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Phospholipase Dζ enhances diacylglycerol flux into triacylglycerol

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One-sentence summary: Phospholipase D increases the production of triacylglycerol in *Camelina sativa* seeds.

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Keywords: *Camelina sativa*, oilseed, lipid metabolism, 14C-labeling, phospholipase, metabolic engineering

SUMMARY

Plant seeds are the primary source of triacylglycerols (TAG) for food, feed, fuel, and industrial applications. As TAG is produced from diacylglycerol (DAG) successful engineering strategies to enhance TAG levels have focused on the conversion of DAG to TAG. However, the production of TAG can be limited by flux through the enzymatic reactions that supply DAG. In this study, two *Arabidopsis phospholipase* D_ζ genes (*AtPLD*$_{\zeta 1}$ and *AtPLD*$_{\zeta 2}$) were co-expressed in *Camelina sativa* to test whether the conversion of phosphatidylcholine (PC) to DAG impacts TAG levels in seeds. The resulting transgenic plants produced 2-3% more TAG as a component of total seed biomass and had increased 18:3 and 20:1 fatty acid levels relative to wild type. Increased DAG and decreased PC levels were examined through the kinetics of lipid assembly by [^{14}C]acetate and [^{14}C]glycerol incorporation into glycerolipids. [^{14}C]acetate was rapidly incorporated into TAG in both WT and overexpression lines, indicating a significant flux of nascent and elongated acyl-CoAs into the *sn*-3 position of TAG. Stereochemical analysis revealed that newly synthesized fatty acids were preferentially incorporated into the *sn*-2 position of PC, but the *sn*-1 position of *de novo* DAG and indicated similar rates of nascent acyl groups into the Kennedy pathway and acyl editing. [^{14}C]glycerol studies demonstrated PC-derived DAG is the major source of DAG for TAG synthesis in both tissues. The results emphasize that the interconversions of DAG and PC pools can impact oil production and composition.

SIGNIFICANCE STATEMENT: Triacylglycerols (TAG) from oilseeds are a significant percentage of human and animal diets. Phospholipase D_ζ overexpression in *Camelina sativa* enhanced the production of TAG in seeds by 2-3%.

ABBREVIATIONS

DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; FA, non-esterified fatty acid; LPA, lysophosphatidic acid; acyl-CoA, acyl-coenzyme A; LPC, lysophosphatidylcholine; LPCAT, lysophosphatidylcholine acyltransferase; MAG, monoacylglycerol; OE, overexpression; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PC, phosphatidylcholine; PDAT, phosphatidylcholine:diacylglycerol acyltransferase; PDCT, phosphatidylcholine:diacylglycerol

cholinephosphotransferase; PLD$_\zeta$, phospholipase D$_\zeta$; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol; WT, wild type

INTRODUCTION

Plant oils are an important source of energy for human and animal diets and are regularly used in production of biofuels and industrial products (Lu et al., 2011). Therefore, there is considerable effort to increase the oil quality and yield in plants through breeding and application of biotechnology (Tan et al., 2011; Shi et al., 2012; Haslam et al., 2013; Vanhercke et al., 2013; van Erp et al., 2014; Li et al., 2015). Though many of the involved genes have been catalogued and enzyme activities measured, a better understanding of the coordinated metabolic network (Figure 1) that produces both membrane lipids and storage oils is necessary. Plant oil (triacylglycerol, TAG) biosynthesis begins with generation of 16 and 18 carbon fatty acids (FA) in the plastid (Li-Beisson et al., 2013; Allen et al., 2015). Acyl chains are transported across the plastid envelope (Koo et al., 2004; Li et al., 2015) and activated to acyl-CoAs (Shockey et al., 2002). Then they can be elongated to lengths of 20 carbons or more (Kunst et al., 1992), shuttled directly into the endoplasmic reticulum (ER) for desaturation on phosphatidylcholine (PC) (Stymne and Glad, 1981; Bates et al., 2009), or used by Kennedy pathway enzymes to generate lysophosphatidic acid (LPA) and subsequently phosphatidic acid (PA) (Kornberg and Pricer, 1953; Weiss et al., 1960; Kennedy, 1961). PA is converted to diacylglycerol (DAG) by PA phosphatase (Eastmond et al., 2010; Mietkiewska et al., 2011; Pascual and Carman, 2013; Craddock et al., 2015). This "*de novo*" synthesized DAG can then be utilized for membrane lipid synthesis or converted to TAG in plants by a further acylation at *sn*-3 using either the acyl-CoA dependent diacylglycerol acyltransferase (DGAT) (Barron and Stumpf, 1962; Griffiths et al., 1985; Katavic et al., 1995; Zou et al., 1999), or by the transfer of the *sn*-2 FA from PC to DAG by phosphatidylcholine:diacylglycerol acyltransferase (PDAT) (Dahlqvist et al., 2000). These mechanisms of plant TAG biosynthesis summarized in Fig. 1. have recently been reviewed in extensive detail (Weselake et al., 2009; Zhang et al., 2009; Chapman and Ohlrogge, 2012; Li-Beisson et al., 2013; Allen et al., 2015; Allen, 2016; Bates, 2016).

The DAG for TAG synthesis can also be derived from the ER lipid, PC (Bates, 2016). FA desaturation reactions modify acyl chains that are esterified to PC, thus the flux through PC leads to an enhanced degree of unsaturated FA in the PC-derived DAG pool relative to *de novo* DAG and can provide an increased source of polyunsaturated fatty acid (PUFA) for TAG biosynthesis (Griffiths et al., 1988; Bates et al., 2009; Bates and Browse, 2012). PC is produced from *de novo* DAG by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT-DAG), which may be reversible and consequently produce PC-derived DAG (Slack et al., 1983; Slack et al., 1985). Alternatively, flux from *de novo* DAG through PC and back to PC-derived DAG may occur through a phosphocholine headgroup exchange mechanism with phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) (Lu et al., 2009). In Arabidopsis, the PDCT mutant (*rod1*) reduces the PUFA content of TAG by 40%, emphasizing the important role of DAG flux through PC for acyl desaturation prior to incorporation into TAG (Lu et al., 2009).

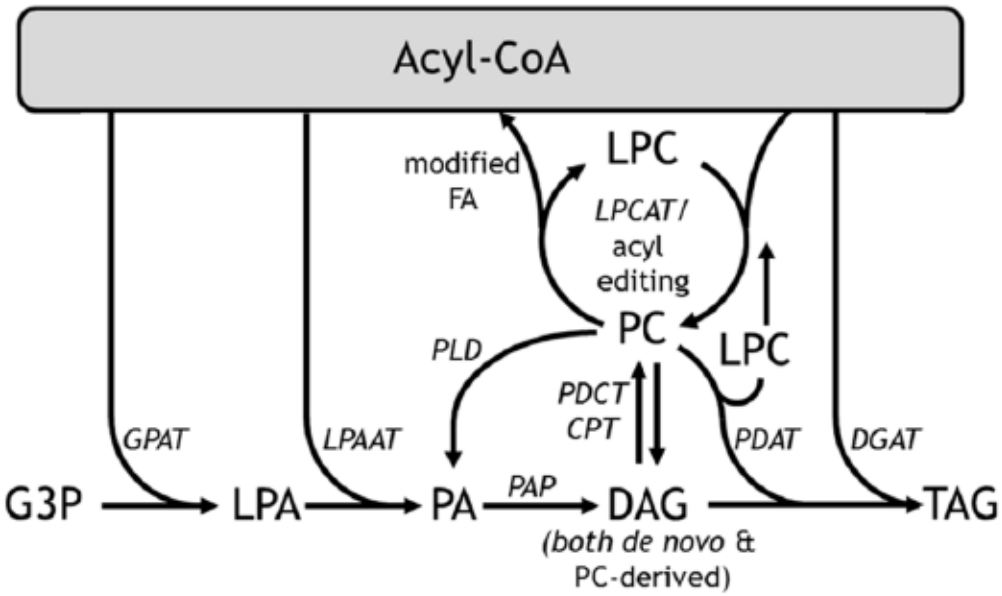

Fig. 1. Simplified metabolic network description of acyl chain incorporation into TAG in oilseeds. Enzymes are labeled in italics. G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; DAG, diacylglycerol; TAG, triacylglycerol; LPCAT, lyso-PC acyltransferase; PLA, phospholipase A; GPAT, glycerol-3-phosphate acyltransferase; LPAAT, lyso-PA acyltransferase; PAP, phosphatidic acid phosphatase; PLD, phospholipase D; CPT, CDP-choline:diacylglycerol cholinephosphotransferase; PDCT, phosphatidylcholine:diacylglycerol cholinephosphotransferase; DGAT, acyl-CoA:diacylglycerol acyltransferase; PDAT, phospholipid:diacylglycerol acyltransferase.

Other mechanisms also participate in shuttling acyl chains through PC to promote DAG and TAG formation with greater concentrations of desaturates. For example, PC can be produced through esterification of FA to lysophosphatidylcholine (LPC) with lysophosphatidylcholine acyltransferase (LPCAT) (Stymne and Stobart, 1984; Bates et al., 2012; Wang et al., 2012) and after modification the FA is released for reentry into the acyl-CoA pool, regenerating LPC and completing the cycle in a process coined acyl editing (Williams et al., 2000; Bates et al., 2009; Bates and Browse, 2012; Tjellstrom et al., 2012). In soybean and Arabidopsis it is estimated that >90% of nascent FA flux through PC by acyl editing or as DAG components prior to incorporation into TAG (Bates et al., 2009; Bates and Browse, 2011; Bates et al., 2012). However, the mechanisms involved in this acyl flux have not been thoroughly explored. For example, knockouts of the major enzymes involved in Arabidopsis PC acyl editing (LPCAT1/2) and PC-DAG exchange (PDCT) only reduced the TAG desaturate content by 2/3(Bates et al., 2012). The suppression of phospholipase D (PLD) in soybeans partially reduced the PUFA content of TAG (Lee et al., 2011), indicating an alternative mechanism to PDCT based PC-derived DAG production. Possibly the flux of *de novo* DAG into PC by CPT, followed by PLD and PA phosphatase activity is necessary to produce the DAG substrate for TAG synthesis. In Arabidopsis, two PLD$_\zeta$ genes, AtPLD$_{\zeta1}$ and AtPLD$_{\zeta2}$, have been identified that hydrolyze PC to produce PA (Qin and Wang, 2002; Cruz-Ramirez et al., 2006). Since altered levels of PA have been shown to stimulate production of PC (Eastmond et al., 2010; Craddock et al., 2015);

123 we hypothesized that increased PLD activity in oilseeds would enhance both the flux of *de novo* DAG into
124 PC and the flux of PC-derived DAG into TAG, leading to enhanced levels of TAG and the accumulation of
125 PUFA in TAG.
126 Here we show that overexpression of PLDζs in *Camelina sativa* (i.e. Camelina), a close relative of
127 Arabidopsis, had enhanced acyl flux to TAG through an increase in the levels of DAG. Camelina was
128 chosen for obvious advantages, it can be floral-dip transformed (Lu et al., 2011), has a short life cycle, and
129 has oil rich seeds (30-40% of seed weight) that contain high levels of PUFAs (>50%), especially α-
130 linolenic acid (18:3, >30% of total fatty acids) (Campbell et al., 2013; Iskandarov et al., 2013). Recently
131 phospholipase A overexpression in Camelina resulted in an increase in total oil (Li et al., 2015), and other
132 transgenics have resulted in TAG containing non-native FAs (Lu and Kang, 2008; Petrie et al., 2014; Ruiz-
133 Lopez et al., 2014; Liu et al., 2015; Nguyen et al., 2015). However, mechanisms that control acyl flux
134 through PC into TAG have not been studied as extensively in Camelina as in other species.
135 In the described work, the heterologous co-expression of two *Arabidopsis thaliana* PLDζ's
136 (AtPLDζ1 and AtPLDζ2) in Camelina seeds resulted in a 3% increase in TAG and fatty acid composition
137 that was elevated in linolenic and eicosenoic acids. Production of active forms of AtPLDζ1 and AtPLDζ2
138 were confirmed by RT-PCR and enzyme activity assays with microsomes. Differences in lipid and acyl
139 chain composition from the wild type were consistently observed throughout seed development.
140 [^{14}C]acetate pulse labeling indicated newly synthesized acyl groups were rapidly incorporated into TAG.
141 The rate of [^{14}C]acetate incorporation as fatty acids into PC and DAG were similar but stereochemically
142 involved different sites of attachment on the glycerol backbone, indicating acyl flux through different
143 branches of lipid metabolism (Fig. 1). [^{14}C]glycerol labeling indicated a prominent role of the PC-derived
144 DAG pathway in Camelina, but significantly more flux through the Kennedy pathway relative to other
145 oilseeds such as Arabidopsis (Bates et al., 2012) or soybean (Bates et al., 2009).
146

RESULTS

AtPLDζ overexpression results in a high oil phenotype

Two PLDζ genes, *AtPLDζ1* and *AtPLDζ2*, have been identified in the *Arabidopsis thaliana* genome (Qin and Wang, 2002; Li et al., 2006). *In vitro* assays indicated that AtPLDζ1 specifically hydrolyzes PC to produce PA and choline (Qin and Wang, 2002). In the present study, *AtPLDζ1* and *AtPLDζ2* cDNA ORFs were placed behind the seed specific glycinin and β-conglycinin promoters, respectively. Camelina plants were transformed with *Agrobacterium* containing a binary vector for co-expression of *AtPLDζ1* and *AtPLDζ2*, and DsRed selection marker. Homozygosity was inspected by screening with DsRed and the expression of both *AtPLDζ* genes was confirmed in developing seeds through RT-PCR (Fig. 2). No visual differences in plant phenotypes were observed between WT and transgenic plants. *In vitro* assays of PLDζ activity within microsomes prepared from developing seeds indicated increased PLD activity in the transgenic lines (86 to 269 pmol/h/g) relative to WT controls (65.74 pmol/h/g; Table I). Measureable activity in total protein extracts was not detected, indicating that AtPLDζ is highly enriched in microsomes and is likely associated with the endoplasmic reticulum in developing Camelina seeds, however, the mechanism of ER association is unclear due to the lack of a predicted transmembrane domain in either AtPLDζ isoform. Four transgenic lines overexpressing both PLDζ isoforms (subsequently referred to as PLDζ 1-4) contained 1.9-3.5% more oil than WT on a dry weight basis across seed development, and 1.9-3% in mature seeds (Table 1). This is equivalent to an average 12.7% increase in total oil content relative to WT at 12 DAF, and 9.5% increase in mature seeds.

PLDζs alter acyl lipid and fatty acid profiles

Total lipid extracted from the developing seeds was analyzed for changes in glycerolipid distribution and fatty acid composition. Polar lipids (phospholipids and galactolipids, PL/GL) and neutral lipids including DAG and TAG were separated using silica thin layer chromatography (TLC) and the fatty acid profiles were analyzed in WT and the transgenic PLDζ line 1 (subsequently referred to as PLDζ). TAG and DAG levels increased throughout development in the transgenic lines (Fig. 3A, 3B) whereas polar lipids decreased (Fig. 3C). The results indicated that PLDζ altered the equilibrium between steady state pool levels to favor the production of DAG and TAG relative to polar lipids such as PC which is the primary phospholipid in seeds. In addition, the composition of fatty acids in total lipid extracts was altered in PLDζ throughout seed development (Fig. 4). Palmitic (16:0), stearic (18:0), and linoleic acids (18:2) were reduced in the transgenic line whereas linolenic (18:3) and eicosenoic acids (20:1) increased. Oleic acid (18:1) (Fig. 4C) and minor fatty acids including arachidic acid (20:0), eicosadienioc acid (20:2), eicosatrienioc acid (20:3), docosanoic acid (22:0), and erucic acid (22:1) (Fig. S1) showed smaller differences between the lines.

The fatty acid compositions of individual acyl lipids were also evaluated during development to establish the relationship between glycerolipid intermediates. The acyl lipid fatty acid profiles of TAG, DAG and phospholipids between developing WT and PLDζ seeds (Fig. 5) were qualitatively similar to the

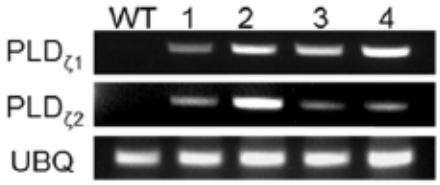

Fig. 2. Transcript level of AtPLD$_{\zeta 1}$ and AtPLD$_{\zeta 2}$ in developing seeds at 22 DAF of Camelina WT and PLD$_{\zeta}$ overexpression lines. UBQ, ubiquitin.

183 total lipid fatty acid changes (Fig. 4), though showed some statistical differences ($p<0.05$) with the most
184 notable changes in 18:3 and 18:2 lipid fractions of each lipid class.

185 **In vivo [^{14}C]acetate labeling of Camelina WT and PLD$_{\zeta}$ embryos indicates**
186 **significant PC acyl editing and DGAT activity utilizing a PC-derived DAG pool**

187 Production of plant oil containing PUFAs requires acyl flux through PC, the site for ER localized
188 fatty acid desaturation. Based on the PUFA amounts in TAG harvested from developing seeds (20 DAF),
189 approximately 56% of acyl groups flux through PC in both WT and PLD$_{\zeta}$ Camelina. However, not all fatty
190 acids are further desaturated whilst on PC, thus the calculated flux through this metabolic pool is an
191 underestimate (Bates and Browse, 2012; Bates, 2016). To determine the effect of PLD$_{\zeta}$ on acyl flux
192 through PC, eukaryotic glycerolipid assembly was investigated with [^{14}C]acetate labeling (Allen et al.,
193 2015). Developing embryos of WT and PLD$_{\zeta}$ incorporated [^{14}C]acetate-derived fatty acids linearly into
194 glycerolipids over a 3 h period (Fig. S2) including PC, DAG and TAG (Fig. 6A, 6B). The initial rate of
195 nascent [^{14}C]fatty acid incorporation into TAG was the same or greater than for PC and DAG in both WT
196 and the PLD$_{\zeta}$, indicating that labeled PC or DAG was not the initially labeled precursor for TAG
197 biosynthesis in Camelina seeds (Fig. 6C, 6D). Thus, the TAG was likely enriched by direct incorporation of
198 [^{14}C]acyl chains into the *sn*-3 position of unlabeled DAG with DGAT. TAG and DAG labeling was greater
199 in the transgenics by the conclusion of the experiment and may reflect the larger pool of DAG (Fig. 3) in
200 the transgenic line that is available for *sn*-3 acylation with nascent fatty acids whereas PC enrichment was
201 not statistically different between lines, and likely represents the intermediate role of PC within fatty acid
202 flux into TAG. Other minor phospholipids including PA, PI, PE/PG and the galactolipid
203 monogalactosyldiacylglycerol (MGDG) were also labeled in both lines though generally less enriched in
204 PLD$_{\zeta}$ (Fig. S3).

205 [^{14}C]fatty acids were preferentially incorporated into the *sn*-2 position of PC which is consistent
206 with initial incorporation through acyl editing rather than through the Kennedy pathway as has been
207 previously described in pea leaves, soybean developing embryos, Arabidopsis developing seeds and cell
208 suspensions (Bates et al., 2007; Bates et al., 2009; Bates et al., 2012; Tjellstrom et al., 2012).
209 Stereochemical analyses indicated similar values for WT and PLD$_{\zeta}$, initially ~60% of nascent fatty acids at
210 the *sn*-2 position of PC at 5 min which equilibrated to ~55% by 60 min (Fig. 7A, 7B). This regiospecific
211 incorporation of acyl chains on the glycerol backbone is more balanced between *sn*-1 and *sn*-2 in Camelina

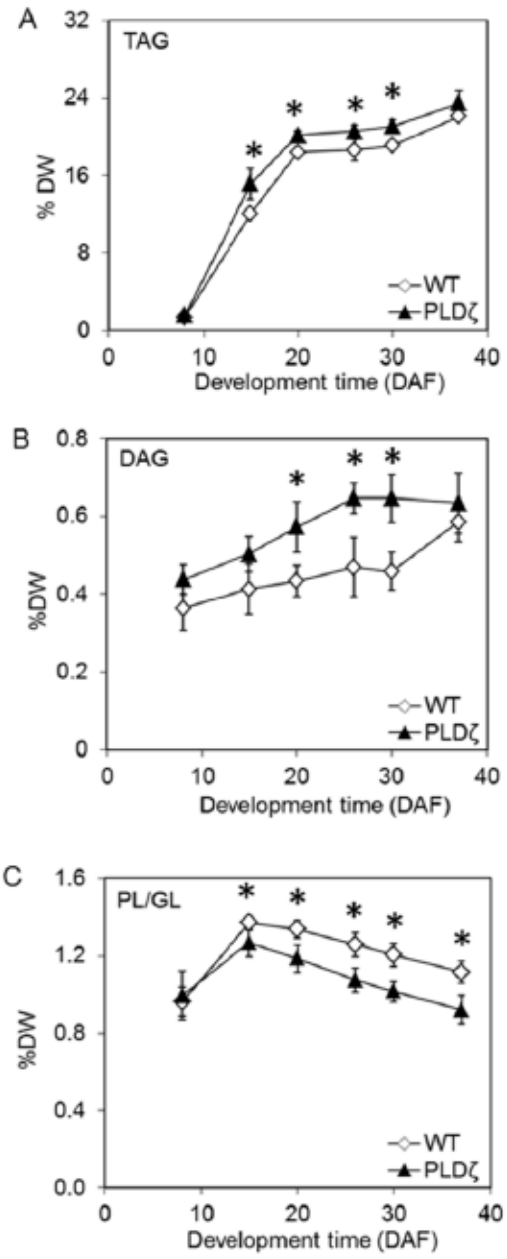

Fig. 3. Lipid composition in Camelina seeds. Relative lipid composition including: TAG (A), DAG (B), PL/GL (C) in seeds of WT and PLD$_\zeta$ line 1 (henceforth referred to as PLD$_\zeta$) during development. TAG, DAG, PL/GL were quantified by GC-FID using internal standard after they were eluted from TLC plate of total lipid separation (SD, n=3). Significant differences (*T* test, $P < 0.05$) between PLD$_\zeta$ and WT are denoted with an asterisk.

relative to soybeans or Arabidopsis seeds that initially label the PC *sn*-2 position at over 70-80% but then equilibrate to 50-60% with time (Bates et al., 2009; Bates et al., 2012). DAG showed a different trend unlike PC, the stereochemical incorporation of [^{14}C]fatty acids into DAG (Fig. 7C, 7D) indicated a greater preference for the sn-1 position. 55-60% of nascent [^{14}C]fatty acids were incorporated into the *sn*-1 position of DAG in WT and PLD$_\zeta$ lines. The preference for *sn*-1 labeling in Camelina DAG is comparable with

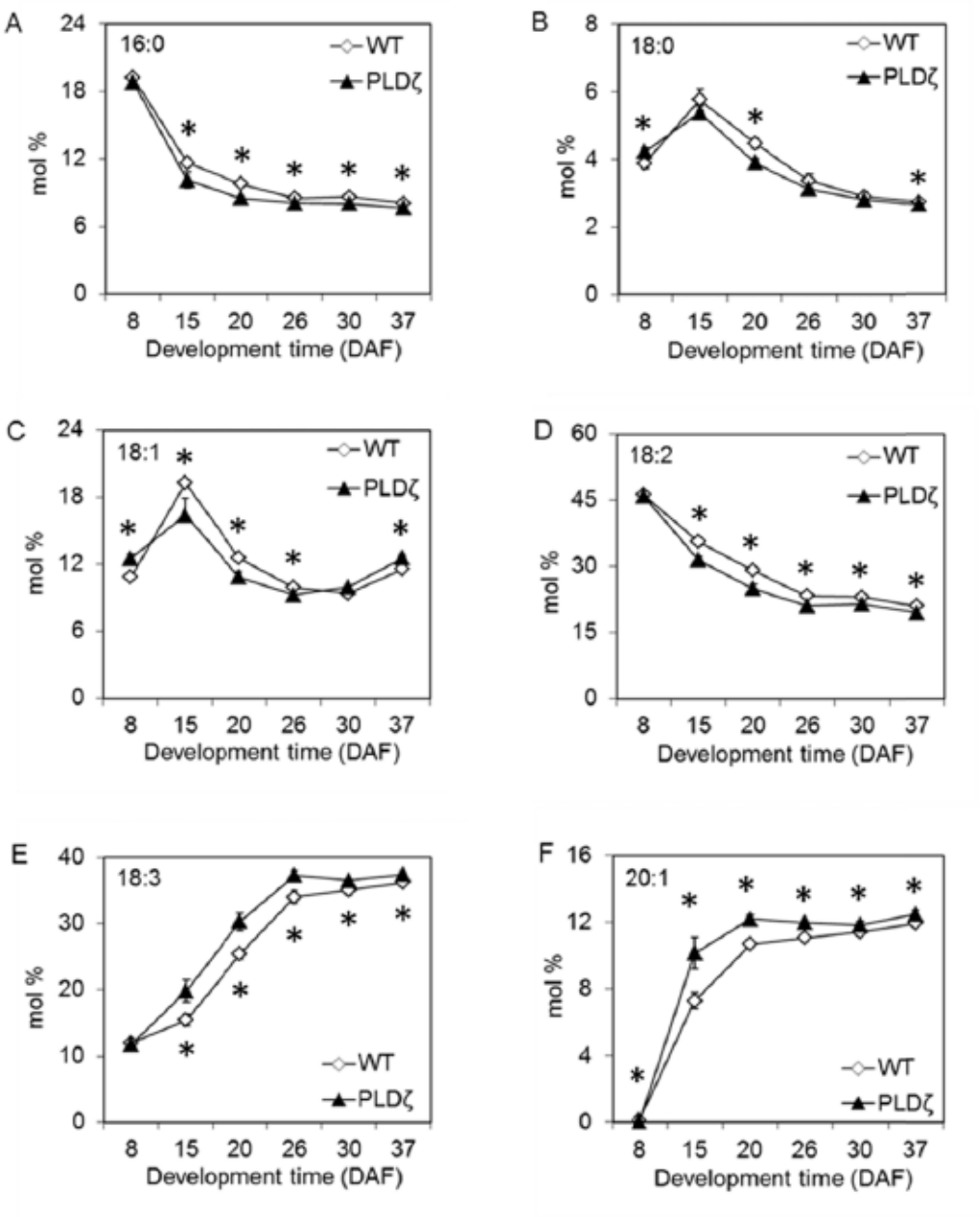

Fig. 4. Changes in fatty acid composition of major fatty acids in seed lipid during seed development of WT and a PLD$_\zeta$. A, Palmitic acid (16:0). B, Stearic acid (18:0). C, Oleic acid (18:1). D, Linoleic acid (18:2). E, Linolenic acid (18:3). F, Eicosenoic acid (20:1) (SD, n=3). Significant differences (*T* test, $P < 0.05$) between PLD$_\zeta$ and WT are denoted with an asterisk.

observations in Arabidopsis, where *sn*-1 is over 60% labeled (Taylor et al., 1995; Bates et al., 2012) but distinct from developing soybean embryos that incorporate nearly equal amounts of nascent [^{14}C]fatty acids into *sn*-1 and *sn*-2 positions (Bates et al., 2009). PA labeling was qualitatively similar to DAG, suggesting that at these time points the labeled PA and DAG measured is likely produced consecutively within the Kennedy pathway (Fig. 7E, 7F). The contrast in labeling between PC and PA suggests that PC contributes

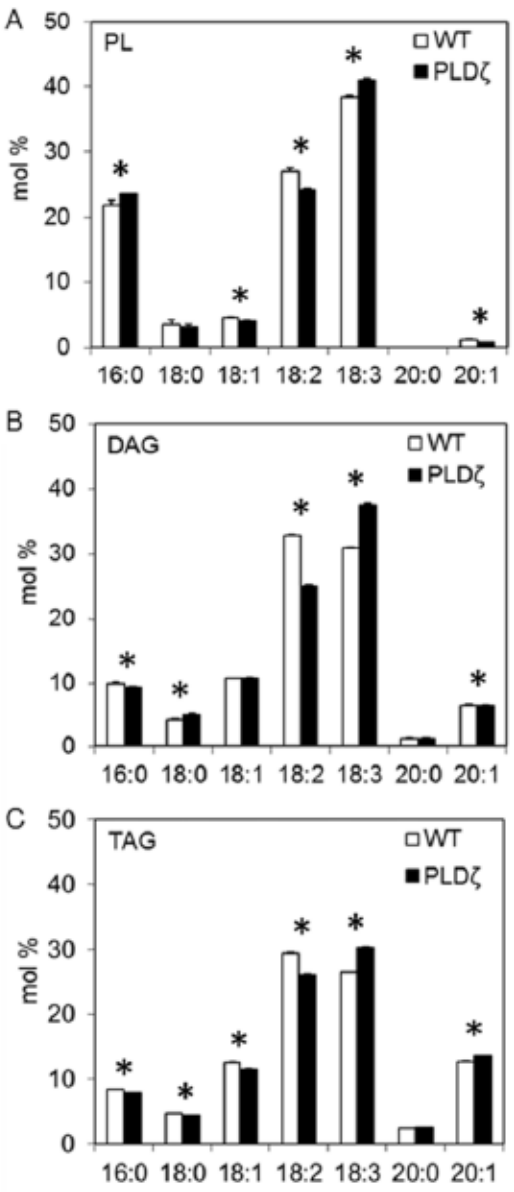

Fig. 5. Fatty acid composition in PL, DAG and TAG of WT and PLD$_\zeta$ developing seeds at 20 DAF. A, Fatty acid profile of PL in developing seeds of WT and PLD$_\zeta$ at 20 DAF. B, Fatty acid profile of DAG in developing seeds of WT and PLD$_\zeta$ at 20 DAF. C, Fatty acid profile of TAG in developing seeds of WT and PLD$_\zeta$ at 20 DAF (SD, n=3). Significant differences (*T* test, $P < 0.05$) between PLD$_\zeta$ and WT are denoted with an asterisk.

less to overall PA labeling probably because the pool of PA derived from PC is quite small and is mixed with the *de novo* pool derived directly from LPA or from other parts of membranes.

[^{14}C]-labeled TAG was analyzed regiochemically (Fig. S4) to establish whether the rapid initial labeling (Fig. 6) was due to direct incorporation of nascent [^{14}C]fatty acids into *sn*-3 position of non-labeled DAG or from labeled *de novo* DAG produced by the Kennedy pathway (Fig. 7). Cleavage of *sn*-1 and *sn*-3 positions of TAG with *Rhizomucor miehei* lipase produced *sn*-2 monoacylglycerol (MAG) which was labeled at low levels relative to the released fatty acids (Fig. S4). When considered with the stereochemical

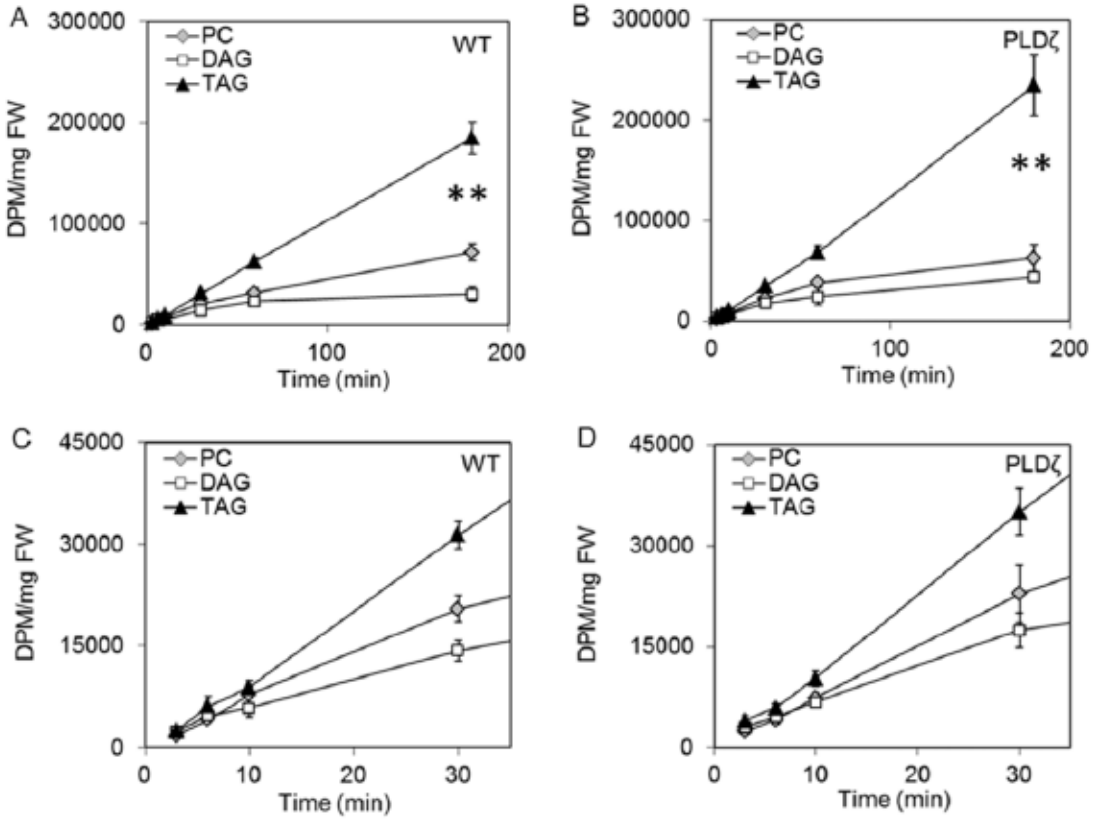

Fig. 6. Incorporation of [^{14}C]-fatty acid into glycerolipids during [^{14}C]-acetate labeling of WT and PLD$_\zeta$ developing embryos. A and C, [^{14}C]-fatty acid into TAG, DAG and PC in WT embryos. B and D, [^{14}C]-fatty acid into TAG, DAG and PC in PLD$_\zeta$ embryos (SD, n=3, time points: 3, 6, 10, 30, 60, 180 min). Significant differences (*T* test, $P < 0.05$) between PLD$_\zeta$ (A) and WT (B) were limited to differences in TAG and differences in DAG at 180 min as indicated with two asterisks.

analyses of labeled DAG that indicated 55/45 *sn*-1/*sn*-2 ratio of labeling (Fig. 7C, 7D), these results suggest that most labeling in TAG is due to incorporation of a [^{14}C]fatty acid at the *sn-3* position of an unlabeled DAG. Camelina seeds contain approximately 12% eicosenoic acid (Fig. 4F), and very long chain fatty acids such as C20:1 have higher specific activities from labeling experiments because they are elongated in the cytosol which contains an acyl-CoA with a higher ^{14}C specific activity than in the plastid (Bao et al., 2000). The rapid *sn*-3 TAG labeling is qualitatively consistent with previous studies in Camelina (Pollard et al., 2015) and *Arabidopsis thaliana* developing seeds (Bates et al., 2012) that also contain highly labeled 20:1 in the *sn*-3 position of TAG (Taylor et al., 1995; Bates et al., 2012). Very long chain fatty acids are predominantly incorporated into TAG by DGAT rather than PDAT (Katavic et al., 1995; Zhang et al., 2009; Xu et al., 2012).

In vivo glycerolipid backbone labeling indicates PLD$_\zeta$ enhances flux through the PC-derived DAG pathway of TAG biosynthesis

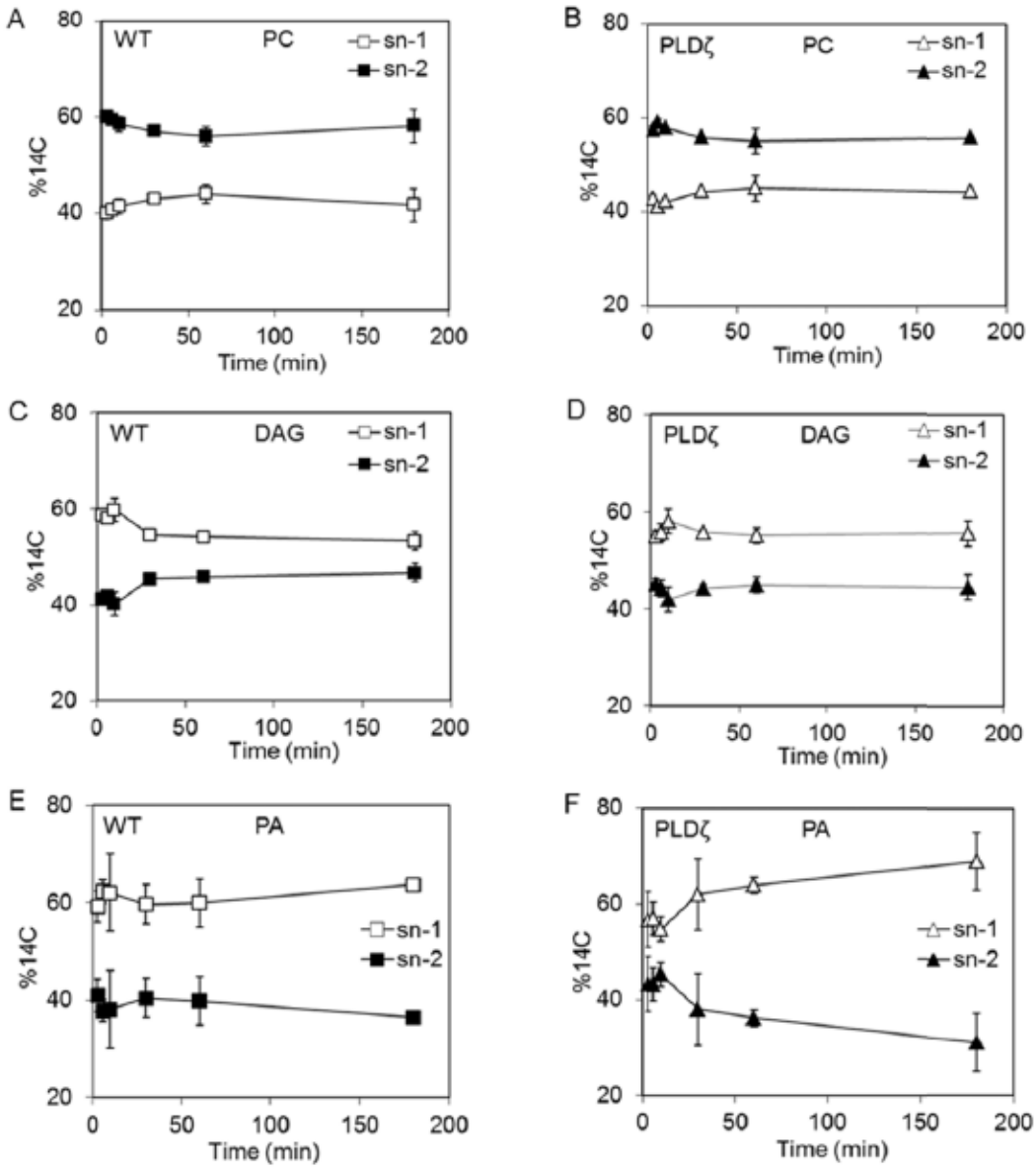

Fig.7. Stereochemical incorporation of [^{14}C]-fatty acids into PC, PA and DAG of WT and PLD$_\zeta$ developing embryos. A, PC of WT. B, PC of AtPLD$_\zeta$-OE1-12. C, DAG of WT. D, DAG of PLD$_\zeta$. E, PA of WT. F, PA of PLD$_\zeta$ (SD, n=3, time points: 3, 6, 10, 30, 60, 180 min).

The initial steps in eukaryotic glycerolipid assembly in WT and PLD$_\zeta$ developing embryos were further investigated with [^{14}C]glycerol kinetic labeling. Developing embryos of WT and PLD$_\zeta$ incorporated [^{14}C]glycerol linearly into glycerolipids at similar rates over a three hour time course (Fig. S5). The major labeled lipids were DAG, PC, and TAG (Fig. S6), and other phospholipids including PA/PI, PE/PG and MGDG, were labeled to low levels in both lines (Fig. S7). [^{14}C]glycerol metabolized by the developing embryos was incorporated into the backbone of lipids through carbons in glycerol-3-phosphate (G3P) and to a lesser extent into the acyl chains by glycolytic reactions that convert glycerol to acetyl-CoA. In both WT and PLD$_\zeta$, DAG was backbone labeled from [^{14}C]glycerol at a faster rate than PC or TAG (Fig. 8).

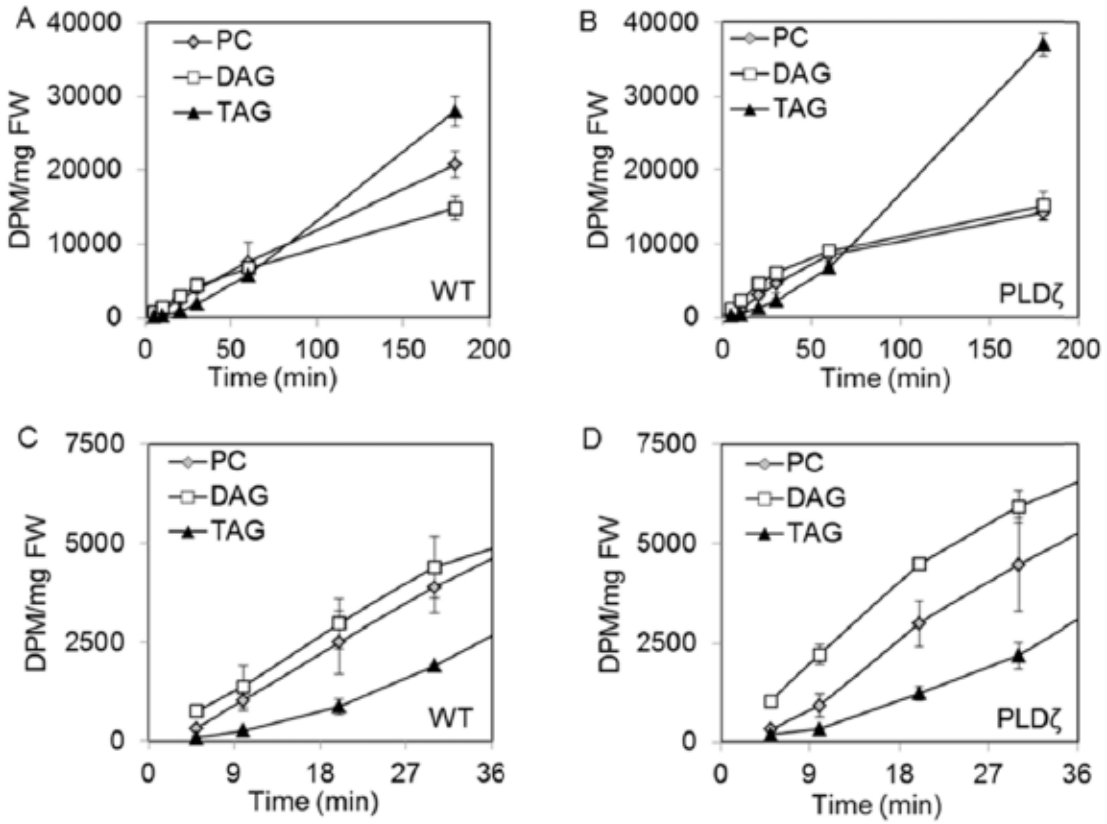

Fig. 8. Labeling of backbones in PC, DAG or TAG in [^{14}C]glycerol labeled WT and PLD$_\zeta$ developing embryos. A and C [^{14}C]backbone incorporation into TAG, DAT and PC in WT embryos. B and D, [^{14}C]backbone incorporation into TAG, DAG and PC in PLD$_\zeta$ embryos (SD, n=3, time points: 5, 10, 20, 30, 60, 180). Significant differences (*T* test, P <0.05) between PC and DAG (WT time points: 5, 180; PLD $_\zeta$ time points: 5, 10, 20) and between PC and TAG (WT time points: 5, 10, 20, 30, 180; PLD $_\zeta$ time points: 10, 20, 30, 180) were observed within but not between lines.

This description is consistent with the role of *de novo* DAG for the production of both PC and TAG in developing Camelina seeds [Fig. 1, (Bates and Browse, 2012)]. DAG labeling was more rapid in PLD$_\zeta$ possibly reflecting the increased Kenney pathway flux into *de novo* DAG that is necessary to produce more TAG. The initial labeling of PC was linear indicating the *de novo* DAG precursor pool to PC synthesis was rapidly filled, and that the continual increase in DAG accumulation represents filling of a DAG pool other than that required for PC synthesis. TAG labeling followed an exponential pattern with slow labeling initially because precursor supplies of labeled DAG had not been turned over sufficiently to label TAG. After 60 min TAG labeling approached a level that was similar to DAG and by 3 h TAG labeling was twice that of DAG. The delay in TAG labeling relative to PC indicates a larger precursor pool for the flux of enriched backbone into TAG. Such a pattern is consistent with PC synthesis directly from initially labeled *de novo* DAG and TAG labeling at a reduced rate as the [^{14}C]glycerol-backbone transitions through PC, and PC-derived DAG pools (Allen et al., 2015).

[^{14}C]glycerol was also incorporated into fatty acids by conversion to [^{14}C]acetyl-CoA through glycolysis and pyruvate dehydrogenase (Fig. 9, S8). The [^{14}C]acyl chains obtained from lipids were

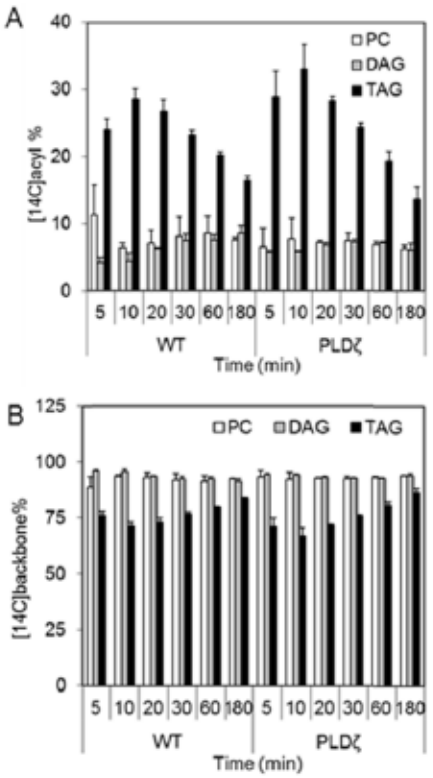

Fig. 9. Incorporation of [^{14}C]-glycerol into backbones of glycerolipids during labeling of WT and PLD$_\zeta$ developing embryos. A, [^{14}C]-glycerol into acyl chains of TAG, DAG and PC in WT and PLD$_\zeta$ embryos. B , [^{14}C]-glycerol into backbone of TAG, DAG and PC in WT and PLD$_\zeta$ embryos (SD, n=3, time points: 5, 10, 20, 30, 60, 180).

analyzed independent of the glycerol-backbone. Relative to the total label incorporation, approximately 5-35% was incorporated into fatty acids in a lipid class specific manner during the time course experiments with the remainder going into glycerolipid backbone metabolism involving the Kennedy pathway (Fig. 9). The labeling in the acyl chain relative to the glycerol backbone was similar when PC was compared to DAG (~5-10% across the time course) and indicated that the precursor pools for these intermediates (i.e. G3P, LPA, PA) are rapidly equilibrated with both forms of label (Bates et al., 2009). However, the fraction of acyl labeling in TAG (relative to TAG backbone labeling) changed more dramatically (decreasing from ~30% to ~15% during the time course), indicating that over time the glycerol backbone represented more of the total labeling. The distinct labeling in TAG reflects the initial incorporation of *sn*-3 labeled fatty acids onto an unlabeled DAG backbone as previously described. Over time this contribution was diminished as the [^{14}C]glycerol-backbone labeling increased in precursor pools for TAG synthesis. These observed changes in TAG labeling relative to PC are consistent with the presence of a larger precursor pool for backbone labeling of TAG than that of PC and are thus in agreement with both the [^{14}C]acetate acyl labeling (Fig. 6) and rate of [^{14}C]glycerol incorporation into the backbone of TAG (Fig. 8).

Both PC and TAG can be synthesized from *de novo* DAG, yet TAG can also be produced from PC-derived DAG (Fig. 1). The relative initial rates of PC and TAG [^{14}C]glycerol backbone labeling (i.e. without acyl labeling) at short time points (e.g. $\leq$ 10 min) predominantly represent the competition for

initially synthesized *de novo* DAG, whereas at longer time points the labeling in TAG also includes contributions from PC-derived DAG. The initial relative rate of PC/TAG synthesis from *de novo* DAG was approximately 3.9/1 and 4.3/1, in wild-type and PLD_{ζ} respectively (Table S1). Considering that PLD_{ζ} may be rapidly turning over the labeled PC, the actual ratio of PC/TAG synthesis in PLD_{ζ} line could be an underestimate. Developing embryos predominantly accumulate TAG not PC, thus the higher rate of PC synthesis suggests that most of the PC will eventually turnover for production of PC-derived DAG, and then TAG. The higher ratio of initial PC/TAG synthesis rates from *de novo* DAG in PLD_{ζ} suggests that turnover of PC by PLD_{ζ} activity may induce flux through the PC-derived DAG pathway, leading to the higher overall oil levels.

DISCUSSION

The objective of this study was to investigate the endogenous pathways of acyl flux into TAG of Camelina seeds, and to examine how TAG accumulation is affected in seeds of transgenic Camelina expressing *Arabidopsis thaliana AtPLD$_{\zeta 1}$* and *AtPLD$_{\zeta 2}$* to alter acyl flux through PC. In oilseeds, two families of enzymes are mainly responsible for TAG assembly: (1) DGATs that catalyze production of TAG by using DAG and acyl-CoAs, and (2) PDATs that produce TAG using DAG with an additional acyl group donated by PC. How much each of these two groups of enzymes contributes to TAG assembly varies with plant species and tissues (Stymne and Stobart, 1987; Zhang et al., 2009; Banas et al., 2013). Since both enzymes require a supply of DAG as a common precursor for TAG synthesis, *de novo* DAG must be produced by the Kennedy pathway through sequential acylation of G3P. However given the high percentage of unsaturated acyl chains in TAG, additional steps that move fatty acids through PC are necessary. Acyl editing and the interconversion of DAG with PC results in a second pool of DAG that is PC-derived and is the direct source for TAG production (Bates and Browse, 2012). The production of higher levels of TAG in oilseeds requires a balance of: i) enhanced synthesis of nascent fatty acids, ii) increased production of DAG and iii) enough flux through PC to produce a viable combination of saturated and unsaturated chains.

PLD$_{\zeta}$ alters the steady state pool sizes of PC and DAG leading to enhanced DAG accumulation

This study indicated that co-expression of two phospholipases, PLD$_{\zeta 1}$ and PLD$_{\zeta 2}$, that convert PC to PA (Table 1) has the consequence of altering the steady state pool concentrations of DAG and PC in transgenic lines (Fig. 3). The enhanced DAG pool size leads to an increase in TAG accumulation throughout development and results in 3% more TAG (as a percent of total biomass) in mature Camelina seeds (Table 1). In addition, the fatty acid profile was altered with increased 18:3 and 20:1 and reduced levels of other acyl groups. Time course labeling experiments with [^{14}C]acetate resulted in a labeled PC pool that was approaching an asymptotic maximum in the transgenic line, indicating the active PC pool was nearly completely labeled within the time period (Fig. 6). [^{14}C]glycerol kinetic incorporation (Fig. 8) indicated that DAG was more labeled than PC at early time points and was only surpassed by PC in the WT later in time due to the larger active PC pool size. PC in the transgenics became maximally labeled more quickly and corresponded to a more rapid accumulation of glycerol-labeled TAG. Taken together with the measured changes in DAG and PC pool sizes, and [^{14}C]acetate acyl labeling, the results indicate that PC was being turned over more rapidly to produce DAG. Thus the role of the PLD$_{\zeta}$ enzyme may be to increase the available substrate for TAG production. Along with a limited number of descriptions of significantly enhanced TAG in oilseeds (Lardizabal et al., 2008; Weselake et al., 2009; Oakes et al., 2011), the current study suggests that the last steps in TAG biosynthesis are bottlenecks to its production. By converting PC to DAG more effectively, PLD$_{\zeta}$ may enhance DGAT activity for TAG production in Camelina seeds. It is unclear from these experiments if the increase in DAG concentration observed in PLD$_{\zeta}$ is a consequence of

DGAT activity that may now be limiting further increases in oil production. Possibly the co-expression of a DGAT with PLD$_\zeta$ could lead to more significant oil levels in the future.

Carbon flux between DAG and PC are highly interconnected in Camelia, but have distinct differences from other oilseeds and are enhanced by PLD

Interconversion of the DAG moiety between PC and DAG pools is a central process in TAG assembly of many oilseeds and provides a pool of DAG for TAG synthesis that contains more unsaturated fatty acids than *de novo* DAG (Bates and Browse, 2012); however the quantitative roles of enzymes that provide this connection have been difficult to assess and cannot be determined based on TAG fatty acid composition alone. The rapid incorporation of [^{14}C]acetate labeled fatty acids predominantly into *sn*-3 position of TAG, the PC-TAG precursor-product kinetics of [^{14}C]glycerol labeling, and the ratio of glycerol backbone to acyl group labeling from [^{14}C]glycerol all support a predominantly PC-derived DAG pathway of TAG synthesis. Quantitatively, the initial relative rate of PC/TAG synthesis from [^{14}C]glycerol backbone labeled *de novo* DAG was approximately 3.9/1 and 4.3/1, in wild-type and PLD$_\zeta$ respectively (Table S1). Based on the initial rates of [^{14}C]glycerol labeling into PC/TAG this would suggest that approximately 80% of TAG in wild-type Camelina is produced from PC-derived DAG with the remaining flux directly through the Kennedy pathway using *de novo* DAG for TAG biosynthesis. For comparison, the relative initial rates of PC/TAG synthesis are approximately 14/1 in Arabidopsis, indicating greater than 93% of TAG is synthesized from PC-derived DAG (Bates and Browse, 2011). Considering that the membrane lipid PC typically does not accumulate unusual FAs (Millar et al., 2000), and the flux of unusual FAs through PC has been indicated as a bottleneck in oilseed engineering (Bates and Browse, 2011; Bates et al., 2014), the relatively larger proportion of acyl flux through the Kennedy pathway in Camelina may explain the enhanced accumulation of some unusual FAs in TAG of transgenic Camelina (Ruiz-Lopez et al., 2014) relative to the transgenic Arabidopsis (Ruiz-Lopez et al., 2013) engineered with the same genes.

Fluxes between DAG and PC are achieved reversibly through DAG-CPT and PDCT and unidirectionally from PC to DAG by phospholipase C or through a combination of PLD and PAP. In *Arabidopsis thaliana*, PDCT is responsible for approximately 40% of the flux of PUFAs from PC into PC-derived DAG for TAG synthesis (Lu et al., 2009), but the Arabidopsis *lpcat1 lpcat2 rod1* triple mutant which abolishes both acyl editing and PDCT activity still contains 1/3 of wild-type PUFA levels in TAG (Bates et al., 2012). The remaining flux of PUFA from PC to TAG may involve PC associated with phospholipase-based production of PC-derived DAG. Widespread evidence for the concerted action of PLD and PAP includes reports in mammalian literature that demonstrate DAG production from endothelial-derived PC (Martin, 1988) and endogenous DAG generation in human polymorphonuclear leukocytes where DAG stimulated 5-lipoxygenase enzyme activity and function (Albert et al., 2008). In plants, isolated protein bodies from seedlings show both activities of PLD and PAP (Herman and Chrispeels, 1980) and studies of AtPLD$_{\zeta1}$ and AtPLD$_{\zeta2}$ suggest the supply of DAG for galactolipid synthesis is dependent on this pathway and also subverts phosphorus starvation (Cruz-Ramirez et al., 2006; Li et al., 2006). It has been shown that knock-down of PLDα in soybean leads to reduced PUFAs in TAG

(Lee et al., 2011) implicating a flux from PC to TAG through PLD-PAP without a requirement for PDCT involvement. Thus a number of mechanisms exist that contribute DAG derived from PC that is rich in PUFA which accumulates in TAG. Our results cannot conclusively distinguish between the mechanistic differences in WT Camelina and Arabidopsis or other well-studied oilseeds but together the studies suggest a combination of PLD and possibly PDCT enzyme activities may be responsible.

Here, we demonstrated that PLD$_\zeta$ enhances steady state levels of DAG from PC, thus apparently homeostatic mechanisms involving PAP favors conversion of the produced PA to DAG. The increase in PC-derived DAG (Fig. 8) considerably altered TAG accumulation (up to 3% oil increase) supporting that acyl flux through PC may be a regulator of total fatty acid synthesis (Bates et al., 2014; Bates, 2016). However, it was surprising that no significant difference was observed in the stereochemical labeling of PC, which is a marker of acyl flux through acyl editing vs the Kennedy pathway. Stereospecific analyses of the acyl chains from [^{14}C]acetate labeling revealed that DAG (as well as PA) was more enriched at the *sn*-1 position (Fig. 7) whereas PC favored *sn-2* incorporation. A lack of significant changes in regiochemical lipid labeling between the lines indicates that the distribution of nascent fatty acids into different branches of the lipid metabolic network (Fig. 1) may not be significantly altered by PLD$_\zeta$ activity. In this case an increased flux of acyl groups into *de novo* DAG for subsequent PC, and PC-derived DAG synthesis must be balanced by an increased flux of acyl groups into PC by acyl editing. Then the regiochemical labeling of PC would not change significantly between lines. This result is consistent with previous hypotheses that suggest PC is a central intermediate, and "fatty acid or DAG carrier" through the ER membrane prior to TAG synthesis (Allen et al., 2015; Shockey et al., 2016). Together these acyl lipid flux experiments provide novel results that Camelina utilizes a higher proportion of direct Kennedy pathway than other related plants, and that the flux through the PC-derived DAG pathway can be enhanced through modulation of PLD activity.

Increased PLD$_\zeta$ activity may enhance DGAT over PDAT activity for TAG synthesis.

The acylation of DAG to make TAG involves one of two enzymatic routes. PDAT uses the *sn*-2 acyl chain of PC along with DAG to make TAG, whereas DGAT produces TAG from acyl-CoA and DAG substrates. PDAT activity can lead to synthesis of TAG rich in unsaturated fatty acids at *sn*-3 position (Dahlqvist et al., 2000; Stahl et al., 2004; Xu et al., 2012) though the differences in substrate specificities for DGAT and PDAT enzymes may not be significant and have not been characterized in a number of oilseeds. Our results indicate changes in 18:3 and 20:1 invoked by PLD$_\zeta$ activity. DGAT1 is apparently responsible for much of the 20:1 incorporation into *sn*-3 of TAG in *Arabidopsis thaliana* whereas DGAT2 has been implicated in the incorporation of other modified fatty acids into TAG (Kroon et al., 2006; Shockey et al., 2006). When Camelina embryos were labeled with [^{14}C]acetate the most rapid labeling was seen in TAG (Fig. 6). This is consistent with studies of Arabidopsis seeds (Bates et al 2012) that also contain a significant fraction of elongated fatty acids. It is well-known that the elongation process that occurs outside of the plastid results in higher specific activities for these fatty acids relative to those of 18 carbons or shorter chains (Bao et al., 2000). In contrast, soybean seeds have very low levels of elongated

fatty acids and in combination with a high degree of acyl editing result in more rapid PC labeling with nascent FA than TAG (Bates et al., 2009). When TAG labeled from [^{14}C]acetate was regiochemically analyzed through enzymatic cleavage of the *sn*-1 and *sn*-3 positions (Fig. S4) and compared to the *sn*-1 and *sn*-2 positions of DAG, the labeling difference was attributable to *sn*-3, consistent with both the incorporation of elongated fatty acids at this position and the greater level of 20:1 in TAG relative to PC or DAG (Fig. 5). If the *sn*-3 labeling was due primarily to PDAT we would expect to see a precursor-product relationship with *sn*-2 PC to *sn*-3 TAG, which was not evident.

A significant challenge to producing increased levels of lipids in plants is that many of the genes putatively assigned by genome studies as being involved in lipid metabolism have not been characterized. Furthermore the operation of an enzyme within a metabolic network is context-specific (Allen, 2016) and may differ between species, tissues, environments, or when introduced transgenically. Therefore, the operation of a cellular network *in planta* requires dynamic analyses of flux with isotopes to assess the underlying changes in metabolism responsible for an altered phenotype. Given the central role of PC in lipid metabolism (Allen, 2016; Bates, 2016), we hypothesized that overexpressing a phospholipase that acts specifically on PC (PLD$_\zeta$) would influence the exchange and flux between PC and DAG and potentially alter TAG production. Through combined overexpression of two Arabidopsis PLD$_\zeta$ genes in Camelina, TAG levels, PUFA concentration and elongated fatty acid content were all increased. PC levels were reduced and DAG levels were increased presumably due to the altered interchange of these lipids by the PLD$_\zeta$ activity. Labeling with [^{14}C]acetate and [^{14}C]glycerol provided new insights into lipid metabolism in Camelina specifically indicating a higher flux through the Kennedy pathway as compared to Arabidopsis, but still predominantly composed of a PC-derived DAG pathway of TAG synthesis which was further enhanced by PLD$_\zeta$ expression. When combined with other changes that could further alter the FA profile of TAG (e.g. suppression of FAD2/3 for accumulation of monosaturates), the overexpression of PLD$_\zeta$ may be part of an engineering strategy to enhance seed oil content for biofuels or industrial chemicals.

MATERIALS AND METHODS

Plant Materials and Chemicals

Camelina sativa wild type 'Suneson' (MT5) and AtPLD$_\zeta$ overexpressing lines were grown in greenhouses at 20°C/21°C under supplemental light to ensure a consistent 16 hr/8 hr day/night cycle at >500 µmoles/cm^2/sec in St Louis, Missouri (38.63°N, 90.20°W). Developing siliques were harvested from plants at various times throughout development for lipid analysis or radioactive labeling.

Organic solvents, primuline, phospholipase A$_2$ from *Naja mossambica mossambica*, and *Rhizomucor miehei* lipase, were purchased from Sigma-Aldrich (http://www.sigmaaldrich.com/); thin layer chromatography (TLC) plates silica gel 60 Å were from EMD Millipore (http://www.emdmillipore.com/); Hionic Fluor liquid scintillation cocktail was from PerkinElmer (www.perkinelmer.com/); butylated

hydroxytoluene was from MP Biochemicals, LLC (www.mpbiochemicals.com/); [1-^{14}C]acetate (specific activity, 59 mCi mmol^{-1}) and [1,3-^{14}C] glycerol (specific activity 56 mCi/mmol) were purchased from American Radiolabeled Chemicals (http://www.arc-inc.com/).

Vector Construction and Plant Transformation

The full-length cDNA coding regions of *AtPLD*$_{\zeta 1}$ and *AtPLD*$_{\zeta 2}$ were amplified by PCR using the cDNA library prepared from *Arabidopsis thaliana* Col-1. The *AtPLD*$_{\zeta 1}$ coding region was placed behind a glycinin promoter on vector pGly-DsRed (generating pGly-DsRed-ζ1). The *AtPLD*$_{\zeta 2}$ coding region was inserted behind a β-conglycinin promoter on a separate cloning vector. Then, the *AtPLD*$_{\zeta 2}$ expression cassette including the β-conglycinin promoter, the coding region of *AtPLD*$_{\zeta 2}$ and the terminator was amplified with PCR, and the product was then cloned into the binary vector pGly-DsRed-ζ1 containing DsRed and hygromycin selection markers (generating pGly-DsRed-ζ1- *ζ2*). The binary vector containing both *AtPLD*$_{\zeta 1}$ and *AtPLD*$_{\zeta 2}$ cassettes, pGly-DsRed-ζ1- *ζ2,* was introduced into *Agrobacterium* strain GV3101 by a freezing and thawing method. *Camelina sativa* 'Suneson' was transformed with the above *Agrobacterium* GV3101 by floral dipping (Lu and Kang, 2008). Transgenic plants were selected on 10 mg/L hygromycin growth media and confirmed by digital imaging of DsRed expression.

RNA Expression Analysis

Total RNA was isolated from developing seeds using TriPure Isolation Reagent (Roche). cDNA was obtained using Transcriptor First Strand cDNA Synthesis Kit (Roche). PCR reactions were performed using Ex Taq Premix polymerase (Clontech Laboratories Inc) with 30 cycles of amplification. Primers used for *AtPLD*$_{\zeta 1}$, *AtPLD*$_{\zeta 2}$ and ubiquitin were as follows: *AtPLD*$_{\zeta 1}$, forward 5'- ATG GCA TCT GAG CAG TTG ATG TCT CCC -3', reverse 5'- CTG GTG AGA ATG ACA ACA TCG AAA CCT CC -3'; *AtPLD*$_{\zeta 2}$, forward 5'- TAA CGG CGT TAA GTC AGA CGG AGT CAT C -3', reverse 5'- GGA ACT TGC AGA CCT CTT TGG AGT T -3'; *ubiquitin* (homolog of Arabidopsis ubiquitin 10 gene), forward: 5'- AAG ATG GCC GCA CCT TGG CTG ATT AC -3', reverse 5'- TCT CAA CCT CCA AAG TGA TGG TTT TAC -3'.

AtPLD$_{\zeta 1}$ Activity Assay

Developing seeds were ground in liquid nitrogen, and then extracted with buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM KCl, 1 mM EDTA, 2 mM DTT and 0.5 mM PMSF. After centrifugation at 12,000 *g* for 10 min at 4°C, the supernatant containing the soluble protein fraction was centrifuged at 100,000 *g* for 30 min at 4°C twice to obtain the microsomal protein fraction. AtPLD$_{\zeta 1}$ activity was assayed using radiolabeled PC, L-a-dipalmitoyl [2-palmitoyl-9,10-^{3}H(N) (1 mCi/ml and 60 Ci/mmol) (American Radiolabeled Chemicals) as described in (Qin and Wang, 2002). After two hour reactions at 30°C, lipids were extracted from the reaction mixture with 1 mL chloroform/methanol (2:1, v/v), and then separated on silica gel 60 Å TLC plates with a developing solvent system of chloroform/methanol/30% ammonium hydroxide (65:25:4, v/v/v). Lipid spots for PA were scraped from the plate and counted by scintillation.

[^{14}C]Acetate and [^{14}C]Glycerol Labeling

Camelina seeds were removed from developing pods (20 days after flowering) and embryos were dissected from the seed coats and cultured in medium containing 5 mM MES, pH 5.8, 0.5% sucrose, and 0.5x Murashige and Skoog salts (Bates and Browse, 2012). After preincubation of embryos for 20 min under light of 35 μmoles/cm^2/sec, temperature at 24°C, relative humidity of 35% with constant shaking, the labeling of twenty six embryos per sample was started by replacing the 1 mL preincubation medium with 1 mL fresh culture medium containing [1-^{14}C]-acetate (0.5 mM) or [1,3-^{14}C] glycerol (0.2 mM) in a 2-mL Eppendorf tube. For each time point, the labeling reaction was stopped by removing the 1.0 mL culture medium containing radioactive substrate and freezing the embryos in the tube immediately in liquid nitrogen. For [^{14}C]glycerol-labeled lipid classes including PC, DAG and TAG, the proportion of label in the acyl chains versus the backbone was determined by base-catalyzed transmethylation of TLC-separated and purified lipids and scintillation counting of the separated organic and aqueous phases (Ichihara et al., 1996).

Lipid Analysis

Total lipids of developing seeds or embryos were extracted using a modified method based on a protocol by Kansas Lipidomics Research Center (http://www.k-state.edu/lipid/lipidomics/protocols.htm). Briefly, seeds or embryos were quickly transferred to an 8-mL glass tube containing hot isopropanol with 0.01% butylated hydroxytoluene (at 75°C), and incubated at 75°C for 15 min. Then, seeds or embryos in isopropanol were homogenized thoroughly with glass rod before adding 1.0 mL chloroform and 1.0 mL methanol and 0.8 mL water. After vortexing, 1.0 mL chloroform and 1.0 mL water were added. Then the mixture was partitioned into two phases by centrifugation. The chloroform phase with lipids was moved to a separate glass tube, and the remaining mixture was twice extracted by adding 1.0 mL chloroform, shaking, centrifugation, and combining the chloroform phases. The total lipid extract was washed once by adding a small amount (0.5 mL) of 1 M KCl. Lipids were dried under nitrogen gas flow, and suspended to a small volume in chloroform. Fatty acid methyl esters were prepared by the acid transmethylation procedure as described (Cahoon et al., 2002), and quantified by gas chromatography using a flame ionization detection (Focus GC, Thermo Scientific) and a HP-INNOWAX capillary column (30 m length x 0.25 mm i.d., 0.25 μm film thickness, Agilent J&W GC Columns, Agilent Technologies).

Radioactivity in the total lipid samples, and individually purified lipids, were quantified by liquid scintillation counting. Radioactivity on TLC plates was visualized and imaged by electronic radiography (Packard A20240 Instant Imager; Packard Instrument). Polar lipid separation by TLC, recovery of polar lipids from TLC plates, and positional analysis of PC and PA acyl groups using phospholipase A_2 were conducted as described (Bates et al., 2007). For acyl chain fatty acid composition in DAG and TAG, *Rhizomucor miehei* lipase was used for digestion as described similar to protocol described for porcine pancreatic lipase hydrolysis of TAG (Christie and Han, 2003; Cahoon et al., 2006). Briefly, an aliquot of DAG or TAG was dried under nitrogen gas flow, and suspended in 1.0 mL diethyl ether. Then, 0.60 mL of a reaction buffer containing 50 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$, and 200 μL *Rhizomucor miehei* lipase were added to the lipid in diethyl ether. Reactions were incubated at 37°C for 1~3 h with shaking. Reactions were stopped by adding 0.3 mL 6N hydrochloric acid and the diethyl ether phase was evaporated

under nitrogen gas flow. Lipids in the reaction were extracted with chloroform/methanol (2:1, v/v). The lipid extract was dried under nitrogen gas flow, and suspended in small volume of chloroform before being separated by TLC plate Silica gel 60 Å together with standards. TLC plates were developed with hexane/diethyl ether/acetic acid (70:30:1, v/v/v) to separate neutral lipid classes and individual neutral lipids were eluted from TLC silica with chloroform/methanol (2:1, v/v) and back-extracted with chloroform. All TLC solvents contained 0.01% (w/v) butylated hydroxytoluene. Phospholipids from the TLC plate were eluted with chloroform/methanol/0.9% KCl (2:1:1, v/v/v), and then the aqueous phase was re-extracted with chloroform and lipids combined prior to further analysis.

Polar lipid classes were separated by a solvent system of chloroform/methanol/30% ammonium hydroxide (65:25:4, v/v/v) that was used to establish the fraction of labeling in PC relative to other polar components. Lipid spots on the TLC plate were visualized under UV light after staining with primuline. Each lipid was eluted from the silica plate as described (Bates et al., 2007). PC and PA stereochemistry was determined by a modified method of phospholipase A_2 digestion based on methods described previously (Bates et al., 2007). Briefly, PC or PA in 1 mL diethyl ether was incubated with 0.5 mL of reaction buffer containing 50 mM borate, pH 7.5, 4 mM $CaCl_2$ and 5 U PLA_2 at 25 °C for 20 min. Products were extracted and separated by TLC using a developing system of chloroform/methanol/30% ammonium hydroxide (65:25:4, v/v/v). DAG and TAG regiochemistry was determined by *Rhizomucor miehei* lipase digestion and TLC (Cahoon et al., 2006). GraphPad Prizm (version 6) was used to perform linear regression analysis on initial rate data (Table S1).

Seed oil content was determined by fatty acid methylation analysis. The developing seeds were lyophilized then transmethylated by the acid transmeythlation procedure as described (Cahoon et al., 2002). Briefly, methanol containing 2.5% sulfuric acid, 0.01% (w/v) butylated hydroxytoluene, 20% toluene, and TAG-17:0 internal standard were added to a glass vial containing seeds. The seeds were crushed with glass rod and then heated at 90 C for 1 h. Transmethylation was quenched through addition of 1 M NaCl, prior to extraction with hexane. The hexane fraction was transferred to a GC vial and the fatty acid methyl esters were analyzed by GC-FID. The oil content was determined by comparison of the detector response from seed-derived fatty acid methyl esters relative to methyl heptadecanoate from the triheptadecanoin internal standard.

SUPPLEMENTAL DATA

The following supplemental materials are available.

Supplemental Fig. S1. Minor fatty acid composition in WT and *PLDζ*

Supplemental Fig. S2. Total [^{14}C] incorporation from labeled acetate

Supplemental Fig. S3. [^{14}C] incorporation in to minor polar lipids from labeled acetate

Supplemental Fig. S4. [^{14}C] regiochemical analysis of TAG from acetate

Supplemental Fig. S5. Total [^{14}C] incorporation from labeled glycerol

Supplemental Fig. S6. [^{14}C] incorporation in to PC, DAG and TAG from labeled glycerol

Supplemental Fig. S7. [^{14}C] incorporation in to minor polar lipids from labeled glycerol

Supplemental Fig. S8. Incorporation of [^{14}C]-glycerol into acyl chains of glycerolipids

Supplemental Table S1. Initial labeling of glycerol backbone

ACKNOWLEDGEMENTS

Any product or trademark mentioned here does not imply a warranty, guarantee, or endorsement by the authors or their affiliations over other suitable products.

TABLES

Table I. Oil content in developing and mature seeds of WT and PLD$_\zeta$ lines, and PLD$_\zeta$ activity measured in developing seeds of WT and PLD$_\zeta$ lines at 20 DAF. Values are means ± SD (n=3). Asterisks indicate that the values were statistically significantly different from the wild type at $p<0.05$ based on Student's *T* test.

	WT	1	2	3	4
Oil level (%DW)					
12 DAF	15.76 ± 0.91	17.67 ± 0.48*	17.00 ± 0.13*	17.21 ± 0.08*	19.30 ± 0.11*
20 DAF	22.64 ± 0.71	25.84 ± 0.42*	24.61 ± 0.35*	27.03 ± 0.66*	25.92 ± 0.65*
mature	24.90 ± 0.60	27.96 ± 0.60*	26.86 ± 1.00*	27.47 ± 1.86*	26.80 ± 0.68*
PLD$_\zeta$ activity (pmol/h/g)					
20 DAF	65.74 ± 12.58	104.67 ±19.92*	86.01 ± 10.30*	269.13 ± 41.97*	101.24 ± 20.59*

FIGURE LEGENDS

Fig. 1. Simplified metabolic network description of acyl chain incorporation into TAG in oilseeds. Enzymes are labeled in italics. G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; DAG, diacylglycerol; TAG, triacylglycerol; LPCAT, lyso-PC acyltransferase; PLA, phospholipase A; GPAT, glycerol-3-phosphate acyltransferase; LPAAT, lyso-PA acyltransferase; PAP, phosphatidic acid phosphatase; PLD, phospholipase D; CPT, CDP-choline:diacylglycerol cholinephosphotransferase; PDCT, phosphatidylcholine:diacylglycerol cholinephosphotransferase; DGAT, acyl-CoA:diacylglycerol acyltransferase; PDAT, phospholipid:diacylglycerol acyltransferase.

Fig. 2. Transcript level of AtPLD$_{\zeta 1}$ and AtPLD$_{\zeta 2}$ in developing seeds at 22 DAF of Camelina WT and PLD$_\zeta$ overexpression lines. UBQ, ubiquitin.

Fig. 3. Lipid composition in Camelina seeds. Relative lipid composition including: TAG (A), DAG (B), PL/GL (C) in seeds of WT and PLD$_\zeta$ line 1 (henceforth referred to as PLD$_\zeta$) during development. TAG, DAG, PL/GL were quantified by GC-FID using internal standard after they were eluted from TLC plate of total lipid separation (SD, n=3). Significant differences (*T* test, $P < 0.05$) between PLD$_\zeta$ and WT are denoted with an asterisk.

Fig. 4. Changes in fatty acid composition of major fatty acids in seed lipid during seed development of WT and a PLD$_\zeta$. A, Palmitic acid (16:0). B, Stearic acid (18:0). C, Oleic acid (18:1). D, Linoleic acid (18:2). E, Linolenic acid (18:3). F, Eicosenoic acid (20:1) (SD, n=3). Significant differences (*T* test, $P < 0.05$) between PLD$_\zeta$ and WT are denoted with an asterisk.

Fig. 5. Fatty acid composition in PL, DAG and TAG of WT and PLD$_\zeta$ developing seeds at 20 DAF. A, Fatty acid profile of PL in developing seeds of WT and PLD$_\zeta$ at 20 DAF. B, Fatty acid profile of DAG in developing seeds of WT and PLD$_\zeta$ at 20 DAF. C, Fatty acid profile of TAG in developing seeds of WT and PLD$_\zeta$ at 20 DAF (SD, n=3). Significant differences (*T* test, $P < 0.05$) between PLD$_\zeta$ and WT are denoted with an asterisk.

Fig. 6. Incorporation of [^{14}C]fatty acid into glycerolipids during [^{14}C]acetate labeling of WT and PLD$_\zeta$ developing embryos. A and C, [^{14}C]fatty acid into TAG, DAG and PC in WT embryos. B and D, [^{14}C]fatty acid into TAG, DAG and PC in PLD$_\zeta$ embryos (SD, n=3, time points: 3, 6, 10, 30, 60, 180 min). Significant differences (*T* test, $P < 0.05$) between PLD$_\zeta$ (A) and WT (B) were limited to differences in TAG and differences in DAG at 180 min as indicated with two asterisks.

Fig.7. Stereochemical incorporation of [^{14}C]fatty acids into PC, PA and DAG of WT and PLD$_\zeta$ developing embryos. A, PC of WT. B, PC of AtPLD$_\zeta$-OE1-12. C, DAG of WT. D, DAG of PLD$_\zeta$. E, PA of WT. F, PA of PLD$_\zeta$ (SD, n=3, time points: 3, 6, 10, 30, 60, 180 min).

Fig. 8. Labeling of backbones in PC, DAG or TAG in [^{14}C]glycerol labeled WT and PLD$_\zeta$ developing embryos. A and C [^{14}C]backbone incorporation into TAG, DAT and PC in WT embryos. B and D, [^{14}C]backbone incorporation into TAG, DAG and PC in PLD$_\zeta$ embryos (SD, n=3, time points: 5, 10, 20, 30, 60, 180). Significant differences (*T* test, $P < 0.05$) between PC and DAG (WT time points: 5, 180; PLD$_\zeta$ time points: 5, 10, 20) and between PC and TAG (WT time points: 5, 10, 20, 30, 180; PLD$_\zeta$ time points: 10, 20, 30, 180) were observed within but not between lines.

Fig. 9. Incorporation of [^{14}C]glycerol into glycerolipids during labeling of WT and PLD$_\zeta$ developing embryos. A, [^{14}C]glycerol into acyl chains of TAG, DAG and PC in WT and PLD$_\zeta$ embryos. B , [^{14}C]-glycerol into backbone of TAG, DAG and PC in WT and PLD$_\zeta$ embryos (SD, n=3, time points: 5, 10, 20, 30, 60, 180).

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