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ORIGINAL RESEARCH

Arabidopsis cytosolic acyl-CoA-binding proteins function in determining seed oil composition

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Abstract

As plant seed oils provide animals with essential fatty acids (FAs), genes that regulate plant lipid metabolism have been used in genetic manipulation to improve dietary seed oil composition and benefit human health. Herein, the *Arabidopsis thaliana* cytosolic acyl-CoA-binding proteins (AtACBPs), AtACBP4, AtACBP5, and AtACBP6 were shown to play a role in determining seed oil content by analysis of *atacbp* (*atacbp4*, *atacbp5*, *atacbp6*, *atacbp4atacbp5*, *atacbp4atacbp6*, *atacbp5atacbp6*, and *atacbp4atacbp5atacbp6*) seed oil content in comparison with the Col-0 wild type (WT). Triacylglycerol (TAG) composition in electrospray ionization-mass spectrometer (ESI-MS) analysis on *atacbp6* seed oil showed a reduction (−50%) of C58-TAGs in comparison with the WT. Investigations on fatty acid composition of *atacbp* mutants indicated that 18:2-FA accumulated in *atacbp6* and 18:3-FA in *atacbp4*, both at the expense of 20:1-FA. As TAG composition can be modified by acyl editing through phosphatidylcholines (PC) and lysophosphatidylcholines (LPC), total PC and LPC content in *atacbp6* mature seeds was determined and ESI-MS analysis revealed that LPC had increased (+300%) at the expense of PC. Among all the 14 tested PC species, all (34:1-, 34:2-, 34:3-, 34:4-, 34:5-, 34:6-, 36:2-, 36:3-, 36:5-, 36:6-, 38:2-, 38:3-, and 38:4-PCs) but 36:4-PC were lower in *atacbp6* than the WT. In contrast, all LPC species (16:0-, 18:1-, 18:2-, 18:3-, and 20:1-LPC) examined were elevated in *atacbp6*. LPC abundance also increased in *atacbp4atacbp5*, but not *atacbp4* and *atacbp5*. Interestingly, when LPC composition in *atacbp4atacbp5* was compared with *atacbp4* and *atacbp5*, significant differences were observed between *atacbp4atacbp5* and each single mutant, implying that AtACBP4 and AtACBP5 play combinatory roles by affecting LPC (but not PC) biosynthesis. Furthermore, PC-related genes such as those encoding acyl-CoA:lysophphosphatidylcholine acyltransferase (LPCAT1) and phospholipase A2 alpha (PLA2α) were upregulated in *atacbp6* developing seeds. A model on the role of AtACBP6 in modulating TAG through regulating *LPCAT1* and *PLA2α* expression is proposed. Taken together, cytosolic AtACBPs appear to affect

Guo and Ye contributed equally to this work.

unsaturated TAG content and are good candidates for engineering oil crops to enhance seed oil composition.

KEYWORDS
developing seeds, lysophosphatidylcholine, phosphatidylcholine, reversible lysophosphatidylcholine acyltransferase, triacylglycerols

1 | INTRODUCTION

In animals, two dietary essential fatty acids (EFAs), linoleic acid (LA; 18:2Δ*9*, 12), and α-linolenic acid (18:3Δ*9*, 12, 15) act as precursors to very-long-chain-polyunsaturated fatty acids (VLC-PUFAs), such as eicosatetraenoic acid (EPA) and docosahexaenoic acid (DHA) that are known to benefit human health (Nakamura & Nara, 2003). To obtain these VLC-PUFAs, humans and animals need to ingest plant seed oil, which is rich in the two EFAs (Ohlrogge & Browse, 1995), thereby making plant seed oil essential in human and animal diets. The major lipid component in plant seed oil, triacylglycerols (TAGs), is synthesized by the acylation of diacylglycerol (DAG) molecules (reviewed in Weselake et al. (2009)). DAG molecules are derived from either de novo biosynthesis through the Kennedy pathway, or from phosphatidylcholine (PC) (Bates, 2016). During acyl editing, *via* either the Lands Cycle or reversible acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT) activity, acyl groups are exchanged from PC without affecting net PC synthesis or turnover, allowing PC to support an acyl flux leading to the biosynthesis of other membrane lipids and TAGs (Bates, 2016).

In the Lands Cycle, fatty acids (FAs) from PC are thio-esterified by long-chain acyl-CoA synthetase (LACS) to form acyl-CoA esters before utilization in lipid biosynthesis. In contrast, FAs from PC are preferentially transferred to CoA by reversible LPCAT (Bates, 2016). LPCAT has been reported to play central roles in acyl editing of PC (Wang et al., 2012), and its reversible activity has been confirmed by studies on AtLPCAT2, which catalyzes acyl exchange between the acyl-CoA pool and PC (Jasieniecka-Gazarkiewicz, Demski, Lager, Stymne, & Banaś, 2016). In the *lpcat1lpcat2* double mutant, LPC accumulated over the WT, and the mRNAs corresponding to several *PHOSPHOLIPASE A* (*PLA*) genes were observed induced (Wang et al., 2012). Correspondingly, the rate of de novo PC synthesis as well as its turnover increased (Wang et al., 2012), indicating compensation in the lack of acyl editing by acyl flux increase through PC (Bates, 2016). Reverse genetics have also been used to demonstrate that, in the Kennedy pathway, the genes encoding GPAT, LPAAT, and DGAT regulate seed oil content (Jain, Coffey, Lai, Kumar, & MacKenzie, 2000; Jako et al., 2001; Maisonneuve, Bessoule, Lessire, Delseny, & Roscoe, 2010; Shrestha et al., 2018; Xu, Falarz, & Chen, 2019).

In plant seeds, fatty acids (FAs) must be thio-esterified to Coenzyme-A derivatives by acyl-CoA synthase before they can be used as precursors for the biosynthesis of other lipids or stored as TAGs (Ohlrogge & Browse, 1995). Acyl-CoA-binding proteins (ACBPs), present in eukaryotes and some prokaryotes (Burton, Rose, Faergeman, & Knudsen, 2005; Du, Arias, Meng, & Chye, 2016; Lung & Chye, 2016; Xiao & Chye, 2011; Ye & Chye, 2016), bind acyl-CoA esters to maintain an intracellular acyl-CoA pool as well as transport acyl-CoA esters in lipid metabolism (Du et al., 2016; Lung & Chye, 2016; Xiao & Chye, 2011; Ye & Chye, 2016). In plants, ACBPs have been reported to control the enzyme activities in the Kennedy pathway and affect seed oil lipid composition (Brown, Johnson, Rawsthorne, & Hills, 1998; Brown, Slabas, & Denton, 2002; Yurchenko & Weselake, 2011). Typical examples include the modulation of GPAT, LPAAT, and DGAT enzyme activities by *Brassica napus* ACBP (BnACBP) (reviewed in Yurchenko & Weselake, 2011). It has been demonstrated that the incubation of yeast microsomes with small amounts of BnACBP elevated DGAT activity by 20%, but further addition of BnACBP impaired TAG formation (Yurchenko & Weselake, 2011).

In *Arabidopsis thaliana*, three cytosolic *ACBPs* (*AtACBP4*, *AtACBP5*, and *AtACBP6*) are highly expressed in siliques (Hsiao, Yeung, Ye, & Chye, 2015). They play combinatory roles in floral and seed development (Hsiao et al., 2014, 2015; Ye, Xu, Shi, Zhang, & Chye, 2017). *AtACBP6pro::GUS* is highly expressed in cotyledonary-staged embryos, and knockout of *AtACBP6* caused C18:1-CoA accumulation in these embryos (Hsiao et al., 2014). Recombinant AtACBP6 was reported to bind long-chain acyl-CoA esters (C16- and C18-CoA) in vitro, while AtACBP4 and AtACBP5 showed lower affinity to these acyl-CoA esters (Hsiao et al., 2014).

The overexpression of BnACBP in Arabidopsis seeds caused an increase in total polyunsaturated fatty acids (PUFAs) and long-chain FAs, but led to a reduction in VLC-FAs in both the acyl-CoA pool and seed oil (Yurchenko et al., 2009, 2014). Herein, we followed up on the initial findings of Yurchenko et al. (2009) using Arabidopsis cytosolic ACBP knockout mutants to compare their seed oil to the WT. C58-TAGs and lysophosphatidylcoline (LPC) abundance declined in *atacbp6* seeds, while phosphatidylcoline (PC) increased. Furthermore, TAG-related marker gene expression was induced in *atacbp6* developing seeds.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

Arabidopsis thaliana single mutants *atacbp4* (SALK_040164) and *atacbp5* (SALK_134337) mutants were purchased from the Arabidopsis Biological Resource Center (ABRC) and were previously characterized in Xiao, Li, Zhang, Chan, and Chye (2008) and Hsiao et al. (2015), respectively. The double mutants *atacbp4atacbp5*, *atacbp4atacbp6*, *and atacbp5atacbp6*, and triple mutant

atacbp4atacbp5atacbp6 were generated by Hsiao et al. (2015). Seeds from the WT and mutant Arabidopsis were surface sterilized and plated on MS medium followed by cold stratification for 4 d. Seedlings were potted in soil and raised in a growth chamber with 23°C/21°C (day/night) cycles, followed by a day length regime of 16 hr light.

2.2 | β-glucuronidase (GUS) histochemical assays

Transgenic Arabidopsis *AtACBP4pro::GUS* and *AtACBP5pro::GUS* have been reported in Hsiao et al. (2015). Histochemical GUS assays were performed as previously described in Hsiao et al. (2015). Transgenic Arabidopsis were inoculated in GUS staining solution (100 mM sodium phosphate buffer, pH 7.0, 0.1% Triton X-100, 2 mM $K_3[Fe(CN)_6]$, 2 mM $K_4[Fe(CN)_6]\cdot 3H_2O$, and 1 mg/ml X-glucuronide), together with the vector pBI101.3-transformed control. Samples were vacuum infiltrated for 1 hr, followed by a 2-hr incubation at room temperature. Chlorophyll was removed by washing in 70% ethanol, and the samples were imaged under a dissection microscope.

2.3 | Lipid extraction and analysis

Three hundred mg of Arabidopsis seeds were heated for 10 min at 95°C in 1 ml isopropanol and homogenized using a mortar and pestle. The homogenate was centrifuged at 300 g for 15 min at room temperature, supernatant was collected, and the pellet was re-extracted with isopropanol/chloroform (1:1 *v/v*). Both extracts were pooled, evaporated, and dissolved in acetic acid/chloroform (1:100 *v/v*). Quantitative analyses of TAGs and phospholipids (PCs and LPCs) were carried out using electrospray ionization tandem triple-quadrupole mass spectrometry (API 4000 QTRAP; Applied Biosystems; ESI-MS/MS) (Lee, Welti, Schapaugh, & Trick, 2011; Ruiz-Lopez, Haslam, Napier, & Sayanova, 2014). The lipid extracts were infused at 15 μl/min with an autosampler (HTS-xt PAL, CTC-PAL Analytics AG). Data acquisition and acyl group identification of the polar lipids were as described in Ruiz-Lopez et al. (2014) with modifications. The internal standards for polar lipids were supplied by Avanti, incorporated as 0.857 nmol of 13:0-LPC and 0.086 nmol of di24:1-PC. The standards dissolved in chloroform and 25 μL of the samples in chloroform were combined with chloroform/methanol/300 mM ammonium acetate (300:665:3.5 *v/v/v*) to make a final volume of 1 ml.

The ESI-MS/MS method described by Ruiz-Lopez et al. (2014) was used to quantify TAGs. For quantifying TAGs, 15 μl of lipid extract and 0.857 nmol of tri15:0-TAG (Nu-Chek-Prep) were combined with chloroform/methanol/300 mM ammonium acetate (24:24:1.75 *v/v/v*), to final volumes of 1 ml for direct infusion into the mass spectrometer. TAGs were detected as $[M + NH_4]^+$ ions by a series of different neutral loss scans, targeting losses of FAs.

The data were processed using the program Lipid View Software (AB Sciex) where isotope corrections were applied. The peak area of each lipid was normalized to the internal standard and further normalized to the weight of the initial sample. There was variation in ionization efficiency among acyl glycerol species with different fatty acyl groups, and no response factors for individual species were determined in this study; therefore, the values were not directly proportional to the TAG abundance of each species. However, the approach did allow a realistic comparison of TAG species across samples in this study. Limit of detection was estimated at ~1% for FA analysis, ~0.5 nmol/mg for TAG analysis, ~0.1 nmol/mg for PC analysis, and ~0.002 nmol/mg for LPC analysis.

2.4 | RNA analysis

Arabidopsis developing seeds were isolated from 7-DAF siliques. TRIzol reagent (Invitrogen) was used for extraction of total RNA from 0.1 g of homogenized sample following the manufacturer's protocol. Subsequently, the total RNA was reverse transcribed using the SuperScript First-strand Synthesis System (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was conducted on a StepOne Plus Real-time PCR system using SYBR Green Mix (Applied Biosystems), and the program was as follows: 10 min at 95°C followed by 40 cycles of 95°C (15 s) and 56°C (1 min). For each reaction, three experimental replicates were performed with gene-specific primers (Table S5), and *Arabidopsis thaliana ACTIN2* was used as an internal control. The relative expression of the targeted genes was normalized using the *ACTIN2* control.

3 | RESULTS

3.1 | Differential *AtACBP4* and *AtACBP5* expression in Arabidopsis developing embryos

When 10-week-old transgenic Arabidopsis *AtACBP4pro::GUS* embryos at different developmental stages were analyzed, GUS expression was observed at the heart-staged embryo (Figure 1a), while embryos from torpedo to cotyledonary stages did not show obvious GUS activity (Figure 1b-d). In contrast, GUS expression was detected only at the cotyledonary-staged *AtACBP5pro::GUS* embryos (Figure 1e-h). These results suggest that *AtACBP4pro::GUS* expression does not overlap with *AtACBP5pro::GUS* in embryo development, consistent with microarray data from the EFP browser (http://bar.utoronto.ca/efp2/Arabidopsis/Arabidopsis_eFPBrowser2.html) (Figure S1).

3.2 | Seed oil composition was modified in Arabidopsis *atacbp6*

When seed oil content was examined using electrospray ionization-mass spectrometry (ESI-MS/MS), total TAG abundance in *atacbp4*, *atacbp5*,and *atacbp6* did not show any significant differences from the Col-0 wild type (WT, Table S1 and Figure S2). However, analysis on TAG composition on *atacbp6* seed oil revealed a reduction (−50%) of C58-TAGs in comparison with the WT (Figure 2 and Table S1). Although

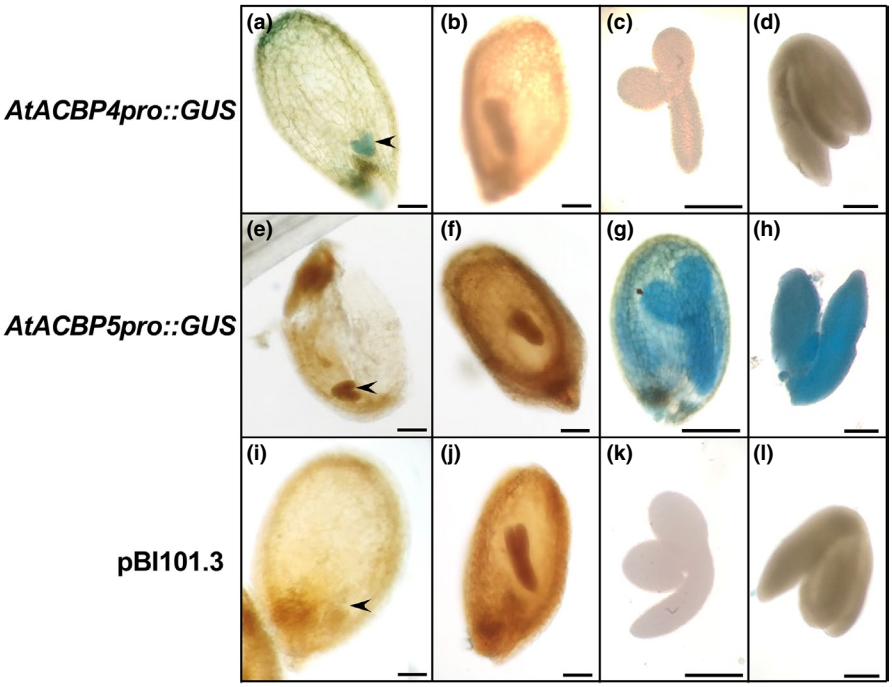

FIGURE 1 Histochemical GUS stains on transgenic Arabidopsis *AtACBP4pro::GUS* and *AtACBP5pro::GUS* developing embryos. Developing embryos from *AtACBP4pro::GUS* (a–d) and *AtACBP5pro::GUS* (e–h) were assayed for GUS activity. Same-staged embryos of the vector-transformed Arabidopsis (pBI101.3) (i–l) were used as the controls. (a) Heart-staged embryo from *AtACBP4pro::GUS*; (b) Torpedo-staged embryo from *AtACBP4pro::GUS*; (c) Mature cotyledonary-staged embryo excised from *AtACBP4pro::GUS*; (d) Mature embryo from *AtACBP4pro::GUS* dry seeds; (e) Heart-staged embryo from *AtACBP5pro::GUS*; (f) Torpedo-staged embryo from *AtACBP5pro::GUS*; (g) Mature cotyledonary-staged embryo from *AtACBP5pro::GUS*; (h) Mature embryo from *AtACBP5pro::GUS* dry seeds; (i) Heart-staged embryo from pBI101.3-transformant; (j) Torpedo-staged embryo from pBI101.3-transformant; (k) Mature cotyledonary-staged embryo from pBI101.3-transformant; (l) Mature embryo from pBI101.3 dry seed. Arrowheads indicate heart-staged embryo. Bars = 0.25 mm

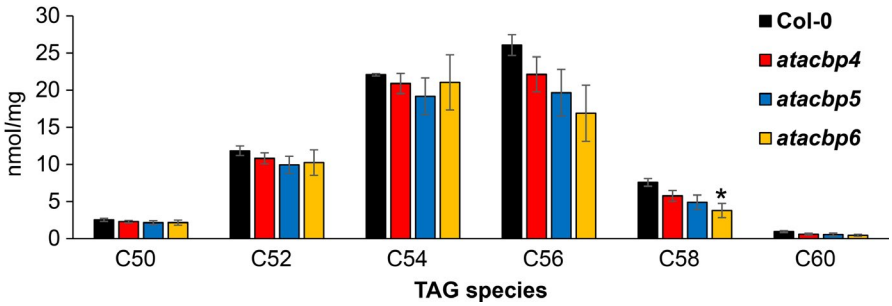

FIGURE 2 TAG composition in imbibed Arabidopsis seeds of *atacbp4*, *atacbp5*, and *atacbp6*. ESI-MS was carried out to determine TAG composition in 1-day-old *atacbp4*, *atacbp5*, and *atacbp6* imbibed seeds. Col-0 was used as a control. For each line, ~200 mg seed was used for each extraction. TAGs are grouped according to their carbon length. Values are mean ± *SE* of measurements made on three independent batches of samples. Asterisks indicate significant differences from Col-0 (*$p < .05$)

C54:2-TAG abundance was lower in *atacbp4* than Col-0 (Table S1), C50-, C52, C54-, C56-, C58-, and C60-TAG abundance did not differ between *atacbp4* or*atacbp5* and the WT (Figure 2). Also, significant changes in TAG abundance were not evident among *atacbp4atacbp5*, *atacbp4atacbp6*, and *atacbp4atacbp5atacbp6*, while *atacbp5atacbp6* showed higher total TAG abundance over the WT (Table S1).

Investigations on fatty acid (FA) composition of the *atacbp* mutants indicated that 18:2-FA accumulated in *atacbp6* at the expense of 20:1-FA, while 18:3-FA was elevated in *atacbp4* (Figure 3 and Table S2). When FA composition of the double mutant *atacbp4atacbp6* was analyzed, 18:2- or 18:3-FA content was not significantly different from the WT. Although 20:0- and 20:1-FAs had declined, 16:0-FA was elevated in the *atacbp4atacbp6* double mutant in comparison with the WT (Figure S3 and Table S2). However, FA composition in *atacbp4atacbp6* did not differ from either *atacbp4* or *atacbp6* (Figure S3).

3.3 | Cytosolic ACBPs modulate PC and LPC composition in Arabidopsis seeds

As TAG composition can be modified through acyl editing using PC and LPC, the total PC and LPC abundance of *atacbp4*, *atacbp5*,

and *atacbp6* seeds was determined using ESI-MS/MS (Figure 4). The results obtained revealed that in *atacbp6* mature seeds LPC was elevated (+300%) over the WT at the expense of PC (−33%) (Figure 4). As *atacbp6* showed increase in total LPC and reduction in total PC, PC and LPC composition in *atacbp6* imbibed seeds were subsequently analyzed (Figures 5 and 6). Among the 14 tested species, 34:1-, 34:2-, 34:3-, 34:4-, 34:5-, 34:6-, 36:2-, 36:3-, 36:5-, 36:6-, 38:2-, 38:3-, and 38:4-PCs (with the exception of 36:4-PC) were lower in *atacbp6* than the WT ($p > .05$; Figure 5). In contrast, LPC (16:0-, 18:1-, 18:2-, 18:3-, and 20:1-LPC) abundance increased in *atacbp6* over the WT (Figure 6).

Total PC composition was not affected in *atacbp4*, *atacbp5*, or *atacbp4atacbp5* in comparison with the WT (Table S3). However, LPC abundance increased in *atacbp4atacbp5*, but not *atacbp4* and *atacbp5*, in comparison with the WT (Table S4). Also, significant differences in LPC abundance were observed between *atacbp4atacbp5* and each single mutant (Figure 7).

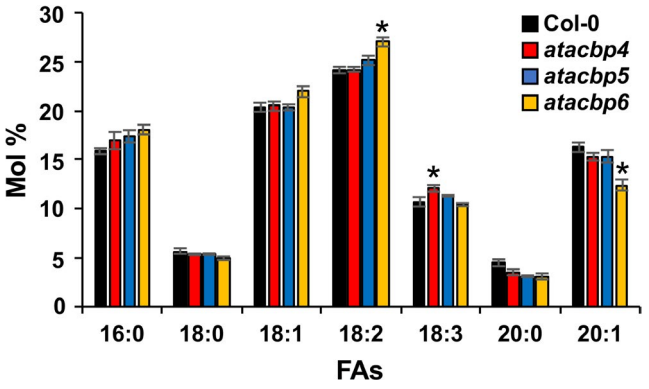

FIGURE 3 FA composition in imbibed Arabidopsis seeds of *atacbp4*, *atacbp5*, and *atacbp6*. ESI-MS was carried out to determine TAG FA composition in *atacbp4*, *atacbp5*, and *atacbp6* imbibed seeds. For each line, ~200 mg seed was used for each extraction. TAGs are grouped regarding their carbon length. Values are mean ± *SE* of measurements made on three independent batches of samples. Asterisks indicate significant differences from Col-0 (*$p < .05$) based on the Student's *t* test

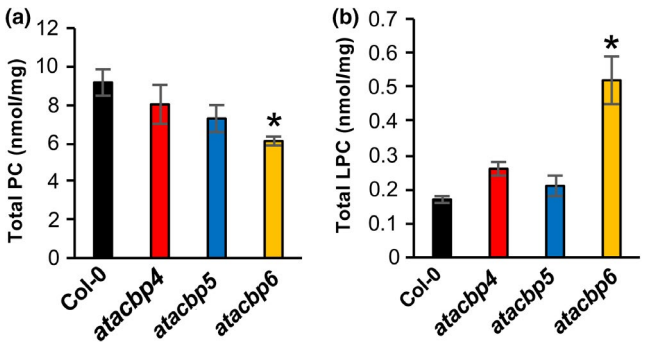

FIGURE 4 Analysis of phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) from imbibed Arabidopsis seeds of *atacbp4*, *atacbp5*, and *atacbp6*. ESI-MS was carried out to determine total PC (a) and LPC (b) content in *atacbp4*, *atacbp5*, and *atacbp6* imbibed seeds. Col-0 was used as a control. Values are mean ± *SE* of measurements made on three independent batches of samples. Asterisks indicate significant differences from Col-0 (*$p < .05$)

3.4 | Enhanced expression of acyl editing-related enzymes in Arabidopsis *atacbp* developing seeds

As LPCAT1 (Ståhl, Stålberg, Stymne, & Ronne, 2008) and PLA2α (Chen, Snyder, Greer, & Weselake, 2011) catalyzes PC formation and degradation, the expression of their corresponding genes in *atacbp* developing seeds was investigated. When the mRNAs from *atacbp6* 7 days after flowering (DAF), Arabidopsis developing seeds were analyzed using qRT-PCR, *LPCAT1*, and *PLA2α* expression was upregulated in *atacbp6* in comparison with the WT (Figure 8a). In contrast, *PLA2α* expression was elevated in *atacbp4 and atacbp5*, with even higher expression in the *atacbp4atacbp5* double mutant, while *LPCAT1* was not affected in any of these mutants (Figure 8b). Taken together, a model of AtACBP6 regulating TAG abundance and composition by the modulation of *LPCAT1* and *PLA2α* expression is proposed (Figure 9).

4 | DISCUSSION

4.1 | Cytosolic AtACBPs play combinatory roles in reproductive development

In this study, *AtACBP4* was demonstrated to be expressed at the early stages of embryo development using histochemical GUS staining, while *AtACBP5* was expressed later (Figure 1). In contrast, *AtACBP6* was found to be highly expressed throughout development in embryos (Hsiao et al., 2015; Ye et al., 2016). These profiles are consistent with *AtACBP4*, *AtACBP5*, and *AtACBP6* expression in siliques by qRT-PCR (Hsiao et al., 2015) and microarray analysis (Figure S1). Besides embryos, *AtACBP4* and *AtACBP5* mRNAs were both reported to be highly expressed in inflorescences (Hsiao et al., 2015). In histochemical GUS stains, *AtACBP5pro::GUS* was highly expressed in the early floral stages and *AtACBP4pro::GUS* at a later stage (Ye et al., 2017). In *atacbp5* flowers, *AtACBP4* mRNA was induced, whereas *AtACBP5* was upregulated in *atacbp4* flowers (Ye et al., 2017). The complementary functions of AtACBP4 and AtACBP5 in pollen development (Ye et al., 2017) were consistent to microarray prediction (https://www.arabidopsis.org/). Similar to *AtACBP4* and *AtACBP5*, *AtACBP6* was also expressed in anthers (Hsiao et al., 2015) besides embryos. In pollen grains, gas chromatography-mass spectrometry (GC-MS) from *atacbp4* and *atacbp4atacbp5* revealed that C18:0-FA content decreased but C18:3-FA accumulated in both mutants in comparison with the WT (Ye et al., 2017). Both *atacbp4* and *atacbp4atacbp5* mutants showed an increase in C16:0-dicarboxylic fatty acid, and in *atacbp5* and *atacbp4atacbp5* flower buds, hydroxyl fatty acids were elevated (Ye et al., 2017). However, pollen tube length was not affected in *atacbp4* and *atacbp4atacbp5*, while the *atacbp4atacbp5atacbp6* triple mutant displayed a significant reduction in pollen tube length (Ye et al., 2017). These results taken

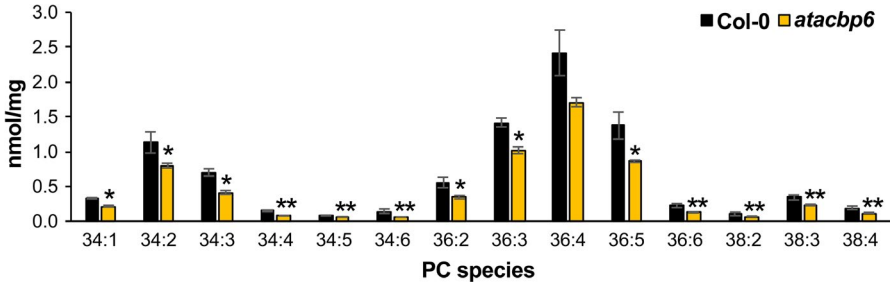

FIGURE 5 Analysis of phosphatidylcholine (PC) from imbibed Arabidopsis seeds of *atacbp6* and Col-0. Quantitative analysis of PCs (34:1, 34:2, 34:3, 34:4, 34:5, 34:6, 36:2, 36:3, 36:4, 36:5, 36:6, 38:2, and 38:4) from *atacbp6* and Col-0 imbibed seeds. Values (nmol/mg) are mean ± *SE* of measurements made on three independent batches of samples. Asterisks indicate significant differences from Col-0 (*$p < .05$, **$p < .01$)

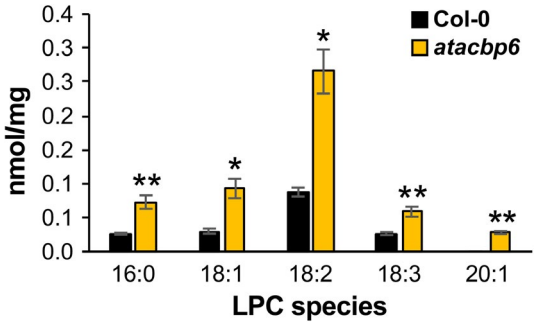

FIGURE 6 Analysis of lysophosphatidylcholine (LPC) from imbibed Arabidopsis seeds of *atacbp6* and Col-0. Quantitative analysis of LPCs (16:0, 18:1, 18:2, 18:3 and 20:1) from *atacbp6* and Col-0 Arabidopsis imbibed seeds. Values (nmol/mg) are mean ± *SE* of measurements made on three independent batches of samples. Asterisks indicate significant differences from Col-0 (*$p < .05$, **$p < .01$)

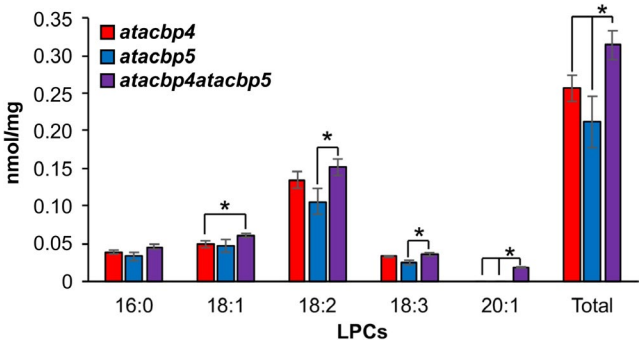

FIGURE 7 Analysis of lysophosphatidylcholine (LPC) from imbibed Arabidopsis seeds of *atacbp4*, *atacbp5*, and *atacbp4atacbp5*. Quantitative analysis of LPCs (16:0, 18:1, 18:2, 18:3, and 20:1) from *atacbp4*, *atacbp5*, and *atacbp4atacbp5* imbibed seeds. LPC composition in *atacbp4atacbp5* seeds was used as a control. Values (nmol/mg) are mean ± S.E. of measurements made on three independent batches of samples. Asterisks indicate significant differences (*$p < .05$)

together indicate that AtACBP4 and AtACBP5 possess overlapping roles in floral development.

The complementary functions of the Kelch-motif containing AtACBP4 and AtACBP5 in modulating LPC biosynthesis in seeds (Figure 7) are reminiscent of their collaborative role in seeds, affecting seed weight and germination rate (Hsiao et al., 2014), and in floral development, in regulating wax, and cutin composition in buds and FA composition in pollen grains (Ye et al., 2017). Complementation in function has also been observed between the two ankyrin repeat containing members in the AtACBP family (Chen et al., 2010). Although *atacbp1atacbp2* was embryo lethal, the two single mutants (*atacbp1* and *atacbp2*) germinated normally (Chen et al., 2010). Furthermore, in rosettes, *AtACBP1* mRNA in *atacbp2* and *AtACBP2* mRNA in *atacbp1* appeared upregulated in RT-PCR analysis (Chen et al., 2010), implying that these ankyrin repeat containing AtACBPs play overlapping roles in embryogenesis.

4.2 | ACBPs modulate gene expression in manipulating lipid metabolism

Phospholipase has also been reported to be required for Arabidopsis embryo development (Di Fino et al., 2017). Further to our observations that *PLA2α* was upregulated in *atacbp4*, *atacbp5*, *atacbp6*, and *atacbp4atacbp5* developing seeds (Figure 8), causing LPC increase in *atacbp6* and *atacbp4atacbp5* (Table S4), it has been reported that ACBPs affect the expression of *PHOSPHOLIPASE D (PLD)* to modify lipid metabolism in plants (Du, Chen, Chen, Xiao, & Chye, 2013; Du, Xiao, Chen, & Chye, 2010; Lung et al., 2017; Xiao et al., 2010).

PA-related *PLDα1* (Li, Hong, & Wang, 2009) mRNA was induced in 12-day-old Arabidopsis seedlings (Du et al., 2013) and 5-week-old transgenic Arabidopsis AtACBP1-OE rosettes (Du et al., 2010). AtACBP1-OE seeds were more sensitive to abscisic acid (ABA) inhibition and thus exhibited greater dormancy in comparison with the WT (Du et al., 2013), while 5-week-old AtACBP1-OE plants were more susceptible to frost than the WT (Du et al., 2010). In contrast, *PLDα1* was found to be upregulated in *atacbp1* seeds and siliques at 7-DAF (Lung et al., 2017). *PLDβ1*, *PLDγ*, and *PLDζ2* were upregulated in AtACBP3-OE rosettes, while membrane lipids from transgenic Arabidopsis AtACBP3-OEs were reduced in comparison with the WT, causing accelerated leaf senescence in Arabidopsis AtACBP3-OEs (Xiao et al., 2010). In AtACBP6-OE rosettes with and without cold acclimation, *PLDα1* was upregulated in cold-treated AtACBP6-OE rosettes, while *PLDδ* was highly expressed (Chen, Xiao, & Chye,

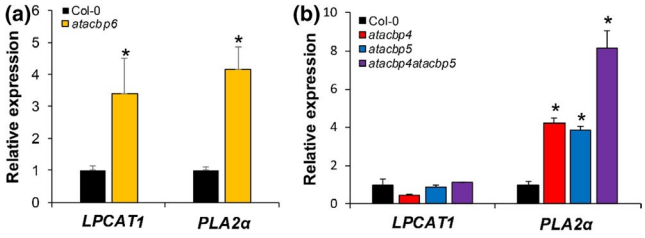

FIGURE 8 Expression of*LPCAT1* and *PLA2α* was affected in 7-DAF Arabidopsis *atacbp* mutant developing seeds. Siliques were dissected under microscope to release developing seeds at 7 DAF. Relative expression of *LPCAT1* (AT1G12640) and *PLA2α* (AT2G06925) from Arabidopsis Col-0 and *atacbp4*, *atacbp5*, *atacbp6* and *atacbp4*, *and atacbp5* were determined. The relative mRNA amount was normalized against the expression of Arabidopsis *ACTIN2* (AT3G18780). Expression of the indicated genes in Col-0 was set as 1 to normalize expression levels. Asterisks indicate significant differences from the Col-0 by the Student's *t* test ($p < .05$, $n = 3$)

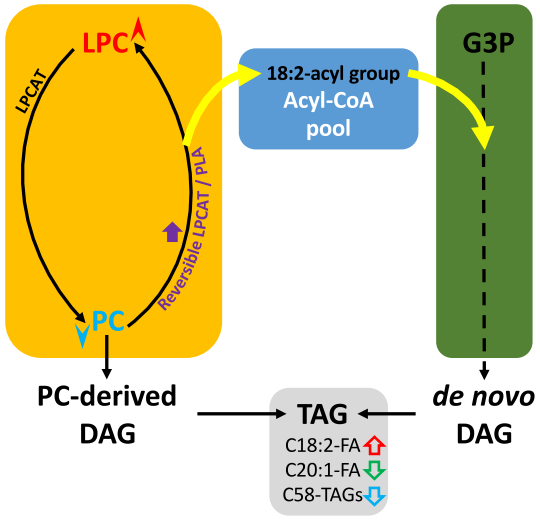

FIGURE 9 Proposed model on lipid metabolism in Arabidopsis *atacbp6* developing seeds. Proposed changes in the lipid metabolic network are displayed. PC-derived DAG production (yellow box) and the Kennedy pathway (green box) represent two routes for DAG biosynthesis (Bates, 2016). In *atacbp6*, *LPCAT1* and *PLA2α* were upregulated (Figure 8), boosting reversible LPCAT activity (purple arrow) with decrease in PC (blue arrowhead) and increase in LPC (red arrowhead) (Figure 4). Given that 18:2 (open red arrow) increased at the cost of 20:1 (open green arrow), leading to reduction of C58-TAGs (open blue arrow), and one possibility might be that the increased reverse LPCAT1 activity and/or PLA2α activity had higher preference to 18:2 on PC, thus leading to more 18:2 for TAG synthesis. G3P, glycerol-3-phosphate; LPC, lysophosphatidylcholine; LPCAT, lysophosphatidylcholine acyltransferase; DAG, diacylglycerol; PC, phosphatidylcholine; PLA, phospholipase A; and TAG, triacylglycerol

2008) and PC-related genes downregulated (Liao, Chen, & Chye, 2014).

4.3 | ACBPS affect seed TAG and phospholipid composition

Besides *PLA2α*, *LPCAT1* mRNA expression was upregulated in *atacbp6* (Figure 8). As 18:2 increased at the cost of 20:1 (Figure 3 and Table S2), leading to decrease in C58-TAGs (Figure 2 and Table S1), reverse LPCAT1 activity and/or PLA2α activity may have demonstrated higher preference to 18:2 on PC, leading to greater 18:2 utilization in TAG production. On the other hand, a reduction in PC implies enhanced PC turnover, thereby providing 18:2 through PC-derived DAG to TAG. PC had actually decreased (Figure 4a) to ~3 nmol/mg despite a mere (~0.3 nmol/mg) increase in LPC (Figure 4), implying a degradation of overaccumulated LPC by lysophospholipase to prevent LPC toxicity to cell membranes (Gao, Li, Xiao, & Chye, 2010).

Although C16:0-, C20:0-, C20:1-, and C22:0-FA content in *atacbp4atacbp6* seeds differed from the WT (Table S2), they did not vary from those FA species in *atacbp6* (Figure 7), indicating that FA content changes in *atacbp4atacbp6* resulted from *AtACBP6* knockout. Variation in seed oil fatty acid composition (Table S2) among the *atacbp* mutants (*atacbp4*, *atacbp5*, *atacbp6*, *atacbp4atacbp5*, *atacbp4atacbp6*, and *atacbp5atacbp6*) may have arisen from the differential binding affinities of AtACBP4, AtACBP5, and AtACBP6 to acyl-CoA esters (Hsiao et al., 2014).

Many other plant ACBPs have been reported to affect seed lipid composition (Chen et al., 2008; Du et al., 2013; Guo et al., 2019; Lung et al., 2017; Xiao et al., 2010; Yurchenko et al., 2014). Expression of a *BnACBP* cDNA in Arabidopsis developing seeds caused an increase in PUFAs at the expense of eicosenoic acid (20:1*cis*Δ11) and saturated FAs in seed oil (Yurchenko et al., 2014). In Arabidopsis, PA overaccumulated in 5-week-old AtACBP1-OE rosettes in comparison with the WT, while PC content decreased (Du et al., 2013). PA content also increased in AtACBP1-OE germinating seeds, while 32:0-PA declined detected in *atacbp1* seeds (Du et al., 2013). Although PA in 5-week-old rosettes and seeds of *atacbp1* Arabidopsis was not affected (Du et al., 2013), total FA was elevated in mature green *atacbp1* siliques (Lung et al., 2017). In *atacbp1* mature seeds, total FA content was higher than the WT (Lung et al., 2017). PC and phosphatidylethanolamine (PE) accumulated in 3-week-old Arabidopsis AtACBP3-OE leaves, while PC and PE were reduced in *atacbp3* and *AtACBP3*-RNAi leaves in comparison with the WT (Xiao et al., 2010). Cold-treated Arabidopsis *AtACBP6*-OE rosettes showed higher PA and lower PC content in comparison with the WT (Chen et al., 2008). Taken together, in *atacbp6* seeds, changes in LPC, PC, and TAG composition in comparison with the WT, may be associated with the alteration in the expression of *LPCAT* and *PLA2α* (Figure 9).

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CONFLICT OF INTEREST

The authors declare no conflict of interest associated with the work described in this manuscript.

AUTHOR CONTRIBUTION

ZHG performed qRT-PCR, ZWY collected seed samples, and RPH and LVM performed lipid analyses, supported by JAN. ZHG, ZWY, RPH, and MLC evaluated data. ZHG, ZWY, and MLC designed the research and wrote the manuscript with contribution from all authors.

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SUPPORTING INFORMATION

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