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Corresponding Author: John Browse
Address: Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164-6340
Tel: +1(509)3352293
Email: jab@wsu.edu

Research Area: Biochemistry and Metabolism
Secondary Research Area: Systems and Synthetic Biology
A Small Phospholipase A$_2$-α from Castor Catalyzes the Removal of Hydroxy Fatty Acids from Phosphatidylcholine in Transgenic Arabidopsis Seeds$^1$

Shen Bayon$^2$, Guanqun Chen, Randall J. Weselake, John Browse$^*$

Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164-6340, USA (S.B., J.B.); and Alberta Innovates Phytola Centre, Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada T6G 2P5 (G.C., R.J.W.)

A Castor phospholipase is involved in editing ricinoleic acid from the membrane lipids in transgenic Arabidopsis seeds.
This work was supported by the U.S. National Science Foundation (grants no. DBI-0701919 and IOS-1339385) and by the Agricultural Research Center at Washington State University (J.B.), and Alberta Innovates Bio Solutions, AVAC Ltd. and the Canada Research Chairs Program (R.J.W.).

Present address: Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, NE, USA

*Corresponding author; email jab@wsu.edu

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantpathology.org) is: John Browse (jab@wsu.edu).
Abstract

Ricinoleic acid, an industrially useful hydroxy fatty acid (HFA), only accumulates to high levels in the triacylglycerol fraction of castor bean 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 identity of the proteins involved in this process, however, are unknown. A phospholipase A₂ (PLA₂) is thought to be involved in the acyl editing process. We show here a role RcsPLA₂α in the acyl editing of HFA esterified to PC. RcsPLA₂α was identified by its high relative expression in the castor (Ricinus communis) endosperm transcriptome. Co-expression in Arabidopsis (Arabidopsis thaliana) seeds of RcsPLA₂α with castor hydroxylase RcFAH12 led to a dramatic decrease in seed HFA content when compared to RcFAH12 expression alone, both in 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 RcsPLA₂α functions in vivo as a PLA₂ with HFA specificity. Activity assays with yeast microsomes showed a high specificity of RcsPLA₂α for ricinoleic acid, superior to that of the endogenous AtsPLA₂α. These results point to RcsPLA₂α as a phospholipase involved in acyl editing, adapted to specifically removing HFA from membrane lipids in seeds.
INTRODUCTION

Phospholipase A enzymes (PLAs) catalyze the hydrolysis of either the *sn*-1 (PLA₁) or the *sn*-2 (PLA₂) 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 evidenced 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. Up to now, in plants, however, evidence suggests 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 acyl editing includes a broader set of enzymatic activities than originally envisioned by Lands. Despite the recent description of a patatin-related phospholipase, pPLAIⅢδ, 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 has also 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 may also be involved in maintaining cellular membrane integrity through hydrolysis of oxidized lipid. Banás et al. (1992) showed in a variety of plants that both lipid peroxides and lipid hydroxides were preferentially hydrolyzed over non-oxidized lipids.

In plants, three major groups of PLAs have been identified: the patatin related pPLAs, the DAD-like PLA1, and the secretory/small sPLA2s (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 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 weight PLAs of around 14 kDa, sPLA2α, β, γ and δ (Lee et al., 2005; Matos and Pham-Thi, 2009). Evidence for the involvement of sPLA2 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 sPLA2 expression in Arabidopsis plants led to golgi disruption and 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 LCAT-like4 (Chen et al., 2012) unrelated to these three main families, have also been identified as PLAs.

With the aim of gaining more insights into the involvement and identities of PLAs involved in acyl editing and HFA metabolism, with potential downstream industrial applications, we made use of the available castor bean endosperm transcriptome database (Troncoso-Ponce et al., 2011) and the transgenic Arabidopsis seed line CL37 expressing the castor fatty acid hydroxylase12 (FAH12) (Lu et al., 2006). Amongst the PLA genes examined from the castor
endosperm transcriptome, \textit{RcsPLA}_{2}\alpha was the most highly expressed, indicating possible involvement in ricinoleate metabolism in castor. Expression of \textit{RcsPLA}_{2}\alpha in CL37 seeds led to a drastic reduction in HFA levels both in TAG and PC fractions. Also, microsomal fractions from yeast expressing \textit{RcsPLA}_{2}\alpha showed strong activity with \textit{sn}-2-ricinoleoyl-PC as a substrate. Our results suggest roles for \textit{RcsPLA}_{2}\alpha in: 1) the acyl editing to generate LPC for re-acylation through LPCAT activity, 2) freeing HFAs for activation to acyl-CoA and incorporation into TAG and 3) contributing to membrane integrity though the removal of potentially deleterious ricinoleate from membrane lipids.

\textbf{RESULTS}

\textit{PLA}_{2}\alpha is the Most Highly Expressed \textit{PLA} in Developing Castor Endosperm

To identify which of the \textit{PLA} genes are potentially involved in castor lipid metabolism, protein sequences of Arabidopsis PLAs were used in the BLASTP program search against the 4X draft castor bean genome accessible from the J. Craig Venter institute (http://www.jcvi.org). The gene sequences identified were then used in a BLASTN program search against the MSU castor EST database (http://glbrc.bch.msu.edu/castor/); (Troncoso-Ponce et al., 2011) to determine gene expression levels. The 13 \textit{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 \textit{PC-PLA}_{1}, one \textit{PA-PLA}_{1}, one \textit{LCAT-like3}, 12 \textit{pPLA}, two \textit{sPLA}_{2}, one \textit{SOBER1} and one \textit{LCAT-like4} were identified in the castor genome. Four \textit{PLA}s were found to have substantial expression in developing castor endosperm; the highest being \textit{sPLA}_{2}\alpha (12.1 EST/10^5 av.), followed by \textit{LCAT-like4} (7.7 EST/10^5 av.), \textit{pPLA}_{1} (4.6 EST/10^5 av.) and \textit{sPLA}_{2}\beta (3.2 EST/10^5 av.). 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 \textit{RcsPLA}_{2}\alpha and \textit{RcsPLA}_{2}\beta. Both Arabidopsis homologues, \textit{AtsPLA}_{2}\alpha and \textit{AtsPLA}_{2}\beta, have been shown to catalyze the hydrolysis of FAs specifically from the \textit{sn}-2 position of PC and phosphatidylethanolamine (PE) (Lee et al., 2003; Ryu et al., 2005; Mansfeld and Ulbrich-Hofmann, 2007), whereas \textit{AtpPLA}_{1} is selective for galactolipid acyl-chains at both \textit{sn}-1 and \textit{sn}-2
positions (Yang et al., 2007) and the PLA$_2$ AtLCAT-like displays the acyl preference oleoyl>linoleoyl>ricinoleoyl and is mostly cytosolic (Chen et al., 2012).

Phylogenetic and Sequence Analysis of sPLA$_2$

Phylogenetic analysis shows the evolutionary relatedness of sPLA$_2$ protein sequences from castor, Arabidopsis and rice (Fig. 1). Both castor RcsPLA$_2$ and –β, identified by BLAST search in TIGR, show a close relatedness with Arabidopsis AtsPLA$_2$ and –β, with a degree of identity at the amino acid level, respectively, of 73.5% and 52.9%. No castor homologues were identified for AtsPLA$_2$¥ or –δ. Multiple sequence alignment analysis confirmed, in both RcsPLA$_2$α and RcsPLA$_2$β, the presence of highly conserved regulatory and catalytically important motifs characteristic of sPLA$_2$ proteins. Both RcsPLA$_2$α and –β harbor a defined PA2c (SMART acc. #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 cysteine 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 homologues AtsPLA$_2$α, AtsPLA$_2$β and the rice homologue OssPLA$_2$α possess signal sequences as predicted by PSORT (http://psort.hgc.jp) and have previously been localized 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$_2$α sequences, constituting a putative ER retention signal (Singh et al., 2012).

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

RcsPLA$_2$α and RcsPLA$_2$β were cloned from a castor endosperm cDNA library, introduced into the expression vector pGate-DsRed-Phas (Lu et al., 2006) and expressed under 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 is also mutated in its fatty acid elongase (fae1) gene (Kunst et al., 1992) and is thus devoid of the very long chain HFAs lesquerolic acid (20:1-OH) and auricolic acid (20:2-
simplifying the FA profile analysis by gas chromatography. 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*:RcsPLA₂α or *phaseolin*:RcsPLA₂β transformation events. Samples of seed expressing the RcsPLA₂β construct showed no change in HFA content compared with CL37 control samples and subsequent analysis of T2 seed from 30 additional T1 plants also failed to provide any evidence that expression of RcsPLA₂β altered HFA accumulation, or other aspects of TAG metabolism in seeds. T1 seed samples expressing RcsPLA₂α, however, exhibited an average 20% reduction in HFA relative to segregating and control CL37 samples (14.8 ± 0.1% vs. 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 seed with a >25% reduction in HFA relative to three CL37 controls with several lines having approximately 5% HFA.

Segregation of the DsRed marker indicated both single insert (75% red seed) and multiple insert (>90% red seed) lines were present. By genetic analysis in the T2 and subsequent generations, we identified five homozygous, single-insert lines (designated RcsPLA₂α-CL37 #1 to #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 RcsPLA₂α enzyme acting to selectively remove HFA from the pathway of TAG synthesis, possibly through 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 PLA₂ enzymes are presumably rapidly metabolized since their accumulation can disrupt membrane and cell function.
Overexpression of the Arabidopsis sPLA₂α Homologue Does Not Alter HFA Levels in CL37 Seeds

To ascertain whether the reduction in HFA in RcsPLA₂α-CL37 lines was not simply due to increased non-specific PLA₂ activity, but is in indeed due to ResPLA₂α having specificity for HFAs, the endogenous Arabidopsis gene AtsPLA₂α, was cloned into the pGate-DsRed-Phas vector and used to transform CL37 plants. Samples of T2 seed from 24 independent T1 transformants were analyzed for FA composition. None of the samples had HFA content beyond two standard deviations from the mean 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 the controls at 18.5 ± 0.3% (se). The proportions of other FAs in the AtsPLA₂α transgenics were also very similar to the CL37 controls (Supplemental Table S2). We also overexpressed AtsPLA₂β 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 AtsPLA₂α-CL37 seeds may indicate a lack of specificity of AtsPLA₂α for HFA, or possibly a low degree of activity of the AtsPLA₂α isozyme in the seed tissues. We also cannot exclude the possibility that our transgene for some reason failed to express high amounts of the protein. In any case, our results indicate that the castor sPLA₂α enzyme likely possesses specificity for HFA above that of AtsPLA₂α and the HFA reductions observed are not solely due to a general increase in PLA₂ activity.

RcsPLA₂α is More Efficient than AtsPLA₂α in Catalyzing the Liberation of Ricinoleate from Ricinoleoyl-PC

To further investigate the castor and Arabidopsis sPLA₂α isozymes, we cloned cDNAs encoding RcsPLA₂α and AtsPLA₂α into a pYES2 vector to allow strong inducible expression in yeast. An empty-vector construct was used as control. Separate PLA₂ assays were performed using both cytosolic and microsomal fractions derived from yeast producing recombinant RcsPLA₂α or AtsPLA₂α. Yeast-derived fractions after ultracentrifugation were used in PLA₂ assays to catalyze the hydrolysis of the substrates sn-1-palmitoyl(16:0)-sn-2-[¹⁴C]oleoyl- PC ([¹⁴C]18:1-PC) and sn-1-palmitoyl-sn-2-[¹⁴C]ricinoleoyl-PC ([¹⁴C]Ric-PC) to produce, respectively, free [¹⁴C]oleic acid or [¹⁴C]ricinoleic acid. From the time course of the reaction plots (Supplemental Fig. S1), it appears that at early time points both microsomal fractions
containing, respectively, RcsPLA2α or AtsPLA2α displayed higher activity against 18:1-PC than the cytosolic fractions. Contrasting with AtsPLA2α, RcsPLA2α showed substantial cytosolic activity. PLA2 activity, however, was primarily associated with the microsomal fraction for both isozymes suggesting a potential ER localization for RcsPLA2α and AtsPLA2α.

We first studied the substrate specificity of RcsPLA2α and AtsPLA2α in yeast microsomes supplied with either labeled [14C]18:1-PC or [14C]Ric-PC. RcsPLA2α specific activity was almost double that of AtsPLA2α using [14C]18:1-PC as substrate (52.3±1.4 and 28.5±2.8 pmol/min/mg protein, respectively). Moreover, when using [14C]Ric-PC, RcsPLA2α was nine times more efficient in catalyzing the hydrolysis of the substrate than AtsPLA2α (32.7±0.1 and 3.4±0.1, pmol/min/mg protein, respectively) (Fig. 3A). We then determined the selectivity of both enzymes by using an equimolar mixture of [14C]18:1-PC and [14C]Ric-PC (Fig. 3B). In this case, hydrolysis of [14C]18:1-PC was much more effective relative to [14C]Ric-PC, with oleic acid representing over 75% of product for both enzymes. However, RcsPLA2α was still twice as efficient as AtsPLA2α in catalyzing the hydrolysis of [14C]Ric-PC, even in the presence of mixed substrates: with RcsPLA2α, 23.5% of hydrolyzed FAs were ricinoleate whereas with AtsPLA2α only 11.8% were ricinoleate (Fig. 3B). It appears that RcsPLA2α is a more efficient enzyme than AtsPLA2α in catalyzing the hydrolysis of [14C]Ric-PC, even if both enzymes display higher specificities and selectivities for [14C]18:1-PC than for [14C]Ric-PC.

Changes in FA Composition of TAG and PC during Seed Development of RcsPLA2α-CL37 Lines

Total lipids were extracted from CL37 and RcsPLA2α-CL37 seeds at different stages of development from 7 to 18 days after flowering (DAF). Total lipid fractions were separated into neutral lipids (mostly TAG) and PC (Fig. 3) by thin-layer chromatography. The neutral lipid fraction from CL37 seeds showed an increase in HFAs which rose from zero to 20% of total FAs in mid-development (Fig. 4A). Developing RcsPLA2α-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 in CL37 seeds (Fig. 4C). The ~80% HFA reduction in RcsPLA2α-CL37 seed TAG relative to CL37 was not confined to mature seed 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 by almost 50% due to expression of the RcsPLA2α transgene.
(Fig. 4E). The relative amount of 18:1 in TAG only shows a slight increase during development, whereas both 16:0 and 18:0 were reduced. The differences in TAG FA composition between RcsPLA2α-CL37 and CL37 seeds remained largely constant beyond 13+14 DAF (Fig. 4E).

In the PC fraction derived from CL37 developing seeds, HFA levels increased up to 9.5% in mid-developmental 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 RcsPLA2α-CL37 seeds, but HFA only marginally increased during development to 2.9% (Fig. 4D). When comparing differences in PC FA composition between CL37 and RcsPLA2α-CL37 seeds, the reduction in HFA observed in PC (~70%) was similar to the reduction observed in TAG (~80%), and was constant throughout development (Fig. 4F). The changes in other FAs were modest throughout seed development, with the exception of 18:3 that showed large differences (up to 70%) during early to mid-developmental stages before reducing to 20% at a later stage of development (Fig. 4F). 18:1 and 18:2 both show slight increases of ~15% during later stages of development, whereas both 18:0 and 16:0 were reduced by ~50% and ~30%, respectively, during seed development. The differences in FA composition in PC between the two lines can be considered mostly constant after the mid-developmental stage (Fig. 4F).

**Total Fatty Acid Content is Increased Slightly and Germination Rate is Restored in RcsPLA2α-CL37 Seeds**

Total FA content of homozygous seeds from five RcsPLA2α-CL37 lines was compared to that of the brown null-segregant seeds from the same lines and to that of fae1 seeds (Fig. 5). Previous analysis by Bates and Browse (2011) and by van Erp et al. (2011) showed a reductions in seed total FA content, respectively of 30% and 20% in CL37 plants expressing RcFAH12, compared to parental fae1, and the difference between fae1 and the CL37 null segregants in Fig. 5 (25.6%) is in this range. Expression of RcsPLA2α in CL37 seed increased total average FA content in homozygote seeds up to 5.63 μg/seed, compared to brown null-segregating CL37 seed (5.05 μg/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/seed). Seed germination rates were also restored to near wild type levels from an average of 24% for co-segregating T2 CL37 seeds to 95% for the transgenic T2 RcsPLA2α-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 seed (in lines CL7 and CL37), 18:1-OH and 18:2-OH HFA accumulate to 10-11% 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 RcDGAT2 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 PLA2 plays a major role in removing newly synthesized HFA from PC (Bafor et al., 1991). We therefore sought to identify a suitable PLA2 gene in castor and test its effectiveness in supporting metabolism and accumulation of HFA when co-expressed with RcFAH12 in transgenic Arabidopsis.

RNAseq results (Table I) indicate that the RcsPLA2 gene is the most highly-expressed PLA2 isoform throughout the stages of oil accumulation in developing castor endosperm (Troncoso-Ponce et al., 2011). This gene encodes an enzyme of the small PLA2 family, RcsPLA2α. sPLA2α homologues 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 sPLA2α is strongly expressed in seeds and other tissues (Singh et al., 2012), while the Arabidopsis homologue is not highly expressed in developing seeds (Supplementary Table S1). AtPLA2α 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 AtPLA2α in developing Arabidopsis seeds, the high expression of RcsPLA2α in developing castor endosperm suggest that it may encode an enzyme responsible for efficient removal 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\textsubscript{2}α protein fused to green fluorescence protein was shown to co-localize with an ER marker (Singh et al., 2012). This result is consistent with our finding that recombinant RcsPLA\textsubscript{2}α produced in yeast showed high activity in the microsomal fraction of cell homogenates (Supplemental Fig. S1), but the activity in the high-speed supernatant was also high. The RcsPLA\textsubscript{2}α 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 (Stahl et al., 1999).

The enzymatic properties of the Arabidopsis family of sPLA\textsubscript{2}s have been well characterized, with a regiospecificity for \textit{sn}-2 acyl chains and with a head group selectivity for PC and PE over other phospholipids (Chen et al., 2011). In the current study, the \textit{in vitro} assays using yeast microsomes show that both castor and Arabidopsis sPLA\textsubscript{2}α are active in the picomolar range against [\textsuperscript{14}C]18:1-PC demonstrating the \textit{sn}-2 regiospecificity of PLA\textsubscript{2}. Enzymes from both castor and Arabidopsis displayed higher specificity for [\textsuperscript{14}C]18:1-PC than for [\textsuperscript{14}C]Ric-PC (Fig.3A). However, this loss of oleate from PC through preferential hydrolysis of 18:1-PC over Ric-PC may be mitigated to some extent through the selective re-acylation 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-CoA synthetase (LACS) (Shockey et al., 2002). Both specificity and selectivity assays showed RcsPLA\textsubscript{2}α was a more efficient enzyme than AtsPLA\textsubscript{2}α in catalyzing the hydrolysis of [\textsuperscript{14}C]Ric-PC. Assuming comparable levels of protein expression in the yeast, the specific activity of RcsPLA\textsubscript{2}α was nine times that of AtsPLA\textsubscript{2}α (32.7±0.1 versus 3.4±0.1, pmol/min/mg protein) with [\textsuperscript{14}C]Ric-PC as sole substrate, and RcsPLA\textsubscript{2}α was twice as efficient as AtsPLA\textsubscript{2}α in a selectivity assay with the presence of an equimolar mixture of [\textsuperscript{14}C]18:1-PC and [\textsuperscript{14}C]Ric-PC (Fig. 3A and B).

Consistent with the high activity of recombinant RcsPLA\textsubscript{2}α on Ric-PC, developing CL37 seeds expressing RcsPLA\textsubscript{2}α showed a dramatic decrease in HFA in PC to less than 3% of total FAs during the period of oil accumulation, compared to 10% in CL37 controls (Fig. 4). In mature CL37 seeds, approximately 70% of HFA in TAG is esterified to the \textit{sn}-2 position of the glycerol backbone, and is produced from PC via PC-derived diacylglycerol (DAG) (Bates and Browse, 2011). Thus, activity of the castor lipase in RcsPLA\textsubscript{2}α-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.

Expression of \( RcsPLA_{2\alpha} \) in CL37 also resulted in substantial changes in the proportions of other FAs in the seed. Compared to the \( fae1 \) parental line, CL37 seed had a 66% decrease in 18:2 + 18:3 fatty acids (from 59.2% to 26.2%) (Table II). Co-expression 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 a FA composition similar to \( fae1 \). It has been proposed that HFA 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 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 \textit{de novo} DAG into TAG. Because \textit{de novo} DAG contains much higher 18:1 and lower 18:2 + 18:3 than PC-derived DAG (Bates and Browse, 2011), this shift would also 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 removal of HFA from \textit{sn}-2 of PC catalyzed by RcsPLA\(_{2\alpha}\), the expected route of HFA breakdown is via \( \beta \)-oxidation in the peroxisome. The current view of \( \beta \)-oxidation would anticipate conversion of HFA to the HFA-CoA thioester via activity of a LACS (Shockey et al., 2002), subsequent hydrolysis of the HFA-CoA during transport into the peroxisome through the PXA1 ABC-cassette transporter (De Marcos Lousa et al., 2013) and re-synthesis 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 or not the plant PXA1 can also 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 (PHA) synthase to capture \( \beta \)-oxidation intermediates and allow assessment of the relative flux of HFA and other fatty acids through the \( \beta \)-oxidation pathway. Expression of PHA synthase in CL37 and CL37-RcsPLA\(_{2\alpha}\) plants could be used to confirm our
expectation that HFA degradation occurs through \(\beta\)-oxidation. Interestingly, while this technique indicated increased rates of \(\beta\)-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 \(\beta\)-oxidation pathway (Moire et al., 2004).

The question of whether HFA released by RcsPLA\(\alpha\) 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\(\alpha\)-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 acyltransferase) 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 reincorporation of HFA, we identified three castor LACS genes (homologues of Arabidopsis LACS1, LACS4 and LACS8, respectively) that are highly expressed in developing endosperm tissue (Troncoso-Ponce et al., 2011). However, when we co-expressed each of these isoforms in RcsPLA\(\alpha\)-CL37 Line #3, we observed no increase in seed HFA content relative to that shown for Line #3 in Table II. It will also be worthwhile investigating the biochemistry and HFA content of CL37-RcsPLA\(\alpha\) 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\(\alpha\), 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 \(RcsPLA\alpha\) in CL37 does result in efficient removal of HFA from PC;
however there is little or no re-incorporation into TAG and instead HFA appears to be degraded, likely by β-oxidation. These results imply that any release of HFA by endogenous Arabidopsis PLA₂ would also be degraded and unavailable for incorporation into TAG. AtsPLA₂α and AtsPLA₂β are poorly expressed in developing Arabidopsis seeds, and the physiological function of the AtsPLA₂α enzyme is thought to be in membrane trafficking in vegetative tissues (Lee et al., 2005). One possible explanation for why expression of AtsPLA₂α has no effect on the HFA content of seeds (Table S2), is that the enzyme is inactive in the absence of upstream regulatory processes in seeds. However, two other genes, pPLAIIfβ (At4g37050) and pPLAIIfγ (At4g29800) are highly expressed in developing Arabidopsis seeds (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 thaliana protein sequences were obtained from TAIR (http://www.arabidopsis.org/) and the castor bean homologues were obtained by performing a protein BLAST against the 4X draft castor bean protein database hosted by the JCVI (http://www.jcvi.org). The castor gene model identities were identified using a cutoff score of a 1000. The protein sequence of the castor genes and their Arabidopsis homologues used as query 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 identification of castor homologues of the Arabidopsis proteins. The high degree of homology (>60% pairwise id) was confirmed by aligning the castor protein sequence with that of its homologue using the alignment function in Geneious (www.geneious.com). (Only 12% of selected protein sequences had a pairwise id. of less than 50%). Castor bean genes were named after their closest Arabidopsis homologue based on % id, if there is more than one homologue, 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 JCVI, to obtain the nucleotide sequence. The castor nucleotide sequence was subsequently used to perform a BLASTN at the MSU Castor 454 expression database (~70,000 contigs assembled from
the expression data of the stages was obtained from *R. communis* 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 267,141 ESTs, Stage VI 152,462 ESTs, Stage VII+VIII 151,964 ESTs. With only 77,518 and 84,170 ESTs respectively, Stages V and IVb were ignored, so was stage III-V with 184,642 ESTs. Data containing: the contig number, the number of ESTs per contig, the best Arabidopsis homologue, and the number of ESTs per 100,000 per embryo stage were compiled in Table I. This table also includes: the Gene family, the Arabidopsis gene id, Arabidopsis gene name, the name of the castor homologue, the castor gene model accession from JCVI and the % of homology as measured by % of pairwise identical amino acids in the aligned sequences. Additional methodology is available from Troncoso-Ponce et al., (2011).

**Isolation and Cloning of RcsPLA2α**

The protein sequences corresponding to *RcsPLA2α* (XP_002523659) ORF obtained after translation using Expasy translate tool (http://web.expasy.org/translate) of DNA sequences identified from a) the castor database at the Craig Venter institute (http://www.jvci.org), GI: 29840.m000629, b) the NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/), XM_002523613, c) the MSU 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 AtsPLA2α (At2g06925). *RcsPLA2α* amplicons were isolated from an *R. communis* seed cDNA library, using primers: RcPLA21/f: CACC ATG GCA AAT TTA AGC CAG CCA TTG AAT TTA GTA GCT TAT C and RcPLA21/r: TCA TGG TTT ATG AAG GTA TCT ACC AGC AA TCA AAG CAG C, and a hot-start DNA polymerase, KOD, (Novagen, Madison, WI, USA) used in a touch-down PCR reaction: 94 °C, 2 min, 5x [94 °C, 30 s; 72°C, 2 min], 5x [94 °C, 30 s; 70 °C, 2 min], 25x [94 °C, 30 s; 68 °C, 2 min], 7 °C hold. The amplicon was purified using the QIAquick Gel Extraction Kit (Quiagen, USA), and ligated into PCR-Script-Amp (Agilent, USA). Positive colonies resulting from the heat-shock transformation of XL10-Gold *E. coli* cells (Stratagene, USA), were determined by colony PCR using GoTAQ (Promega, USA) and the above primers. A PCR reaction using KOD (Novagen, USA) with was used to amplify the *RcsPLA2α* sequence that was then ligated into the entry vector of the gateway system pENTR/D-TOPO (Life
Technologies, USA). Subsequent transformation of Top10 cells, and colony PCR screening allowed the isolation DNA from three colonies, PerfectPrep Spin Mini (5PRIME, USA). The $RcsPLA_2\alpha$ sequence in pENTR was then sequenced using the BigDye sequencing kit (ABI, Foster City, CA, USA), and primers RcPLA21/f, RcPLA21/r and the additional primers: M13(-21): TGT AAA ACG ACG GCC AGT and M13r : AAC AGC TAT GAC CAT G. pENTR-RcPLA21-2 was combined with pGate-DsRed-Phas (Lu et al., 2006) after an LR clonase reaction (Life technologies, USA). DNA resulting from minipreps of positive XL10-Gold colonies was again sequenced with the above mentioned primers, and used to electroporate $Agrobacterium$ $tumefaciens$ GV3101 strain for dipping Arabidopsis flowers (Clough and Bent, 1998). For plant transformation, the separate $Agrobacterium$ cultures were grown overnight to stationary phase in 400 mL LB medium at 28 °C with the appropriate antibiotic selection, spun down 6000 rpm for 15 min and resuspended in 400 mL of 5% sucrose and 0.05% Silwett L-77 prior to dipping. Similarly to $RcsPLA_2\alpha$, $AtsPLA_2\alpha$ (TAIR: 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: CACC ATG GCG GCT CCG ATC ATA CTT TTC TC and AtPLA21/1r: TTA GGG TTT CTT GAG GAC TTT GCC GAC TTT GCC GGC G.

**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): CL37, is derived from $fae1$, expresses $RcFAH12$ and accumulates ~17% 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 incubation in sterilization solution (0.5% NaOCl (Clorox), 30% ethanol, and 1 g/L SDS), followed by five washes with sterile water and 24 h of stratification at 4 °C. Seeds were germinated on half-strength Murashige and Skoog medium (4.4 g/L), 1% agar plates containing 1% sucrose, 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 for 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 FTA cards were incubated in 10 μL H₂O at 95 °C for 10 min, and 0.5 μL was used for PCR amplification (30 cycles, Tm: 60 °C) using the KOD polymerase.

**Preparation of Fatty Acid Methyl Esters (FAMES) and GC Analysis**

FAMEs were isolated from 30 whole seed 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 H₂O were added and vortexed, spun down at 2,000g and 100 μL was transferred to a GC vial. For total seed FA quantification, 10 μL of glyceryl triheptadecanoate (tri-17:0-TAG, 3 μg/μL) were added prior to derivatization. FAMEs were quantified by GC with flame ionization detection on a wax column (EC Wax; 30 m x 0.53 mm i.d. x 1.20 μm; Alltech). GC parameters were as follows: 220 °C for 2 min followed by a ramp (10 °C/min) up to 245 °C with a 6 min final temperature hold. The significance of difference between FA content from transgenic seeds and co-segregating seeds was determined by Student t-test.

**Developmental Time Course and Seed Lipid Extraction**

Flowers and siliques from lines CL37 and RcsPLA₂α-CL37 #3 were counted as they appeared on a daily basis to determine silique age. After 20 days, siliques were harvested according to their age, frozen with liquid N₂ and stored at -80 °C. Lipid extraction followed the protocol based 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 thin layer chromatography (TLC), lipid content was determined by GC analysis using tri-17:0-TAG standard (3 μg/μL). Lipids were separated on Whatman Partisil® K6 silica gel 60Å 20 X 20 cm glass plates with mobile phase CHCl₃: methanol:acetic acid (75:25:8, v/v/v) ; Plates were dried in a vacuum for 20 min, prior to staining with 0.005% primulin in (80/20, v/v) acetone/water. Lipid bands were visualized by UV, 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 (ORF) of \( AtsPLA_2\alpha \) was amplified with the cDNA in the Gateway pENTR/D-TOPO entry vector as the template using primers GC164 (5′-ACT ATG GCG GCT CCG ATC-3′) and GC165 (5′-TTA GGG TTT CTT GAG GAC TTT GC-3′). Similarly, \( RcsPLA_2\alpha \) was amplified using primers GC166 (5′-ACA ATG GCA AAT TTA AGC CAG-3′) and GC167 (5′-TCA TGG TTT ATG AAG GTA TCT ACC AG-3′). The PCR products were individually sub-cloned into the yeast expression vector pYES2.1/V5-His-TOPO (Invitrogen, Burlington, ON, Canada). The plasmids were then transformed into wild type Saccharomyces cerevisiae (Inv Sc1 strain; Invitrogen). A yeast strain transformed with a null pYES2.1/V5-His-TOPO vector was used as negative control.

Transformed yeast cells were grown at 30 °C and 200 rpm in synthetic uracil drop-out medium containing 1% (w/v) raffinose and 2% (w/v) galactose for 20 hours. Yeast cells were then harvested, washed with 50 mM Tric-HCl pH 7.6, and suspended in 1 mL of breaking buffer (50 mM Tris-HCl pH 7.6, 600 mM sorbitol, 1 mM EDTA) containing protease inhibitor (Complete, Roche). The cells were then broken with a Mini-Beadbeater (BioSpec Products, Bartlesville, OK, USA) 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 were 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 (St. Louis, MO, USA) and \( sn1\)-palmitoyl-\( sn2\)-[\(^{14}\)C]ricinoleoyl-PC (Ric-PC) was synthesized according to Banaś et al. (1992). The PLA enzyme assay was performed at 30 °C with 750 rpm agitation 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 8.0 with incubation times indicated in the figures. The reaction was terminated by addition of 1 mL of chloroform: methanol (1:1, v/v) and 200 µL of 0.15 M acetic acid.
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 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, QC, Canada). Spots corresponding to free FA were scraped and analyzed for radioactivity by scintillation counting.

For the experiment of substrate selectivity, 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 on TLC plates. The plates were developed in 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 results represented as mean ± SE.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: At2g06925 (AtsPLA₂α), At2g19690 (AtsPLA₂β), At4g29460 (AtsPLA₂γ), At4g29470 (AtsPLA₂δ), XM_002523613 (RcsPLA₂α), XM_002514118 (RcsPLA₂β), NM_001057569 (OssPLA₂α), XM_002523613 (RcsPLA₂α), XM_002514118 (RcsPLA₂β), XM_001057569 (OssPLA₂α), XM_002528081 (RcFAH12), XM_002515198 (RcLACS1), XM_002509911 (RcLACS4), XM_002532166 (RcLACS8).

Supplemental Data

Supplementary Table S1: Expression levels of PLA genes in castor endosperm stages III-VIII (number of EST per 10⁵) and expression levels from the BAR expression browser (www.bar.utoronto.ca) of Arabidopsis Col-0 seed and silique. Data for Arabidopsis correspond to Log2 transformed ratios, thus 4 in the table indicates a 64-fold increase relative to the control.

Supplementary Table S2. Fatty acid compositions of seed from CL37 control line and T1 CL37 lines expressing Arabidopsis genes AtsPLA₂α and AtsPLA₂β. Data are mean ± SE for 12 to 24 samples.

Supplementary Figure S1. Enzyme reaction progress curve. [¹⁴C]oleic acid produced from hydrolysis of sn1-palmitoyl-sn2-[¹⁴C]oleoyl-phosphatidylcholine (18:1-PC) over time in
presence of microsomal and cytosolic fractions from yeast heterologously expressing RcsPLA2α and AtsPLA2α. Initial reaction rates at 30 min incubation time with 100 μg protein were subsequently used for determining specific enzyme activity.

ACKNOWLEDGMENTS
We would like to thank all the members of the Browse laboratory who contributed to this article and the greenhouse staff for their help with growing plants.
LITERATURE CITED


biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. Plant Cell 13: 2191-2209


Zhang M, Fan J, Taylor DC, Ohlrogge JB (2009) DGAT1 and PDAT1 acyltransferases have overlapping functions in Arabidopsis triacylglycerol biosynthesis and are essential for normal pollen and seed development. Plant Cell 21: 3885-3901
Table I: Expression data for 13 genes encoding phospholipase isozymes in developing castor endosperm.

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

<table>
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<tr>
<th>Castor Name</th>
<th>Gene Identifier</th>
<th>Arabidopsis Homologue</th>
<th>a.a. id%</th>
<th>Total ESTs</th>
<th>Stage III</th>
<th>Stage IVa</th>
<th>Stage VI</th>
<th>Stage VII-VIII</th>
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Table II: Fatty acid composition of T3 and T4 homozygous seeds co-expressing castor RcsPLA₂α with castor hydroxylase (CL37 background).

All samples are derived from lines with a single transgenic insert, as determined by pedigree analysis. Data are mean ± SE for 3-6 samples of 30 seeds each.

<table>
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<th>Line</th>
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<th>Sum of HFAs</th>
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<td>18:0</td>
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<td>fae1</td>
<td>9.7±0.1</td>
<td>3.5±0.1</td>
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<tr>
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<td>#4</td>
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<tr>
<td>#5</td>
<td>10.1±0.1</td>
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</tbody>
</table>
FIGURE LEGENDS

**Figure 1.** Phylogenetic relationship of sPLA$_2$ family in castor (Rc) Arabidopsis (At) and rice (Os). 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 parenthesis. 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 cysteine residues are indicated by an asterisk. The putative signal sequences as predicted by the PSORT prediction program (http://psort.hgc.jp), are underlined. An arrow marks the conserved histidine/aspartate dyad of the active site.

**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 A, $sn$-1-palmitoyl-$sn$2-[14C]oleoyl-phosphatidylcholine (18:1-PC) or $sn$-1-palmitoyl-$sn$-2-[14C]ricinoleoyl-PC (Ric-PC) as substrates to determine the specific activity at 30 min reaction time, or B, 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 incubation time.

**Figure 4.** Changes in fatty acid (FA) composition in TAG and PC from 7 to 18 days after flower opening (DAF) in CL37 (A,D) and transgenic line RcsPLA$_2$$\alpha$-CL37 #3 (B,E). A,B: FA compositions of seed TAG. C: Calculated difference between line #3 and CL37 for TAG. D,E: FA composition of seed PC. F: Calculated difference between line #3 and CL37 for PC. Data are mean +/- SE for three replicates.

**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 eleven CL37
segregants (null-seg) and three \textit{fiae1} plants. Data are mean ± SE for total fatty acids for five transgenic and five null-segregant lines.