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

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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 functions in plants remain unclear. Patatin-related phospholipase A3 (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 (Arabidopsis thaliana). Of the four pPLAIIIδs (α, β, γ, δ), only pPLAIIIδ gene knockout results in a decrease in seed oil content, and pPLAIIIδ is most highly expressed in developing embryos. The overexpression of pPLAIIIδ increases the 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-coenzyme A to release fatty acids. pPLAIIIδ-overexpressing plants have a lower level, whereas pPLAIIIδ knockout plants have a higher level, of acyl-coenzyme A than the wild type. Whereas seed yield decreases in transgenic plants that ubiquitously overexpress pPLAIIIδ, seed-specific overexpression of pPLAIIIδ increases seed oil content without any detrimental effect on overall seed yield. These results indicate that pPLAIIIδ-mediated phospholipid turnover plays a role in fatty acid remodeling and glycerolipid production.

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 (TAGs) 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 the 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 (Bates et al., 2012; Chapman and Ohlrogge, 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 (FFA). This reaction has been supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences (grant no. DE-SC0001295 to J.L., J.J., and X.W.), by the National Science Foundation (grant no. MCB-0922879 to M.L., S.B., T.P., and M.O.), and by the National Natural Science Foundation of China (grant no. 30900787 to C.F.); Instrument acquisition and method development at the Kansas Lipidomics Research Center were supported by the National Science Foundation (grant nos. MCB-0920663 and DBI-0521587 to R.W.) and by the Kansas Experimental Program to Stimulate Competitive Research (award no. EPS-0236913 subaward to X.W. and R.W.).

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1 This work was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences (grant no. DE-SC0001295 to J.L., J.J., and X.W.), by the National Science Foundation (grant no. MCB-0922879 to M.L., S.B., T.P., and M.O.), and by the National Natural Science Foundation of China (grant no. 30900787 to C.F.). Instrument acquisition and method development at the Kansas Lipidomics Research Center were supported by the National Science Foundation (grant nos. MCB-0920663 and DBI-0521587 to R.W.) and by the Kansas Experimental Program to Stimulate Competitive Research (award no. EPS-0236913 subaward to X.W. and R.W.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.113.216994
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 10-gene pPLA family in Arabidopsis (Arabidopsis thaliana) is grouped into three subfamilies, pPLAI, pPLAII (α, β, γ, δ, ε), and pPLAIII (α, β, γ, δ). pPLAI has been shown to contribute to 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 involvement in auxin responses (Rietz et al., 2004, 2010). Activation tagging of pPLAIIδ and overexpression (OE) of pPLAIIβ 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 (*Elaeis guineensis* 'Jacq') and date palm (*Phoenix dactylifera*) 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 with 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 typically contains Asp-Gly-Gly (Scherer et al., 2010). The pPLAIII subfamily differs from pPLAI and pPLAII 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 FFAs (Li et al., 2011). Moreover, OE of pPLAIIIβ increases membrane glycerolipid content in vegetative tissues, whereas its gene knockout (KO) has the opposite effect. These observations prompted us to determine the role of pPLAIIIs in seed oil production. Here, we show that pPLAIIIβ promotes TAG production with increased accumulation of long-chain fatty acids in Arabidopsis seeds.

### Results

**pPLAIIIβ Increases Seed Oil Content**

To investigate the function of pPLAIIIs in seed oil production, we isolated transfer DNA (T-DNA) insertional KO mutants for all four pPLAIIIs (Supplemental Fig. S1). The T-DNA insertion sites of pPLAIIIα and pPLAIIIβ are in the first exon, while the insertion sites of pPLAIIIγ and pPLAIIIδ locate in the 5’ untranslated region (Supplemental Fig. S1B). All of these insertional mutants have a negligible level of transcript as measured by real-time PCR of pPLAIIIα, pPLAIIIγ, and pPLAIIIδ (Fig. 1A). The loss of pPLAIIIβ expression in pPLAIIIβ-KO was described previously (Li et al., 2011). However, only the pPLAIIIβ-KO seeds, not the other pPLAIIIs, displayed a significant change in oil content compared with wild-type seeds; the oil contents of pPLAIIIβ-KO and wild-type seeds were 33% and 35.5% of the seed weight, respectively (Fig. 1B). To confirm the effect of pPLAIIIβ on seed oil production, we genetically complemented the KO by transferring pPLAIIIβ with its native promoter and terminator sequences into the KO mutant (designated as COM; Supplemental Fig. S1C). Expression of pPLAIIIβ in the COM lines was restored to the wild-type level (Fig. 1C), and the oil content in COM seeds was the same as that of the wild type (Fig. 1D).

**Figure 2.** pPLAIIIβ increases 20C fatty acid content at the expense of 18C fatty acids. A. Fatty acid (FA) compositions of pPLAIIIβ KO, OE, COM, and wild-type (WT) seeds. Values are means ± SE (n = 3). HSignificantly higher and Lsignificantly lower, each at P < 0.05 compared with the wild type, based on Student’s t test. B, 20C/18C ratio in pPLAIIIβ KO, OE, COM, and wild-type 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 wild type, based on Student’s t test.
Analysis of mRNA accumulation patterns for pPLAIIIs in seeds indicates that pPLAIIIA, pPLAIIIB, and pPLAIIIC were expressed in tissues that do not accumulate large amounts of TAG in developing seeds (Supplemental Fig. S2, A–E). In mature green seeds, pPLAIIIC was expressed mostly in seed coat, pPLAIIIB mostly in chalazal seed coat, and pPLAIIIA mostly in seed coats and peripheral endosperm (Supplemental Fig. S2, C–E). In contrast, pPLAIIIA was expressed in developing radicle and in cotyledons, the major storage tissue for seed oil in Arabidopsis (Supplemental Fig. S2F). The mRNA accumulation pattern of the pPLAIII genes is consistent with a pPLAIIIA-specific effect on seed oil content; thus, further analysis was focused on pPLAIIIA.

To further investigate pPLAIIIA function, we produced multiple OE Arabidopsis lines by placing pPLAIIIA under the control of cauliflower mosaic virus 35S promoter (35S::pPLAIIIA-OE; Supplemental Fig. S1C). The mRNA level of pPLAIIIA was increased substantially in OE over wild-type plants (Fig. 1C). The presence of the introduced GFP-tagged pPLAIIIA was detected by immunoblotting with a GFP antibody (Fig. 1E). Seed oil content in two OE lines was approximately 40.5%, which was 5% higher than that of the wild type (35.5%; Fig. 1D). Taken together, these data indicate that pPLAIIIA plays a positive role in seed oil accumulation.

pPLAIIIA Increases 20-Carbon Fatty Acid Content at the Expense of 18-Carbon Fatty Acids

The fatty acid composition was significantly altered in pPLAIIIA-KO and 35S::pPLAIIIA-OE seeds (Fig. 2A). The levels of 18-carbon fatty acids tended to increase in KO and decrease in OE seeds compared with the wild type. 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

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**Figure 3.** pPLAIIIA promotes the accumulation of 20C-containing TAG species. A, Normalized mass spectra (as a percentage of the total) from TAG species with the indicated fatty acyl combinations in wild-type, 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 the 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).

B, Normalized mass spectra (as a percentage of the wild type) from TAGs, grouped by total acyl carbons, were classified as C50, C52, C54, C56, C58, and C60. The major components in C50, C52, and C54 are 18C fatty acyl-containing TAGs, while C56, C58, and C60 are TAGs containing one or more 20C fatty acyl-containing TAGs. Values are means ± se (n = 5).

**A**

| TAG level (%) of total TAG MS signal | WT | KO | OE1 | OE2 |
|-----------------------------------|----|----|-----|-----|
| C50                               |    |    |     |     |
| C52                               |    |    |     |     |
| C54                               |    |    |     |     |
| C56                               |    |    |     |     |
| C58                               |    |    |     |     |
| C60                               |    |    |     |     |

**B**

| Relative TAG level (%) of WT | WT | KO | OE1 | OE2 |
|-----------------------------|----|----|-----|-----|
| C50                         |    |    |     |     |
| C52                         |    |    |     |     |
| C54                         |    |    |     |     |
| C56                         |    |    |     |     |
| C58                         |    |    |     |     |
| C60                         |    |    |     |     |

**Significantly higher and significantly lower, each at P < 0.05 compared with the wild type, based on Student’s t test.**
12% and 12% in KO, while 20:0 and 20:1 were increased by 12% and 15% in OE lines, compared with the wild type. 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 the wild type (Fig. 2B). The fatty acid composition in COM seeds was similar to that of wild-type seeds (Fig. 2B). Thus, the increased mRNA level of pPLAIII促进了 the accumulation of longer chain fatty acids at the expense of 18-carbon fatty acids, 18:1 and 18:2, whereas pPLAIII-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 lengths and degrees of unsaturation in the three acyl chains. Three acyl chains in TAG are not randomly distributed. Since pPLAIII affects 18:1 and 20:1 accumulation in TAG, we wondered if pPLAIII alters the distribution of three acyl chains and thus produces some unique TAG molecule species. Therefore, we analyzed the TAG species in wild-type, KO, and OE seeds by electrospray ionization-tandem mass spectrometry (ESI-MS/MS). 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; Fig. 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 and lower in OE mutants when compared with the wild type, while the levels of C56, C58, and C60 TAG species were changed in the opposite manner in KO and OE lines of pPLAIII (Fig. 3; Supplemental Fig. S3). 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 the wild type (Fig. 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 the wild type (Fig. 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 with the wild type (Fig. 3B). Measurement of 113 additional TAG species and eight TAG species groups confirmed the trend for the percentages of 18C-containing TAGs to be lower and the 20C-containing TAGs to be higher in OE lines compared with the wild type (Supplemental Fig. S3, A–E).

Taken together, these data indicate that pPLAIII promotes the accumulation of 20C-containing TAG species.

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

To gain insight into how pPLAIII facilitates TAG accumulation and modification, we measured the mRNA levels of selected genes in TAG and PC synthesis and metabolism in developing Arabidopsis siliques (Fig. 4). In the Kennedy pathway of TAG

**Figure 4.** pPLAIII increases the RNA levels of genes in TAG and PC synthesis in siliques. RNA levels were determined by real-time PCR and normalized in comparison with UBQ10. Values are means ± se (n = 3 technical replicates). **Significantly higher at P < 0.05 compared with the wild type, based on Student’s t test.** WT, Wild type.
biosynthesis, glycerol-3-P is sequentially acylated by glycerol phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAT), followed by phosphatidic acid phosphohydrodrolase (PAH) and diacylglycerol acyltransferase (DGAT). The transcript levels for the genes in the Kennedy pathway, including GPAT, LPAT2, LPAT3, PAH, and DGAT1, were increased 2- to 5-fold in pPLAIIIb-OE lines (Fig. 4A). By comparison, mRNA levels of both DGAT2 and LPAT5 were the same in wild-type, pPLAIIIb-KO, and 35S::pPLAIIIb-OE siliques (Fig. 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 3-fold in OE lines compared with the wild type (Fig. 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 AAPTs in Arabidopsis. Compared with the wild type, the mRNA levels of CCT2 and AAPT1 were increased almost by 10-fold, whereas the increase in CCT1 and AAPT2 was about 2-fold in pPLAIIIb-OE siliques (Fig. 4B). The mRNA abundance of Lyso phosphatidylcholine: acyl-coenzyme A acyltransferase1 (LPCAT1) was also increased 3-fold in OE lines (Fig. 4B). These data demonstrate that OE of pPLAIIIb increases the mRNA levels of genes involved in TAG and PC synthesis. On the other hand, in KO siliques, the mRNA levels for the lipid-metabolizing genes were not significantly different from that of the wild type, even though the mRNA levels for several of these genes tended to be lower than that of the wild type (Fig. 4). These results suggest that the loss of pPLAIIIb may be partially compensated for by other pPLAs.

pPLAIIIb Hydrolyzes PC and Acyl-CoA and Affects Acyl-CoA Levels in Arabidopsis

pPLAIIIb is more distantly related to the other three pPLAIIIs than they are to each other (Supplemental Fig. S1A). pPLAIIIb has an Asp in the Asp-Gly-Gly catalytic dyad motif, similar to pPLAs in the other groups, whereas in pPLAIIIb and pPLAIIIy, the Asp is replaced by Gly (Li et al., 2011). To determine the enzymatic function of pPLAIIIb, we expressed 6XHis-tagged pPLAIIIb in Escherichia coli and purified it to near homogeneity (Fig. 5A). The PC-hydrolyzing activity of pPLAIIIb was examined because PC is the most abundant phospholipid and serves as a key intermediate for TAG synthesis. Incubation of pPLAIIIb with 16:0-18:2-PC resulted in the production of FFA and LPC. pPLAIIIb hydrolysis at the sn-1 position produces 16:0-FFA and 18:2-LPC (Fig. 5B), whereas pPLAIIIb hydrolysis at the sn-2 position produces 18:2-FFA and 16:0-LPC (Fig. 5C). The production of 18:2-FFA was approximately 5-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 pPLAIIIb hydrolyzes PC at both the sn-1 and sn-2 positions and that pPLAIIIb preferentially releases 18:2 from the sn-2 position.

In addition, we determined whether pPLAIIIb could hydrolyze acyl-CoA, because our previous study showed that another pPLAIII member, pPLAIIIb, has thioesterase activity (Li et al., 2011). Incubation of pPLAIIIb with 18:3-CoA resulted in the steady production of 18:3-FFA with increasing reaction time (Fig. 6A), indicating that pPLAIIIb possesses a thioesterase activity. We then determined whether the alterations of pPLAIIIb 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 the wild type (Fig. 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 the levels of 16:0-CoA and 18:2-CoA were significantly lower in OE, than in wild-type siliques (Fig. 6C). These data are consistent with pPLAIIIb functioning as an acyl-CoA thioesterase activity in vivo.
pPLAIII6 Is Associated with the Plasma and Intracellular Membranes

To determine its subcellular association, a GFP-tagged pPLAIII6 was expressed in Arabidopsis, and the green fluorescence signal of pPLAIII6-GFP was mostly detected on the inner cell boundary of leaf epidermal cells (Fig. 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 (Fig. 7B). To further analyze the intracellular association, total leaf proteins were fractionated into cytosolic and microsomal fractions. All intracellular association, total leaf proteins were fractionated into the plasma membrane (Fig. 7B). To further analyze the intracellular association, total leaf proteins were fractionated into cytosolic and microsomal fractions. All intracellular association, total leaf proteins were fractionated into the plasma membrane and intracellular membrane fractions. Approximately 80% of pPLAIII6-GFP was associated with the plasma membrane, whereas 20% was associated with intracellular membranes based on the intensity of the protein bands (Fig. 7C). These data indicate that pPLAIII6 is associated with both the plasma and intracellular membranes.

Seed-Specific OE of pPLAIII6 Increases Oil Content

The increased oil content in seeds raises the question of whether increased pPLAIII6 expression can be used to increase seed oil production. However, constitutive OE of pPLAIII6 resulted in a decrease in plant height and overall seed yield (Fig. 8, A and B). The seed yield per 35S::pPLAIII6-OE plants was approximately 50% of that of wild-type plants (Fig. 8B). To explore whether the improved oil content could be uncoupled from decreased seed production, we placed pPLAIII6 under the control of the seed-specific promoter of soybean (Glycine max) β-conglycinin (CON::pPLAIII6; Supplemental Fig. S4A). The level of pPLAIII6 expression in developing siliques was 25-fold higher in CON::pPLAIII6 than that in the wild type (Fig. 8C). The presence of the pPLAIII6-GFP protein was detectable by visualizing the GFP fluorescence (Supplemental Fig. S4B). CON::pPLAIII6 plant height and seed yield were comparable with the wild type (Fig. 8, A and B). In three CON::pPLAIII6 lines tested, seed oil content was increased over wild-type seeds (39% versus 35%; Fig. 8D). While oil content per CON::pPLAIII6 seed weight was lower than that per 35S::pPLAIII6 seed weight (Figs. 1D and 8D), the overall seed oil production per CON::pPLAIII6 plant was significantly higher than that per 35S::pPLAIII6, due to the higher seed yield per plant (Fig. 8B), and per wild-type plant, due to the increased oil content without change in seed yield (Fig. 8, B and D).

The seed-specific overexpression of pPLAIII6 resulted in changes in fatty acid composition, and the changes in CON::pPLAIII6 were similar to those in 35S::pPLAIII6 seeds. The percentages of 18:1 and 18:2 were lower, while those of 20:0, 20:1, 20:2, and 22:1 were higher, in CON::pPLAIII6 lines than in the wild type (Fig. 8E). The ratio of 20:1 to 18:1 was 30% higher in CON::pPLAIII6 lines than in the wild type (Supplemental Fig. S4C), and the same pattern was observed when total 20-carbon fatty acids were compared.

Figure 6. pPLAIII6 hydrolyzes acyl-CoA and affects acyl-CoA levels in Arabidopsis. A, FFA 18:3 (18:3-FFA) released from 18:3-CoA in 16:0-18:2 PC vesicles by purified pPLAIII6. Vector refers to a control in which proteins from E. coli transformed with an empty vector were isolated using the same immunopurification procedure for purifying pPLAIII6-6×His. Values are means ± se (n = 3). B, Total acyl-CoA contents in developing Arabidopsis siliques of wild-type (WT), pPLAIII6 KO, OE, and COM plants. FW, Fresh weight. C, Levels of acyl-CoA molecular species in developing siliques of wild-type, pPLAIII6 KO, OE, and COM plants. Acyl-CoAs were extracted from developing siliques 7 d post pollination and analyzed by liquid chromatography–ESI-MS/MS. Values are means ± se (n = 5). *Significantly higher and †significantly lower, each at P < 0.05 compared with the wild type, based on Student’s t test.

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with total 18-carbon fatty acids (Supplemental Fig. S4D). These results indicate that pPLAIIId affects TAG metabolism in the same manner regardless of the promoter used and that the use of seed-specific expression of pPLAIIId has the potential to be applied for increased seed oil production.

DISCUSSION

These data show that pPLAIIId positively impacts seed oil content. Whereas pPLAIIId-KO decreases seed oil content, pPLAIIId-OE, driven either by a constitutive or a seed-specific promoter, increases seed oil content. pPLAIIId hydrolyzes PC to generate FFA and LPC. pPLAIIId may accelerate acyl flux from the plastid to the 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 (Fig. 9). Lipid trafficking between organelles is a fundamental, yet poorly understood, process in plants. In recent years, excellent progress has been made toward understanding lipid transport from the ER to the plastid for the synthesis of galactolipids (Wang et al., 2012b). Phosphatidic acid (PA) is imported into the plastid through a protein complex (Wang and Benning, 2012). In contrast, the metabolic and regulatory mechanisms by which fatty acids in the plastid are trafficked to the ER are unknown.

16:0 and 18:1 are two major fatty acids exported from the plastid in Arabidopsis (Pidkowich et al., 2007; Li-Beisson et al., 2010). FFAs 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 convert these fatty acids to acyl-CoA. In the conventional Kennedy pathway, acyl-CoA is used for the sequential acylation of glycerol-3-P → LPA → PA → DAG → TAG (Fig. 9). However, kinetic labeling data indicate that fatty acids exported from the plastid are first incorporated into PC and then channeled to TAG in soybean embryos (Bates et al., 2009, 2012; Bates and Browse, 2011). The presence of highly active LPCAT on the Arabidopsis plastid outer envelope membrane is consistent with the formation of PC using fatty acids from the plastids (Tjellström et al., 2012; Wang et al., 2012a). Recent data indicate that LPCAT1 and LPCAT2 catalyze the incorporation of fatty acids into PC in Arabidopsis seeds (Bates et al., 2012; Wang et al., 2012a). However, knowledge is lacking about...
what enzyme produces LPC that impacts 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 remains unclear (Chapman and Ohlrogge, 2012). pPLAIII could be one of the enzymes hydrolyzing PC to produce an FFA and LPC that LPCAT uses to accept fatty acids from the plastid (Fig. 9). The combined activity of pPLAIII and LPCATs may modulate the rate of fatty acid trafficking from the plastid to the ER in Arabidopsis seeds.

Fatty acids, such as 18:1, released from PC by pPLAIII may enter the acyl-CoA pool for elongation (Fig. 9). KO and OE of the pPLAIII gene displayed opposite effects on the levels of 18:1 and 20:1 fatty acids in seed oil. Detailed profiling of TAG molecules

Figure 8. Seed-specific OE of pPLAIII increases oil content without compromising seed production. A, Plant heights of mature wild-type (WT), pPLAIII-KO, 35S::pPLAIII, and CON::pPLAIII plants. 35S represents the 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 wild-type, pPLAIII-KO, 35S::pPLAIII, and CON::pPLAIII plants. Values are means ± se (n = 5). C, Transcript levels of pPLAIII in developing siliques of the wild type and three independent CON::pPLAIII lines. The RNA level was determined by realtime PCR and normalized in comparison with UBQ10. Values are means ± se (n = 3). D, Seed oil contents in the wild type and three independent CON::pPLAIII lines. Values are means ± se (n = 3). E, Fatty acid (FA) composition in wild-type and CON::pPLAIII seeds. Values are means ± se (n = 3). HSignificantly higher and Lsignificantly lower, each at P < 0.05 compared with the wild type, based on Student’s t test.
also showed the opposite effects on the levels of 18:1-containing and 20:1-containing TAGs by KO and OE of the 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 (Li-Beisson et al., 2010), whereas acyl-CoA is used for fatty acid elongation to form longer chain fatty acids, such as 20:1 (Joubès et al., 2008; Li-Beisson et al., 2010). The effect of pPLAIIIδ on fatty acid composition is distinctively different from that of the recently described PC:DAG cholinephosphotransferase that transfers phosphocholine from PC to DAG, and a mutation of PC:DAG cholinephosphotransferase decreases the 18:2 and 18:3 level in Arabidopsis seed TAG by 40% (Lu et al., 2009). Thus, increased pPLAIIIδ expression may facilitate the release of 18:1 from PC for 20:1 production (Fig. 9).

Compared with the wild type, OE of pPLAIIIδ had a lower acyl-CoA pool size in developing siliques 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 fatty acids 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, PA phosphatase, 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 are connected to the altered mRNA levels and potentially gene expression requires further investigation. In budding yeast (Saccharomyces cerevisiae), 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 LCAT, 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 that genetic alterations of pPLAIIIδ expression change seed oil content and fatty acid composition in Arabidopsis seeds. Our large-scale 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 the ER and/or PC fatty acyl remodeling for TAG synthesis. Furthermore, these results indicate that the use of seed-specific expression of pPLAIIIδ has the potential to improve seed oil production in crops.
Phospholipid Turnover in Triacylglycerol Production

MATERIALS AND METHODS

Generation of pPLAIII KO, OE, and Complementation Plants

Arabidopsis (Arabidopsis thaliana) T-DNA insertional mutants for pPLAIII (Salk_040563), pPLAIIIb (Salk_057212), pPLAIIIi (Salk_088404), and pPLAIIIb (Salk_024970) were identified from the Salk Arabidopsis T-DNA KO collection obtained from the Arabidopsis Biological Resource Center. The homozygous T-DNA insertion mutant for individual pPLAIII was verified by PCR-based screening using a T-DNA left border primer and gene-specific primers as listed in Supplemental Table S1. The isolation of pPLAIIIb-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 (pPLAIII-COM), the genomic DNA sequence of pPLAIII from the promoter region to the terminator region was cloned using two primers as listed in Supplemental Table S1 and fused into binary vector pEC291 for plant transformation.

To overexpress pPLAIIIb, the genomic sequence of pPLAIIIb was obtained by PCR using ecotype Columbia Arabidopsis genomic DNA as a template and primers listed with bars. The cDNA was cloned into the pET28a vector before the GFP-His coding sequence. The expression of the pMDC83 vector before the GFP-His fusion protein was verified by PCR-based screening using a T-DNA left border primer and gene-specific primers as listed in Supplemental Table S1. The isolation of pPLAIIIb-KO was reported previously (Li et al., 2011). The isolation of pPLAIII-KO was confirmed by real-time PCR. To generate the complementation lines (pPLAIII-COM), the genomic DNA sequence of pPLAIII from the promoter region to the terminator region was cloned using two primers as listed in Supplemental Table S1 and fused into binary vector pEC291 for plant transformation.

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 cetyltrimethylammonium bromide method (Stewart and Via, 1993). DNA contamination in RNA samples was removed with RNase-free DNase. cDNA was synthesized using iScript premix and a cDNA template. SYBR Green real-time PCR was performed as described previously (Li et al., 2006, 2011). The 60°C melting curve was used to detect primer dimer formation. The data were expressed as 2-ΔΔCt (n = 3 replicates). Each experiment was performed in triplicate. The expression of the target gene was normalized to a control gene, UBIQ10. The real-time PCR primers are listed in Supplemental Table S2. The data were presented as means ± 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 milligrams of Arabidopsis seeds was placed in glass tubes with Teflon-lined screw caps, and 1.5 mL of 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 separated using a SUPELCOWAX-10 (0.25 mm × 30 m) column with helium as a carrier gas at 20 mL min−1 detection by flame ionization. The oven temperature was maintained at 170°C for 1 min and then ramped to 210°C at 3°C min−1. FAMEs from TAG were identified by comparing their retention times with FAMEs in a standard mixture. Hepatideneic acid (17:0) was used as the internal standard to standardize the quantities of individual fatty acids. Fatty acid composition was expressed in weight percentage.

pPLAIIIb Cloning and Protein Purification from Escherichia coli

The full-length cDNA of pPLAIIIb was obtained by PCR using an Arabidopsis cDNA library as a template and a pair of primers as listed in Supplemental Table S1. The cDNA was cloned into the pET28a vector before the 6×His coding sequence. The 6×His 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 optical density at 600 nm of 0.7 and induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside for 16 h at 16°C. The pPLAIIIb-6×His fusion protein was purified as described previously (Pappan et al., 2004). Briefly, the bacterial pellet was resuspended in STE buffer containing 1 mg mL−1 lysozyme (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, and 1 mM EDTA). The samples were kept on ice for 30 min. Dithiothreitol and N-laurylsarcosine (Sarkosyl) were added to a final concentration of 5 mM and 1.5% (v/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). 6×His agarse beads were added (10%, v/v). The solution was gently rotated at 25°C for 1 h. The fusion proteins bound to agarse 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 of 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 μM 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 the indicated times and stopped by adding 2 mL of chloroform:methanol (2:1, v/v) and 500 μL of 25 μM 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 vitro enzyme assays, lipids were extracted for analysis as described previously (Li et al., 2011). Twenty microliters of lipid sample was combined with 340 μL of chloroform and 840 μL of chloroform:methanol:300 mM ammonium acetate in water (300:665:35). FAs were determined by ESI-MS on an 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 mass-to-charge ratio 500 to 350 (Li et al., 2011). LPC was determined with the same instrument as described previously (Li et al., 2011). Plants for acyl-CoA measurement were grown in glass chambers with a 12-h light/12-h dark cycle, at 23°C/21°C, 50% humidity, and 200 μmol m−2 s−1 light intensity, and watered with fertilizer once per week. Acyl-CoA was extracted and analyzed by liquid chromatography-ESI-MS/MS as described previously (Magnes et al., 2005; Han et al., 2010). TAG and glycerol 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 and Methods S1.

Microscopy Imaging and Subcellular Fractionation

The subcellular location of GFP-tagged protein was determined using a Zeiss LSM 510 confocal microscope equipped with a ×40 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- to 550-nm band-pass emission filter. Plasma-membrane in primary root cells was induced by immersing roots in 0.5 μM NaCl for 1, 3, and 5 min. Developing seeds from Arabidopsis siliques were imaged using a Nikon Eclipse 800 wide-field microscope and a ×60 differential interference contrast, 1.2-numerical aperture objective, with mercury lamp excitation and a 492/18 band pass excitation filter and a 535/40 band pass 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 resulting 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).

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SDS-PAGE and Immunoblotting

Leaf samples, each weighing approximately 1 g, were harvested and ground in 3 mL of buffer of 30 mM HEPES, pH 7.5, 400 mM NaCl, 1.0 mM phenylmethylsulfonyl fluoride, and 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.

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, At1g32220; LPAT1, At5g57150; LPAT3, At1g51260; LPAT4, At1g75200; LPAT5, At3g18850; LPCAT1, At1g6350; PAH1, At5g09560; PDAT1, At5g13640; pPLAILI, At1g61850; pPLAILII, At2g26560; pPLAILI, At4g37070; pPLAILI, At4g37060; pPLAILI, At4g37050; pPLAILI, At4g37040; pPLAILI, At4g37030; pPLAILI, At4g37020; pPLAILI, At4g37010; pPLAILI, At4g37000; pPLAILI, At4g36990; pPLAILI, At4g36980; pPLAILI, At4g363200; and LIPQ10, At4g05320.

Supplemental Data

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

Supplemental Figure S1. Generation of KO, OE, and complementation mutants of pPLAILI.

Supplemental Figure S2. RNA accumulation patterns of four pPLAILI genes in developing Arabidopsis seeds.

Supplemental Figure S3. pPLAILI 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 OE of pPLAILI in Arabidopsis.

Supplemental Table S1. PCR primers for mutant screening and molecular cloning.

Supplemental Table S2. Real-time PCR primers for quantitative measurement of transcript levels.

Supplemental Materials and Methods S1. Mass spectral analysis of TAG.

ACKNOWLEDGMENTS

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

Received February 27, 2013; accepted March 27, 2013; published March 29, 2013.

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