Acyl Editing and Headgroup Exchange Are the Major Mechanisms That Direct Polyunsaturated Fatty Acid Flux into Triacylglycerols[1][W][OA]

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Triacylglycerols (TAG) in seeds of Arabidopsis (*Arabidopsis thaliana*) and many plant species contain large amounts of polyunsaturated fatty acids (PUFA). These PUFA are synthesized on the membrane lipid phosphatidylcholine (PC). However, the exact mechanisms of how fatty acids enter PC and how they are removed from PC after being modified to participate in the TAG assembly are unclear, nor are the identities of the key enzymes/genes that control these fluxes known. By reverse genetics and metabolic labeling experiments, we demonstrate that two genes encoding the lysophosphatidylcholine acyltransferases LPCAT1 and LPCAT2 in Arabidopsis control the previously identified "acyl-editing" process, the main entry of fatty acids into PC. The *lpcat1/lpcat2* mutant showed increased contents of very-long-chain fatty acids and decreased PUFA in TAG and the accumulation of small amounts of lysophosphatidylcholine in developing seeds revealed by [^{14}C]acetate-labeling experiments. We also showed that mutations in LPCATs and the PC diacylglycerol cholinephosphotransferase in the *reduced oleate desaturation1* (*rod1*)*/lpcat1/lpcat2* mutant resulted in a drastic reduction of PUFA content in seed TAG, accumulating only one-third of the wild-type level. These results indicate that PC acyl editing and phosphocholine headgroup exchange between PC and diacylglycerols control the majority of acyl fluxes through PC to provide PUFA for TAG synthesis.

Plant oils are an important natural resource to meet the increasing demands of food, feed, biofuel, and industrial applications (Lu et al., 2011; Snapp and Lu, 2012). The fatty acid composition in the triacylglycerols (TAG), especially the contents of polyunsaturated fatty acids (PUFA) or other specialized structures, such as hydroxy, epoxy, or conjugated groups, determines the properties and thus the uses of plant oils (Dyer and Mullen, 2008; Dyer et al., 2008; Pinzi et al., 2009; Riediger et al., 2009). To effectively modify seed oils tailored for different uses, it is necessary to understand the fundamental aspects of how plant fatty acids are synthesized and accumulated in seed oils.

In developing oilseeds, fatty acids are synthesized in plastids and are exported into the cytosol mainly as oleic acid, 18:1 (carbon number:double bonds), and a small amount of palmitic acid (16:0) and stearic acid (18:0; Ohlrogge and Browse, 1995). Further modification of 18:1 occurs on the endoplasmic reticulum in two major pathways (Fig. 1): (1) the 18:1-CoA may be elongated into 20:1- to 22:1-CoA esters by a fatty acid elongase, FAE1 (Kunst et al., 1992); (2) the dominant flux of 18:1 in many oilseeds is to enter the membrane lipid phosphatidylcholine (PC; Shanklin and Cahoon, 1998; Bates and Browse, 2012), where they can be desaturated by the endoplasmic reticulum-localized fatty acid desaturases including the oleate desaturase, FAD2, and the linoleate desaturase, FAD3, to produce the polyunsaturated linoleic acid (18:2) and α-linolenic acid (18:3; Browse et al., 1993; Okuley et al., 1994). The PUFA may be removed from PC to enter the acyl-CoA pool, or PUFA-rich diacylglycerol (DAG) may be derived from PC by removal of the phosphocholine headgroup (Bates and Browse, 2012). The PUFA-rich TAG are then produced from de novo-synthesized DAG or PC-derived DAG (Bates and Browse, 2012) and PUFA-CoA by the acyl-CoA:diacylglycerol acyltransferases (DGAT; Hobbs et al., 1999; Zou et al., 1999). Alternatively, PUFA may be directly transferred from PC onto DAG to form TAG by an acyl-CoA-independent phospholipid:diacylglycerol acyltransferase (PDAT; Dahlqvist et al., 2000). Recent results demonstrated that DGAT and PDAT are responsible for the majority of TAG synthesized in Arabidopsis (*Arabidopsis thaliana*) seeds (Zhang et al., 2009).

The above TAG synthesis model highlights the importance of acyl fluxes through PC for PUFA enrichment

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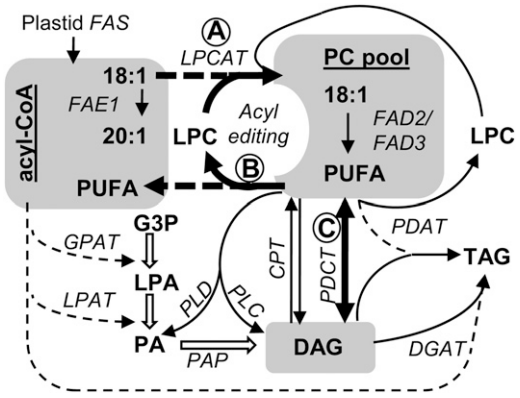

Figure 1. Reactions involved in the flux of fatty acids into TAG. De novo glycerolipid synthesis is shown in white arrows, acyl transfer reactions are indicated by dashed lines, and the movement of the lipid glycerol backbone through the pathway is shown in solid lines. Major reactions (in thick lines) controlling the flux of fatty acid from PC into TAG are as follows: LPC acylation reaction of acyl editing by LPCAT (A); PC deacylation reaction of acyl editing by the reverse action of LPCAT or phospholipase A (B); and the interconversion of DAG and PC by PDCT (C). Substrates are in boldface, enzymatic reactions are in italics. FAD, Fatty acid desaturase; FAS, fatty acid synthase; GPAT, acyl-CoA:G3P acyltransferase; LPA, lysophosphatidic acid; LPAT, acyl-CoA:LPA acyltransferase; PA, phosphatidic acid; PLC, phospholipase C; PLD, phospholipase D.

in plant oils. However, the exact mechanisms of how fatty acids enter PC and how they are removed from PC after being modified to participate in the TAG assembly are unclear, nor are the identities of the enzymes/genes that control these fluxes known. The traditional view is that 18:1 enters PC through de novo glycerolipid synthesis (Fig. 1; Kennedy, 1961): the sequential acylation of glycerol-3-phosphate (G3P) at the *sn*-1 and *sn*-2 positions produces phosphatidic acid; subsequent removal of the phosphate group at the *sn*-3 position of phosphatidic acid by phosphatidic acid phosphatases (PAPs) produces de novo DAG; finally, PC is formed from DAG by a cytidine-5′-diphosphocholine:diacylglycerol cholinephosphotransferase (CPT; Slack et al., 1983; Goode and Dewey, 1999). However, metabolic labeling experiments in many different plant tissues by us and others (Williams et al., 2000; Bates et al., 2007, 2009; Bates and Browse, 2012; Tjellström et al., 2012) have demonstrated that the majority of newly synthesized fatty acids (e.g. 18:1) enter PC by a process termed "acyl editing" rather than by proceeding through de novo PC synthesis. Acyl editing is a deacylation-reacylation cycle of PC that exchanges the fatty acids on PC with fatty acids in the acyl-CoA pool (Fig. 1, A and B). Through acyl editing, newly synthesized 18:1 can be incorporated into PC for desaturation and PUFA can be released from PC to the acyl-CoA pool to be utilized for glycerolipid synthesis.

Additionally, there is accumulating evidence that many plants utilize PC-derived DAG to synthesize TAG laden with PUFA (Bates and Browse, 2012). PC-derived DAG may be synthesized through the reverse reaction of the CPT (Slack et al., 1983, 1985) or by the phospholipases C and D (followed by PAP). However, our recent discovery indicates that the main PC-to-DAG conversion is catalyzed by a phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) through the phosphocholine headgroup exchange between PC and DAG (Fig. 1C; Lu et al., 2009; Hu et al., 2012). The PDCT is encoded by the *REDUCED OLEATE DESATURATION1* (*ROD1*) gene (At3g15820) in Arabidopsis, which is responsible for about 40% of the flux of PUFA from PC through DAG into TAG synthesis (Lu et al., 2009). Acyl editing and PC-DAG interconversion through PDCT may work together to generate PUFA-rich TAG in oilseed plants (Bates and Browse, 2012).

The enzymes/genes involved in the incorporation of 18:1 into PC through acyl editing are not known. However, stereochemical localization of newly synthesized fatty acid incorporation into PC predominantly at the *sn*-2 position (Bates et al., 2007, 2009; Tjellström et al., 2012) strongly suggest that the acyl editing cycle proceeds through the acylation of lysophosphatidylcholine (LPC) by acyl-CoA:lysophosphatidylcholine acyltransferases (LPCATs [Enzyme Commission 2.3.1.23]; Fig. 1A). High LPCAT activity has been detected in many different oilseed plants that accumulate large amounts of PUFA in TAG (Stymne and Stobart, 1987; Bates and Browse, 2012), suggesting the potential ubiquitous involvement of LPCAT in the generation of PUFA-rich TAG. Several possible pathways for the removal of acyl groups from PC to generate the lysophosphatidylcholine within the acyl editing cycle have been proposed. The acyl groups may be released from PC to enter the acyl-CoA pool via the reverse reactions of LPCATs (Stymne and Stobart, 1984) or by reactions of phospholipase A (Chen et al., 2011) followed by the acyl-CoA synthetases (Shockey et al., 2002). The main focus of this study was to identify the genes and enzymes involved in the incorporation of fatty acids into PC through acyl editing in Arabidopsis and to quantify the contribution of acyl editing and PDCT-based PC-DAG interconversion to controlling the flux of PUFA from PC into TAG. Herein, we demonstrate that mutants of two Arabidopsis genes encoding LPCATs (At1g12640 [*LPCAT1*] and At1g63050 [*LPCAT2*]) have reduced TAG PUFA content. Analysis of the acyl-editing cycle through metabolic labeling of developing seeds with [^{14}C]acetate indicate that the *lpcat1/lpcat2* double mutant was devoid of acyl editing-based incorporation of newly synthesized fatty acids into PC, indicating that these two genes are responsible for the acylation of LPC during acyl editing. Additionally, the triple mutant *rod1/lpcat1/lpcat2* indicated that PDCT-based PC-DAG interconversion and acyl editing together provide two-thirds of the flux of PUFA from PC to TAG in Arabidopsis seeds.

RESULTS

Identification of Candidate Genes for LPCATs

Previous studies identified two putative *LPCAT* genes, *LPCAT1* (At1g12640) and *LPCAT2* (At1g63050), in Arabidopsis that encode lysophospholipid acyltransferases

with a wide range of substrate specificity but strongly prefer LPC (Ståhl et al., 2008). Two other genes (At1g80950 and At2g45670) with sequence homology to the mouse LPCATs were found to prefer lysophosphatidylethanolamine as substrate (Stålberg et al., 2009). Moreover, the putative gene for LPCAT2 (At1g63050) was found up-regulated in the *dgat1* mutant to supply PC for TAG synthesis through the PDAT pathway (Xu et al., 2012). Additionally, metabolic labeling studies indicated that acyl editing predominantly involves PC and not phosphatidylethanolamine (PE; Bates et al., 2007, 2009). Therefore, we focused on the two putative *LPCAT* genes in this study.

We reasoned that if the two *LPCAT* genes are involved in acyl editing, the fatty acid composition may be changed in seed TAG of the mutants. The T-DNA insert mutants in the ecotype Columbia (Col-0) wild-type background for *LPCAT1* (At1g12640) and *LPCAT2* (At1g63050) had been isolated; however, the fatty acid composition in the *lpcat1* mutant was nearly unaffected, while the *lpcat2* mutant showed only a slight increase in 20:1 (Xu et al., 2012). The changes were statistically insignificant compared with the wild type for both mutants by our analysis (Table I). Therefore, we generated the *lpcat1/lpcat2* double mutant and analyzed the fatty acid composition in seeds by gas chromatography. As shown in Table I, compared with the wild type, the double mutant showed significantly increased 20:1 (from approximately 20% to approximately 25%) and decreased 18:2 + 18:3 PUFA (from approximately 48% to approximately 43%). These results suggest that less flux of 18:1 is toward PC for desaturation, while more 18:1 is used for elongation (Fig. 1). Therefore, the LPCATs encoded by the two Arabidopsis genes are strong candidates for enzymes involved in acyl editing in Arabidopsis seeds.

LPCAT1 and LPCAT2 Are Involved in Acyl Editing

Acyl editing has been characterized in plant tissue through in vivo metabolic labeling experiments that label fatty acids with [^{14}C]acetate as they are synthesized in the plastid and follow the corresponding newly synthesized [^{14}C]fatty acids through metabolism as they are incorporated into different glycerolipids (Bates et al., 2007, 2009; Bates and Browse, 2012). Key facets of the metabolic tracer studies of acyl editing include the following: (1) rapid incorporation of nascent fatty acids directly into PC by the acyl-editing cycle prior to the incorporation of nascent fatty acids into DAG through de novo glycerolipid synthesis, thus nascent fatty acids do not have a DAG-PC precursor-product relationship as does glycerol backbone incorporation into DAG and PC (Fig. 1); and (2) incorporation of the newly synthesized fatty acids primarily into the *sn*-2 position of PC, whereas de novo glycerolipid synthesis incorporates nascent fatty acids into both positions of DAG. To determine if the fatty acid composition phenotype of TAG in *lpcat1/lpcat2* is due to a change in the acyl-editing cycle, these metabolic labeling facets of acyl editing were investigated by comparing [^{14}C]acetate labeling of developing Col-0 and *lpcat1/lpcat2* seeds.

Developing Col-0 and *lpcat1/lpcat2* seeds excised from siliques incorporated [^{14}C]acetate linearly into glycerolipids at similar rates over a 60-min period of incubation (Fig. 2, A and C). The composition of [^{14}C]acetate-labeled fatty acids was similar in both Col-0 and *lpcat1/lpcat2* over the incubation period, with most radiolabel incorporated initially into newly synthesized 18:1 > > 20:1 > saturated fatty acids. [^{14}C]PUFA slowly accumulated over the time course (Fig. 2, B and D). The amount of radiolabel in 20:1 accumulated over time to a larger proportion of total labeled fatty acids than the mass proportion of 20:1 in the developing seeds, due to 30% to 40% of total 20:1 radioactivity residing in the cytosolic elongated portion of the fatty acid (Fig. 1; Supplemental Discussion S1; Supplemental Fig. S1). The unequal distribution of radioactivity across 20:1 is due to differences in the specific activities of the cytosolic and plastid acetyl-CoA pools and the utilization of [^{12}C]18:1-CoA for elongation with [^{14}C]acetyl-CoA that was liberated from a nonlabeled pool (such as PC), as demonstrated previously in *Brassica rapa* (Bao et al., 1998). The very similar labeling rates and labeled fatty acid composition of Col-0 and *lpcat1/lpcat2* indicate that initial production of [^{14}C]fatty acids from [^{14}C]acetate is not significantly affected by the *lpcat1/lpcat2* mutant.

To assess if the precursor-product relationship facets of acyl editing are affected in the *lpcat1/lpcat2* mutant, the amount of nascent [^{14}C]fatty acid incorporation into the major labeled glycerolipids was quantified over the 1-h time course (Fig. 3). In developing oilseeds, greater

Table I. *Comparison of fatty acid composition of Arabidopsis mutant lines with the wild type*

Data represent measurements of three to five homozygous lines (means ± SE). Student's *t* test was applied to the data; asterisks indicate that these values were statistically significantly different from the wild type (Col-0) at $P < 0.05$.

Line	FA Composition											
	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:3	22:0	22:1
	weight % of total											
Col-0	7.6 ± 0.4	0.4 ± 0.1	3.1 ± 0.2	14.7 ± 1.2	27.8 ± 0.4	20.0 ± 0.7	2.2 ± 0.1	19.9 ± 1.1	1.8 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	1.7 ± 0.1
lpcat1	8.0 ± 0.4	0.5 ± 0.1	3.5 ± 0.4	16.4 ± 0.8	28.6 ± 0.9	17.4 ± 1.1	2.3 ± 0.3	18.2 ± 0.9	2.0 ± 0.7	1.1 ± 0.6	0.5 ± 0.2	1.5 ± 0.2
lpcat2	7.7 ± 0.5	0.3 ± 0.2	3.2 ± 0.1	13.4 ± 0.7	26.5 ± 0.7	18.9 ± 1.1	2.3 ± 0.2	21.2 ± 1.0	2.5 ± 0.8	1.1 ± 0.4	0.6 ± 0.2	2.0 ± 0.2
lpcat1/2	6.8 ± 0.6	0.3 ± 0.2	3.1 ± 0.2	13.0 ± 1.1	25.3 ± 0.8*	18.0 ± 1.2	1.6 ± 0.2	25.3 ± 1.6*	3.3 ± 0.4*	1.1 ± 0.4	0.4 ± 0.2	1.8 ± 0.3
rod1	8.0 ± 0.2	0.3 ± 0.2	3.3 ± 0.1	33.8 ± 1.2*	13.9 ± 0.5*	14.4 ± 1.0*	2.2 ± 0.2	20.6 ± 0.6	0.7 ± 0.5*	0.7 ± 0.3	0.3 ± 0.1	1.7 ± 0.1
rod1/lpcat1/2	6.7 ± 0.6	0.2 ± 0.2	3.6 ± 0.1	42.4 ± 1.2*	7.4 ± 0.4*	9.0 ± 0.7*	1.7 ± 0.2	26.1 ± 1.5*	0.6 ± 0.1*	0.7 ± 0.2	0.2 ± 0.0	1.5 ± 0.1

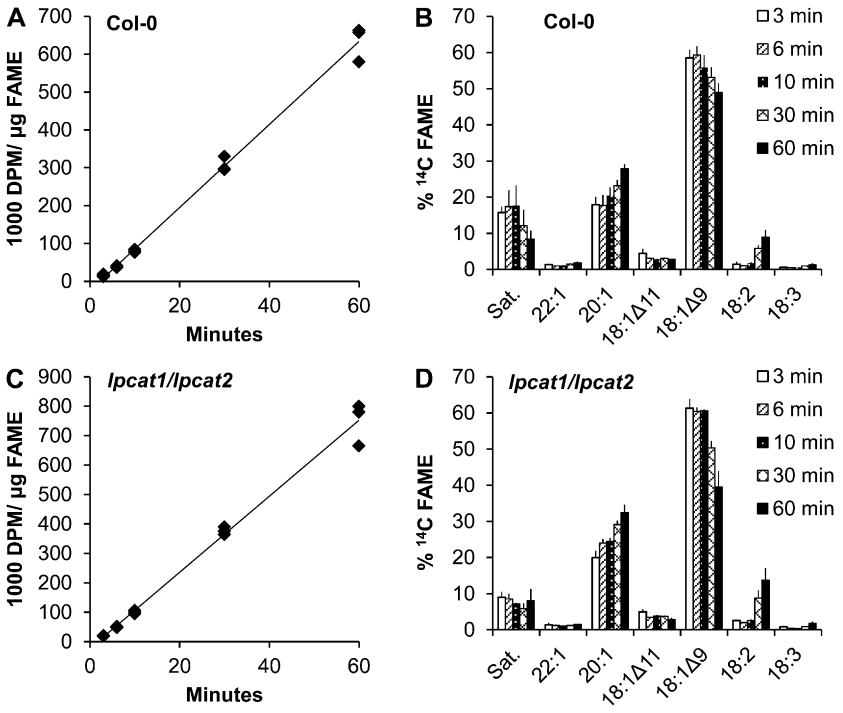

Figure 2. [^{14}C]Acetate incorporation into fatty acids of Col-0 and *lpcat1/lpcat2* developing seeds. A and C, Total radioactivity incorporation into seed fatty acids at 3, 6, 10, 30, and 60 min of incubation. Triplicate labelings for each time point and best fit line are shown. A, Col-0. C, *lpcat1/lpcat2*. B and D, Composition of radiolabeled fatty acids. Average values plus SE of triplicate labeled samples are shown. Sat., Total saturated fatty acids. B, Col-0. D, *lpcat1/lpcat2*.

than 95% of fatty acids synthesized eventually accumulate in TAG (Li-Beisson et al., 2010). Therefore, metabolic tracer studies of newly synthesized fatty acid incorporation into glycerolipids of developing oilseeds will primarily label the glycerolipids involved in the flux of fatty acids from synthesis to accumulation in TAG. In both Col-0 and *lpcat1/lpcat2*, TAG, PC, and DAG contained the most [^{14}C]fatty acids by 60 min of labeling (Fig. 3, A and C). The initial very high labeling of TAG with nascent [^{14}C]fatty acids was almost completely due to *sn*-3 acylation of [^{12}C]DAG synthesized prior to the start of labeling (Supplemental Fig. S2 and Supplemental Discussion S2); thus, the initial TAG labeling does not represent the flux of fatty acids through DAG or PC prior to TAG synthesis. Similar initial *sn*-3 labeling of TAG was also found in soybean (*Glycine max*; Bates et al., 2009). However, in soybean, initial TAG labeling was quantitatively similar to DAG, whereas in Arabidopsis, TAG labeling is much greater than DAG (Fig. 3). The differences between soybean and Arabidopsis [^{14}C]acetate-labeling experiments is most likely due to the highly labeled 20:1 in Arabidopsis (and is not present in soybean), which primarily accumulates at the *sn*-3 position of TAG (Taylor et al., 1995). Other major seed membrane lipids, such as PE, only contained minor amounts of the nascent [^{14}C] fatty acids in both lines, indicating that PE is not a major intermediate in the flux of fatty acids into TAG synthesis in Arabidopsis, as also demonstrated previously in soybean (Bates et al., 2009). Interestingly, radiolabeled LPC was detected in *lpcat1/lpcat2* but not in Col-0. This result suggests that newly synthesized PC with [^{14}C]acyl groups can be broken down to LPC in both Col-0 and *lpcat1/lpcat2*, but in *lpcat1/lpcat2* LPC was not reacylated to PC due to the lack of LPCAT activity.

Even though the major products of [^{14}C]fatty acid accumulation into glycerolipids by 60 min of labeling were similar between Col-0 and *lpcat1/lpcat2* (Fig. 3, A and C), the initial precursor-product relationships of nascent [^{14}C]fatty acid incorporation into DAG and PC differed significantly between Col-0 and *lpcat1/lpcat2* (Fig. 3, B and D). In Col-0, PC contained more labeled fatty acids than DAG at all time points (Fig. 3, A and B). However, in *lpcat1/lpcat2*, DAG initially contained more label than PC, but it was overcome by PC labeling within 10 min of incubation (Fig. 3D). The relative initial labeling of DAG and PC with nascent [^{14}C] fatty acids in *lpcat1/lpcat2* is very similar to the initial relative labeling of DAG and PC with [^{14}C]glycerol in developing Col-0 seeds (Bates and Browse, 2011). In wild-type seeds, [^{14}C]acetate-labeled fatty acids can be incorporated into PC directly through acyl editing or from de novo-synthesized DAG (Bates and Browse, 2012). However, [^{14}C]glycerol can only enter PC through G3P labeling of the glycerol backbone during de novo glycerolipid synthesis (Fig. 1). Therefore, in Col-0, the lack of a DAG-PC precursor-product relationship of nascent [^{14}C]fatty acid incorporation into PC is consistent with nascent fatty acid incorporation into PC through acyl editing faster than de novo DAG synthesis, as described previously (Bates et al., 2007, 2009). However, the *lpcat1/lpcat2* mutant showed a clear DAG-PC precursor-product relationship of [^{14}C] fatty acid incorporation into PC (Fig. 3D), similar to the kinetics of de novo glycerolipid synthesis measured by [^{14}C]glycerol labeling in Col-0 (Bates and Browse, 2011). This indicates that nascent [^{14}C]fatty acids are incorporated into PC primarily through de novo glycerolipid synthesis within the *lpcat1/lpcat2* mutant.

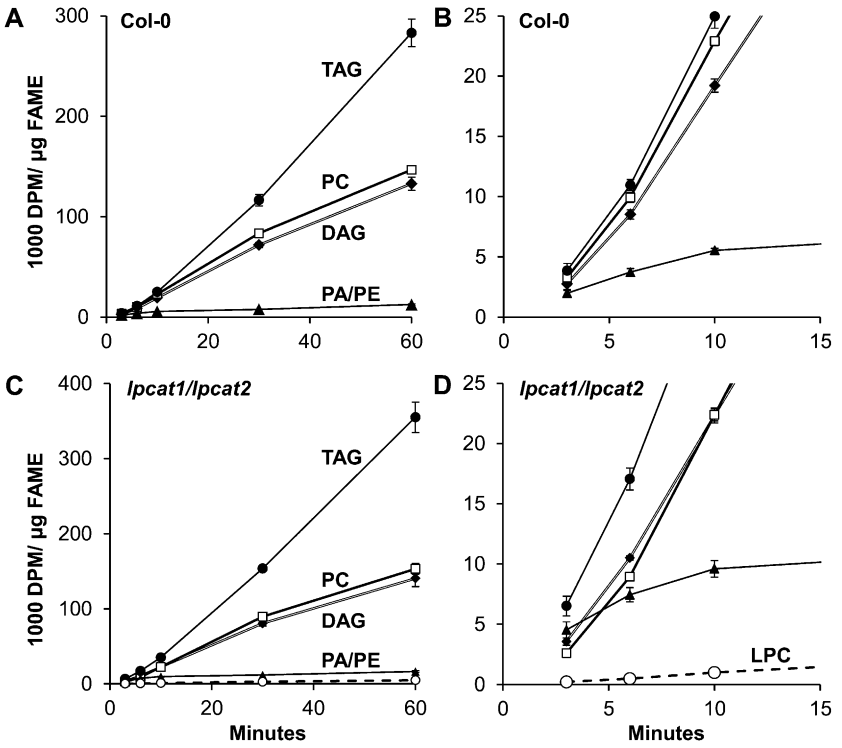

Figure 3. Accumulation of [^{14}C]fatty acids into developing seed glycerolipids of Col-0 and *lpcat1/lpcat2*. A and C, Full 60-min time course. B and D, Initial 15-min of time course. A and B, Col-0. C and D, *lpcat1/lpcat2*. Average values and SE of triplicate labeled samples are shown. Radiolabeled glycerolipids are indicated as follows: TAG (closed circles and solid line); PC (open squares and solid line); DAG (closed diamonds and double line); phosphatidic acid (PA)/PE (closed triangles and solid line); LPC (open circles and dashed line).

Additionally, the similarity in accumulation of radiolabeled PC and DAG by 60 min between Col-0 and *lpcat1/lpcat2* suggests that the flux of de novo-synthesized DAG into PC for eventual PC-derived DAG and TAG production by PDCT is not affected in the *lpcat1/lpcat2* mutant.

To further assess whether the change in DAG-PC precursor-product labeling in the *lpcat1/lpcat2* mutant is due to a reduction in the acyl-editing cycle, we investigated the stereochemical incorporation of nascent [^{14}C]fatty acids into DAG and PC in Col-0 and *lpcat1/lpcat2* (Fig. 4). In Col-0, nascent [^{14}C]fatty acids were initially incorporated into DAG at approximately 65% *sn*-1 and approximately 35% *sn*-2 (Fig. 4A). The unequal distribution of [^{14}C]fatty acids at the *sn*-1 and *sn*-2 positions of the glycerol backbone of DAG is due to the incorporation of a [^{14}C]fatty acid next to a [^{12}C]fatty acid in different molecular species of DAG and is controlled by the acyl group selectivity of the enzymes involved in de novo DAG synthesis (Fig. 1). Previous results in soybean indicated that nascent fatty acids were utilized approximately equally for incorporation next to a [^{12}C]fatty acid in both the *sn*-1 and *sn*-2 positions of DAG (Bates et al., 2009). The higher *sn*-1 labeling in Arabidopsis is also most likely due to the presence of the highly labeled 20:1, of which approximately one-third accumulates at the *sn*-1 position of TAG (Taylor et al., 1995) through de novo DAG synthesis. In great contrast to the stereochemical incorporation of [^{14}C]fatty acids into DAG of Col-0, the incorporation of [^{14}C]fatty acids into PC at the earliest time points was approximately 30% *sn*-1 and 70% *sn*-2 (Fig. 4B). Extrapolation to time 0 indicates that initial incorporation of nascent fatty acids into PC is almost entirely at the *sn*-2 position in Col-0. The very high *sn*-2 labeling of PC is consistent with the rapid incorporation of nascent [^{14}C]fatty acids into PC by acyl editing in Col-0 (Bates et al., 2007, 2009; Tjellström et al., 2012).

The initial stereochemical incorporation of newly synthesized [^{14}C]fatty acids into DAG of *lpcat1/lpcat2* was very similar to that of Col-0 (Fig. 4C), indicating that the reactions involved in de novo DAG synthesis are not affected in the *lpcat1/lpcat2* mutant. However, the stereochemical incorporation of nascent fatty acids into PC in *lpcat1/lpcat2* was drastically different from that in Col-0. Initially, [^{14}C]fatty acids were incorporated into PC at the same stereochemistry as in DAG (approximately 65% *sn*-1 and approximately 35% *sn*-2), indicating that labeled PC was produced from labeled DAG through de novo glycerolipid synthesis and not acyl editing. Together, the stereochemistry of nascent [^{14}C]fatty acid incorporation into DAG and PC (Fig. 4) and the kinetics of DAG and PC labeling (Fig. 3) suggest that the acyl-editing cycle is almost completely impaired in the *lpcat1/lpcat2* mutant. Therefore, these data implicate LPCAT1 and LPCAT2 as the enzymes responsible for the incorporation of nascent fatty acids into PC through acyl editing in Arabidopsis seeds.

Acyl Editing and PDCT-Meditated PC-DAG Conversion Provide the Major Fluxes of PUFA from PC to TAG

The above results strongly support the roles of LPCAT-mediated acyl editing for PUFA accumulation in seed TAG. We have previously shown through metabolic labeling that the PDCT encoded by the *ROD1*

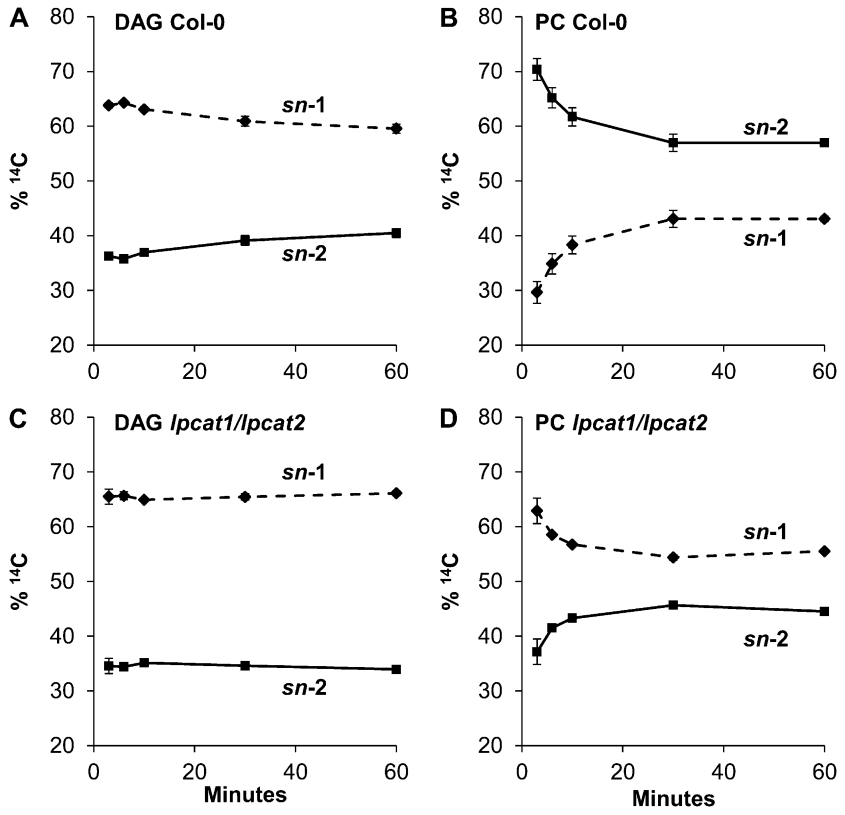

Figure 4. Stereochemical incorporation of [^{14}C] fatty acids into DAG and PC of Col-0 and *lpcat1/lpcat2* developing seeds. Percentage of total lipid radiolabeled fatty acids at the *sn*-1 position of the glycerol backbone, diamonds and dashed line; percentage of total lipid radiolabeled fatty acids at the *sn*-2 position of the glycerol backbone, squares and solid line. A, Col-0 DAG. B, Col-0 PC. C, *lpcat1/lpcat2* DAG. D, *lpcat1/lpcat2* PC. Average values and SE of triplicate labeled samples are shown.

gene is responsible for the major PC-DAG interconversion flux in developing Arabidopsis seeds and that the PUFA levels in the *rod1* mutant seed TAG are reduced approximately 40% from wild-type levels (Lu et al., 2009). To assess the combinatorial effects of these two important mechanisms during TAG synthesis, we generated the *rod1/lpcat1/lpcat2* triple mutant (Table I). The reduction in PUFA (18:2 + 18:3) content in seed TAG of *lpcat1/lpcat2* and *rod1* was approximately 10% and approximately 40%, respectively. However, the *rod1/lpcat1/lpcat2* triple mutant had an approximately 66% reduction in total seed TAG PUFA, suggesting that these three genes are responsible for the major fluxes of 18:1 into PC and of PUFA out of PC for TAG synthesis. The greater than additive reduction in PUFA content in the triple mutant suggests that Arabidopsis can partially compensate for the loss of acyl editing or PDCT by increasing the flux of fatty acid through either pathway in the *lpcat1/lpcat2* and *rod1* backgrounds, respectively. However, in the triple mutant, the major routes of fatty acid flux into and out of PC are lost and the remaining mechanisms for fatty acid flux into and out PC cannot keep up with the rate of TAG synthesis.

To further assess the mechanisms that may provide the remaining one-third (approximately 34%) of wild-type PUFA content to seed TAG in the *rod1/lpcat1/lpcat2* triple mutant, PC fatty acid composition (Fig. 5) and regiochemical incorporation of fatty acids into TAG (Table II; Supplemental Fig. S3) of seeds were analyzed in Col-0 and the *lpcat1/lpcat2*, *rod1*, and *rod1/lpcat1/lpcat2* mutants. The LPCAT and PDCT mutants only have a slight reduction in PC total PUFA (18:2, 18:3, 20:2) content. The greatest effect on PC PUFA is that there is a significant increase in the 18:3/18:2 ratio in *rod1/lpcat1/lpcat2*, although total PUFA stays similar to the *rod1* background (Fig. 5). This result most likely implies that the reduced flux of 18:2 out of PC by acyl editing and PDCT allows for increased desaturation of 18:2 to 18:3 by FAD3. TAG also has an increased 18:3/18:2 ratio in *rod1* and *rod1/lpcat1/lpcat2*, even though total PUFA are greatly decreased (Table II). Additionally, the proportion of 18:2 and 18:3 at the *sn*-2 position of TAG is increased to approximately 66% in the *rod1/lpcat1/lpcat2* mutant from approximately 59% in Col-0 (Table II), indicating that the remaining mechanisms of transferring PUFA from PC to TAG have a preference to locate PUFA at the *sn*-2 position of TAG.

The reduction in TAG PUFA content caused by the loss of LPCAT activity in the acyl-editing cycle may be a factor of both reduced incorporation of 18:1 into PC for desaturation and reduced transfer of PUFA to the CoA pool by the reverse action of LPCAT. Therefore, the analysis of 18:2 and 18:3 content of TAG alone does not indicate if LPCAT1 and LPCAT2 are working in both the forward and reverse directions in Col-0. In both the *lpcat1/lpcat2* and *rod1/lpcat1/lpcat2* mutants, the fatty acid composition of PC had decreased 16:1 and increased 18:0, 20:1, and 20:2 as compared with the respective Col-0 and *rod1* controls. This result implies that LPCAT1/LPCAT2 may be responsible for the incorporation of 16:1 into PC and also the removal of 18:0, 20:1, and 20:2 from PC during the acyl-editing

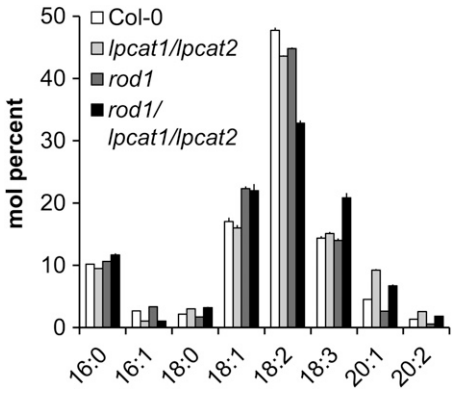

Figure 5. Fatty acid composition of seed PC. The mol % fatty acid composition of seed PC from Col-0, *lpcat1/lpcat2*, *rod1*, and *rod1/lpcat1/lpcat2* is shown. Average values and SE of triplicate samples are shown.

cycle. The increase in 20:1 in PC is not due to a general increase in the cell, because the increase in 20:1 of TAG is only approximately 27%; however, the increase in PC is over 100%, indicating that the loss of LPCAT activity had a more specific effect on 20:1 levels in PC than in TAG. Additionally, 20:2 increases in both *lpcat1/lpcat2* and *rod1/lpcat1/lpcat2* backgrounds in PC, but only in the *lpcat1/lpcat2* background in TAG, and the amount of 20:2 in TAG is greatly reduced in both *rod1* background lines. This result suggests that the mechanism for 20:2 incorporation into TAG is 20:1 incorporation into PC through CPT or PDCT, subsequent desaturation of 20:1 on PC, and then 20:2 incorporation into TAG through the PDCT-mediated PC-derived DAG. However, in Col-0, reverse LPCAT activity keeps 20:1 levels in PC low, reducing 20:2 production. Together, these results suggest that the reverse activity of LPCAT (Stymne and Stobart, 1984) is active in vivo during the acyl-editing cycle for removal of at least some fatty acids from PC.

DISCUSSION

The production of TAG abundant in modified fatty acids (e.g. PUFA) requires substantial fluxes of newly synthesized fatty acids (predominantly 18:1) into PC for modification and subsequent flux of the modified fatty acid out of PC for eventual TAG synthesis. Two main pathways involved in the fluxes of fatty acids into and out of PC are acyl editing and PC-DAG interconversion, producing a PC-derived DAG for TAG synthesis (Bates and Browse, 2012). Acyl editing has been characterized extensively through metabolic labeling studies (Bates et al., 2007, 2009; Tjellström et al., 2012); however, the enzymes/genes involved have not been previously identified. Additionally, the relative contributions of acyl editing and PC-DAG interconversion for the production of PUFA-rich TAG had not been quantified. Our goals in this study were to utilize metabolic labeling and reverse genetics in Arabidopsis to identify enzymes involved in acyl editing and to assess their contribution to providing PUFA for TAG synthesis along with PDCT, the main enzyme involved in the PC-DAG interconversion that we previously identified (Lu et al., 2009). Through in vivo metabolic tracer studies, we tested the involvement of LPCAT1 and LPCAT2 in previously identified facets of acyl editing: (1) rapid incorporation of nascent fatty acids directly into PC by the acyl-editing cycle prior to the incorporation of nascent fatty acids into de novo DAG; and (2) incorporation of the nascent fatty acids primarily into the *sn*-2 position of PC through acyl editing but into both positions of de novo-synthesized DAG (Bates et al., 2007, 2009). By comparing the flux of newly synthesized [^{14}C]acetate-labeled fatty acids into PC and DAG in developing seeds of Col-0 and the *lpcat1/lpcat2* double mutant, we demonstrate that LPCAT1 and LPCAT2 are required for the direct *sn*-2 acylation of LPC during acyl editing to produce PC containing a nascent fatty acid at the *sn*-2 position and a previously synthesized fatty acid at the *sn*-1 position (Fig. 4). Additionally, without LPCAT1/LPCAT2, the kinetics of nascent fatty acid incorporation into DAG and PC is consistent with the de novo synthesis of DAG from G3P and subsequent PC synthesis (Bates and Browse, 2011; Fig. 3D) rather than with direct incorporation of nascent fatty acids into PC through the acyl-editing cycle, as in Col-0 (Fig. 3B). Together, these results indicate that LPCAT1 and LPCAT2 are required for the acylation of LPC within the acyl-editing cycle (Fig. 1). Analysis of the effect of *lpcat1/lpcat2* on the acyl composition of PC in both the Col-0 and *rod1* backgrounds (Fig. 5) suggests that LPCAT1/LPCAT2 are involved in at least part of the deacylation of PC reaction within the acyl-editing cycle to provide fatty acids to the acyl-CoA pool. Additionally, analysis of the *rod1/lpcat1/lpcat2* mutant seeds indicates that acyl editing and PDCT-mediated PC-DAG interconversion together provide about two-thirds (approximately 66%) of the flux of PUFA from PC to TAG in wild-type Arabidopsis seeds.

PC-DAG Interconversion Fluxes Revealed by Metabolic Labeling in the *lpcat1/lpcat2* Mutant

The shape of the time course curves for the stereochemical incorporation of [^{14}C]fatty acids into DAG and PC (Fig. 4) highlights the contributions of acyl

Table II. *TAG 18:2 and 18:3 content and regiochemistry*
The results are calculated from Table I and Supplemental Figure S3.

Line	Total	18:3/18:2 Ratio	Positional Distribution	
			sn-2	*sn*-1/3
Col-0	47.8	0.7	58.7	41.3
lpcat1/lpcat2	43.3	0.7	64.4	35.6
rod1	28.3	1.0	63.0	37.0
rod1/lpcat1/lpcat2	16.4	1.2	66.4	33.6

editing and PC-DAG interconversion for the flux of newly synthesized fatty acids into and out of PC. In Col-0, acyl editing initially incorporates nascent fatty acids almost completely into the *sn*-2 position of PC at time 0. However, at the same time, de novo DAG is synthesized with approximately 65% of nascent fatty acids at *sn*-1. As highly *sn*-1-labeled de novo DAG is incorporated into PC through PC-DAG interconversion, the proportion of [^{14}C]fatty acids at the *sn*-2 position of PC drops to approximately 58% by 30 min (Fig. 4B). This marks the point at which the fluxes of acyl editing and synthesis of PC from de novo DAG reach equilibration to produce PC with a constant stereochemical labeling ratio. Short-time-point [^{14}C] acetate labeling of DAG primarily labels de novo DAG (Bates and Browse, 2012). However, as the highly *sn*-2-labeled PC is converted to PC-derived DAG over time for TAG synthesis, the labeling of the total DAG pool *sn*-2 position is increased (Fig. 4A). The impairment of the acyl-editing cycle in *lpcat1/lpcat2* does not show the increase in PC-derived DAG *sn*-2 labeling over time (Fig. 4C), indicating that stereochemical incorporation of nascent fatty acids in DAG stays the same as it fluxes through PC. The reduction in *sn*-1 labeling of PC of *lpcat1/lpcat2* to approximately 55% by 30 min most likely represents the rapid movement of highly *sn*-1-labeled de novo DAG through the PC pool for the production of PC-derived DAG, leaving behind PC molecular species with more of an equilibrated *sn*-1/*sn*-2 stereochemistry. The most likely DAG molecular species candidates for this rapid flux through PC are DAG molecular species containing the highly labeled 20:1 at the *sn*-1 position, which does not accumulate in PC but must flux through PC, since more than 90% of TAG is synthesized from PC-derived DAG (Bates and Browse, 2011). Thus, analysis of the *lpcat1/lpcat2* mutant labeling stereochemistry and kinetics is able to highlight the rapid fluxes of de novo-synthesized DAG molecular species through the PC-DAG interconversion, which are masked by the acyl-editing cycle in Col-0.

LPCAT Specificity and Functions Ascertained from *lpcat1/lpcat2* Metabolic Labeling

The Arabidopsis LPCAT1 and LPCAT2 enzymes were previously identified in vitro as broad substrate lysophospholipid acyltransferases with a preference for LPC (Ståhl et al., 2008). Our in vivo metabolic labeling experiments did not indicate a change in the incorporation of newly synthesized fatty acids into phospholipids, other than PC, between developing seeds of *lpcat1/lpcat2* and Col-0 (Fig. 3). This result suggests that LPCAT1/LPCAT2 function primarily as LPCATs for the utilization of nascent fatty acids. Two other Arabidopsis genes encoding lysophospholipid acyltransferases with a preference for lysophosphatidylethanolamine also show broad specificity for other lysophospholipids in vitro, including LPC (Stålberg et al., 2009). Our stereochemical analysis of radiolabeled DAG and PC shows very little or no acylation of LPC with nascent fatty acids in *lpcat1/lpcat2* developing seeds (Fig. 4), indicating that other lysophospholipid acyltransferases with broad in vitro activity cannot compensate for the loss of LPCAT1/LPCAT2 activity. This conclusion is also supported by the fact that radiolabeled LPC accumulated in *lpcat1/lpcat2* but not in Col-0 (Fig. 3). The LPC may have been generated by a phospholipase A portion of the acyl-editing cycle or by PDAT activity (Fig. 1). LPC has strong detergent properties that can be detrimental to cellular membranes. Therefore, without LPCAT activity, the *lpcat1/lpcat2* mutant may need to fully break down LPC into fatty acid, G3P, and choline to detoxify the cell. The generation of LPC and the requirement to detoxify it may require *lpcat1/lpcat2* mutants to increase total PC synthesis to replenish the loss of membrane components.

The Triple Mutant Implies Additional Minor Fluxes of Fatty Acid through PC for TAG Synthesis

The remaining one-third of wild-type PUFA content in TAG of the *rod1/lpcat1/lpcat2* triple mutant may be removed from PC and incorporated into TAG by three possible mechanisms: (1) direct transfer of PUFA from *sn*-2 PC into the *sn*-3 position of TAG by PDAT; (2) removal of fatty acids from PC by a phospholipase A and utilization of the corresponding acyl-CoA by de novo DAG and subsequent TAG synthesis; and (3) a residual PC-derived DAG flux produced by the reverse action of CPT, phospholipase C, or phospholipase D/PAP (Fig. 1). The stereochemical analysis of TAG indicated that two-thirds of the PUFA in *rod1/lpcat1/lpcat2* TAG was at the *sn*-2 position (Table II), indicating that, at most, PDAT could be involved in transferring one-third of the remaining PUFA from PC to TAG. The PDAT reaction generates LPC as a by-product, and LPCAT2 is required to regenerate PC for efficient PDAT activity in the *dgat1* mutant background (Xu et al., 2012). Thus, PDAT activity most likely does not contribute much to the PUFA content of *rod1/lpcat1/lpcat2*. The LPC that accumulates in the *lpcat1/lpcat2* mutant could be produced by a phospholipase A portion of the acyl-editing cycle and could possibly account for the removal of PUFA from PC. For the PUFA to have preferential accumulation in the *sn*-2 position of TAG, it would require lysophosphatidic acid acyltransferase to contain a higher specific activity with PUFA than either glycerol-3-phosphate acyltransferase or DGAT, which incorporate the PUFA into the *sn*-1 or *sn*-3 position of TAG, respectively (Fig. 1). The most likely explanation for the accumulation of two-thirds of the PUFA in *rod1/lpcat1/lpcat2* at the *sn*-2 position of TAG may be through a residual PC-derived DAG flux, since the *sn*-2 position of PC is the site of 18:1 desaturation (Sperling et al., 1993). Recently, the analysis of a phospholipase D mutant in soybean indicated a reduction of PUFA in TAG (Lee et al., 2011), further supporting the possibility of a small flux of PC-derived DAG synthesis independent of PDCT in *rod1/lpcat1/lpcat2*. For PUFA to be distributed across the *sn*-2 and *sn*-1/3 positions of the TAG backbone, it will require

the combination of the residual PC-derived DAG flux along with either a phospholipase A activity or PDAT, or both.

To effectively tailor seed oil fatty acid compositions to meet our nutritional or industrial needs, we need to not only understand the enzymes that produce the fatty acids of interest but also the mechanisms required for the flux of the fatty acid from the site of synthesis to accumulation in TAG. We have identified LPCAT1 and LPCAT2 as key components of the acyl-editing cycle in Arabidopsis that together with PDCT control the major fluxes of fatty acids into and out of PC for fatty acid modification and TAG synthesis. Further research should be focused on identifying the remaining mechanisms that control the minor fluxes of fatty acids into and out of PC so that we can increase our metabolic toolbox for bioengineering designer vegetable oils.

MATERIALS AND METHODS

Materials

Arabidopsis (*Arabidopsis thaliana*) lines, wild type (Col-0), and the *rod1* ethyl methanesulfonate-induced mutant, are the same lines as used in the previous study (Lu et al., 2009). The *lpcat1* (SALK_123480) and *lpcat2* (SAIL_357_H01) T-DNA insertion mutant lines (both in the Col-0 background) were described previously (Xu et al., 2012). Seeds of these lines and progeny from genetic crosses were grown in a growth chamber at 22°C with a photoperiod of 16 h of light (120 μE m^{-2} s^{-1}) and 8 h of dark.

HPLC-grade organic solvents were purchased from Fisher Scientific (http://www.fishersci.com). Other materials were from these vendors: thin-layer chromatography (TLC) plates (Partisil K6 silica gel 60 Å 20- × 20-cm glass plates), Whatman (http://www.whatman.com/); [1-^{14}C]acetate (specific activity, 58 mCi $mmol^{-1}$), American Radiolabeled Chemicals (http://www.arc-inc.com/); Ecoscint liquid scintillation cocktail, National Diagnostics (http://www.nationaldiagnostics.com/); butylated hydroxytoluene, primuline, porcine pancreas phospholipase A_2 ammonium sulfate suspension (800 units mg^{-1}, 1.6 mg mL^{-1} protein), and TAG lipase (*Rhizomucor miehei*), Sigma-Aldrich (http://www.sigmaaldrich.com).

Metabolic Labeling

All plants for labeling experiments were grown randomized across two flats in a growth chamber with 24 h of constant light (approximately 110–150 μE), 22°C to 24°C, and approximately 50% to 70% humidity. Silique ages were determined by trimming each plant to one main shoot and counting the new open flowers/siliques produced each day. Seeds of 9- to 10-d-after-flowering siliques were harvested directly into 5 mM MES, pH 5.8, 0.5% Suc, and 0.5× Murashige and Skoog salts on ice as described (Bates and Browse, 2011). For each plant line, approximately 150 siliques worth of seeds from approximately 30 plants were combined, mixed, and divided into aliquots in three individual tubes prior to adding radiolabel. After an approximately 20-min preincubation in a lighted water bath at 23°C, the labeling was initiated by removing the medium and replacing it with the same medium containing approximately 1 mM [^{14}C]acetate for each of the individual seed incubations. At 3, 6, 10, 30, and 60 min, an aliquot of seeds was removed from each individual labeled sample and quenched in 85°C isopropanol + 0.01% butylated hydroxytoluene for 10 min. Therefore, three individual Col-0 and three individual *lpcat1*/*lpcat2* labeling time-course replicates, with five time points each (30 total individual labeled samples), were performed. Lipid extraction was done as described (Bates and Browse, 2011). An aliquot of each total extract was analyzed for incorporation of radioactivity on a Tri-CARB liquid scintillation analyzer (Packard Instrument) and total lipid quantity by conversion to fatty acid methyl esters (FAME; Miquel and Browse, 1992) and quantification against a 17:0 internal standard by gas chromatography with flame ionization detection on a wax column (Enzyme Commission Wax; 30 m × 0.53 mm i.d. × 1.20 mm; Alltech). Total radioactivity in each extract was normalized to the total μg of FAME to correct for different numbers of seeds in each time-point aliquot.

Analysis of Lipids

The fatty acid composition of seed TAG was analyzed using gas chromatography as descried previously (Hu et al., 2012).

Lipid classes were separated by a dual-development TLC system. First, develop 12 cm in chloroform:methanol:acetic acid:water (75:25:4:4, v/v/v/v), dried in a vacuum for 10 min, then fully developed in hexane:diethyl ether:acetic acid (70:30:1, v/v/v). Total ^{14}C in each lipid was determined by the percentage ^{14}C of each lipid band in the TLC lane and the total ^{14}C in each extract. For collection of PC, DAG, and TAG, 200 μg of carrier PC and DAG was separated with the labeled extracts as above, and the mass bands were visualized under UV light after staining with 0.005% primuline in acetone:water (80:20, v/v). Each lipid was eluted from the silica as described (Bates et al., 2007). DAG and TAG regiochemistry was determined by TAG lipase digestion and TLC (Cahoon et al., 2006). PC stereochemistry was determined by phospholipase A_2 digestion modified from Bates et al. (2007). In brief, PC in 1 mL of diethyl ether was vortexed with 0.1 mL of phospholipase A_2 stock 1:1 in 100 mM Tris-HCl, pH 8.7, 10 mM $CaCl_2$ at 35°C for 30 min. Products were extracted and separated by TLC (Bates et al., 2007). Individual radiolabeled fatty acids were separated as FAME by argentation TLC (Bao et al., 1998) and eluted from the $AgNO_3$ silica (Bates and Browse, 2011). Permanganate-periodate oxidation was performed as described (Christie, 2003), and products were separated as described (Bao et al., 1998). All TLC systems contained 0.005% butylated hydroxytoluene. Radioactive lipids separated by TLC were identified by comigration with lipid standards, and radioactivity was quantified by phosphor imaging on a GE Typhoon FLA 7000 (http://www.gelifesciences.com).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Permanganate-periodate oxidation of radiolabeled fatty acid methyl esters.

Supplemental Figure S2. Regiochemistry of [^{14}C]fatty acid incorporation into TAG.

Supplemental Figure S3. Seed TAG *sn*-2 composition.

Supplemental Discussion S1. Permanganate-periodate oxidation of radiolabeled fatty acid methyl esters.

Supplemental Discussion S2. Regiochemistry of [^{14}C]fatty acid incorporation into TAG.

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