Running head: Phospholipid Turnover in TAG Production

Corresponding author: Xuemin Wang, Department of Biology, University of Missouri, St. Louis, Missouri 63121; telephone: 314-587-1419; fax: 314-587-1519; email: swang@danforthcenter.org.

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Patatin-Related Phospholipase pPLAIIIδ Increases Seed Oil Content with Long Chain Fatty Acids in Arabidopsis

Authors:
Maoyin Li, Sung Chul Bahn, Chuchuan Fan, Jia Li, Tien Phan, Michael Ortiz, Mary R. Roth, Ruth Welti, Jan Jaworski, and Xuemin Wang*

Institution address:
Department of Biology, University of Missouri, St. Louis, Missouri 63121 (M.L., S.C.B., T.P., X.W.); Donald Danforth Plant Science Center, St. Louis, Missouri 63132 (M.L., S.C.B., J.L., M.O., J.J., X.W.); National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China (C. F); and Kansas Lipidomics Research Center, Division of Biology, Kansas State University, Manhattan, Kansas 66506 (M.R.R., R.W.)

One sentence summary:
Phospholipase pPLAIIIδ hydrolyzes phospholipids and acyl-CoA, modifies seed fatty acid composition, and enhances seed oil accumulation, and the study also identifies that pPLAIIIδ among four pPLAIIIs specifically plays a positive role in promoting triacylglycerol accumulation and changing fatty acid composition.
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Corresponding author

Xuemin Wang, email: swang@danforthcenter.org. The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Xuemin Wang (swang@danforthcenter.org).
ABSTRACT

The release of fatty acids from membrane lipids has been implicated in various metabolic and physiological processes, but in many cases, the enzymes involved and their function in plants remain unclear. Patatin-related phospholipase As (pPLAs) constitute a major family of acyl-hydrolyzing enzymes in plants. Here we show that pPLAIIIδ promotes the production of triacylglycerols with 20- and 22-carbon fatty acids in *Arabidopsis thaliana*. Of the four pPLAIIIs (α, β, γ, δ), only pPLAIIIδ’s gene knockout results in a decrease in seed oil content, and pPLAIIIδ is most highly expressed in developing embryos. The overexpression of pPLAIIIδ increases content of triacylglycerol and 20- and 22-carbon fatty acids in seeds with a corresponding decrease in 18-carbon fatty acids. Several genes in the glycerolipid biosynthetic pathways are up-regulated in pPLAIIIδ-overexpressing siliques. pPLAIIIδ hydrolyzes phosphatidylcholine and also acyl-CoA to release fatty acids. pPLAIIIδ-overexpressing plants have a lower, whereas pPLAIIIδ-knockout plants have a higher, level of acyl-CoA than wild type. Whereas seed yield decreases in transgenic plants that ubiquitously overexpress pPLAIIIδ, seed-specific overexpression of pPLAIIIδ increases seed oil content without detrimental effect on overall seed yield. The results indicate that pPLAIIIδ-mediated phospholipid turnover plays a role in fatty acid remodeling and glycerolipid production.
INTRODUCTION

Lipids play essential structural, metabolic, and regulatory roles in plant growth, development, and stress responses. In addition, plant lipids are a major source of food and renewable materials for various industrial and energy applications (Dyer et al., 2008; Hayden et al., 2011; Rogalski and Carrer, 2011; Bates and Browse, 2012). Substantial progress has been made toward a basic understanding of the biochemical reactions of lipid biosynthesis in plants, but many fundamental questions about lipid metabolism remain unanswered (Weselake et al., 2009; Chapman and Ohlrogge, 2012). Recent results suggest that the metabolism of phosphatidylcholine (PC) plays multiple, important roles in glycerolipid production. An increasing line of research shows that storage lipid triacylglycerols (TAG) are not synthesized primarily via the Kennedy pathway, but are derived from PC through acyl editing (Bates et al., 2009; 2012; Tjellström et al., 2012). PC is also hypothesized to be involved in trafficking of fatty acids from the plastid to the endoplasmic reticulum (ER), where glycerolipids, including TAG, are assembled (Wang and Benning, 2012). It is proposed that plastidial fatty acids are transferred to lysophosphatidylcholine (LPC) to form PC, which serves as a substrate for fatty acid desaturation and modification. While the importance of PC metabolism in TAG production is clear, the specific enzymes involved in PC turnover are not well elucidated (Chapman and Ohlrogge, 2012; Bates et al., 2012), and the impact of PC turnover on TAG accumulation remains to be determined.

Phospholipase A (PLA) hydrolyzes PC to produce LPC and a free fatty acid. This reaction has been implicated in various cellular functions, including the production of lipid mediators, carbon partitioning, and cell elongation. Patatin-containing PLA, pPLA, is a major family of intracellular acyl-hydrolyzing enzymes in plants (Scherer et al., 2010; Murakami et al., 2011). The ten-gene pPLA family in Arabidopsis are grouped into three subfamilies, pPLAI, pPLAII (α, β, γ, δ, ε), and pPLAIII (α, β, γ, δ). pPLAI has been shown to contribute to the resistance to Botrytis cinerea, possibly by mediating the basal levels of jasmonate production (Yang et al., 2007), whereas pPLAIIα negatively modulates both plant response to bacterial pathogens (La Camera et al., 2005) and oxylipin production (Yang et al., 2012). pPLAIIβ impacts root elongation during phosphate deficiency, and pPLAIIγ and pPLAIIδ have been implicated in auxin responses (Rietz et al., 2004; 2010). Activation-tagging of pPLAIIIδ and overexpression...
of pPLAIIIβ resulted in decreased cell elongation and stunted growth (Huang et al., 2001; Li et al., 2011). These results indicate that the pPLA family plays important, diverse roles in plant growth and stress responses, but their role in seed oil production is not known.

One enigma from recent genomic analysis of Arabidopsis has been that there are as many genes annotated as being involved in lipid catabolism as there are in lipid synthesis (Li-Beisson et al., 2010). While the functions for many genes involved in lipid biosynthesis have been documented, little is known about the role of lipid-hydrolyzing enzymes in lipid metabolism and oil production. A recent study compared the transcriptomes of mesocarp from oil palm and date palm that accumulate approximately 90% and 1% oil, respectively (Bourgis et al., 2011). The mRNA level of key genes in fatty acid synthesis in oil palm mesocarp is 2 to 44 fold higher than in date palm. The mRNA level of palm pPLAIIIβ is 22 fold higher in oil palm compared to date palm mesocarp (Bourgis et al., 2011), but the role for pPLAIII in oil accumulation remains to be determined. Patatin-related enzymes typically contain a catalytic center with the esterase box GXSXG and other specific motifs including a catalytic dyad motif, which is typically contains DGG (Scherer et al., 2010). The pPLAIII subfamily differs from pPLAI and pPLAI in that it does not contain the canonical esterase GXSXG motif, but instead has the sequence GXGXG (Scherer et al., 2010). Our recent analysis of pPLAIIIβ shows that pPLAIIIβ hydrolyzes PC to produce LPC and free fatty acids (Li et al., 2011). Moreover, overexpression of pPLAIIIβ increases membrane glycerolipid content in vegetative tissues whereas its gene knockout has the opposite effect. These observations prompted us to determine the role of pPLAIIIs in seed oil production. Here we show that pPLAIIIδ promotes TAG production with increased accumulation of long chain fatty acids in Arabidopsis seeds.

RESULTS

pPLAIIIδ Increases Seed Oil Content.

To investigate the function of pPLAIIIs in seed oil production, we isolated T-DNA insertional knockout (KO) mutants for all four pPLAIIIs (Supplemental Figure 1). The T-DNA insertion sites of pPLAIIα and pPLAIIIβ are in the first exon while the insertion sites of pPLAIIγ and pPLAIIIδ locate in the 5′-UTR region (Supplemental Figure 1B). All of these insertional mutants have a negligible level of transcript as measured by real-time PCR of pPLAIIα,
pPLAIIIγ, and pPLAIIIδ (Figure 1A). The loss of pPLAIIIβ expression in pPLAIIIβ-KO was described previously (Li et al., 2011). However, only the pPLAIIIδ-KO seeds, not the other pPLAIIIs, displayed a significant change in oil content compared to WT seeds; the oil contents of pPLAIIIδ-KO and WT seeds were 33% and 35.5% of the seed weight, respectively (Figure 1B). To confirm the effect of pPLAIIIδ on seed oil production, we genetically complemented the KO by transferring pPLAIIIδ with its native promoter and terminator sequences into the KO mutant (designated as COM; Supplemental Figure 1C). Expression of pPLAIIIδ in the COM lines was restored to the WT level (Figure 1C), and the oil content in COM seeds was the same as that of WT (Figure 1D).

Analysis of mRNA accumulation patterns for pPLAIIIs in seeds indicate that pPLAIIIα, β, and γ were expressed in tissues that do not accumulate large amounts of TAG in developing seeds (Supplemental Figure 2A-E). In mature green seeds, pPLAIIIγ was expressed mostly in seed coat, pPLAIIIβ mostly in chalazal seed coat, and pPLAIIIα mostly in seed coats and peripheral endosperm (Supplemental Figure 2C-E). In contrast, pPLAIIIδ was expressed in developing radicle and in cotyledons, the major storage tissue for seed oil in Arabidopsis (Supplemental Figure 2F). The mRNA accumulation pattern of the pPLAIII genes is consistent with a pPLAIIIδ-specific effect on seed oil content and, thus, further analysis was focused on pPLAIIIδ.

To further investigate pPLAIIIδ function, we produced multiple overexpressing (OE) Arabidopsis lines by placing pPLAIIIδ under the control of cauliflower mosaic virus 35S promoter (35S::pPLAIIIδ-OE; Supplemental Figure 1C). The mRNA level of pPLAIIIδ was increased substantially in OE over WT plants (Figure 1C). The presence of the introduced green fluorescence protein (GFP) tagged pPLAIIIδ was detected by immunoblotting with a GFP antibody (Figure 1E). Seed oil content in two OE lines was approximately 40.5%, which was 5% higher than that of WT (35.5%; Figure 1D). Taken together, these data indicate that pPLAIIIδ plays a positive role in seed oil accumulation.

**pPLAIIIδ Increases 20-Carbon Fatty Acid Content at the Expense of 18-Carbon Fatty Acids.**
The fatty acid composition was significantly altered in pPLAIIIδ-KO and 35S::pPLAIIIδ-OE seeds (Figure 2A). The levels of 18 carbon-fatty acids tended to increase in KO and decrease in OE seeds compared to WT. For example, 18:1 was increased by 10% in KO but decreased 6% in OE1. Conversely, the amounts of 20-carbon fatty acids 20:0 and 20:2 were decreased by 12% and 12% in KO, while 20:0 and 20:1 were increased by 12% and 15% in OE lines, compared to WT. The 22-carbon species, 22:1, showed a trend similar to the 20-carbon species. The ratio of 20- to 18-carbon fatty acids was decreased by 10% in KO and increased by 19% in OE compared with WT (Figure 2B). The fatty acid composition in COM seeds was similar to that of WT seeds (Figure 2B). Thus, increased mRNA level of pPLAIIIδ promoted accumulation of longer chain fatty acids at the expense of 18-carbon fatty acids, 18:1 and 18:2, whereas pPLAIIIδ KO decreased the production of longer chains with increased accumulation of 18-carbon fatty acids.

Fatty acids in Arabidopsis seeds occur primarily in esterified form in TAGs. TAGs include many different molecular species with varied carbon chain length and degrees of unsaturation in the three acyl chains. Three acyl chains in TAG are not randomly distributed. Since pPLAIIIδ affects 18:1 and 20:1 accumulation in TAG, we wondered if pPLAIIIδ alters the distribution of three acyl chains and thus produces some unique TAG molecule species. Therefore, we analyzed the TAG species in WT, KO, and OE seeds by electrospray ionization-tandem mass spectrometry. The major fatty acyl chain carbon numbers (C) in seed TAGs are 16C, 18C, and 20C, and the major TAG species have total C of C50 (e.g. 16-16-18), C52 (e.g. 16-18-18), C54 (e.g. 18-18-18), C56 (e.g. 18-18-20), C58 (e.g. 18-20-20), and C60 (e.g. 20-20-20) (Figure 3). The percentages of C50, C52, and C54 TAG species in total TAGs, as indicated by their relative mass spectral signals, tended to be higher in KO while lower in OE mutants when compared with WT, while the levels of C56, C58, and C60 TAG species were changed in the opposite manner in KO and OE lines of pPLAIIIδ (Figure 3 and Supplemental Figure 3). For example, the percentages of some 16C and 18C-containing TAGs (16:0-16:0-18:3, 16:0-18:1-18:3, 18:2-18:2-18:3) were significantly lower in OE mutants than in WT (Figure 3A). While certain TAG species could not be quantified individually and thus their compositional percentages were expressed in combination, the percentages of 20C-containing TAGs and TAG groups tended to be or were significantly lower in KO and higher in OE mutants compared with WT (Figure 3A). Overall, the relative amounts of C50, C52, and C54 TAGs tended to be lower, while C56, C58,
and C60 TAGs tended to be higher in OE mutant seeds compared to WT (Figure 3B). Measurement of 113 additional TAG species and 8 TAG species groups confirmed the trend for the percentages of 18C-containing TAGs to be lower and the 20C-containing TAGs higher in OEs compared with WT (Supplemental Figure 3A-E). Taken together, these data indicate that pPLAIId promotes the accumulation of 20C-containing TAG species.

**pPLAIId-OE Increases the Transcript Levels of Genes in TAG and PC Synthesis.**

To gain insight into how pPLAIId facilitates TAG accumulation and modification, we measured the mRNA levels of selected genes in TAG and PC synthesis and metabolism in developing Arabidopsis siliques (Figure 4). In the Kennedy pathway of TAG biosynthesis, glycerol-3-phosphate (G3P) is sequentially acylated by glycerol phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAT), followed by PA phosphohydrolase (PAH) and diacylglycerol acyltransferase (DGAT). The transcript levels for the genes in the Kennedy pathway, including *GPAT*, *LPAT2*, *LPAT3*, *PAH*, and *DGAT1* were increased two to five-fold in *pPLAIId*-OE lines (Figure 4A). By comparison, mRNA levels of both *DGAT2* and *LPAT5* were the same in WT, *pPLAIId-KO*, and *35S::pPLAIId*-OE siliques (Figure 4). Phospholipid:diacylglycerol acyltransferase (PDAT) catalyzes the transfer of a fatty acid from PC to diacylglycerol (DAG) to produce TAG. The mRNA level of *PDAT1* was increased by almost three fold in OE lines compared to WT (Figure 4B).

In the Kennedy pathway of PC biosynthesis, choline phosphate:CTP cytidylyltransferase (CCT) synthesizes CDP-choline using CTP and phosphocholine and aminoalcohol-phosphotransferase (AAPT) catalyzes the last step of PC synthesis by transferring phosphocholine to DAG from CDP-choline. There are two CCTs and AAPT s in Arabidopsis. Compared to WT, the mRNA levels of *CCT2* and *AAPT1* were increased almost by ten-fold, whereas the increase in *CCT1* and *AAPT2* was about two-fold in *pPLAIId*-OE siliques (Figure 4B). The mRNA abundance of LPC:acyl-CoA acyltransferase (*LPCAT1*) was also increased 3-fold in OE lines (Figure 4B). These data demonstrate that *pPLAIId* overexpression increases the mRNA level of genes involved TAG and PC synthesis. On the other hand, in KO siliques the mRNA level for the lipid-metabolizing genes was not significantly different from that of WT, even though the mRNA level for several of these genes tended to be lower than that of WT (Figure 4). These
results suggest that the loss of \( pPLAIII\delta \) may be partially compensated for by other members of pPLAs.

**pPLAIII\( \delta \) Hydrolyzes PC and Acyl-CoA and Affects Acyl-CoA Levels in Arabidopsis.**

\( pPLAIII\delta \) is more distantly related to the other three pPLAIIIs than they are to each other (Supplemental Figure 1A). \( pPLAIII\delta \) has an aspartic acid (D) in the DGG catalytic dyad motif, similar to pPLAs in the other groups, whereas in \( pPLAIII\beta \) and \( pPLAII\gamma \) the aspartic acid is replaced by glycine (G) (Li et al., 2011). To determine the enzymatic function of \( pPLAIII\delta \), we expressed 6xHis-tagged \( pPLAIII\delta \) in *E. coli* and purified it to near homogeneity (Figure 5A). The PC-hydrolyzing activity of \( pPLAIII\delta \) was examined because PC is the most abundant phospholipid and serves as a key intermediate for TAG synthesis. Incubation of \( pPLAIII\delta \) with 16:0-18:2-PC resulted in the production of free fatty acid (FFA) and LPC. \( pPLAIII\delta \) hydrolysis at the sn-1 position produces 16:0-FFA and 18:2-LPC (Figure 5B) whereas \( pPLAIII\delta \) hydrolysis at the sn-2 position produces 18:2-FFA and 16:0-LPC (Figure 5C). The production of 18:2-FFA was approximately five-fold more than that of 16:0-FFA, and correspondingly much more 16:0-LPC was formed than 18:2-LPC. These data indicate that \( pPLAIII\delta \) hydrolyzes PC at both sn-1 and sn-2 positions and that \( pPLAIII\delta \) preferentially releases 18:2 from the sn-2 position.

In addition, we determined whether \( pPLAIII\delta \) could hydrolyze acyl-CoA because our previous study showed that another pPLAI member, \( pPLAIII\beta \), has thioesterase activity (Li et al., 2011). Incubation of \( pPLAIII\delta \) with 18:3-CoA resulted in the steady production of 18:3-FFA with increasing reaction time (Figure 6A), indicating that \( pPLAIII\delta \) possesses a thioesterase activity. We then determined whether the alterations of \( pPLAIII\delta \) expression impacted the acyl-CoA content in Arabidopsis. In siliques that included developing seeds with active storage lipid biosynthesis, the level of total acyl-CoA was 19% higher in KO and 18% lower in OE mutants than in WT (Figure 6B). The major acyl-CoA species are 18:3-CoA and 18:2-CoA, followed by 16:0-CoA. The levels of 18:1-CoA, 18:2-CoA and 18:3-CoA were significantly higher in KO and 16:0-CoA and 18:2-CoA were significantly lower in OE than in WT siliques (Figure 6C). The data are consistent with \( pPLAIII\delta \) functioning as an acyl-CoA thioesterase activity in vivo.

**pPLAIII\( \delta \) Is Associated with the Plasma and Intracellular Membranes.**
To determine its subcellular association, a GFP-tagged pPLAIIIδ was expressed in Arabidopsis, and the green fluorescence signal of pPLAIIIδ-GFP was mostly detected on the inner cell boundary of leaf epidermal cells (Figure 7A). Plasmolysis by applying saline solution to the roots showed that the GFP signal in root epidermal cells was shrinking along with the plasma membrane (Figure 7B). To further analyze the intracellular association, total leaf proteins were fractionated into cytosolic and microsomal fractions. All pPLAIIIδ-GFP was associated with the microsomal membranes but not cytosol (Figure 7C). The microsomal proteins were further partitioned into the plasma membrane and intracellular membrane fractions. Approximately 80% pPLAIIIδ-GFP was associated with the plasma membrane whereas 20% was associated with intracellular membranes based on the intensity of the protein bands (Figure 7C). These data indicate pPLAIIIδ is associated with both the plasma and intracellular membranes.

**Seed-Specific Overexpression of pPLAIIIδ Increases Oil Content.**

The increased oil content in seeds raises the question of whether increased pPLAIIIδ expression can be used to increase seed oil production. However, constitutive overexpression of pPLAIIIδ resulted in a decrease in plant height and overall seed yield (Figure 8A and B). The seed yield per 35S::pPLAIIIδ-OE plants was approximately 50% of that of WT plants (Figure 8B). To explore whether the improved oil content could be uncoupled from decreased seed production, we placed pPLAIIIδ under the control of the seed-specific promoter of soybean β-conglycinin (CON::pPLAIIIδ; Supplemental Figure 4A). The level of pPLAIIIδ expression in developing siliques was 25 fold higher in CON::pPLAIIIδ than that in WT (Figure 8C). The presence of the pPLAIIIδ-GFP protein was detectable by visualizing the GFP fluorescence (Supplemental Figure 4B). CON::pPLAIIIδ plant height and seed yield were comparable with WT (Figure 8A and B). In three CON::pPLAIIIδ lines tested, seed oil content was increased over WT seeds (39% vs 35%; Figure 8D). While oil content per CON::pPLAIIIδ seed weight was lower than that per 35S::pPLAIIIδ seed weight (Figure 1D vs. 8D), the overall seed oil production per CON::pPLAIIIδ plant was significantly higher than that per 35S::pPLAIIIδ, due to the higher seed yield per plant (Figure 8B), and per WT plant, due to the increased oil content without change in seed yield (Figure 8B and D).
The seed specific overexpression of pPLAIIIδ resulted in changes in fatty acid composition, and the changes in CON::pPLAIIIδ were similar to those in 35S::pPLAIIIδ seeds. The percentages of 18:1 and 18:2 were lower while those of 20:0, 20:1, 20:1, and 22:1 were higher in CON::pPLAIIIδ lines than WT (Figure 8E). The ratio of 20:1 to 18:1 was 30% higher in CON::pPLAIIIδ lines than WT (Supplemental Figure 4C), and the same pattern was observed when total 20 carbon-fatty acids were compared with total 18-carbon fatty acids (Supplemental Figure 4D). The results indicate that pPLAIIIδ affects TAG metabolism in the same manner regardless of the promoter used, and that the use of seed specific expression of pPLAIIIδ has the potential to be applied for increased seed oil production.

DISCUSSION

The present data show that pPLAIIIδ positively impacts seed oil content. Whereas pPLAIIIδ-KO decreases seed oil content, pPLAIIIδ-OE, driven either by a constitutive or seed-specific promoter, increases seed oil content. pPLAIIIδ hydrolyzes PC to generate FFA and LPC. pPLAIIIδ may accelerate acyl flux from the plastid to ER and therefore enhance glycerolipid synthesis. Fatty acids in higher plants are synthesized exclusively in the plastid and have to be exported to the ER where glycerolipids are synthesized (Figure 9). Lipid trafficking between organelles is a fundamental, yet poorly understood process in plants. In recent years, excellent progress has been made toward the understanding lipid transport from the ER to the plastid for the synthesis of galactolipids (Wang et al., 2012). PA is imported into the plastid through a protein complex (Wang and Benning, 2012). In contrast, the metabolic and regulatory mechanism by which fatty acids in the plastid are trafficked to the ER is unknown.

Palmitic acid (16:0) and oleic acid (18:1) are two major FAs exported from the plastid in Arabidopsis (Pidkowich et al., 2007; Andersson and Kelly, 2010). Free FAs are thought to be able to cross membrane bilayers through diffusion and possibly protein-mediated translocation (Wang and Benning, 2012). After reaching the plastid outer envelope, long chain acyl-CoA synthetases (LACS) convert these FAs to acyl-CoA. In the conventional Kennedy pathway, acyl-CoA is used for sequential acylation of G3P → LPA → PA → DAG → TAG (Figure 9). However, kinetic labeling data indicate that FAs exported from the plastid are first incorporated into PC and then channeled to TAG in soybean embryos (Bates et al., 2009; 2011; 2012). The
presence of highly active LPCAT on the Arabidopsis plastid outer envelope membrane is consistent with the formation of PC using FAs from the plastids (Tjellström et al., 2012; Wang et al., 2012). Recent data indicate that LPCAT1 and LPCAT2 catalyze incorporation of fatty acids into PC in Arabidopsis seeds (Bates et al., 2012; Wang et al., 2012). However, knowledge is lacking about what enzyme produces LPC that impact TAG synthesis. PDAT can transfer a fatty acid from PC to DAG to produce TAG and LPC, but its role in TAG production in seeds remain unclear (Chapman and Ohlrogge, 2012). pPLAIIIδ could be one of the enzymes hydrolyzing PC to produce a free fatty acid and LPC that LPCAT uses to accept fatty acids from the plastid (Figure 9). The combined activity of pPLAIII and LPCATs may modulate the rate of FA trafficking from the plastid to ER in Arabidopsis seeds.

Fatty acids, such as 18:1, released from PC by pPLAIIIδ may enter acyl-CoA pool for elongation (Figure 9). Knockout and overexpression of the pPLAIIIδ gene displayed opposite effects on the levels of 18:1 and 20:1 fatty acids in seed oil. Detailed profiling of TAG molecules also show the opposite effects on the levels of 18:1-containing and 20:1-containing TAGs by knockout and overexpression of pPLAIIIδ gene. In Arabidopsis, the major fatty acids exported from plastids to the ER are 16:0, 18:0, and 18:1. In the ER, 18:1 on PC is desaturated to 18:2 and 18:3 (Nishida and Bates, 2010), whereas acyl-CoA is used for FA elongation to form longer chain fatty acids, such as 20:1 (Joubès et al., 2008; Rowland and Bird, 2010). The effect of pPLAIIIδ on fatty acid composition is distinctively different from that of the recently described PC:DAG cholinephosphotransferase (PDCT) that transfers phosphocholine from PC to DAG, and a mutation of PDCT decreases the 18:2 and 18:3 level in Arabidopsis seed TAG by 40% (Lu et al., 2009). Thus, the increased pPLAIIIδ expression may facilitate the release of 18:1 from PC for 20:1 production (Figure 9).

Compared to WT, OE of pPLAIIIδ had a lower acyl-CoA pool size in developing silique and higher seed oil content. The decrease in the acyl-CoA pool size could result from the thioesterase activity of pPLAIIIδ and/or increased PC turnover and TAG synthesis. The exchange of modified acyl groups between PC and the acyl-CoA pool requires extensive acyl editing cycles (Harwood, 1996). Through the acyl editing cycles, modified FAs enter the acyl-CoA pool to be utilized for glycerolipid synthesis and acyl-CoA can be channeled into PC for further
modification and directly for TAG production (Stymne and Stobart, 1984; Bafor et al., 1991; Bates et al., 2007; 2009). The inverse association between acyl-CoA pool and TAG contents could mean that the pPLAIIIδ-catalyzed turnover of acyl-CoA and PC promotes seed oil accumulation.

The enhanced mRNA level of genes, such as AAPT and CCT, in PC-biosynthesis in pPLAIIIδ-OE plants indicates that increased pPLAIIIδ-mediated PC hydrolysis leads to an increase in PC biosynthesis and, thus increased PC turnover. Meanwhile, RNA levels are higher for genes in the Kennedy pathway, such as GPAT, LPAT, PAP, and DGAT in developing pPLAIIIδ-OE siliques. The increased transcript levels of glycerolipid-producing genes may be a feed-forward stimulation by enhanced substrate supplies as the increased pPLAIIIδ expression leads to elevated levels of FFAs and LPC. How the metabolic changes in FFAs and LPC is connected to the altered mRNA levels and potentially gene expression requires further investigation. In yeast, it has been shown that the transcriptional factor directly binds to PA, senses cellular PA levels, and regulates the expression of many genes involved in membrane lipid synthesis (Loewen et al., 2004). In addition, there is an increase in the mRNA level of LPC:acyl-CoA acyltransferase (LPCAT) which catalyzes the acylation of LPC using fatty acids from the plastid. This could mean an increase in fatty acid trafficking from the plastid to the ER where glycerolipids are synthesized. Further studies are needed to determine the mechanism by which increased pPLAIIIδ expression promotes TAG production. Such investigation of how a lipid-hydrolyzing enzyme, such as pPLAIIIδ, promotes lipid accumulation has the potential to better our understanding of lipid metabolism and accumulation.

In summary, our study shows that pPLAIIIδ hydrolyzes PC to generate FFA and LPC and genetic alterations of pPLAIIIδ expression change seed oil content and fatty acid composition in Arabidopsis seeds. Our large scale of TAG species analysis reveals that pPLAIIIδ promotes the production of 20:1-TAG. We propose that pPLAIIIδ plays a role in fatty acyl flux from the plastid to ER and/or PC fatty acyl remodeling for TAG synthesis. Furthermore, the results indicate that the use of seed-specific expression of pPLAIIIδ has the potential to improve seed oil production in crops.
MATERIALS AND METHODS

Generation of pPLAIII Knockouts, Overexpression, and Complementation Plants

T-DNA insertional mutants for pPLAIIIα (Salk_040363), β (Salk_057212), γ (Salk_088404), and δ (Salk_029470) were identified from the Salk Arabidopsis T-DNA knockout collection obtained from the Ohio State University ABRC. The homozygous T-DNA insertion mutant for individual pPLAIIIIs was verified by PCR-based screening using a T-DNA left border primer and gene-specific primers as listed in Supplemental Table 1. The isolation of pPLAIIIβ-KO was reported previously (Li et al., 2011). The loss of gene transcripts in pPLAIII-KO was confirmed by real-time PCR. To generate the complementation lines (pPLAIIIδ-COM), the genomic DNA sequence of pPLAIIIδ from the promoter region to the terminator region was cloned using two primers as listed in Supplemental Table 1 and fused into a binary vector pEC291 for plant transformation.

To overexpress pPLAIIIδ, the genomic sequence of pPLAIIIδ was obtained by PCR using Col-0 Arabidopsis genomic DNA as a template and primers listed in Supplemental Table 1. The genomic DNA was cloned into the pMDC83 vector before the GFP-His coding sequence. The expression of pPLAIIIδ was under the control of the 35S cauliflower mosaic virus promoter or the promoter of soybean β-conglycinin. The sequences of the fusion constructs were verified by sequencing before they were introduced into the Agrobacterium tumefaciens strain C58C1. Col-0 Arabidopsis plants were transformed; and transgenic plants were screened and confirmed by antibiotic selection and PCR. Over 15 independent transgenic lines were obtained (pPLAIIIδ-OE) with similar plant stature. Five independent lines of pPLAIIIδ-OE were further verified by immunoblotting with anti-GFP antibody.

RNA Extraction and Real-Time PCR

Real-time PCR was performed as described previously (Li et al., 2006; 2011). Briefly, total RNA was extracted from different tissues using the cetyl-trimethylammonium bromide method (Stewart and Via, 1993). DNA contamination in RNA samples was removed with RNase-free DNase. An iScript kit (Bio-Rad) was used to synthesize cDNA from isolated RNA template by reverse transcription. The MyiQ sequence detection system (Bio-Rad) was used to detect products during quantitative real-time PCR by monitoring SYBR green fluorescent labeling of
double-stranded DNA. Efficiency was normalized to a control gene *UBQ10*. The real time PCR primers are listed in Supplemental Table 2. The data were expressed as mean ± SE (n = 3 replicates). PCR conditions were as follows: one cycle of 95°C for 1 min; 40 cycles of DNA melting at 95°C for 30 s, DNA annealing at 55°C for 30 s, and DNA extension at 72°C for 30 s; and final extension of DNA at 72°C for 10 min.

**Analysis of Fatty Acid Composition and Oil Content**

Ten milligram of Arabidopsis seeds were placed in glass tubes with Teflon-lined screw caps and 1.5 mL 5% (v/v) H₂SO₄ in methanol with 0.2% butylated hydroxytoluene was added. The samples were incubated for 1 h at 90°C for oil extraction and transmethylation. Fatty acid methyl esters (FAMEs) were extracted with hexane. FAMEs were quantified using gas chromatography on a SUPELCOWAX-10 (0.25 mm x 30 m) column with helium as a carrier gas at 20 mL/min and detection by flame ionization. The oven temperature was maintained at 170°C for 1 min and then ramped to 210°C at 3°C per min. FAMEs from TAG were identified by comparing their retention times with FAMEs in a standard mixture. Heptadecanoic acid (17:0) was used as the internal standard to quantify the amounts of individual fatty acids. Fatty acid composition is expressed in weight percentage.

**pPLAIIIδ Cloning and Protein Purification from *Escherichia coli***

The full-length cDNA of *pPLAIIIδ* was obtained by PCR using an *Arabidopsis thaliana* cDNA library as a template and a pair of primers listed in Supplemental Table 1. The cDNA was cloned into the pET28a vector before the 6xHis coding sequence. The 6xHis fusion construct was sequenced and confirmed to be error free before it was introduced into *E. coli* strain Rosetta (DE3) (Amersham Biosciences). The bacteria were grown to an OD₆₀₀ of 0.7 and induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside for 16 h at 16°C. The pPLAIIIδ-6xHis fusion protein was purified as described previously (Pappan et al., 2004). Briefly, the bacterial pellet was resuspended in STE buffer containing 1 mg/mL lysozyme (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA). The samples were kept on ice for 30 min. DTT and N-laurylsarcosine (Sarkosyl) were added to a final concentration of 5 mM and 1.5% (w/v), respectively. The suspension was vortexed and sonicated on ice for 5 min. After centrifugation at 10,000g for 20 min, the supernatant was transferred to a new tube. Triton X-100 was added to
a final concentration of 4% (v/v) and 6xHis agarose beads were added (10%, w/v). The solution was gently rotated at 25°C for 1 h. The fusion proteins bound to agarose beads were washed with 20 volumes of STE buffer. The amount of purified protein was measured with a protein assay kit (Bio-Rad).

**Enzyme Assays**

Phospholipids and acyl-CoAs were purchased from Avanti Polar Lipids. PC or 18:3-CoA in chloroform was dried under a nitrogen stream and emulsified in reaction buffer (25 mM HEPES, pH 7.5, 10 mM CaCl₂, and 10 mM MgCl₂) by vortexing, followed by 5 min sonication on ice. Acyl hydrolyzing activities were assayed in a reaction mixture containing 25 mM HEPES, pH 7.5, 10 mM CaCl₂, 10 mM MgCl₂, and 60 µmol PC as substrate. Ten micrograms of purified protein was added to the mixture in a final volume of 500 µL. The reaction was incubated at 30°C for indicated minutes, and stopped by adding 2 mL of chloroform/methanol (2:1, v/v) and 500 µL 25 mM LiCl. After vortexing and separation by centrifugation, the lower phase was transferred to a new glass tube. The upper phase was extracted twice more by adding 1 mL of chloroform each time, and the three lower phases were combined. Lipid internal standards were added, and lipid quantification was performed by mass spectrometry as described below.

**Lipid Quantification**

In *in vitro* enzyme assays, lipids were extracted for analysis as previously described (Li et al., 2011). Twenty microliters of lipid sample were combined with 340 µL chloroform, and 840 µL of chloroform/methanol/300 mM ammonium acetate in water (300:665:35). FFAs were determined by ESI-MS on a electrospray ionization triple quadruple mass spectrometer (API4000, Applied Biosystems), using the deuterated internal standard (7,7,8,8-d4-16:0 fatty acid) (Sigma-Aldrich), by scanning in negative ion mode from *m/z* 200 to *m/z* 350 (Li et al., 2011). LPC was determined with the same instrument as previously described (Li et al., 2011). Plants for acyl-CoA measurement were grown in growth chambers with a 12 h-light/12 h-dark cycle, at 23/21°C, 50% humidity, at 200 µmol m⁻² s⁻¹ of light intensity, and watered with fertilizer once a week. Acyl-CoAs were extracted and analyzed by LC-ESI-MS/MS as described previously (Magnes et al., 2005; Han et al., 2010). TAG molecular species were analyzed by
ESI-MS/MS using neutral loss scan modes (Lee et al., 2011; 2012). The TAG analysis is described in detail in Supplemental materials.

**Microscopy Imaging and Subcellular Fractionation**

The subcellular location of GFP-tagged protein was determined using a Zeiss LSM 510 confocal microscope equipped with a X40 differential interference contrast, 1.2-numerical aperture water immersion lens, with excitation using the 488 nm line of an argon gas laser and a 500-550 nm band pass emission filter. Plasmolysis in primary root cells was induced by immersing roots in 0.5 M NaCl for 1, 3, and 5 min. Developing seeds from Arabidopsis siliques were imaged using a Nikon Eclipse 800 widefield microscope and a X60 differential interference contrast, 1.2-numerical aperture objective, with mercury lamp excitation and a 492/18 BP excitation filter and a 535/40 B emission filter. For subcellular fractionation, proteins were extracted from leaves of 4-week-old plants using buffer (30 mM HEPES, pH 7.5, 400 mM NaCl, and 1 mM phenylmethanesulfonyl fluoride), followed by centrifugation at 6,000g for 10 min. The supernatant was centrifuged at 100,000g for 60 min. The resultant supernatant is referred to as the soluble cytosol fraction, and the pellet is referred to as the microsomal fraction. The microsomal fraction was separated further into the plasma and intracellular membrane fractions, using two-phase partitioning as described previously (Fan et al., 1999).

**SDS-PAGE and immunoblotting**

Leaf samples, each weighing approximately 1 g, were harvested and ground in 3 mL buffer of 30 mM HEPES, pH 7.5, 400 mM NaCl, 1.0 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol. Proteins were separated by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was visualized with alkaline phosphatase conjugated to a secondary anti-mouse antibody after blotting with GFP antibody.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: *AAPT1*, At1g13560; *AAPT2*, At3g25585; *CCT1*, At2g32260; *CCT2*, At4g15130; *DGAT1*, At2g19450; *DGAT2*, At3g51520; *GPAT*, At1g32200; *LPAT2*, At3g57650; *LPAT3*, At1g51260; *LPAT4*, At1g75020; *LPAT5*, At3g18850; *LPCAT1*,...
At1g63050; \textit{PAH1}, At3g09560; \textit{PDAT1}, At5g13640; \textit{pPLAII}, At1g61850; \textit{pPLAII\alpha}, At2g26560; \textit{pPLAII\beta}, At4g37050; \textit{pPLAII\gamma}, At4g37070; \textit{pPLAII\delta}, At4g37060; \textit{pPLAII\epsilon}, At5g43590; \textit{pPLAIII\alpha}, At2g39220; \textit{pPLAIII\beta}, At3g54950; \textit{pPLAIII\gamma}, At4g29800; \textit{pPLAIII\delta}, At3g63200; and \textit{UBQ10}, At4g05320.

**Supplemental Data**

**Supplemental Figure S1.** Generation of Knockout, Overexpression, and Complementation Mutants of \textit{pPLAIII}s.

**Supplemental Figure S2.** RNA Accumulation Patterns of Four \textit{pPLAIII} Genes in Developing Arabidopsis Seeds.

**Supplemental Figure S3.** \textit{pPLAIII\delta} Promotes Increased Levels of 20C Fatty Acyl-containing TAG over 18C Fatty Acyl-containing TAG in Arabidopsis Seeds, as Determined by Mass Spectral Analysis.

**Supplemental Figure S4.** Seed Specific Overexpression of \textit{pPLAIII\delta} in Arabidopsis.

**Supplemental Table 1.** PCR Primers for Mutant Screening and Molecular Cloning.

**Supplemental Table 2.** Real Time PCR Primers for Quantitative Measurement of Transcript Levels.

**Supplemental Method 1.** Mass Spectral Analysis of TAG.

**ACKNOWLEDGMENTS**

We thank Howard Berg at the Donald Danforth Plant Science Center’s Integrated Microscopy Facility for imaging assistance.

**REFERENCES**


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FIGURE LEGENDS

Figure 1. Alterations of pPLAIIIδ Change Arabidopsis Seed Oil Content.

(A) Transcript levels of pPLAIIIα, -γ, and -δ in 2-week-old rosettes. The RNA levels were determined by real time PCR and normalized to the level of wild type. Values are means ± SE (n = 3).

(B) Seed oil content in T-DNA insertion mutants of pPLAIIIα-KO, pPLAIIIβ-KO, pPLAIIIγ-KO, and pPLAIIIδ-KO. KO, knockout. Values are means ± SE (n = 3). 1Significantly lower at P < 0.05 compared with the WT, based on Student’s t test.

(C) Transcript levels of pPLAIIIδ in WT, pPLAIIIδ-KO, OE, and COM plants. pPLAIIIδ-OE1, -2 and pPLAIIIδ-COM1, -2 are two independent lines of T3 generation of pPLAIIIδ-OE and pPLAIIIδ-COM. The RNA levels were determined by real time PCR and normalized in comparison to UBQ10. Values are means ± SE (n = 3).

(D) Seed oil content in pPLAIIIδ-OE and pPLAIIIδ-COM T3 seeds. pPLAIIIδ expression in OE lines was under the control of the cauliflower mosaic virus 35S promoter, while in the COM lines, it was under the control of its own promoter. Values are means ± SE (n = 3). HSignificantly higher and 1Significantly lower, each at P < 0.05 compared with the WT, based on Student’s t test.

(E) Immunoblotting of GFP-tagged pPLAIIIIδ in Arabidopsis. Leaf proteins extracted from plants were separated by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane and visualized with alkaline phosphatase conjugated to a secondary anti-mouse antibody after blotting with GFP antibody. Five independent T3 lines of pPLAIIIδ-OE mutants were examined.
Figure 2. pPLAIIIδ Increases 20C Fatty Acid Content at the Expense of 18C Fatty Acids.

(A) Fatty acid compositions of pPLAIIIδ-KO, OE, COM, and WT seeds. Values are means ± SE (n = 3). HSignificantly higher and LSignificantly lower, each at P < 0.05 compared with the WT, based on Student’s t test.

(B) 20C/18C ratio in pPLAIIIδ-KO, OE, COM, and WT seeds. 20C/18C denotes fatty acids with 20 carbons over fatty acids with 18 carbons. Values are means ± SE (n = 3). HSignificantly higher and LSignificantly lower, each at P < 0.05 compared with the WT, based on Student’s t test.

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(A) Normalized mass spectra (as % of total) from TAG species with indicated fatty acyl combinations in WT, KO, and OE seeds. The fatty acids making up each molecular species are indicated, but no positional specificity is implied. The TAG species shown on left side of the dashed lines contain only 16- and 18-carbon chains, while those on the right include one or more 20-carbon chains. Values are means ± SE (n = 5). HSignificantly higher and LSignificantly lower, each at P < 0.05 compared with the WT, based on Student’s t test.

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Figure 4. pPLAIIIδ Increases the RNA Level of Genes in TAG and PC Synthesis in Siliques.

RNA levels were determined by real time PCR and normalized in comparison to UBQ10. Values are means ± SE (n = 3 technical replicates). HSignificantly higher at P < 0.05 compared with the WT, based on Student’s t test. GPAT, Glycerol phosphate acyltransferase; LPAT, lysophosphatidic acid acyltransferase; PAH, PA phosphatase; DGAT, diacylglycerol acyltransferase; PDAT, phospholipid: diacylglycerol acyltransferase; LPCAT, LPC acyltransferase; AAPT, aminoalcohol-phosphotransferase; CCT, choline phosphate:CTP cytidylyltransferase.
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(B) Plasmolysis of root epidermal cells of the pPLAIIIδ-OE:GFP mutant. (a) 1 min after plasmolysis and the green fluorescence signal was located close to cell wall; (b) and (c) 3 min and 5 min after plasmolysis respectively and the green fluorescence signal was co-localized with plasma membrane during cell shrink. Bar = 50 µm.
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(A) Plant heights of mature WT, pPLAIIIδ-KO, 35S::pPLAIIIδ, and CON::pPLAIIIδ plants. 35S represents cauliflower mosaic virus 35S promoter and CON represents the promoter of soybean β-conglycinin. Values are means ± SE (n = 5).

(B) Seed yield per plant of WT, pPLAIIIδ-KO, 35S::pPLAIIIδ, and CON::pPLAIIIδ plants. Values are means ± SE (n = 5).

(C) Transcript levels of pPLAIIIδ in developing siliques of WT and three independent CON::pPLAIIIδ lines. The RNA level was determined by real time PCR and normalized in comparison to UBQ10. Values are means ± SE (n = 3).

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