our *in vivo* results, the enzyme assays confirm that RcLPAT2 utilizes HFA-containing substrates more efficiently than does endogenous AtLPAT2.

Discussion

Most of the fundamental acyltransferase enzymatic reactions required for membrane and storage glycerolipid biosynthesis were determined more than 50 years ago (Lands 1960; Kennedy 1961). Understanding the biochemical properties of each step, how these pathways are regulated, and how they are integrated into total cellular metabolism required identification of the genes that encode the relevant enzymes and accessory proteins. Meaningful progress in this respect began only about 20 years ago, with the identification of mutant *Arabidopsis* genomic loci linked to defects in seed TAG biosynthesis (Katavic et al. 1995); later studies showed that these mutations were located in the *Arabidopsis DGAT1* gene (Routaboul et al. 1999; Zou et al. 1999), the first of its kind to be discovered in plants, and one of the first in nature.

While we, and many others, ultimately hope to learn general truths about the common bottlenecks and other limitations that likely affect all oilseed engineering projects, our specific goal is to achieve high-level production of UFA-containing oils (such as those from *Euphorbiaceae* species) in safe, sustainable, non-food oilseed crops. Our collective interest in the biochemistry and molecular genetics of UFA biosynthesis dates back nearly 30 years (Bafor et al. 1991). Basic proof-of-concept metabolic engineering studies in *Arabidopsis* were established by the creation of strong castor *FAH*-expressing lines (Lu et al. 2006), followed by modest incremental successes in HFA elevation via co-expression of important castor enzymes such as DGAT2, PDAT1A, or both (Burgal et al. 2008; van Erp et al. 2011). Here we focused on LPAT enzymes from two *Euphorbiaceae* species, tung and castor. *In planta* experiments demonstrated that overexpressed VfLPAT2 and RcLPAT2 both have higher selectivity for UFA in transgenic seeds than the endogenous *Arabidopsis* LPAT activities, leading to higher accumulation of the target fatty acids (Figs. 1, 2, 3). These results support the co-evolution of UFA synthesis and UFA-utilizing enzymes, and demonstrate the potential utility of these genes in biotechnological applications. The ESA produced by tung and our transgenic *Arabidopsis* is very labile under typical laboratory conditions, therefore all additional experiments further characterizing the effect of *Euphorbiaceae* LPATs focused on the castor enzyme and relatively stable HFA.

Toward the production of castor type oils

Previous work (Arroyo-Caro et al. 2013; Chen et al. 2016), has also addressed the properties of RcLPAT2. Arroyo-Caro et al. (2013) cloned four candidate *LPAT* genes from castor. Two genes, *RcLPAT2* and *RcLPATB*, were

expressed ubiquitously in castor organs and tissues, including in developing seeds. After expression in *lpat* mutant *E. coli*, both enzymes also possessed significant levels of *in vitro* enzyme activity towards several combinations of substrates, including those containing HFA in the acyl donor, acyl acceptor, or both (Arroyo-Caro et al. 2013). Chen et al. (2016) expressed *RcLPAT2* in seeds of lesquerella (*Physaria fendlerii*), a desert crop plant that produces ~60% HFA, primarily lesquerolic acid (20:1 13-OH) rather than the ricinoleic acid found in castor. RcLPAT2 was an obvious target for overexpression in lesquerella because HFA are largely excluded from the *sn*-2 position of TAG. Positional analysis of *RcLPAT2*-transgenic lesquerella seed oil indicated that RcLPAT2 was able to affect increases in *sn*-2 HFA, but the overall seed HFA content remained essentially unchanged (Chen et al. 2016).

Here we functionally characterized RcLPAT2 both *in vitro* and *in vivo* to assess its potential role in determining castor oil fatty acid composition. Biochemical analyses of yeast-expressed enzymes clearly demonstrated that AtLPAT2 (the endogenous LPAT isozyme that largely controls the *sn*-2 fatty acid composition of *A. thaliana* seed oil) is not well-suited for HFA metabolism, while RcLPAT2 showed strong selectivity for both LPA and acyl-CoA substrates containing HFA (Fig. 7). We also show that RcLPAT2 still retains this selectivity *in planta* in a transgenic system that has not evolved to accumulate HFA-containing TAGs. RcLPAT2 overexpression clearly increases the HFA-TAG component of transgenic seed oil, unlike the results shown previously (Chen et al. 2016). In Fig. 3, RcLPAT2 showed an additive effect when combined with RcDGAT2, leading to significant increases in total seed HFA levels compared to lines transformed with empty vector, or with *RcDGAT2* alone.

In Fig. 5 we demonstrate that RcLPAT2 alleviates the HFA-induced reduced oil phenotype of CL37 (Bates et al. 2014), and almost doubles the seed oil amount by predominantly increasing 1-HFA-TAG (Fig. 4a) that contains HFA at the *sn*-2 position (Fig. 6). Previously, expression of *sn*-3-specific castor acyltransferases (RcDGAT2 or RcPDAT1a) in CL37 caused a shift in the 1-HFA-TAG regiochemistry by reducing the amount of HFA at *sn*-2 and increasing the amount at the *sn*-1/3 positions (van Erp et al. 2011), and which we confirmed in our *RcDGAT2* line (Fig. 6). In addition, *RcLPAT2* expression in lesquerella (which naturally does not accumulate HFA at *sn*-2) increased the *sn*-2 HFA content in TAG (Chen et al. 2016). Therefore, it may have been expected that the proportion of HFA at the *sn*-2 position of 1-HFA-TAG would have increased in RcLPAT2 transgenic lesquerella. However, this simple expectation does not fit with the potential multiple pathways in which 1-HFA-TAG is produced in *Arabidopsis*. Fig. 8 is a schematic of HFA-TAG assembly in transgenic Arabidopsis and demonstrates how RcDGAT2, but not RcLPAT2, leads to a shift in HFA regiochemistry. HFA are synthesized by RcFAH12 at the s*n*-2 position of the ER

membrane lipid phosphatidylcholine (PC). The major pathway of TAG biosynthesis in *Arabidopsis* is through PC-derived DAG (Bates 2016), therefore HFA synthesized at *sn*-2 PC can stay at the s*n*-2 position as PC is turned over to produce the DAG substrate for TAG synthesis. This is the dominant mechanism at work in parental CL37 (Bates and Browse 2011), and produces 1-HFA-TAG with 70% of the HFA at *sn*-2. For HFA to be utilized by RcLPAT2 or RcDGAT2 the HFA are removed from PC and incorporated into the acyl-CoA pool by acyl editing mechanisms (Bates 2016, Bates and Browse 2012). If the HFA-CoA are utilized by RcDGAT2, this leads to a shift in regiochemistry from the *sn*-2 position of PC, to the *sn*-3 position of TAG. However, if the HFA-CoA is utilized by RcLPAT2, it will re-enter the *sn*-2 position of PA and can ultimately end up in *sn*-2 TAG by way of *de novo* DAG or PC-derived DAG. Therefore, the similar proportions of *sn*-2 HFA found in the *RcLPAT2* alone line and CL37 are likely due to the synthesis of the same product (1-HFA-TAG), but by two different pathways. Interestingly, the line containing all three castor acyltransferases had the most 1-HFA-TAG (Fig. 4), and the most HFA located at the *sn*-1/3 position of 1-HFA-TAG (Fig. 6). This result is likely due to the combined action of both RcGPAT9 (*sn*-1-specific) and RcDGAT2 (*sn*-3-specific) acyltransferases contributing to production of predominantly 1-HFA-TAG.

In Figs 4a and 4b, we demonstrate that co-expression of the full suite of all three castor Kennedy pathway acyltransferases (*RcGPAT9*, *RcLPAT2*, and *RcDGAT2*) in CL37 nearly doubles the accumulation of seed TAG HFA, from approximately 17-20% in CL37 to approximately 35-39%. Interestingly, despite such increases in total HFA content, very little 3-HFA-TAG accumulated. Castor oil contains approximately 90% HFA, and 3-HFA-TAG makes up over 70% of total TAG species in castor seeds (Lin et al. 2003). There are two possibilities that may explain the limited accumulation of total HFA and 3-HFA-TAG in transgenic *Arabidopsis* based on our current understanding of the metabolic network of plant TAG biosynthesis (Fig. 8). First, differences may exist between the components and precise location of the TAG assembly pathways in castor and *Arabidopsis*. Current evidence indicates that castor uses a classical Kennedy pathway of sequential GPAT, LPAT, and PAP enzymatic activities to produce *de novo* synthesized diacylglycerols containing two HFA, which is used by castor DGATs to produce 3-HFA-TAG (Bafor et al. 1991). However, *Arabidopsis* utilizes a pathway where *de novo* DAG is first incorporated into the membrane lipid phosphatidylcholine (where the fatty acid composition can be remodeled by acyl editing) prior to conversion to a PC-derived DAG and subsequent incorporation into TAG by DGAT (Bates and Browse 2011; Bates et al. 2012; Bates 2016). Substrate competition and imperfect protein locations/interactions between the endogenous *Arabidopsis* TAG assembly enzymes and transgenic castor enzymes may produce a system that cannot achieve efficient, uninterrupted

flux of HFA-containing substrates into 3-HFA-TAG, even in the presence of all three introduced castor acyltransferases. One possible solution may be to reduce competition from the endogenous TAG biosynthetic pathway enzymes, which on a limited basis has been demonstrated to increase the accumulation of unusual fatty acids in transgenic plants (van Erp et al. 2015; Bansal et al. 2018). Another possible solution would be to enhance HFA flux through the same lipid metabolic network that *Arabidopsis* uses by enhancing TAG synthesis from PC-derived substrates (Fig. 8). Again, on a limited basis this has been successfully demonstrated by increased HFA accumulation in seeds co-expressing *RcFAH12* and *RcPDAT1a* (van Erp et al. 2011), or *RcPDCT* (Hu et al. 2012). The conversion of *Arabidopsis* oil to castor-type oils may require a combination of these approaches.

Insufficient fatty acid hydroxylase activity may also explain the limited production of total HFA and 3-HFA-TAG in CL37 plants that co-express various castor acyltransferases. Overexpression of *RcDGAT2* or *RcPDAT1A* in parental CL37 increased HFA content from ~17-20% to approximately 25-30% (Burgal et al. 2008; van Erp et al. 2011), showing that considerable 'headspace' existed in parental CL37, in the absence of co-evolved castor TAG biosynthetic enzymes. In the lines described in this study, containing two or all three of the castor Kennedy pathway enzymes, it is highly likely that FAH activity has become a limiting factor. RcGPAT9 has not yet been characterized in detail, but biochemical analyses (Kroon et al. 2006; Burgal et al. 2008; and this study) suggest that RcDGAT2 and RcLPAT2 have high selectivity for HFA-containing DAG and/or acyl-CoA substrates, with a certain degree of promiscuity towards other substrates. Therefore, in the presence of limited FAH activity, the castor acyltransferases could quickly deplete the HFA-CoA from developing seed metabolite pools, forcing the collective acyltransferase set (containing both native and transgenic enzymes) in the TAG biosynthetic apparatus to use a mixture of HFA and non-HFA substrates, resulting in production of a mixture of TAG species containing 0-3 HFA. The CL37 background was selected without any additional HFA-selective acyltransferases included (Lu et al. 2006), and significant amounts of HFA accumulated in membrane lipids (van Erp et al. 2011), which can be detrimental to cellular function (Millar et al. 2000). Therefore, the initial selection of CL37 may have been unintentionally biased towards lines expressing relatively low levels of FAH12 activity. The efficient transfer of UFA from membranes into TAG can limit their adverse effects on membrane structure and function. Therefore, one mechanism to test the HFA limitation hypothesis may be to express RcFAH12 in lines previously transformed with HFA-selective acyltransferases. These activities, such as RcDGAT2 or RcPDAT1A, will help to establish neutral lipid metabolic sinks that can accommodate higher levels of HFA TAG products produced from higher-expressing RcFAH12 lines.

Previous studies of HFA production in transgenic Arabidopsis seeds (Kumar et al. 2006) indicated that castor FAH inefficiently competes with endogenous fatty acid desaturases for reduced cytochrome b5 (cb5), an essential cofactor for both desaturation and hydroxylation reactions. Wayne and Browse (2013) expressed various combinations of castor *cb5* and *cytochrome b5 reductase* (*CBR*) genes in CL37, but did not observe increases in seed HFA levels. However, as noted above, the HFA levels in seed lipids of CL37 (lacking other castor 'sink' enzymes such as RcDGAT2), does not fully capitalize on the available metabolic headspace of FAH12, and thus may not have been the ideal vehicle for testing cb5/CBR complementation. Integration of the two approaches described here may provide an ideal blend of genetic components necessary for both production of high levels of HFA from RcFAH12, and assembly of HFA-containing TAGs via overexpression of multiple Kennedy pathway and other 'sink' enzymes.

Overexpression of *RcLPAT2* in CL37 greatly increased total seed oil content (Fig. 5). Previously, it has been demonstrated in multiple species that enhanced LPAT activity is associated with higher seed oil content (Zou et al. 1997; Taylor et al. 2002; Maisonneuve et al. 2010). Detailed biochemical studies indicated plant DGAT1 activity is stimulated by PA, the product of the LPAT reaction (Caldo et al. 2018). Our result fits with these previous studies, however this result was not initially expected. Our previous work has demonstrated that the CL37 seeds contain approximately ~40-50% less oil, compared to wild-type, due to HFA-induced feedback regulation of fatty acid synthesis (Bates et al. 2014). To increase seed oil content, overexpression of RcLPAT2 would have to do more than just activate endogenous DGAT activity, but also alleviate the down-regulation of fatty acid synthesis. Consistent with our previous analyses, CL37 contained 24.2% oil, on a seed dry weight basis (Fig. 5). The near wild-type levels of seed lipid when *RcLPAT2* is overexpressed alone (34.2±1.2%) indicates that RcLPAT2 expression largely eliminates the oil yield penalty created by FAH expression in CL37, likely by alleviating the HFA-induced feedback inhibition directly by enhancing incorporation of HFA into PA (and TAG, thus reducing any possible toxic effects of HFA intermediate buildup) and indirectly through synthesis of PA which upregulates endogenous DGAT activity (Caldo et al. 2018).

Endogenous *Arabidopsis* LPAT2 is a likely bottleneck to efficient HFA accumulation in CL37 plants

There may be several reasons why CL37 *Arabidopsis* is limited to ~17-20% HFA in seed oil. One such reason is substrate incompatibility for one or more of the Kennedy pathway isozymes present in developing seeds. To address this point directly, we expressed RcLPAT2 and AtLPAT2 in yeast, and performed *in vitro* LPAT enzyme assays with

different combinations of hydroxylated and 'normal' (non-hydroxylated) acyl-CoA and LPA substrates. AtLPAT2 and RcLPAT2 differed radically in their respective substrate specificities. Whereas the *Arabidopsis* enzyme could not acylate ricinoleoyl-CoA to ricinoleoyl-LPA, the RcLPAT2 readily utilized this combination of substrates. These data corroborate well with the data from Bates and Browse (2011) showing that, in *in-vivo* labelling experiments, no di-ricinoleoyl DAG but only DAG with no or one ricinoleoyl groups were produced in *Arabidopsis* seeds expressing castor FAH. More unexpectedly, the RcLPAT2 showed very little, if any, ability to produce di-18:1-PA and *sn*-1-ricinoleoyl-*sn*-2-18:1-PA, whereas both enzymes produced similar levels of *sn*-1-18:1-*sn*-2-ricinoleoyl-PA. This indicates that RcLPAT2 can only produce di-ricinoleoyl PA when ricinoleoyl-LPA is a substrate and it likely does not produce meaningful amounts of di-18:1-PA. Given that castor oil is made up of 70 mole% triricinolein, and 90% HFA in total, ricinoleoyl-LPA is likely the major LPA molecular species produced in developing castor seeds. Thus, the particular specificity of RcLPAT2 will ensure that this substrate is efficiently channeled into di-ricinoleoyl PA, and ultimately di-ricinoleoyl DAG, the preferred substrate for RcDGAT2 (Kroon et al. 2006; Burgal et al. 2008). The substrate specificity of RcLPAT2 strongly suggests that it plays a significant role in triricinolein biosynthesis in developing castor seeds, and also makes it a useful tool for production of transgenic HFA as well.

At the same time, our results suggest that RcLPAT2 will not compete with other housekeeping LPAT activities that produce di-oleoyl-PA (and likely other non-HFA PA species) that are necessary for membrane lipid synthesis. *RcLPAT3A* is a closely related ortholog of *RcLPAT2* (Körbes et al. 2016), but when cloned and expressed in our yeast system was inactive against all four combinations of oleoyl- and ricinoleoyl-CoAs/LPAs described above (data not shown). *RcLPAT3A* is preferentially expressed in male parts of castor flowers, and may therefore have evolved specificity for substrates unique to floral lipid metabolism. Therefore, the most likely candidate for the housekeeping role is *RcLPATB*, a member of a separate clade of LPAT enzymes of a more ancient origin than LPAT2 and the other members of that subfamily (Körbes et al. 2016). *RcLPATB* is ubiquitously expressed in castor organs, including developing seeds (Arroyo-Caro et al. 2013). RcLPATB possesses very broad substrate range, effectively utilizing acyl-CoAs containing medium-chain saturated fatty acids (C12:0, C14:0), long-chain monounsaturates, and ricinoleic acid. Arroyo-Caro et al. (2013) state that these unusual substrate specificities argue against RcLPATB involvement in delivering PA species for membrane lipid synthesis. We have not assayed RcLPATB here, and it should be noted that some parts of the RcLPAT2 substrate specificity data presented by these authors contrasts sharply with what is reported here by us. In their assays, as in ours, ricinoleoyl-CoA was efficiently acylated to ricinoleoyl-

LPA by the castor enzyme. However, they also reported that RcLPAT2 efficiently acylated 18:1-CoA to both 18:1-LPA and ricinoleoyl-LPA; our assays showed no or little activity over background with these substrate combinations. We cannot offer any obvious explanation for these discrepancies but note that that their enzyme assays utilized membranes from *E. coli* expressing the enzymes whereas we used yeast membranes. Their substrate and enzyme concentrations differed from ours and their assays were done with an indirect spectrophotometric method whereas ours were based on direct measurement of the radioactive PA products. Regardless of these discrepancies, our data clearly shows that, when using the same assay conditions for both AtLPAT2 and RcLPAT2, the two enzymes showed radically different substrate specificities that support a specialized role for RcLPAT2 in di-ricinoleoyl PA production while the *Arabidopsis* enzyme lacks this capacity. We also feel that the broad substrate specificity of RcLPATB does not rule it out from providing the 'housekeeping' LPAT activity necessary for membrane lipid synthesis in *R. communis*. It displays significant activity towards substrate combinations that would produce fatty acid profiles typical of most plant ER phospholipids (Arroyo-Caro et al. 2013), which we did not observe for RcLPAT2 in our experiments (Fig. 6). Future studies will be focused on clarifying this question.

In summary, we have presented results from a series of experiments that address the potential role of RcLPAT2 in the selective biosynthesis of PA containing HFA, and the contribution that this enzyme activity makes towards increasing total seed oil levels and HFA production specifically in transgenic *Arabidopsis* seeds. Both tung and castor LPATs had a positive effect on UFA accumulation in transgenic *Arabidopsis* when co-expressed with their respective UFA synthesis enzymes, suggesting a similar co-evolution of UFA synthesis with oil assembly enzymes to enhance selective fatty acid incorporation into seed oils, over that of the common fatty acids found in membrane lipids. Together these data support inclusion of LPAT2 enzymes from *Euphorbiaceae* species in future oilseed metabolic engineering strategies to produce high levels of UFA containing oils in sustainable agronomic crops.

Author contribution statement

JS, IL, SS, and PDB conceived and designed research. HKK contributed new resources and analytical tools. JS, IL, SS and PB wrote the manuscript. All authors conducted experiments and analyzed data, and read and approved the manuscript.

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Footnotes

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Figure Legends

Fig. 1 Comparison of α-eleostearic acid (ESA) production in T_2 populations of DsRed fluorescent transgenic *Arabidopsis fad3fae1* double mutant plants expressing tung *FADX* alone *FADX* and *VfDGAT2* or *FADX*, *VfDGAT2*, and *VfLPAT2*. Each data point represents the relative quantity of ESA present in the segregating T_2 seeds derived from a different independent transgenic event. The mean and SEM are shown for each population of samples. Significant differences ($p < 0.05$, unpaired student's t test) from the base *VfFADX* line are marked with an asterisk.

Fig. 2 Comparison of ESA production in T_2 populations of basta herbicide-resistant transgenic *Arabidopsis fad3fae1* double mutant plants expressing tung *FADX* alone, or *FADX* and *VfLPAT2*. Each data point represents the relative quantity of ESA present in the segregating T_2 seeds derived from a different independent transgenic event. The mean and SEM are shown for each population of samples. Significant differences ($p < 0.05$, unpaired student's t test) from the base *VfFADX* line are marked with an asterisk.

Fig. 3 Comparison of hydroxy fatty acid (HFA) production in T_2 populations of *Arabidopsis* CL37 plants retransformed with either empty binary vector or , *RcDGAT2*, or *RcDGAT2* and *RcLPAT2, or RcGPAT9* and *RcLPAT2* and *RcDGAT2.* (a) DsRed selection lines. (b) basta herbicide-resistant selection lines. Each data point represents the relative quantity of HFA present in the segregating T_2 seeds derived from a different independent transgenic event. The mean and SEM are shown for each population of samples. Different letters above each line indicate significant differences ($p < 0.05$), unpaired student's t test).

Fig. 4 Analysis of accumulation of HFA-containing TAGs. (a) relative proportions of different HFA-containing TAG classes in various transgenic lines. (b) total TAG HFA content from the sum of each TAG species. Seeds analyzed from *RcDGAT2* and *RcLPAT2/DGAT2* were homozygous T_3, and *RcLPAT2* and *RcGPAT9/LPAT2/DGAT2* were homozygous T_4. Data represents the mean and standard error of lipids extracted from 2-4 individual plants. Statistical analysis was performed with a 2-way ANOVA in (a) and a 1-way ANOVA in (b). Significant differences ($p < 0.05$) from the control CL37 line are marked with an asterisk.

Fig. 5 Total seed fatty acid content in HFA-producing *Arabidopsis* seeds. Lines are the parental CL37 and those co-expressing either *RcLPAT2,* or *RcLPAT2* and *RcDGAT2*. Seeds are homozygous T_4 seed. Data represents the mean and standard error, $n = 4$ individual plants. Growth conditions: 23 °C, 16 hr white light/ 8 hr dark, ~150 µmole photons m^{-2} s^{-1}.

Fig. 6 Regiochemical analysis of 1-HFA-TAG. 1-HFA-TAG from lipid extracts in Fig. 4 was collected from the parental CL37 line and T_4 seed in each new transgenic line. Data represents the mean and standard error, n = 3-4 individual plants. Significant differences ($p < 0.05$) from the control CL37 line are marked with an asterisk (unpaired Student's t-test). The α indicates that the *RcGPAT9/LPAT2/DGAT2* line is significantly different than all other lines.

Fig. 7 Biochemical analysis of *Arabidopsis* and castor LPAT enzymes. The ORFs for *AtLPAT2, RcLPAT2,* and *RcLPAT3A* were cloned in a yeast expression vector, and enzyme production was induced in an *ale1* mutant of *S. cerevisiae*. Enzyme activity was measured as described in the text. RcLPAT3A was inactive with all four of the substrate combinations tested (data not shown). Means and standard deviations are shown for triplicate assays for each substrate combination. Significant differences ($p < 0.05$) from the control empty vector are marked with an asterisk (unpaired Student's t-test).

Fig. 8 Proposed pathway of RcLPAT2 contribution to HFA-TAG synthesis in transgenic Arabidopsis. Dotted lines indicate acyl transfer reactions, solid lines indicate glycerolipid flux. Relevant castor enzymes that have been used to make transgenic plants are underlined. Castor enzymes used in this study are in red. Abbreviations: G3P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; PC, phosphatidylcholine; PDAT, phospholipid:diacylglycerol acyltransferase; PDCT, phosphatidylcholine:diacylglycerol cholinephosphotransferase; LPC, lyso-phosphatidylcholine; TAG, triacylglycerol; HFA, hydroxy fatty acid.

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Figure 1

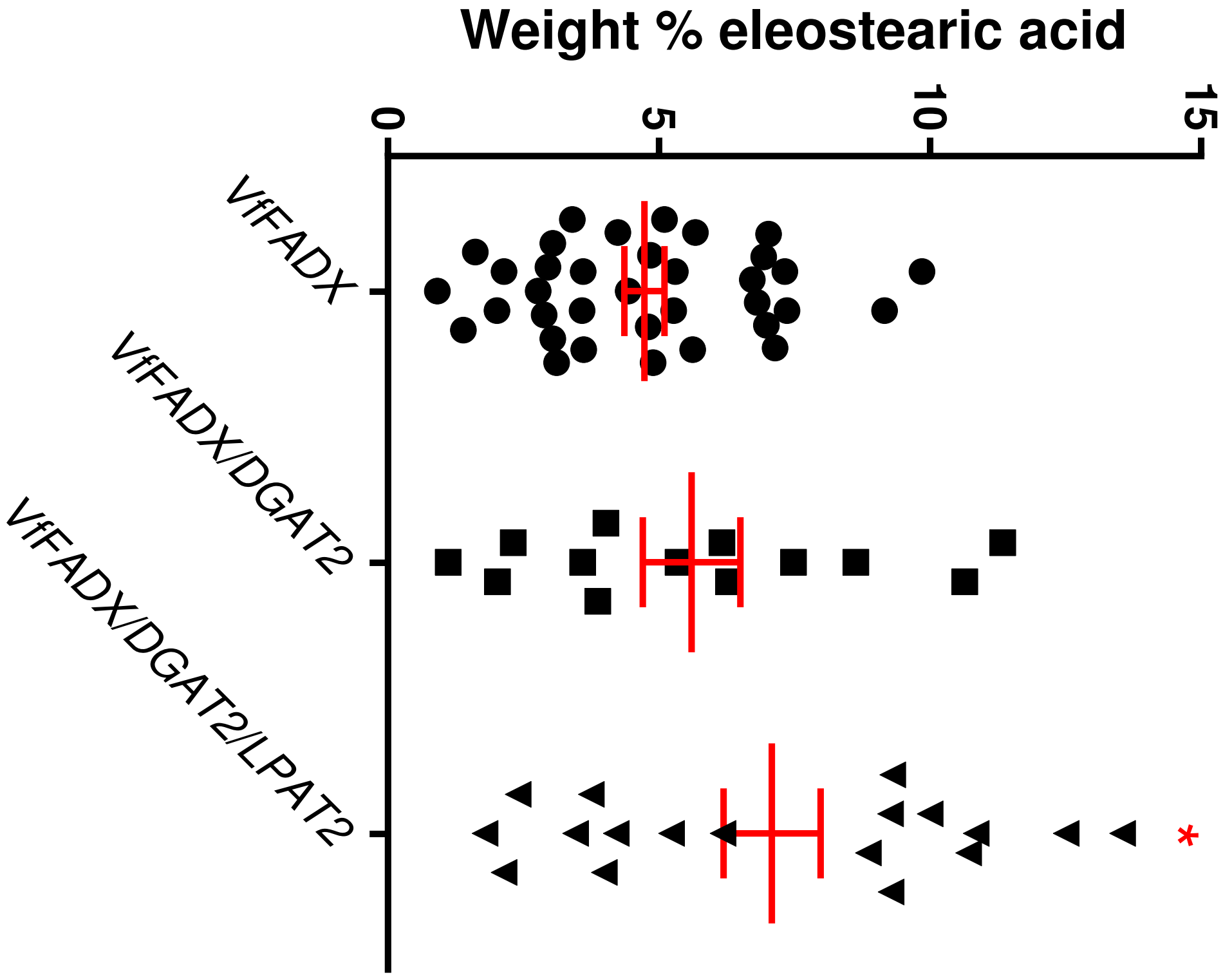

Weight % eleostearic acid
0
5
10
15
VfFADX
VfFADX/DGAT2
VfFADX/DGAT2/LPAT2
*

Figure 2

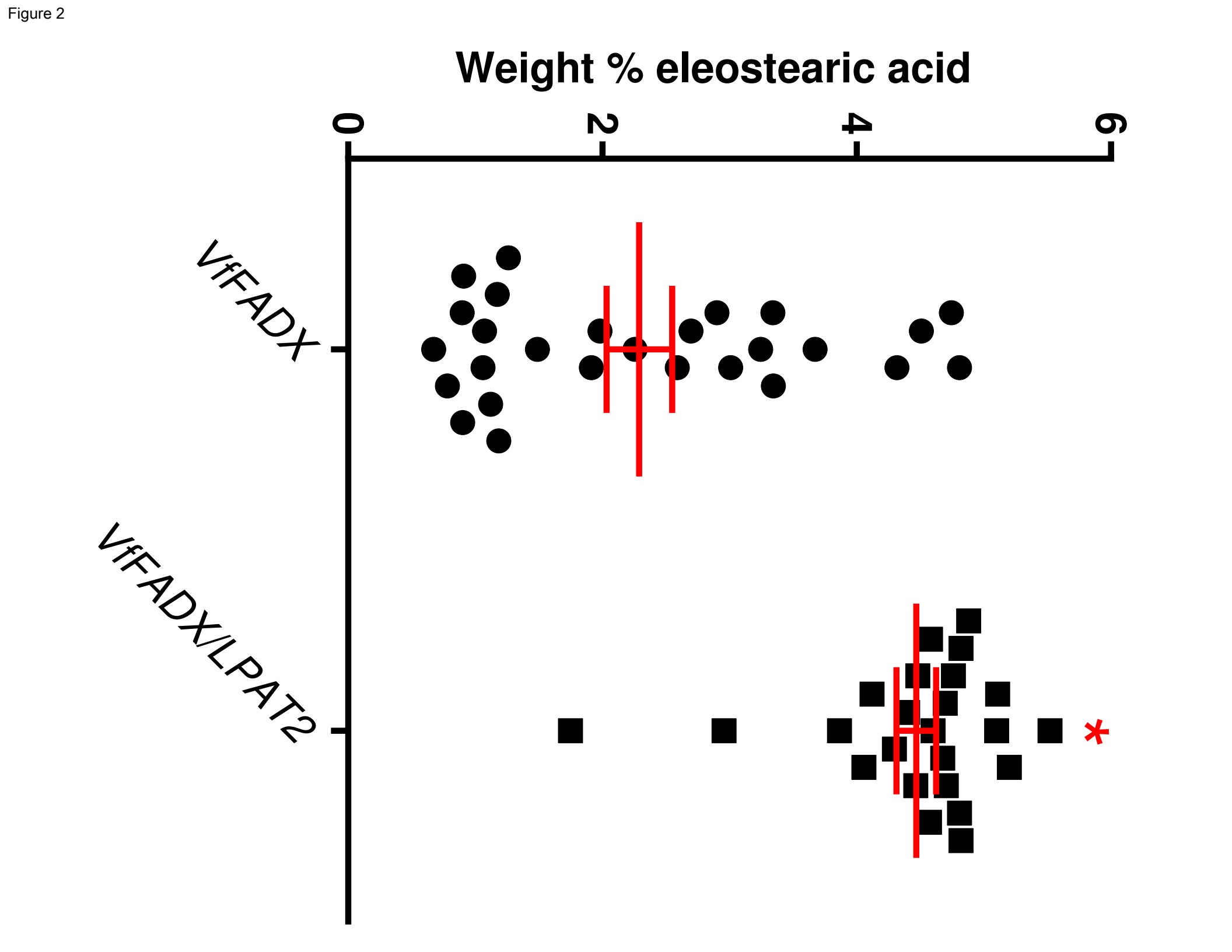


Figure 3

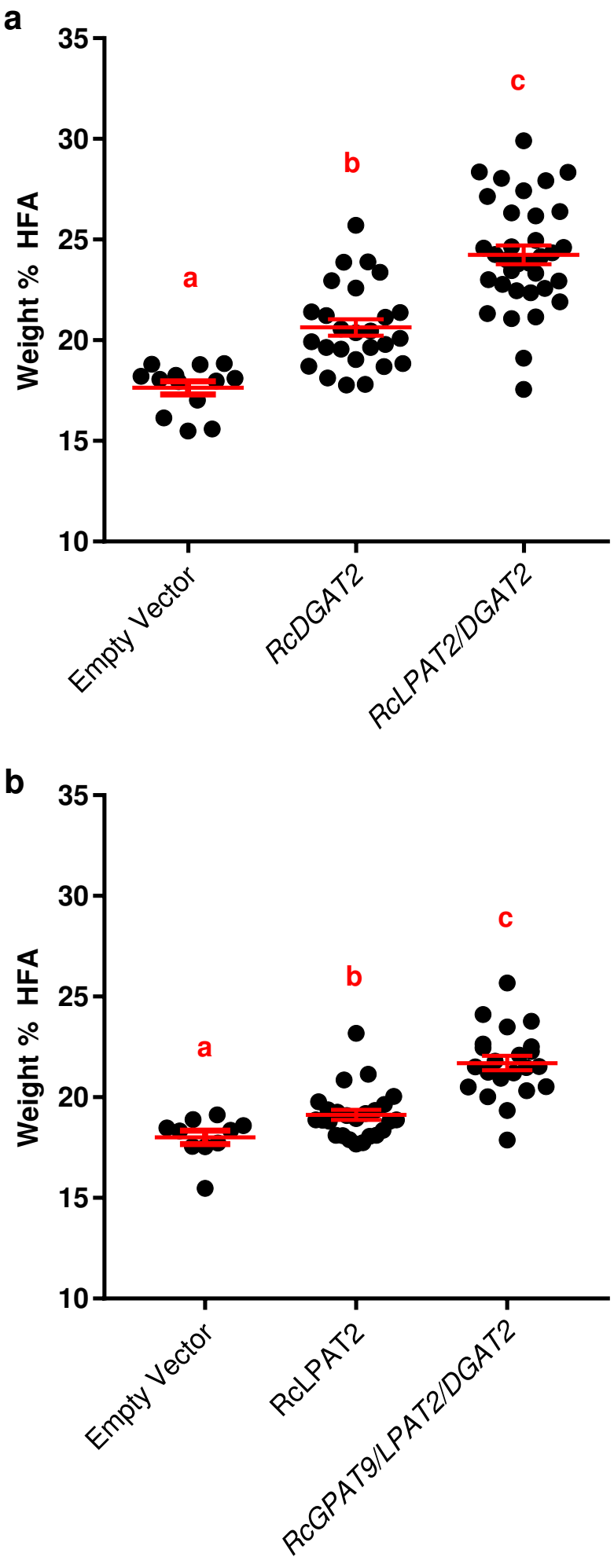

a
Weight % HFA
35
30
25
20
15
10
a
b
c
Empty Vector
RcDGAT2
RcLPAT2/DGAT2
b
Weight % HFA
35
30
25
20
15
10
a
b
c
Empty Vector
RcLPAT2
RcGPAT9/LPAT2/DGAT2

Figure 4

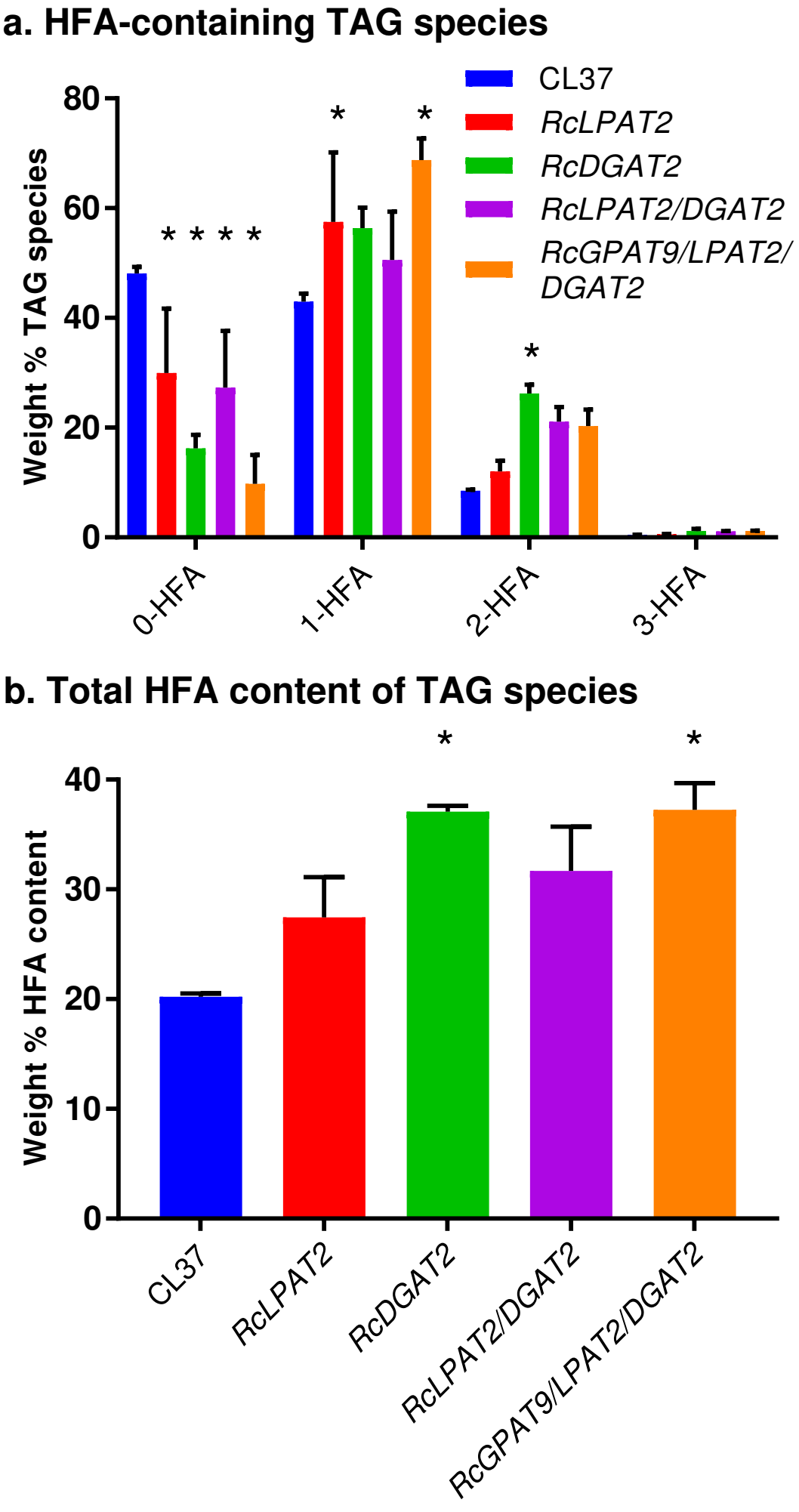

a. HFA-containing TAG species
Weight % TAG species
80
60
40
20
0
CL37
RcLPAT2
RcDGAT2
RcLPAT2/DGAT2
RcGPAT9/LPAT2/
DGAT2
* * * *
*
*
*
0-HFA
1-HFA
2-HFA
3-HFA
b. Total HFA content of TAG species
Weight % HFA content
40
30
20
10
0
*
*
CL37
RcLPAT2
RcDGAT2
RcLPAT2/DGAT2
RcGPAT9/LPAT2/DGAT2

Figure 5

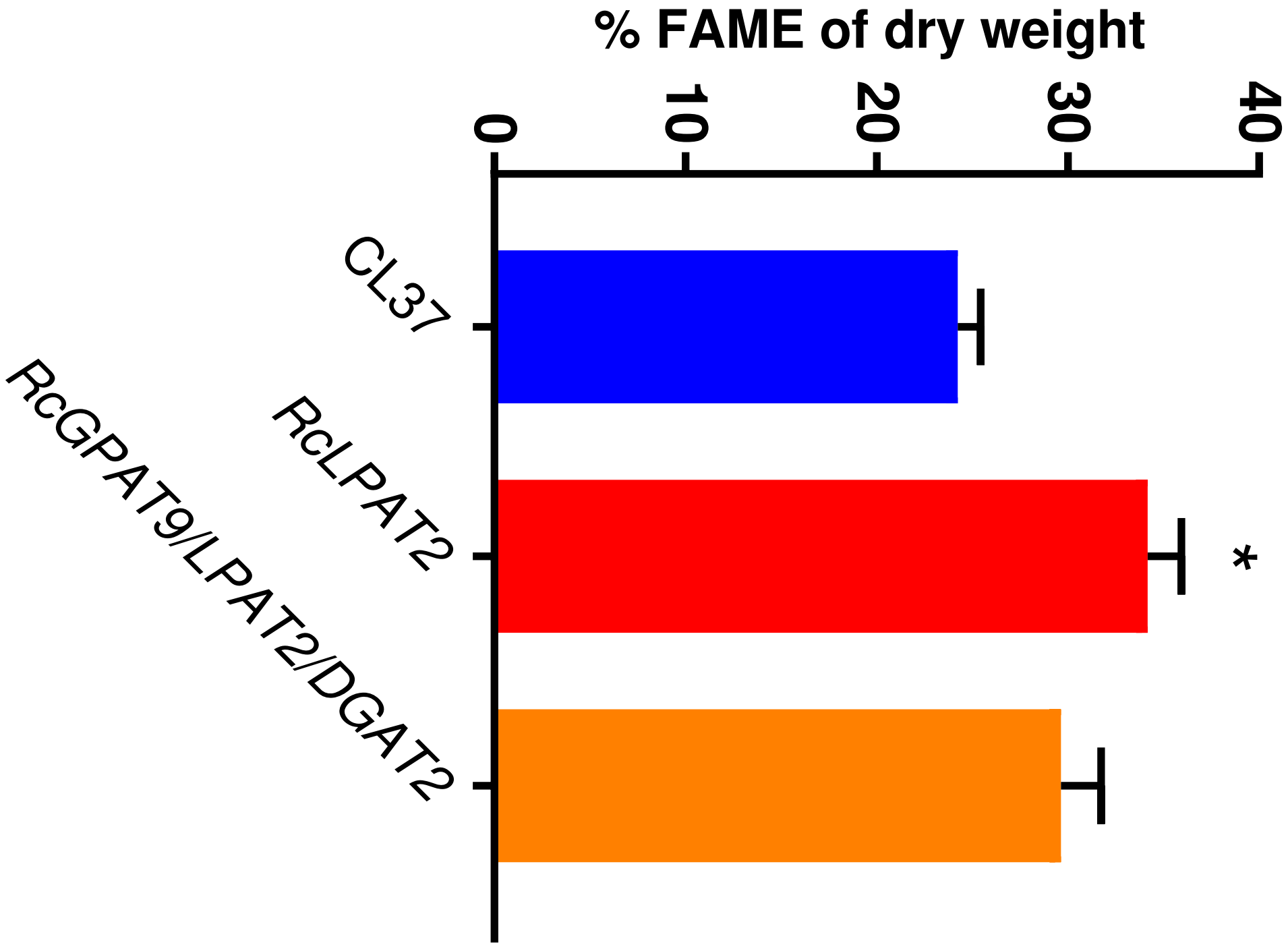

% FAME of dry weight
0
10
20
30
40
CL37
RcLPAT2
RcGPAT9/LPAT2/DGAT2
*

Figure 6

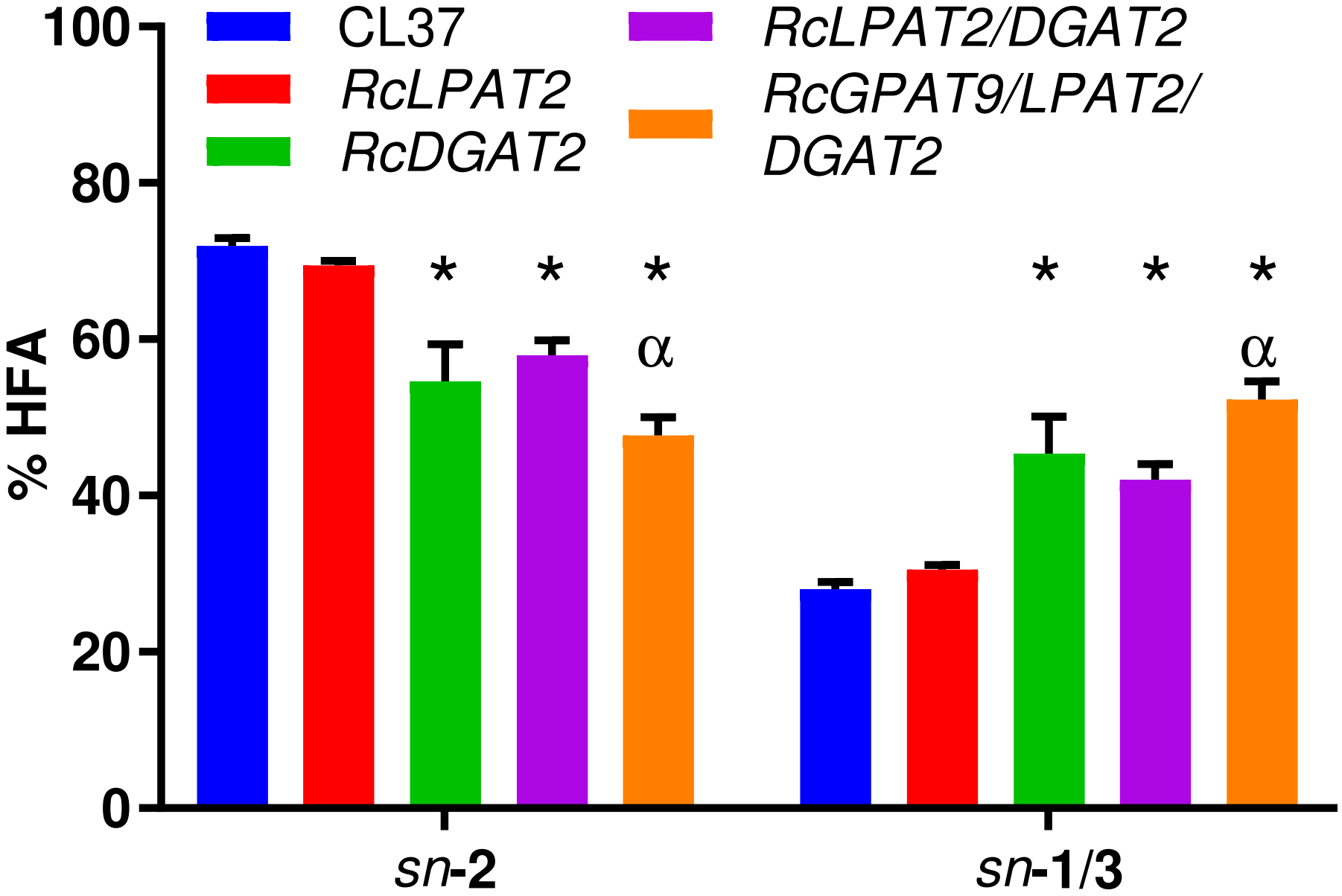

100
80
60
40
20
0
% HFA
CL37
RcLPAT2
RcDGAT2
RcLPAT2/DGAT2
RcGPAT9/LPAT2/
DGAT2
*
*
*
α
*
*
*
α
sn-2
sn-1/3

Figure 7

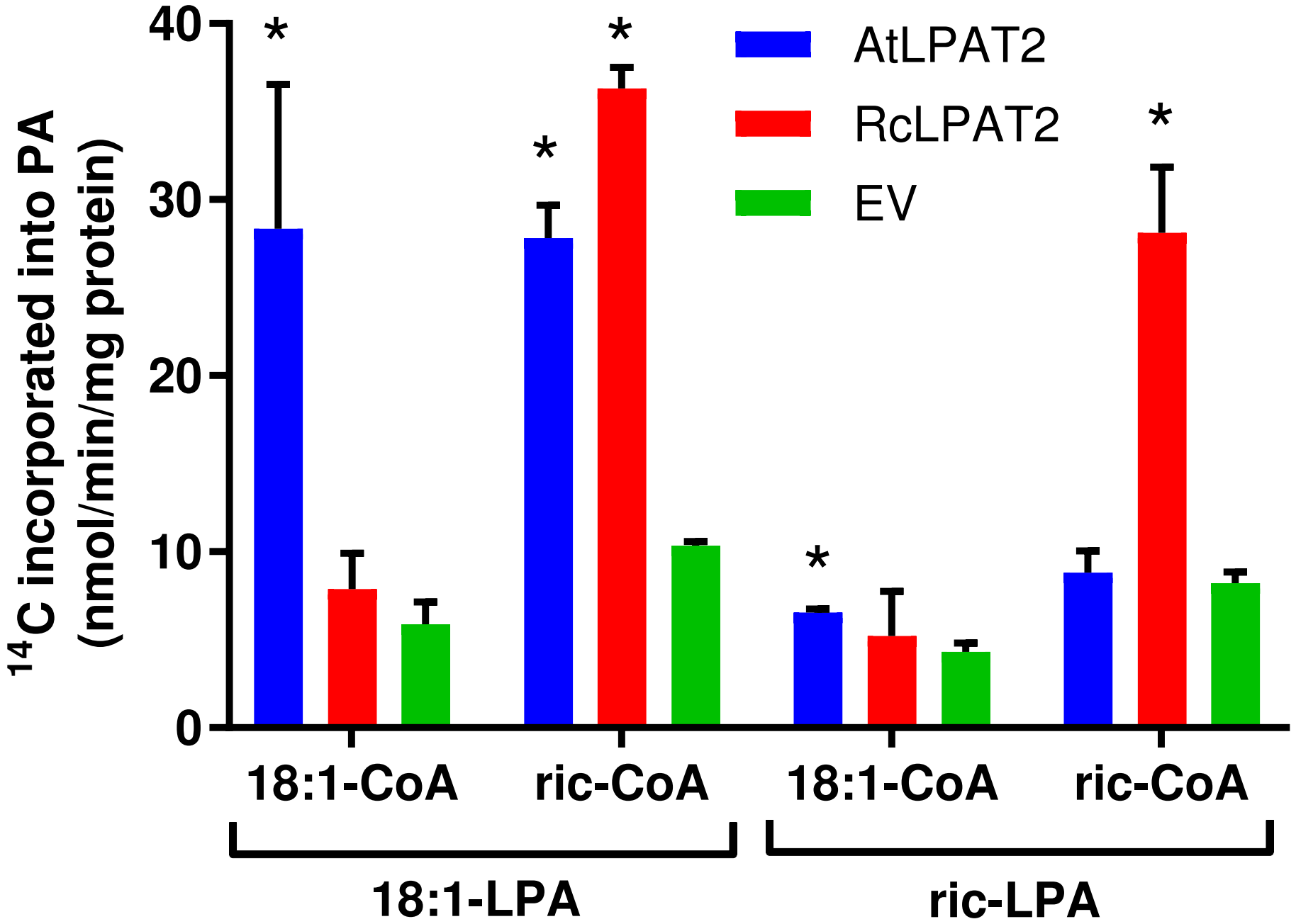
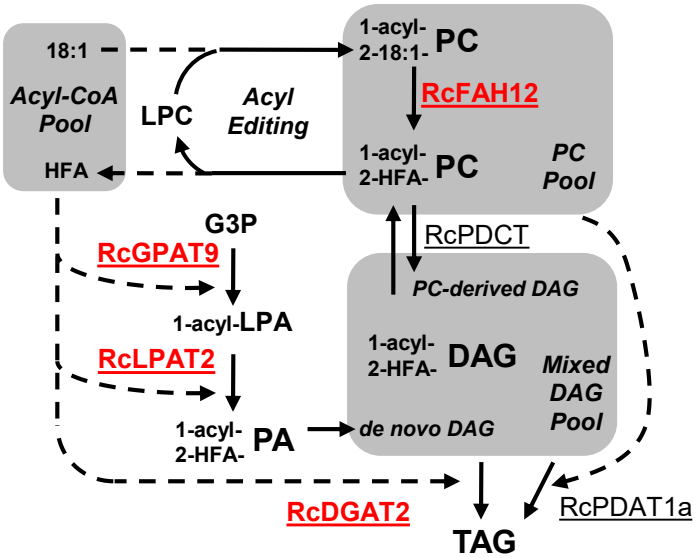

40
30
20
10
0
^{14}C incorporated into PA
(nmol/min/mg protein)
AtLPAT2
RcLPAT2
EV
*
*
*
*
18:1-CoA
ric-CoA
18:1-CoA
ric-CoA
18:1-LPA
ric-LPA

Figure 8


18:1
Acyl-CoA Pool
HFA
LPC
Acyl Editing
1-acyl-2-18:1- PC
RcFAH12
1-acyl-2-HFA- PC
PC Pool
RcPDCT
PC-derived DAG
1-acyl-2-HFA- DAG
Mixed DAG Pool
de novo DAG
G3P
RcGPAT9
1-acyl-LPA
RcLPAT2
1-acyl-2-HFA- PA
RcDGAT2
TAG
RcPDAT1a

Tables

Table 1. Listing of plasmid binary vectors used for plant transformation. ORFs for all genes are driven by strong, seed-specific promoters, see Shockey et al. (2015), Materials and methods, and specific figure legends for more details. All *V. fordii* genes are co-expressed with tung *FADX* (Dyer et al. 2002) to produce eleostearic acid, while all *R. communis* genes are expressed in CL37 (Lu et al. 2006).

Plasmid #	Expressed Genes	Selectable marker
Fig. 1		
E116	*VfFADX*	DsRed fluorescence
E259	*VfFADX+VfDGAT2*	DsRed fluorescence
E318	*VfFADX+VfDGAT2+VfLPAT2*	DsRed fluorescence
Fig. 2		
E29	*VfFADX*	Finale® herbicide
E188	*VfFADX+VfLPAT2*	Finale® herbicide
Figs. 3,4,5		
B9	None; empty vector negative control	Finale® herbicide
E565	*RcDGAT2*	Finale® herbicide
E658	*RcDGAT2+RcLPAT2*	Finale® herbicide
E660	*RcLPAT2*	DsRed fluorescence
E678	*RcGPAT9+RcLPAT2+RcDGAT2*	DsRed fluorescence