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

Ze-Hua Guo1 | Zi-Wei Ye1 | Richard P. Haslam2 | Louise V. Michaelson2 | Johnathan A. Napier2 | Mee-Len Chye1,3

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 atacb (atacbp4, atacb, atacb4atacb5, atacb4atacbp5, atacb5atacb6, and atacb4atacb5atacb6) seed oil content in comparison with the Col-0 wild type (WT). Triacylglycerol (TAG) composition in electrospray ionization-mass spectrometer (ESI-MS) analysis on atacb6 seed oil showed a reduction (~50%) of C58-TAGs in comparison with the WT. Investigations on fatty acid composition of atacb mutants indicated that 18:2-FA accumulated in atacb6 and 18:3-FA in atacb4, 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 atacb6 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 atacb6 than the WT. In contrast, all LPC species (16:0-, 18:1-, 18:2-, 18:3-, and 20:1-LPC) examined were elevated in atacb6. LPC abundance also increased in atacb4atacb5, but not atacb4 and atacb5. Interestingly, when LPC composition in atacb4atacb5 was compared with atacb4 and atacb5, significant differences were observed between atacb4atacb5 and each single mutant, implying that AtACBP4 and AtACBP5 play combinatorial roles by affecting LPC (but not PC) biosynthesis. Furthermore, PC-related genes such as those encoding acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT1) and phospholipase A2 alpha (PLA2α) were upregulated in atacb6 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...
1 | INTRODUCTION

In animals, two dietary essential fatty acids (EFAs), linoleic acid (LA; 18:2 Δ9, 12), and α-linolenic acid (18:3 Δ9, 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-Gazkiewicz, 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 lysophosphatidylcholine (LPC) abundance declined in atacbp6 seeds, while phosphatidylcholine (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 atacbpc5, atacbpc4atacbp5, and atacbpc5atacbp6, and triple mutant

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
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 K3[Fe(CN)6], 2 mM K4[Fe(CN)6]·3H2O, and 1 mg/ml X-glucuronide), together with the vector pB101.3-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 + NH4]+ 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
C54:2-TAG abundance was lower in atacb4 than Col-0 (Table S1), and C50-, C52, C54-, C56-, C58-, and C60-TAG abundance did not differ between atacb4 or atacb5 and the WT (Figure 2). Also, significant changes in TAG abundance were not evident among atacb4atacb5 or atacb4atacb6, while atacb5atacb6 showed higher total TAG abundance over the WT (Table S1).

Investigations on fatty acid (FA) composition of the atacb mutants indicated that 18:2-FA accumulated in atacb6 at the expense of 20:1-FA, while 18:3-FA was elevated in atacb4 (Figure 3 and Table S2). When FA composition of the double mutant atacb4atacb6 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 atacb4atacb6 double mutant in comparison with the WT (Figure S3 and Table S2). However, FA composition in atacb4atacb6 did not differ from either atacb4 or atacb6 (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 atacb4, atacb5,
and atacb6 seeds was determined using ESI-MS/MS (Figure 4). The results obtained revealed that in atacb6 mature seeds LPC was elevated (+300%) over the WT at the expense of PC (−33%) (Figure 4). As atacb6 showed increase in total LPC and reduction in total PC, PC and LPC composition in atacb6 imbibed seeds were subsequently analyzed (Figures 5 and 6). Among the 14 tested species, 34:1-, 34:2-, 34:3-, 34:4-, 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 atacb6 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 atacb6 over the WT (Figure 6).

Total PC composition was not affected in atacb4, atacb5, or atacb4atacb5atacb6 in comparison with the WT (Table S3). However, LPC abundance increased in atacb4atacb5atacb6, but not atacb4 and atacb5, in comparison with the WT (Table S4). Also, significant differences in LPC abundance were observed between atacb4atacb5 and each single mutant (Figure 7).

3.4 Enhanced expression of acyl editing-related enzymes in Arabidopsis atacb 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 atacb developing seeds was investigated. When the mRNAs from atacb7 days after flowering (DAF), Arabidopsis developing seeds were analyzed using qRT-PCR, LPCAT1, and PLA2α expression was upregulated in atacb6 in comparison with the WT (Figure 8a). In contrast, PLA2α expression was elevated in atacb4 and atacb5, with even higher expression in the atacb4atacb5 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 atacb5 flowers, AtACBP4 mRNA was induced, whereas AtACBP5 was upregulated in atacb4 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 atacb4 and atacb4atacb5 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 atacb4 and atacb4atacb5 mutants showed an increase in C16:0-distearoyl fatty acid, and in atacb5 and atacb4atacb5 flower buds, hydroxyl fatty acids were elevated (Ye et al., 2017). However, pollen tube length was not affected in atacb4 and atacb4atacb5, while the atacb4atacb5atacb6 triple mutant displayed a significant reduction in pollen tube length (Ye et al., 2017). These results taken...
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 atacb1atacb2 was embryo lethal, the two single mutants (atacb1 and atacb2) germinated normally (Chen et al., 2010). Furthermore, in rosettes, AtACBP1 mRNA in atacb2 and AtACBP2 mRNA in atacb1 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 atacb4, atacb5, atacb6, and atacb4atacb5 developing seeds (Figure 8), causing LPC increase in atacb6 and atacb4atacb5 (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 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 atacb1 seeds and siliques at 7-DAF (Lung et al., 2017). PLDγ1, PLDγ, and PLDδ 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, PLDα was upregulated in cold-treated AtACBP6-OE rosettes, while PLDδ was highly expressed (Chen, Xiao, & Chye,
Besides composition changes in seed oil fatty acid composition (Table S2) among Arabidopsis atacb p mutants (Chen et al., 2008; Du et al., 2013), total FA was elevated in mature green siliques (Lung et al., 2017). PC and phosphatidylethanolamine (PE) accumulated in 3-week-old Arabidopsis seeds, changes in LPC, PC, and TAG composition in 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 atacb p6, LPCAT1 and PL A2α 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 PL A2α 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; PL A, phospholipid A; and TAG, triacylglycerol also increased in AtACBP1-OE germinating seeds, while 32:0-PA declined detected in atacb p1 seeds (Du et al., 2013). Although PA in 5-week-old rosettes and seeds of atacb p1 Arabidopsis was not affected (Du et al., 2013), total FA was elevated in mature green atacb p1 siliques (Lung et al., 2017). In atacb p1 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 atacb p3 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 atacb p6 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 PL A2α (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.

REFERENCES


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