A Small Phospholipase A_2-α from Castor Catalyzes the Removal of Hydroxy Fatty Acids from Phosphatidylcholine in Transgenic Arabidopsis Seeds[1][OPEN]

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Ricinoleic acid, an industrially useful hydroxy fatty acid (HFA), only accumulates to high levels in the triacylglycerol fraction of castor (*Ricinus communis*) endosperm, even though it is synthesized on the membrane lipid phosphatidylcholine (PC) from an oleoyl ester. The acyl chains of PC undergo intense remodeling through the process of acyl editing. The identities of the proteins involved in this process, however, are unknown. A phospholipase A_2 (PLA_2) is thought to be involved in the acyl-editing process. We show here a role for $RcsPLA_2\alpha$ in the acyl editing of HFA esterified to PC. $RcsPLA_2\alpha$ was identified by its high relative expression in the castor endosperm transcriptome. Coexpression in Arabidopsis (*Arabidopsis thaliana*) seeds of *$RcsPLA_2\alpha$* with the castor fatty acid hydroxylase *RcFAH12* led to a dramatic decrease in seed HFA content when compared with *RcFAH12* expression alone in both PC and the neutral lipid fraction. The low-HFA trait was heritable and gene dosage dependent, with hemizygous lines showing intermediate HFA levels. The low seed HFA levels suggested that $RcsPLA_2\alpha$ functions in vivo as a PLA_2 with HFA specificity. Activity assays with yeast (*Saccharomyces cerevisiae*) microsomes showed a high specificity of $RcsPLA_2\alpha$ for ricinoleic acid, superior to that of the endogenous Arabidopsis $PLA_2\alpha$. These results point to $RcsPLA_2\alpha$ as a phospholipase involved in acyl editing, adapted to specifically removing HFA from membrane lipids in seeds.

Phospholipase A enzymes (PLAs) catalyze the hydrolysis of either the *sn*-1 (PLA_1) or the *sn*-2 (PLA_2) ester bond of phospholipids to produce lysophospholipids and free fatty acids (FAs). They are involved in several biological processes in both animals and plants (Chen et al., 2011): (1) as a component of the acylation/hydrolysis of phospholipids in the so-called Lands cycle involved in primary lipid metabolism in animals (Lands, 1960; Balsinde and Dennis, 1997; Imae et al., 2010) and plants (Li et al., 2013); (2) in membrane architecture and trafficking (Lee et al., 2010; Kim et al., 2011); (3) in the production of bioactive compounds involved in signaling, pathogen defense, and programmed cell death (Munnik and Testerink, 2009; Canonne et al., 2011); (4) in catalyzing the hydrolysis of oxidized lipids (van Kuijk et al., 1987; Banaś et al., 1992); (5) in seed storage lipid mobilization (Rudolph et al., 2011); and (6) in uncommon and modified FA production in seeds (Bafor et al., 1991; Ståhl et al., 1995; Bates and Browse, 2011).

With recent developments in the understanding of lipid biosynthesis, focusing on FA incorporation and turnover in phosphatidylcholine (PC), attention is moving from the traditional Kennedy pathway of lipid synthesis to that of acyl editing (Bates et al., 2012; Wang et al., 2012; Xu et al., 2012), with the relative importance of each pathway in lipid synthesis depending on the plant species (Bates and Browse, 2012). Acyl turnover in membrane lipids, through a cycle of deacylation/acylation of PC to lysophosphatidylcholine (LPC), was first described by Lands (1958). Substantial progress has been made in the understanding of the acylation mechanism of LPC, involving lysophosphatidylcholine acyltransferases (LPCAT; Ståhl et al., 2008; Bates et al., 2012; Wang et al., 2012; Xu et al., 2012). As suggested by Lands (1960), PC deacylation was initially thought to result from the activity of a phospholipase. Until now, in plants, however, evidence suggested that LPC production comes from the action of phospholipid:diacylglycerol acyltransferase (PDAT; Zhang et al., 2009; van Erp et al., 2011; Xu et al., 2012) and through the reverse activity of LPCAT (Stymne and Stobart, 1984; Yurchenko et al., 2009; Bates et al., 2012; Lager et al., 2013). Thus,

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acyl editing includes a broader set of enzymatic activities than originally envisioned by Lands (1960). Despite the recent description of a patatin-related phospholipase, pPLAIIIδ, contributing to acyl turnover in PC (Li et al., 2013), phospholipase involvement in acyl editing in plants remains largely to be determined.

In contrast to common FAs, FAs with unusual functional groups only accumulate to relatively high levels in seed triacylglycerol (TAG) and are excluded from membrane lipids (Millar et al., 2000). Phospholipase activity also has been shown to be involved in the metabolism of the uncommon FAs ricinoleate (12-OH 18:1Δ9cis), vernoleate, and the short-chain FAs caprylate and laurate in plant seeds (Bafor et al., 1991; Ståhl et al., 1995). In castor (*Ricinus communis*), ricinoleate is synthesized from oleate (18:1Δ9cis) esterified to the *sn*-2 position of PC, and the activity of a ricinoleate-specific phospholipase is involved in liberating the newly formed hydroxy fatty acid (HFA) for further incorporation into TAG (Bafor et al., 1991). Phospholipases also may be involved in maintaining cellular membrane integrity through the hydrolysis of oxidized lipid. Banaś et al. (1992) showed in a variety of plants that both lipid peroxides and lipid hydroxides were preferentially hydrolyzed over nonoxidized lipids.

In plants, three major groups of PLAs have been identified: the patatin-related pPLAs, the Delayed Anther Dehiscence1 (DAD1)-like PLA_1, and the secretory/small $sPLA_2$s (Matos and Pham-Thi, 2009; Chen et al., 2011). In the first group, pPLA proteins show homology to patatin, a storage glycoprotein with lipase activity present in potato (*Solanum tuberosum*) tubers, and have been associated with signaling, pathogen defense, and lipid mobilization during seed germination (Scherer et al., 2010). In the second group of PLAs, DAD1 and DONGLE are clearly involved in signaling and jasmonate production (Ishiguro et al., 2001; Seo et al., 2009). The third group contains four paralogous genes coding for low-molecular-mass PLA_2s of around 14 kD, $sPLA_2\alpha$, $sPLA_2$-β, $sPLA_2$-γ, and $sPLA_2$-δ (Lee et al., 2005; Matos and Pham-Thi, 2009). Evidence for the involvement of $sPLA_2$ in signaling and pathogen defense is more limited (Munnik and Testerink, 2009; Canonne et al., 2011). Their involvement in membrane trafficking, however, is emerging: reductions in $sPLA_2$ expression in Arabidopsis (*Arabidopsis thaliana*) plants led to Golgi disruption and the inhibition of PIN auxin efflux transporter traffic to the plasma membrane in roots (Lee et al., 2010) and also to membrane disruption in pollen (Kim et al., 2011). Two other proteins, SOBER1 (Kirik and Mudgett, 2009) and Lecithin Cholesterol Acyltransferase (LCAT)-like4 (Chen et al., 2012), unrelated to these three main families, also have been identified as PLA_2s.

With the aim of gaining more insights into the involvement and identities of PLA_2s involved in acyl editing and HFA metabolism, with potential downstream industrial applications, we made use of the available castor endosperm transcriptome database (Troncoso-Ponce et al., 2011) and the transgenic Arabidopsis seed line CL37 expressing the castor fatty acid hydroxylase FAH12 (Lu et al., 2006). Among the *PLA* genes examined from the castor endosperm transcriptome, $RcsPLA_2\alpha$ was the most highly expressed, indicating possible involvement in ricinoleate metabolism in castor. The expression of $RcsPLA_2\alpha$ in CL37 seeds led to a drastic reduction in HFA levels in both TAG and PC fractions. Also, microsomal fractions from yeast (*Saccharomyces cerevisiae*) expressing $RcsPLA_2\alpha$ showed strong activity with *sn*-2-ricinoleoyl-PC as a substrate. Our results suggest roles for $RcsPLA_2\alpha$ in (1) acyl editing to generate LPC for reacylation through LPCAT activity, (2) freeing HFAs for activation to acyl-CoA and incorporation into TAG, and (3) contributing to membrane integrity through the removal of potentially deleterious ricinoleate from membrane lipids.

RESULTS

$PLA_2\alpha$ Is the Most Highly Expressed *PLA* in Developing Castor Endosperm

To identify which of the *PLA* genes are potentially involved in castor lipid metabolism, protein sequences of Arabidopsis PLAs were used in a BLASTP search against the 4× draft castor genome accessible from the J. Craig Venter Institute (JCVI; http://www.jcvi.org). The gene sequences identified were then used in a BLASTN search against the Michigan State University castor EST database (http://glbrc.bch.msu.edu/castor/; Troncoso-Ponce et al., 2011) to determine gene expression levels. The 13 *PLA* genes found to be expressed in castor endosperm are listed in Table I. Information about 38 known or putative PLA isoforms in castor and Arabidopsis is included in Supplemental Table S1. Twelve *PC-PLA*$_1$, one *phosphatidic acid* (*PA*)-*PLA*$_1$, one *LCAT-like3*, 12 *pPLA*, two *sPLA*$_2$, one *SOBER1*, and one *LCAT-like4* were identified in the castor genome. Four *PLAs* were found to have substantial expression in developing castor endosperm, the highest being $sPLA_2\alpha$ (12.1 ESTs per 10^5), followed by *LCAT-like4* (7.7 ESTs per 10^5), pPLAI (4.6 ESTs per 10^5), and $sPLA_2\beta$ (3.2 ESTs per 10^5). By combining expression data with the knowledge available about the substrate specificity of plant PLA enzymes, it appears that the castor phospholipases most likely involved in catalyzing HFA hydrolysis from PC are $RcsPLA_2\alpha$ and $RcsPLA_2\beta$. Both Arabidopsis homologs, $AtsPLA_2\alpha$ and $AtsPLA_2\beta$, have been shown to catalyze the hydrolysis of FAs specifically from the *sn*-2 position of PC and phosphatidylethanolamine (Lee et al., 2003; Ryu et al., 2005; Mansfeld and Ulbrich-Hofmann, 2007), whereas AtpPLAI is selective for galactolipid acyl chains at both *sn*-1 and *sn*-2 positions (Yang et al., 2007) and the PLA_2 AtLCAT-like4 displays the acyl preference oleoyl > linoleoyl > ricinoleoyl and is mostly cytosolic (Chen et al., 2012).

Phylogenetic and Sequence Analyses of $sPLA_2$

Phylogenetic analysis shows the evolutionary relatedness of $sPLA_2$ protein sequences from castor, Arabidopsis,

Table I. *Expression data for 13 genes encoding phospholipase isozymes in developing castor endosperm*

The most closely related Arabidopsis homolog for each gene is listed along with the pairwise amino acid identity. Expression data include total ESTs identified and the frequency of ESTs (per 10^5) at each development stage (for details, see "Materials and Methods").

Castor Name	Gene Identifier	Arabidopsis Homolog	Amino Acid Identity	Total ESTs	Stage III	Stage IVa	Stage VI	Stages VII + VIII
			%					
RcPLA1-Iβ2	28192.m000243	At4g16820	58.8	1	0	0.4	0	0
RcPLA1-Iγ1	29883.m001996	At1g06800	53.7	5	2.1	0.4	0	0
RcPA-PLA1	29757.m000745	At1g31480	65.3	10	1.4	0.4	1.3	1
RcLCAT3	29929.m004538	At3g03310	69.2	7	0.7	1.2	0.7	1
RcpPLAI	29842.m003623	At1g61850	69.6	47	7	5.6	1.3	5
RcpPLAIIα	30115.m001179	At2g26560	52.0	3	0	0	0	1
RcpPLAIIIβ	28327.m000363	At3g54950	71.4	4	1.4	0.7	0	0
RcpPLAIIIγ	28359.m000278	At4g29800	43.1	3	0.7	0.4	0	0
RcpPLAIIIδ	29647.m002082	At3g63200	72.8	34	2.8	1.9	0	1
$RcsPLA_2\alpha$	29840.m000629	At2g06925	75.0	125	9.9	15.5	18	5
$RcsPLA_2\beta$	29912.m005406	At2g19690	54.1	38	2.8	7.9	2	0
RcSOBER1	29842.m003515	At4g22300	69.1	7	0	0.8	0	0
RcLCAT4	30060.m000520	At4g19860	68.9	98	4.9	8.6	3.3	14

and rice (*Oryza sativa*; Fig. 1). Both castor $RcsPLA_2\alpha$ and $RcsPLA_2\beta$, identified by BLAST search in The Institute for Genomic Research, show a close relatedness with Arabidopsis $AtsPLA_2\alpha$ and $AtsPLA_2\beta$, with a degree of identity at the amino acid level of 73.5% and 52.9%, respectively. No castor homologs were identified for $AtsPLA_2\gamma$ or $AtsPLA_2\delta$. Multiple sequence alignment analysis confirmed, in both $RcsPLA_2\alpha$ and $RcsPLA_2\beta$, the presence of highly conserved regulatory and catalytically important motifs characteristic of $sPLA_2$ proteins. Both $RcsPLA_2\alpha$ and $RcsPLA_2\beta$ harbor a defined PA2c (SMART accession no. SM00085) domain with a conserved Ca^{2+}-binding loop denoted by the YGKYCGxxxxGC motif and the catalytic site LDACCxxHDxCV, with His/Asp residues participating in catalysis (Fig. 2). Twelve Cys residues are completely conserved in the different $sPLA_2$ proteins and are potentially involved in intramolecular disulfide bridges, stabilizing the protein structure (Fig. 2). In relation to subcellular localization, both Arabidopsis homologs $AtsPLA_2\alpha$ and $AtsPLA_2\beta$ and the rice homolog $OssPLA_2\alpha$ possess signal sequences as predicted by PSORT (http://psort.hgc.jp) and have been localized previously to the endoplasmic reticulum (ER) or Golgi (Seo et al., 2008; Matos and Pham-Thi, 2009; Lee et al., 2010; Singh et al., 2012). A common motif (-LHKP) is found at the C terminus of both the rice and castor $sPLA_2\alpha$ sequences, constituting a putative ER retention signal (Singh et al., 2012).

RcsPLA$_2\alpha$ Expression Leads to a Dramatic Reduction of HFA Levels in CL37 Seeds

$RcsPLA_2\alpha$ and *$RcsPLA_2\beta$* were cloned from a castor endosperm complementary DNA (cDNA) library, introduced into the expression vector pGate-DsRed-Phas (Lu et al., 2006), and expressed under the control of the seed-specific phaseolin promoter (Slightom et al., 1983) in the Arabidopsis CL37 line. CL37 is an Arabidopsis line expressing *RcFAH12* and accumulating 17% HFAs, including ricinoleic acid and densipolic acid (12-hydroxy-octadec-cis-9,15-enoic acid [18:2-OH]; Lu et al., 2006). CL37 also is mutated in its *fatty acid elongase* (*fae1*) gene (Kunst et al., 1992) and thus is devoid of the very-long-chain HFAs lesquerolic acid (20:1-OH) and auricolic acid (20:2-OH), simplifying the FA profile analysis by gas chromatography (GC). The marker gene *DsRed* allows the identification of transgenic seeds (Stuitje et al., 2003).

To obtain an initial estimate of the effect of each enzyme on HFA metabolism in transgenic Arabidopsis seeds, we analyzed samples (30 seeds each) of red and brown T1 seeds. Thus, each sample of red seed was hemizygous for 30 distinct *phaseolin:$RcsPLA_2\alpha$* or

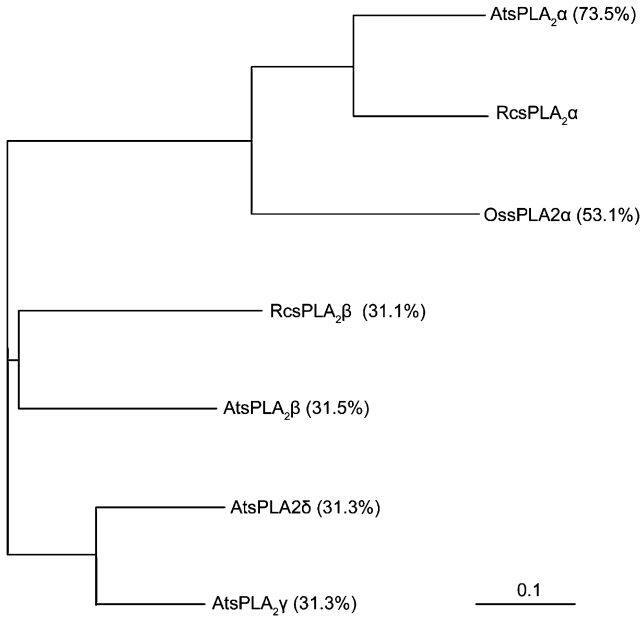

Figure 1. Phylogenetic relationship of the $sPLA_2$ family in castor (Rc), Arabidopsis (At), and rice (Os). The sequence alignment was produced with ClustalX 2.0, and the rectangular cladogram was generated using TreeView. The percentage amino acid pairwise identity between the different protein sequences and $RcsPLA_2\alpha$ is indicated in parentheses. The scale bar represents 0.1 amino acid substitutions per site.

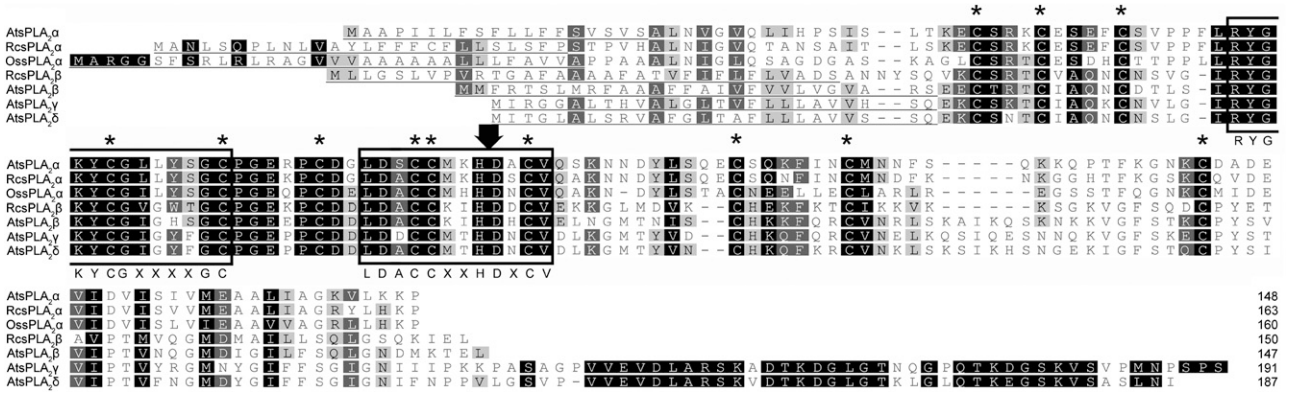

Figure 2. Alignment of amino acid sequences of seven sPLA$_2$s from castor (Rc), Arabidopsis (At), and rice (Os). The conserved domains, Ca^{2+}-binding loop (RYGKYCGxxxxGC) and active site (LDACCxxHDxC) motifs, are boxed. Each of the 12 conserved Cys residues is indicated by an asterisk. The putative signal sequences as predicted by the PSORT prediction program (http://psort.hgc.jp) are underlined. The arrow marks the conserved His/Asp dyad of the active site

phaseolin:RcsPLA$_2\beta$ transformation events. Samples of seeds expressing the *RcsPLA$_2\beta$* construct showed no change in HFA content compared with CL37 control samples, and subsequent analysis of T2 seeds from 30 additional T1 plants also failed to provide any evidence that expression of *RcsPLA$_2\beta$* altered HFA accumulation or other aspects of TAG metabolism in seeds. T1 seed samples expressing *RcsPLA$_2\alpha$*, however, exhibited an average 20% reduction in HFA relative to segregating and control CL37 samples (14.8% ± 0.1% versus 18.8% ± 0.3%). Therefore, we grew 75 T1 plants to maturity and analyzed the FA composition of a sample of T2 (segregating) seeds from each plant. Twenty-four of the 75 plants yielded seeds with a greater than 25% reduction in HFA relative to three CL37 controls, with several lines having approximately 5% HFA.

Segregation of the DsRed marker indicated that both single-insert (75% red seeds) and multiple-insert (more than 90% red seeds) lines were present. By genetic analysis in the T2 and subsequent generations, we identified five homozygous, single-insert lines (designated RcsPLA$_2\alpha$-CL37 line 1–RcsPLA$_2\alpha$-CL37 line 5) for further characterization, along with null-segregant lines that were analyzed and confirmed to have seed FA compositions similar to CL37. The fatty acid compositions of seed samples collected from homozygous plants of each line are shown in Table II. HFA content was decreased by 64% (line 4) to 94% (line 5) relative to CL37 controls. In all five lines, we noted substantial increases in α-linolenic acid (18:3Δ9cis,12cis,15cis) and linoleic acid (18:2Δ9cis,12cis) and a modest decrease in oleic acid. Our results are consistent with the RcsPLA$_2\alpha$ enzyme acting to selectively remove HFA from the pathway of TAG synthesis, possibly through the hydrolysis of HFA from the pathway intermediate, PC. Our analyses of seed lipids did not indicate a decrease in PC content or any substantial increase in free fatty acids or LPC. These two products of PLA$_2$ enzymes presumably are rapidly metabolized, since their accumulation can disrupt membrane and cell function.

Overexpression of the Arabidopsis *sPLA$_2\alpha$* Homolog Does Not Alter HFA Levels in CL37 Seeds

To ascertain whether the reduction in HFA in RcsPLA$_2\alpha$-CL37 lines was not due simply to increased nonspecific PLA$_2$ activity but is indeed due to RcsPLA$_2\alpha$ having specificity for HFAs, the endogenous Arabidopsis gene *AtsPLA$_2\alpha$* was cloned into the pGate-DsRed-Phas vector and used to transform CL37 plants. Samples of T2 seeds from 24 independent T1 transformants were analyzed for FA composition. None of the samples had HFA content beyond 2 SD of 12 CL37 control samples (low, 16.4% HFA; high, 20.3% HFA), and the mean value of all 24 plants, 18.7% ± 0.2% (SE), was indistinguishable from that of the controls at 18.5% ± 0.3% (SE). The proportions of other FAs in the *AtsPLA$_2\alpha$* transgenics also were very similar to the CL37 controls (Supplemental Table S2). We also overexpressed *AtsPLA$_2\beta$* in CL37 using the same pGate-DsRed-Phas vector and observed a similar lack of impact on the HFA content of the resulting transgenic seeds (Supplemental Table S2). The absence of modification in the lipid profile in AtsPLA$_2\alpha$-CL37 seeds may indicate a lack of specificity of AtsPLA$_2\alpha$ for HFA or possibly a low degree of activity of the AtsPLA$_2\alpha$ isozyme in the seed tissues. We also cannot exclude the possibility that, for some reason, our transgene failed to express high amounts of the protein. In any case, our results indicate that the castor sPLA$_2\alpha$ enzyme likely possesses specificity for HFA above that of AtsPLA$_2\alpha$ and that the HFA reductions observed are not due solely to a general increase in PLA$_2$ activity.

RcsPLA$_2\alpha$ Is More Efficient than AtsPLA$_2\alpha$ in Catalyzing the Liberation of Ricinoleate from Ricinoleoyl-PC

To further investigate the castor and Arabidopsis sPLA$_2\alpha$ isozymes, we cloned cDNAs encoding RcsPLA$_2\alpha$ and AtsPLA$_2\alpha$ into a pYES2 vector to allow strong inducible expression in yeast. An empty-vector construct

Table II. *FA composition of T3 and T4 homozygous seeds coexpressing castor RcsPLA$_2\alpha$ with castor hydroxylase (CL37 background)*

All samples were derived from lines with a single transgenic insert, as determined by pedigree analysis. Data are means ± SE for three to six samples of 30 seeds each. –, Not detected.

Line	Fatty Acid Composition							Sum of HFAs
	16:0	18:0	18:1	18:2	18:3	18:1-OH	18:2-OH	
				% of total				
fae1	9.7 ± 0.1	3.5 ± 0.1	27.3 ± 0.5	36.0 ± 0.1	23.2 ± 0.5	–	–	–
CL37	12.6 ± 0.3	6.0 ± 0.2	37.4 ± 0.4	20.0 ± 0.5	6.2 ± 0.1	14.3 ± 0.5	3.6 ± 0.1	17.9 ± 0.5
Line 1	10.0 ± 0.1	2.6 ± 0.1	33.6 ± 0.5	32.5 ± 0.3	17.8 ± 0.4	2.1 ± 0.1	1.3 ± 0.1	3.5 ± 0.2
Line 2	9.9 ± 0.1	3.3 ± 0.0	35.7 ± 0.3	28.5 ± 0.1	18.1 ± 0.2	2.8 ± 0.0	1.7 ± 0.0	4.5 ± 0.1
Line 3	13.7 ± 1.3	2.8 ± 0.1	34.7 ± 0.5	28.1 ± 0.5	16.4 ± 0.4	2.8 ± 0.1	1.1 ± 0.1	3.9 ± 0.1
Line 4	10.1 ± 0.1	4.1 ± 0.1	31.5 ± 0.7	27.7 ± 1.2	20.1 ± 1.4	4.7 ± 1.1	1.8 ± 0.5	6.5 ± 1.6
Line 5	10.1 ± 0.1	3.3 ± 0.0	29.5 ± 0.4	34.5 ± 0.2	21.6 ± 0.1	0.7 ± 0.1	0.4 ± 0.2	1.1 ± 0.2

was used as a control. Separate PLA_2 assays were performed using both cytosolic and microsomal fractions derived from yeast, producing recombinant RcsPLA$_2\alpha$ or AtsPLA$_2\alpha$. Yeast-derived fractions after ultracentrifugation were used in PLA_2 assays to catalyze the hydrolysis of the substrates *sn*-1-palmitoyl(16:0)-*sn*-2-[^{14}C]oleoyl-phosphatidylcholine ([^{14}C]18:1-PC) and *sn*-1-palmitoyl-*sn*-2-[^{14}C]ricinoleoyl-phosphatidylcholine ([^{14}C]Ric-PC) to produce free [^{14}C]oleic acid or [^{14}C]ricinoleic acid, respectively. From the time course of the reaction plots (Supplemental Fig. S1), it appears that at early time points both microsomal fractions containing RcsPLA$_2\alpha$ or AtsPLA$_2\alpha$, respectively, displayed higher activity against *sn*-1-palmitoyl(16:0)-*sn*-2-oleoyl-phosphatidylcholine (18:1-PC) than the cytosolic fractions. Contrasting with AtsPLA$_2\alpha$, RcsPLA$_2\alpha$ showed substantial cytosolic activity. PLA_2 activity, however, was associated primarily with the microsomal fraction for both isozymes, suggesting a potential ER localization for RcsPLA$_2\alpha$ and AtsPLA$_2\alpha$.

We first studied the substrate specificity of RcsPLA$_2\alpha$ and AtsPLA$_2\alpha$ in yeast microsomes supplied with either labeled [^{14}C]18:1-PC or [^{14}C]Ric-PC. RcsPLA$_2\alpha$ specific activity was almost double that of AtsPLA$_2\alpha$ using [^{14}C]18:1-PC as substrate (52.3 ± 1.4 and 28.5 ± 2.8 pmol min^{-1} mg^{-1} protein, respectively). Moreover, when using [^{14}C]Ric-PC, RcsPLA$_2\alpha$ was nine times more efficient in catalyzing the hydrolysis of the substrate than AtsPLA$_2\alpha$ (32.7 ± 0.1 and 3.4 ± 0.1 pmol min^{-1} mg^{-1} protein, respectively; Fig. 3A). We then determined the selectivity of both enzymes by using an equimolar mixture of [^{14}C]18:1-PC and [^{14}C]Ric-PC (Fig. 3B). In this case, the hydrolysis of [^{14}C]18:1-PC was much more effective relative to [^{14}C]Ric-PC, with oleic acid representing over 75% of product for both enzymes. However, RcsPLA$_2\alpha$ was still twice as efficient as AtsPLA$_2\alpha$ in catalyzing the hydrolysis of [^{14}C]Ric-PC, even in the presence of mixed substrates: with RcsPLA$_2\alpha$, 23.5% of hydrolyzed FAs were ricinoleate, whereas with AtsPLA$_2\alpha$, only 11.8% were ricinoleate (Fig. 3B). It appears that RcsPLA$_2\alpha$ is a more efficient enzyme than AtsPLA$_2\alpha$ in catalyzing the hydrolysis of [^{14}C]Ric-PC, even if both enzymes display higher specificities and selectivities for [^{14}C]18:1-PC than for [^{14}C]Ric-PC.

Changes in the FA Composition of TAG and PC during Seed Development of RcsPLA$_2\alpha$-CL37 Lines

Total lipids were extracted from CL37 and RcsPLA$_2\alpha$-CL37 seeds at different stages of development from 7 to 18 d after flowering. Total lipid fractions were separated into neutral lipids (mostly TAG) and PC (Fig. 3) by thin-layer chromatography (TLC). The neutral lipid fraction from CL37 seeds showed an increase in HFAs, which rose from 0% to 20% of total FAs in mid development (Fig. 4A). Developing RcsPLA$_2\alpha$-CL37 seed TAG also showed an increase in HFAs during development, but the maximum of less than 5% of total FAs was much lower than that in CL37 seeds (Fig. 4C). The approximately 80% HFA reduction in RcsPLA$_2\alpha$-CL37 seed TAG relative to CL37 was not confined to mature seeds but was evident throughout development (Fig. 4E). Relative amounts of 18:3 and 18:2 also showed significant increases during development, with 18:3 increasing by 160% and 18:2 increasing by almost 50% due to the expression of the *RcsPLA$_2\alpha$* transgene (Fig. 4E). The relative amount of 18:1 in TAG showed only a slight increase during development, whereas both 16:0 and 18:0 were reduced. The differences in TAG FA composition between RcsPLA$_2\alpha$-CL37 and CL37 seeds remained largely constant beyond 13 or 14 d after flowering (Fig. 4E).

In the PC fraction derived from CL37 developing seeds, HFA levels increased up to 9.5% in the mid development stage, whereas 16:0, 18:0, and 18:3 levels were relatively constant throughout seed development (Fig. 4B). 18:1 showed an increase correlating with a large decrease in 18:2 before stabilizing at mid development. A similar pattern occurred in PC derived from RcsPLA$_2\alpha$-CL37 seeds, but HFA increased only marginally during development to 2.9% (Fig. 4D). When comparing differences in PC FA composition between CL37 and RcsPLA$_2\alpha$-CL37 seeds, the reduction in HFA observed in PC (approximately 70%) was similar to the reduction observed in TAG (approximately 80%) and was constant throughout development (Fig. 4F). The changes in other FAs were modest throughout seed development, with the exception of 18:3, which showed large differences (up to 70%) during early to mid development stages before being reduced to 20% at a later stage of development

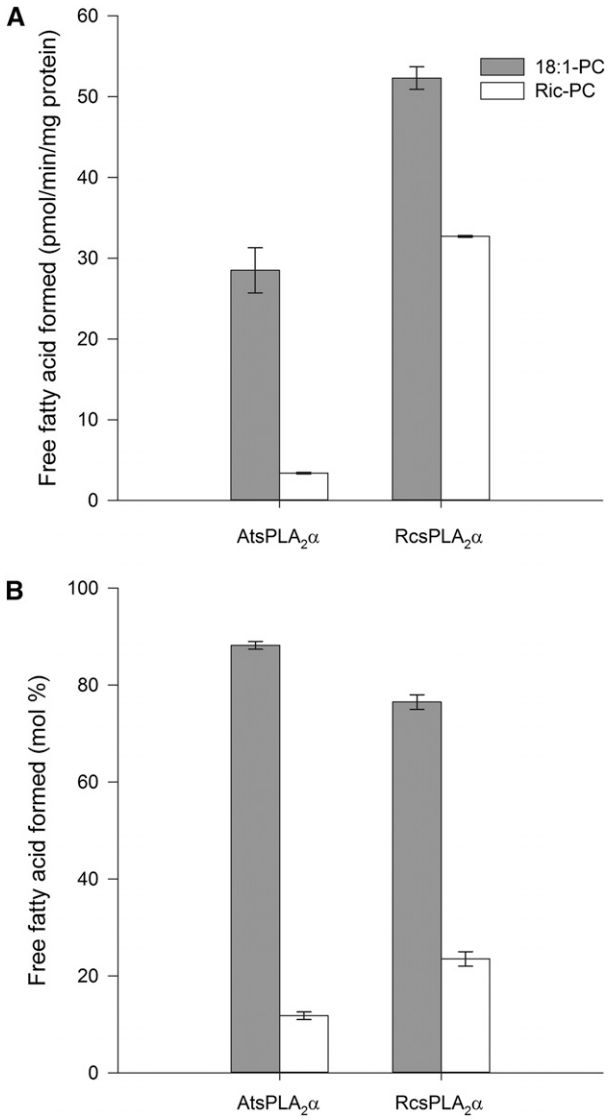

Figure 3. Substrate specificities and selectivities of AtsPLA$_2\alpha$ and RcsPLA$_2\alpha$. Microsomal fractions derived from yeast expressing either AtsPLA$_2\alpha$ or RcsPLA$_2\alpha$ were incubated with 18:1-PC or Ric-PC as substrate to determine the specific activity at 30 min of reaction time (A) or an equimolar mixture of 18:1-PC and Ric-PC used as substrates to determine enzymatic selectivity with ratios of free fatty acids formed after 30 min of incubation time (B).

(Fig. 4F). 18:1 and 18:2 both showed slight increases of approximately 15% during later stages of development, whereas 18:0 and 16:0 were reduced by approximately 50% and approximately 30%, respectively, during seed development. The differences in FA composition in PC between the two lines can be considered mostly constant after the mid development stage (Fig. 4F).

Total Fatty Acid Content Is Increased Slightly and Germination Rate Is Restored in RcsPLA$_2\alpha$-CL37 Seeds

The total FA content of homozygous seeds from five RcsPLA$_2\alpha$-CL37 lines was compared with that of the brown null-segregant seeds from the same lines and that of *fae1* seeds (Fig. 5). Previous analyses by Bates and Browse (2011) and van Erp et al. (2011) showed reductions in seed total FA content of 30% and 20%, respectively, in CL37 plants expressing *RcFAH12*, compared with parental *fae1*, and the difference between *fae1* and the CL37 null segregants in Figure 5 (25.6%) is in this range. The expression of *RcsPLA$_2\alpha$* in CL37 seed increased the total average FA content in homozygote seeds up to 5.63 μg per seed, compared with brown null-segregating CL37 seeds (5.05 μg per seed). This represents a significant increase of 11.5% ($P = 0.024$); however, oil content remained substantially below that of the *fae1* controls (6.79 μg per seed). Seed germination rates also were restored to nearly wild-type levels, from an average of 24% for cosegregating T2 CL37 seeds to 95% for the transgenic T2 RcsPLA$_2\alpha$-CL37 seeds from three different lines.

DISCUSSION

The castor hydroxylase RcFAH12 is an ER enzyme that converts 18:1 to 18:1-OH esterified at the *sn*-2 position of PC (Bafor et al., 1991; Broun and Somerville, 1997). When *RcFAH12* is expressed in Arabidopsis seeds (in lines CL7 and CL37), 18:1-OH and 18:2-OH HFA accumulate to 10% to 11% of the FA in PC and produce a bottleneck in lipid metabolism that reduces FA synthesis and TAG accumulation through feedback inhibition of acetyl-CoA carboxylase (Bates and Browse, 2011; Bates et al., 2014). Expression of castor *Diacylglycerol Acyltransferase2* or *RcPDAT1A* reduced PC HFA levels (van Erp et al., 2011) and substantially alleviated the reductions in FA synthesis and TAG accumulation (Bates et al., 2014). In castor endosperm, it is likely that PLA$_2$ plays a major role in removing newly synthesized HFA from PC (Bafor et al., 1991). Therefore, we sought to identify a suitable *PLA$_2$* gene in castor and test its effectiveness in supporting metabolism and the accumulation of HFA when coexpressed with *RcFAH12* in transgenic Arabidopsis.

RNA sequencing results (Table I) indicate that the *RcsPLA$_2$* gene is the most highly expressed *PLA$_2$* isoform throughout the stages of oil accumulation in developing castor endosperm (Troncoso-Ponce et al., 2011). This gene encodes an enzyme of the small PLA$_2$ family, RcsPLA$_2\alpha$. sPLA$_2\alpha$ homologs have been identified in Arabidopsis, rice, and several other plant species (Ståhl et al., 1999; Lee et al., 2005; Singh et al., 2012). The rice *sPLA$_2\alpha$* is strongly expressed in seeds and other tissues (Singh et al., 2012), while the Arabidopsis homolog is not highly expressed in developing seeds (Supplemental Table S1). *AtPLA$_2\alpha$* is expressed in vegetative tissues, and the encoded enzyme likely acts in lipid signaling (Lee et al., 2005). In contrast to the very low expression of *AtPLA$_2\alpha$* in developing Arabidopsis seeds, the high expression of *RcsPLA$_2\alpha$* in developing castor endosperm suggests that it may encode an enzyme responsible for the efficient removal

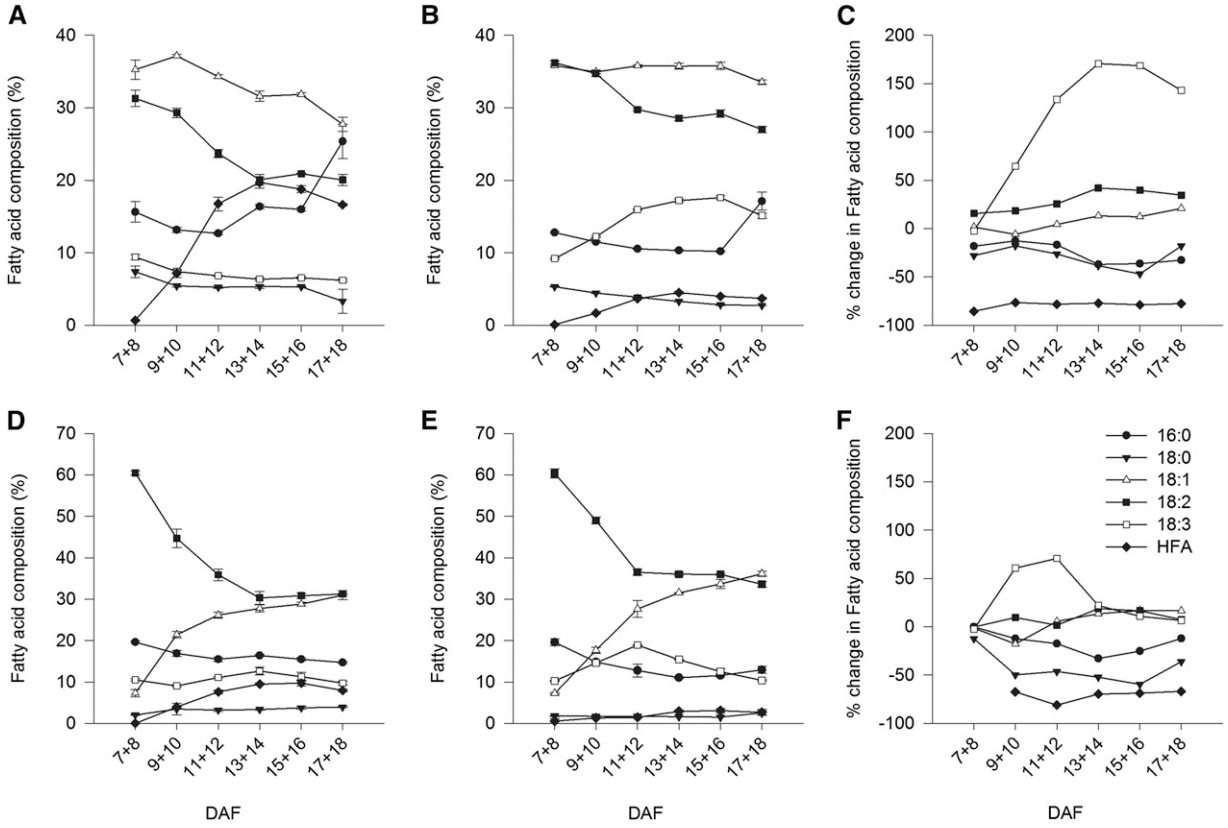

Figure 4. Changes in FA composition in TAG and PC from 7 to 18 d after flower opening (DAF) in CL37 (A and D) and transgenic RcsPLA$_2\alpha$-CL37 line 3 (B and E). A and B, FA composition of seed TAG. C, Calculated difference between line 3 and CL37 for TAG. D and E, FA composition of seed PC. F, Calculated difference between line 3 and CL37 for PC. Data are means ± SE for three replicates.

of HFA from *sn*-2 of PC following its synthesis catalyzed by the hydroxylase during TAG accumulation and seed development in castor. Release of HFA from PC in castor endosperm is thought to allow conversion to the CoA thioester and incorporation into TAG by the three acyltransferases of the Kennedy pathway, *sn*-glycerol-3-phosphate acyltransferase, lysophosphatidic acid acyltransferase, and diacylglycerol acyltransferance (Bafor et al., 1991).

The rice sPLA$_2\alpha$ protein fused to GFP was shown to colocalize with an ER marker (Singh et al., 2012). This result is consistent with our finding that recombinant RcsPLA$_2\alpha$ produced in yeast showed high activity in the microsomal fraction of cell homogenates (Supplemental Fig. S1), but the activity in the high-speed supernatant also was high. The RcsPLA$_2\alpha$ protein has a C-terminal sequence, -LHKP, that is identical to the sequence in the rice protein (Singh et al., 2012), and this may constitute the ER retention signal. Alternatively, protein acylation has been suggested as a potential mechanism for localization to the ER (Ståhl et al., 1999).

The enzymatic properties of the Arabidopsis family of sPLA$_2$s have been well characterized, with a regiospecificity for *sn*-2 acyl chains and with a head group selectivity for PC and phosphatidylethanolamine over other phospholipids (Chen et al., 2011). In this study, the in vitro assays using yeast microsomes show that both castor and Arabidopsis sPLA$_2\alpha$ are active in the picomolar range against [^{14}C]18:1-PC, demonstrating the *sn*-2 regiospecificity of PLA$_2$. Enzymes from both castor and Arabidopsis displayed higher specificity for [^{14}C]18:1-PC than for [^{14}C]Ric-PC (Fig. 3A). However, this loss of oleate from PC through the preferential hydrolysis of 18:1-PC over *sn*-1-palmitoyl-*sn*-2-ricinoleoylphosphatidylcholine (Ric-PC) may be mitigated to some extent through the selective reacylation of LPC by the endogenous Arabidopsis LPCATs (Lager et al., 2013). The newly liberated oleic acid would have to be reactivated to its CoA-thioester, prior to transfer catalyzed by LPCAT into PC, via the catalytic action of long-chain acyl-coenzyme A synthetase (LACS; Shockey et al., 2002). Both specificity and selectivity assays showed that RcsPLA$_2\alpha$ was a more efficient enzyme than AtsPLA$_2\alpha$ in catalyzing the hydrolysis of [^{14}C]Ric-PC. Assuming comparable levels of protein expression in the yeast, the specific activity of RcsPLA$_2\alpha$ was 9 times that of AtsPLA$_2\alpha$ (32.7 ± 0.1 versus 3.4 ± 0.1 pmol min^{-1} mg^{-1} protein) with [^{14}C]Ric-PC as sole substrate, and RcsPLA$_2\alpha$ was twice as efficient as

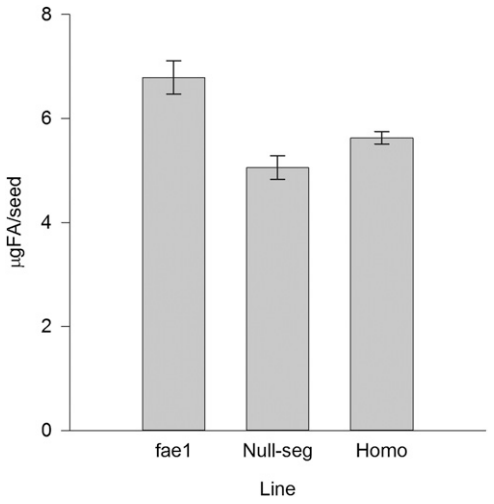

Figure 5. Partial recovery of seed oil content in CL37 lines expressing RcsPLA$_2\alpha$. Five plants from each of the homozygous lines 1 to 5 were sampled (Homo), together with 11 CL37 segregants (Null-seg) and three *fae1* plants. Data are means ± SE for total fatty acids for five transgenic and five null-segregant lines.

AtsPLA$_2\alpha$ in a selectivity assay with the presence of an equimolar mixture of [^{14}C]18:1-PC and [^{14}C]Ric-PC (Fig. 3).

Consistent with the high activity of recombinant RcsPLA$_2\alpha$ on Ric-PC, developing CL37 seeds expressing RcsPLA$_2\alpha$ showed a dramatic decrease in HFA in PC to less than 3% of total FAs during the period of oil accumulation, compared with 10% in CL37 controls (Fig. 4). In mature CL37 seeds, approximately 70% of HFA in TAG is esterified to the *sn*-2 position of the glycerol backbone and is produced from PC via PC-derived diacylglycerol (DAG; Bates and Browse, 2011). Thus, the activity of the castor lipase in RcsPLA$_2\alpha$-CL37 plants provides a reasonable explanation for both the low HFA content in PC and the greatly reduced accumulation of HFA in oil of mature seeds relative to CL37 controls.

The expression of *RcsPLA$_2\alpha$* in CL37 also resulted in substantial changes in the proportions of other FAs in the seed. Compared with the *fae1* parental line, CL37 seeds had a 66% decrease in 18:2 + 18:3 fatty acids (from 59.2% to 26.2%; Table II). Coexpression of the *sPLA$_2\alpha$* in our RcsPLA$_2\alpha$-CL37 lines reduces this change, especially in line 5 with the lowest HFA level, which had an FA composition similar to that of *fae1*. It has been proposed that HFAs inhibit the FAD2 and FAD3 desaturases that catalyze the conversion of 18:1 to 18:2 and 18:3, respectively (Broun and Somerville, 1997). An alternative explanation is that the bottleneck in the conversion of PC to PC-derived DAG that occurs in CL37 plants and causes a substantial decrease in FA synthesis and oil accumulation (Bates and Browse, 2011; Bates et al., 2014) results in increased flux of de novo DAG into TAG. Because de novo DAG contains much higher 18:1 and lower 18:2 + 18:3 than PC-derived DAG (Bates and Browse, 2011), this shift also would contribute to explaining the FA composition observed in CL37 seeds. The reverse changes in FA composition in RcsPLA$_2\alpha$-CL37 seeds suggest that the bottleneck may be substantially alleviated. We did observe an increase in total FA accumulation in lines expressing *RcsPLA$_2\alpha$*, but this was relatively small compared with the difference between CL37 (RcsPLA$_2\alpha$-null) segregants and the *fae1* parental line (Fig. 5).

Following the removal of HFA from *sn*-2 of PC catalyzed by RcsPLA$_2\alpha$, the expected route of HFA breakdown is via β-oxidation in the peroxisome. The current view of β-oxidation would anticipate the conversion of HFA to the HFA-CoA thioester via the activity of a LACS (Shockey et al., 2002), subsequent hydrolysis of the HFA-CoA during transport into the peroxisome through the Peroxisomal ABC transporter1 ATP-binding cassette transporter (De Marcos Lousa et al., 2013), and the resynthesis of HFA-CoA inside the peroxisome catalyzed by the LACS6 and LACS7 isozymes (Fulda et al., 2004). While acyl-CoAs are known to be substrates for the PXA1 transporter, it is not definitively known whether the plant PXA1 also can accept free FAs for transport into the peroxisome and activation catalyzed by LACS6 and LACS7. It may be that HFA released by RcsPLA$_2\alpha$ is transported directly into the peroxisome for degradation. Moire et al. (2004) used a transgene encoding polyhydroxyalkanoate synthase to capture β-oxidation intermediates and allow the assessment of the relative flux of HFA and other fatty acids through the β-oxidation pathway. The expression of polyhydroxyalkanoate synthase in CL37 and RcsPLA$_2\alpha$-CL37 plants could be used to confirm our expectation that HFA degradation occurs through β-oxidation. Interestingly, while this technique indicated increased rates of β-oxidation in seeds of Arabidopsis lines producing HFA or other unusual fatty acids, there was no increase in transcript levels of genes encoding enzymes of the β-oxidation pathway (Moire et al., 2004).

The question of whether HFAs released by RcsPLA$_2\alpha$ are direct substrates of the peroxisomal PXA1 transporter is potentially important, because if HFA-CoA were synthesized in the ER (or cytoplasm), it would be expected to be available for TAG synthesis catalyzed by the acyltransferases of the Kennedy pathway as well as for the reincorporation into PC catalyzed by LPCAT. The very low levels of HFA in both PC and TAG of RcsPLA$_2\alpha$-CL37 seeds indicate that these routes of HFA reincorporation are substantially unused either because HFA-CoA is not synthesized or, possibly, because the three acyltransferases of the Kennedy pathway (glycerol-3-phosphate acyltransferase, lysophosphatidic acid acyltransferase, and diacylglycerol acyltransferance) have low activity with HFA-CoA and preferentially select other 16- and 18-carbon acyl-CoAs for incorporation into glycerolipids. We have considered the possibility that Arabidopsis ER (or cytoplasmic) LACS isozymes are not able to efficiently convert HFA to HFA-CoA. In attempting to overcome this potential barrier to the

reincorporation of HFA, we identified three castor *LACS* genes (homologs of Arabidopsis *LACS1*, *LACS4*, and *LACS8*) that are highly expressed in developing endosperm tissue (Troncoso-Ponce et al., 2011). However, when we coexpressed each of these isoforms in RcsPLA$_2\alpha$-CL37 line 3, we observed no increase in seed HFA content relative to that shown for line 3 in Table II. It also will be worthwhile to investigate the biochemistry and HFA content of RcsPLA$_2\alpha$-CL37 plants that are additionally expressing transgenes encoding the castor isoforms of the Kennedy pathway enzymes, as these lines become available.

Available evidence indicates that, in castor endosperm, HFA synthesized from 18:1 at the *sn*-2 of PC is released by PLA$_2$, activated to the CoA thioester, and incorporated into TAG by acyltransferases of the Kennedy pathway (Bafor et al., 1991). Our goal in this investigation was to test the possibility that RcsPLA$_2\alpha$, which is highly expressed in developing castor endosperm, would catalyze the removal of HFA from PC to relieve the metabolic bottleneck in CL37 Arabidopsis that reduces FA synthesis and TAG accumulation (Bates and Browse, 2011; Bates et al., 2014) while making the HFA available for TAG synthesis via enzymes of the Kennedy pathway. Expression of *RcsPLA$_2\alpha$* in CL37 does result in the efficient removal of HFA from PC; however, there is little or no reincorporation into TAG, and instead, HFA appears to be degraded, likely by β-oxidation. These results imply that any release of HFA by endogenous Arabidopsis PLA$_2$ also would be degraded and unavailable for incorporation into TAG. *AtsPLA$_2\alpha$* and *AtsPLA$_2\beta$* are poorly expressed in developing Arabidopsis seeds, and the physiological function of the AtsPLA$_2\alpha$ enzyme is thought to be in membrane trafficking in vegetative tissues (Lee et al., 2005). One possible explanation for why the expression of AtsPLA$_2\alpha$ has no effect on the HFA content of seeds (Supplemental Table S2) is that the enzyme is inactive in the absence of upstream regulatory processes in seeds. However, two other genes, *pPLAIIβ* (At4g37050) and *pPLAIIIγ* (At4g29800), are highly expressed in developing Arabidopsis seeds (Supplemental Table S1). Our results suggest that blocking the expression of one or both of these genes may result in higher HFA in PC. Whether this would result in greater inhibition of FA synthesis (Bates et al., 2014) or an increase in HFA accumulation in the seed oil will need to be investigated experimentally.

MATERIALS AND METHODS

Analysis of Data from 454 Massively Parallel Pyrosequencing of Castor Endosperm

The corresponding Arabidopsis (*Arabidopsis thaliana*) protein sequences were obtained from The Arabidopsis Information Resource (http://www.arabidopsis.org/), and the castor (*Ricinus communis*) homologs were obtained by performing a protein BLAST against the 4× draft castor protein database hosted by the JCVI (http://www.jcvi.org). The castor gene model identities were identified using a cutoff score of 1,000. The protein sequences of the castor genes and their Arabidopsis homologs used as queries were then aligned using the ClustalX2 algorithm (Larkin et al., 2007) and visualized as a homology tree with TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Analysis of the tree allowed the identification of castor homologs of the Arabidopsis proteins. The high degree of homology (greater than 60% pairwise identity) was confirmed by aligning the castor protein sequence with that of its homolog using the alignment function in Geneious (www.geneious.com). (Only 12% of selected protein sequences had a pairwise identity of less than 50%.) Castor genes were named after their closest Arabidopsis homolog based on percentage identity; if there was more than one homolog, the castor gene name was appended with a letter with decreasing levels of homology. The castor protein sequence was then used in a TBLASTN at the JCVI to obtain the nucleotide sequence. The castor nucleotide sequence was subsequently used to perform a BLASTN at the Michigan State University Castor 454 expression database (approximately 70,000 contigs assembled from approximately 1,060,000 ESTs; http://glbrc.plantbiology.msu.edu/castor). The expression data of the stages were obtained from castor seeds that were harvested from stage III to stage VIII, based on embryo length and testa (seed coat) color, as defined anatomically by Greenwood and Bewley (1982). In the 454 database, the total number of contigs is 69,194, stage III contains 141,964 ESTs, stage IVa contains 267,141 ESTs, stage VI contains 152,462 ESTs, and stages VII and VIII contain 151,964 ESTs. With only 77,518 and 84,170 ESTs, respectively, stages V and IVb were ignored, as were stages III to V with 184,642 ESTs. Data containing the contig number, the number of ESTs per contig, the best Arabidopsis homolog, and the number of ESTs per 100,000 per embryo stage are compiled in Table I. Table I also includes the gene family, the Arabidopsis gene identifier, the Arabidopsis gene name, the name of the castor homolog, the castor gene model accession from the JCVI, and the percentage homology as measured by the percentage of pairwise identical amino acids in the aligned sequences. Additional methodology is available from Troncoso-Ponce et al. (2011).

Isolation and Cloning of RcsPLA$_2\alpha$

The protein sequences corresponding to the *RcsPLA$_2\alpha$* (XP_002523659) open reading frame obtained after translation using the Expasy translate tool (http://web.expasy.org/translate) of DNA sequences identified from the castor database at the JCVI (http://www.jvci.org; GI: 29840.m000629), the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov/genbank/; XM_002523613), and the Michigan State University castor database (http://glbrc.bch.msu.edu/castor/; M01000020055) are identical at the amino acid level and show a high degree of identity (73.5%) with AtsPLA$_2\alpha$ (At2g06925). *RcsPLA$_2\alpha$* amplicons were isolated from a castor seed cDNA library using primers RcPLA21/f (5′-CACCATGGCAAATTTAAGCCAGCCATTGAATTTAGTAGCTTATC-3′) and RcPLA21/r (5′-TCATGGTTTATGAAGGTATCTACCAGCAATCAAAGCAGC-3′) and a hot-start DNA polymerase, KOD (Novagen), used in a touch-down PCR: 94°C for 2 min, five times at 94°C for 30 s and 72°C for 2 min, five times at 94°C for 30 s and 70°C for 2 min, and 25 times at 94°C for 30 s and 68°C for 2 min, followed by a 7°C hold. The amplicon was purified using the QIAquick Gel Extraction Kit (Qiagen) and ligated into PCR-Script-Amp (Agilent). Positive colonies resulting from the heat-shock transformation of XL10-Gold *Escherichia coli* cells (Stratagene) were determined by colony PCR using GoTAQ (Promega) and the above primers. A PCR using KOD (Novagen) was used to amplify the *RcsPLA$_2\alpha$* sequence, which was then ligated into the entry vector of the Gateway system pENTR/D-TOPO (Life Technologies). Subsequent transformation of Top10 cells (Life Technologies) and colony PCR screening allowed the isolation DNA from three colonies (PerfectPrep Spin Mini; 5PRIME). The *RcsPLA$_2\alpha$* sequence in pENTR was then sequenced using the BigDye sequencing kit (ABI), primers RcPLA21/f and RcPLA21/r, and the additional primers M13(-21) (5′-TGTAAAACGACGGCCAGT-3′) and M13r (5′-AACAGCTATGACCATG-3′). pENTR-RcPLA21-2 was combined with pGate-DsRed-Phas (Lu et al., 2006) after an LR clonase reaction (Life Technologies). DNA resulting from minipreparations of positive XL10-Gold colonies was again sequenced with the above-mentioned primers and used to electroporate *Agrobacterium tumefaciens* strain GV3101 for dipping Arabidopsis flowers (Clough and Bent, 1998). For plant transformation, the separate *A. tumefaciens* cultures were grown overnight to stationary phase in 400 mL of Luria-Bertani medium at 28°C with the appropriate antibiotic selection, spun down at 6,000 rpm for 15 min, and resuspended in 400 mL of 5% (w/v) Suc and 0.05% (w/v) Silwet L-77 prior to dipping. Similar to *RcsPLA$_2\alpha$*, *AtsPLA$_2\alpha$* (The Arabidopsis Information Resource, At2g06925; GenBank, NM_126670) was introduced into pGate-DsRed-Phas for seed expression after being amplified from Arabidopsis

leaf cDNA (donated by Dr. Laura Wayne) using primers AtPLA21/1ftopo (5′-CACCATGGCGGCTCCGATCATACTTTTCTC-3′) and AtPLA21/1r (5′-TTAGGGTTTCTTGAGGACTTTGCCGGCG-3′).

Plant Growth and Selection of Transgenic Plants

The different Arabidopsis lines used in the experiments are *fae1*, a fatty acid elongase mutant line (AC56) of Arabidopsis ecotype Columbia (Kunst et al., 1992), and CL37, which is derived from *fae1*, expresses *RcFAH12*, and accumulates approximately 17% (w/w) HFAs (Lu et al., 2006). Plants were grown in growth chambers under continuous fluorescent light (100–200 μmol m^{-2} s^{-1}) at 22°C. Seeds were sterilized following 5 min of incubation in sterilization solution (0.5% [w/v] NaOCl [bleach], 30% [v/v] ethanol, and 1 g L^{-1} SDS), followed by five washes with sterile water and 24 h of stratification at 4°C. Seeds were germinated on one-half-strength Murashige and Skoog medium (4.4 g L^{-1}), 1% (w/v) agar plates containing 1% (w/v) Suc and then transplanted to soil. Transgenic seeds expressing the marker DsRed protein were identified by illumination with green light and a hand-held magnifying glass or microscope equipped with a red filter (Stuitje et al., 2003). Further confirmation of the presence of the transgene in T2 plants was performed by screening DNA obtained from T2 leaf material by PCR using the gene-specific primers. DNA was obtained by using Whatman FTA Classic Cards. Leaf discs crushed onto Whatman FTA cards were incubated in 10 μL of water at 95°C for 10 min, and 0.5 μL was used for PCR amplification (30 cycles; melting temperature of 60°C) using the KOD polymerase.

Preparation of Fatty Acid Methyl Esters and Gas Chromatography Analysis

Fatty acid methyl esters (FAMEs) were isolated from 30 whole seeds by derivatizing the seed lipid in 1 mL of 2.5% (v/v) sulfuric acid in methanol for 1 h, 30 min at 80°C. After cooling, 200 μL of hexane and 1.5 mL of water were added and vortexed, spun down at 2,000*g*, and 100 μL was transferred to a GC vial. For total seed FA quantification, 10 μL of glyceryl triheptadecanoate (3 μg μL^{-1}) was added prior to derivatization. FAMEs were quantified by GC with flame ionization detection on a wax column (EC Wax; 30-m × 0.53-mm i.d. × 1.20 μm; Alltech). GC parameters were as follows: 220°C for 2 min, followed by a ramp (10°C min^{-1}) up to 245°C, with a 6-min final temperature hold. The significance of difference between FA content from transgenic seeds and cosegregating seeds was determined by Student's *t* test.

Developmental Time Course and Seed Lipid Extraction

Flowers and siliques from lines CL37 and RcsPLA$_2\alpha$-CL37 line 3 were counted as they appeared on a daily basis to determine silique age. After 20 d, siliques were harvested according to their age, frozen with liquid N_2, and stored at −80°C. Lipid extraction followed the protocol from the Kansas Lipidomics Center (http://www.k-state.edu/lipid/lipidomics/AT-seed-extraction.html). Triplicate samples of 10 siliques were harvested at each time point. Prior to TLC, lipid content was determined by GC analysis using glyceryl triheptadecanoate standard (3 μg μL^{-1}). Lipids were separated on Whatman Partisil K6 silica gel 60-Å 20- × 20-cm glass plates with mobile phase $CHCl_3$:methanol:acetic acid (75:25:8, v/v/v). Plates were dried in a vacuum for 20 min prior to staining with 0.005% (w/v) primulin in acetone:water (80:20, v/v). Lipid bands were visualized by UV light, and bands corresponding to neutral TAG fraction and PC fraction were scraped into 8-mL vials and used to generate FAMEs for GC analysis as described above.

Heterologous Expression of *RcsPLA$_2\alpha$* and *AtsPLA$_2\alpha$* in Yeast, Yeast Cultivation, and Protein Preparation

The open reading frame of *AtsPLA$_2\alpha$* was amplified with the cDNA in the Gateway pENTR/D-TOPO entry vector as the template using primers GC164 (5′-ACTATGGCGGCTCCGATC-3′) and GC165 (5′-TTAGGGTTTCTTGAGGACTTTGC-3′). Similarly, *RcsPLA$_2\alpha$* was amplified using primers GC166 (5′-ACAATGGCAAATTTAAGCCAG-3′) and GC167 (5′-TCATGGTTTATGAAGGTATCTACCAG-3′). The PCR products were individually subcloned into the yeast (*Saccharomyces cerevisiae*) expression vector pYES2.1/V5-His-TOPO (Invitrogen). The plasmids were then transformed into wild-type yeast (Inv Sc1 strain; Invitrogen). A yeast strain transformed with a null pYES2.1/V5-His-TOPO vector was used as a negative control.

Transformed yeast cells were grown at 30°C and 200 rpm in synthetic uracil dropout medium containing 1% (w/v) raffinose and 2% (w/v) Gal for 20 h. Yeast cells were then harvested, washed with 50 mM Tris-HCl, pH 7.6, and suspended in 1 mL of breaking buffer (50 mM Tris-HCl, pH 7.6, 600 mM sorbitol, and 1 mM EDTA) containing protease inhibitor (Complete; Roche). The cells were then broken with a Mini-Beadbeater (BioSpec Products), and the crude homogenates were centrifuged at 12,000*g* at 4°C for 10 min to remove the cell debris. The supernatant was further centrifuged at 100,000*g* at 4°C for 70 min to separate microsomal and cytosolic fractions. The microsomal pellets were washed and resuspended in breaking buffer. Protein concentration was determined using the Bradford method (Bradford, 1976). The protein samples were flash frozen with liquid nitrogen and stored at −80°C until used in the assays (Chen et al., 2012).

Enzyme Assays

For PLA enzyme assays, *sn*-1-palmitoyl-*sn*-2-[^{14}C]oleoyl-PC was purchased from American Radiolabeled Chemicals, and *sn*-1-palmitoyl-*sn*-2-[^{14}C]ricinoleoyl-PC was synthesized according to Banaś et al. (1992). The PLA enzyme assay was performed at 30°C with agitation at 750 rpm in a 200-μL reaction mixture containing 50 mM Tris-HCl, 10 mM $CaCl_2$, 0.05% (v/v) Triton X-100, 9 nmol of substrate, and 100 μg of protein at pH 8 with the incubation times indicated in the figures. The reaction was terminated by the addition of 1 mL of chloroform:methanol (1:1, v/v) and 200 μL of 0.15 M acetic acid and vortexed. After centrifugation, the chloroform phase (lower phase) was dried under nitrogen, dissolved in 40 μL of chloroform, and applied to a TLC plate with standards (0.25-mm Silica gel; DC-Fertigplatten). The plate was developed in the polar solvent chloroform:methanol:water:acetic acid (65:25:4:1, v/v/v/v). The products were visualized by phosphor imaging (Typhoon Trio Variable Mode Imager; GE Healthcare). Spots corresponding to free FA were scraped and analyzed for radioactivity by scintillation counting. For the substrate selectivity experiment, equal amounts of 18:1-PC and Ric-PC (4.5 nmol each) were mixed. The spots corresponding to free FAs were scraped from TLC plates, extracted with chloroform (Bligh and Dyer, 1959), and loaded onto TLC plates. The plates were developed in the neutral solvent hexane:diethyl ester:acetic acid (50:50:1, v/v/v) to separate oleic acid and ricinoleic acid. The corresponding spots were then scraped for scintillation counting. All assays were repeated in triplicate, and the results are represented as means ± SE.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: At2g06925 (*AtsPLA$_2\alpha$*), At2g19690 (*AtsPLA$_2\beta$*), At4g29460 (*AtsPLA$_2\gamma$*), At4g29470 (*AtsPLA$_2\delta$*), XM_002523613 (*RcsPLA$_2\alpha$*), XM_002514118 (*RcsPLA$_2\beta$*), NM_001057569 (*OssPLA$_2\alpha$*), XM_002528081 (*RcFAH12*), XM_002515198 (*RcLACS1*), XM_002509911 (*RcLACS4*), and XM_002532166 (*RcLACS8*).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Enzyme reaction progress curve.

Supplemental Table S1. Expression levels of *PLA* genes in castor endosperm stages III to VIII (number of ESTs per 10^5) and expression levels from the Bio-Analytical Resource expression browser (www.bar.utoronto.ca) of Arabidopsis Columbia-0 seeds and siliques.

Supplemental Table S2. FA composition of seeds from the CL37 control line and T1 CL37 lines expressing Arabidopsis genes *AtsPLA$_2\alpha$* and *AtsPLA$_2\beta$*.

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