Characterization of a PLDζ2 homology gene from developing castor bean endosperm

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Abbreviations

RA, ricinoleic acid; TAG, triacylglycerols; DAG, diacylglycerol; PLD, phospholipase D; PtdOH, phosphatidic acid; PtdCho, phosphatidylcholine; PAP, phosphatidic acid phosphatase; GPAT,glycerol-3-phosphate acyltransferase; LPAAT, lysophosphatidic acid acyltransferase; DGAT, acyl-CoA:diacylglycerol acyltransferase; lysoPtdOH, lysophosphatidic acid; HFA, hydroxyl fatty acids; LPCAT, lysophosphatidylcholine

26 acyltransferase; PLA2, phospholipase A2; PDCT, phosphatidylcholine:diacylglycerol
27 cholinephosphotransferase; PDAT, phospholipid:diacylglycerol acyltransferase;
28

Abstract

Castor oil contains approximately 90% ricinoleic acid (RA) which is stored mainly in the form of tri-ricinoleic acid containing triacylglycerols (TAG). Ricinoleate is synthesized from oleate (18:1n-9cis) esterified to the sn-2 position of phosphatidylcholine (PtdCho) catalyzed by oleoyl-12-hydroxylase. PtdCho-derived diacylglycerol (DAG) is an important substrate pool for TAG synthesis, and the interconversion between PtdCho and DAG has been shown to play a critical role in channeling hydroxy fatty acids (HFA) to TAG. Although phospholipase D (PLD) has been reported to catalyze the hydrolysis of PtdCho to produce phosphatidic acid which can then be converted to DAG, its potential functions in the channeling of RA from PtdCho to DAG and the assembly of RA on TAG is largely unknown. In the present study, 11 PLD genes were identified from the Castor Bean Genome Database. Gene expression analysis indicated that *RcPLD9* is expressed at relatively high levels in developing seeds compared to other plant tissues. Sequence and phylogenetic analyses revealed that *RcPLD9* is a homolog of Arabidopsis *PLDζ2*. Overexpression of *RcPLD9* in the Arabidopsis CL7 line producing C18-HFA resulted in RA content reductions in the polar lipid fraction (mainly PtdCho) and mono-HFA-TAG, but increased RA content in di-HFA-TAG. Since part of RA in di-HFA-TAG is derived from HFA-DAG, the results indicated that RcPLD9 facilitates the channeling of RA from PtdCho to DAG for its assembly on TAG in developing seeds.

Introduction

Castor bean (*Ricinus communis* L.) is an important oilseed crop for industrial use. Castor bean accumulates approximately 60% of oil mainly in the form of triacylglycerol (TAG), with up to 90% ricinoleic acid (RA, 12-hydroxy-octadeca-9-enoic acid) (da Silva Ramos et al., 1984). The hydroxyl group (-OH) provides unique properties to RA and makes this special fatty acid an attractive feedstock for the production of high-performance lubricants, cosmetics, polymers, surfactants, and coatings (Caupin, 1997; McKeon, 2016). However, castor is not commercially cultivated in many countries due to the presence of toxic ricin and allergenic 2S albumins in seeds (Severino et al., 2012). As a result, the supply of castor oil has fallen short of demand (https://www.castoroilworld.com/statistics-market-demand-future-trend/, accessed on 04 October, 2018). Genetic engineering is a potential strategy for producing RA in existing oil crop species, such as oilseed rape, to meet the existing and future demand. To achieve this goal, it is essential to uncover the mechanism of RA biosynthesis and accumulation in castor bean.

In the past decade, many genes responsible for RA synthesis and its accumulation in castor bean have been identified by functionally characterizing them in the model plant *Arabidopsis* due to the lack of efficient castor transformation methods (van de Loo et al., 1995; Smith et al., 2003; Lu et al., 2006; Burgal et al.,2008; van Erp et al., 2011; Kim et al., 2011; Hu et al., 2012; Bayon et al., 2015; Chen et al., 2016; Aryal and Lu, 2018; Lunn et al., 2019). Fatty acid hydroxylase 12 (FAH12) catalyzes RA production by hydroxylating oleic acid (18:1n-9) at the *sn-2* position of phosphatidylcholine (PtdCho) (Bafor et al., 1991). However, hydroxyl fatty acids (HFA), including RA and 18:2n-6-OH fatty acid, only accounted for 17 % of the total fatty acids in the seed oil of Arabidopsis lines expressing the *RcFAH12* gene (Smith et al., 2003; Lu et al., 2006). Subsequent studies indicated that RA produced on PtdCho can be released into the acyl-CoA pool as RA-CoA catalyzed by lysophosphatidylcholine acyltransferase (LPCAT) or phospholipase A2 (PLA2) (Arroyo-Caro et al., 2013; Bayon et al., 2015). RA produced on PtdCho can also be

used to produce PtdCho-derived diacylglycerol (DAG) by phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) (Hu et al., 2012), or directly assembled to TAG by phospholipid:diacylglycerol acyltransferase (PDAT) (van Erp et al., 2011 ; Kim et al., 2011). RA-CoA may be assembled to TAG via the Kennedy pathway, in which glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT), phosphatidic acid phosphatase (PAP), and acyl-CoA:diacylglycerol acyltransferase (DGAT) sequentially produce lysophosphatidic acid (lysoPtdOH), phosphatidic acid (PtdOH), DAG, and TAG, respectively (Lunn et al., 2019). PtdCho-derived DAG can also be utilized to produce TAG by DGAT (Bates, 2016).

Nevertheless, coexpression of *RcFAH12* with genes from the Kennedy pathway and/or acyl editing in Arabidopsis resulted in up to 34% HFA in seed oil, which is still much less than in castor oil (Lunn et al., 2019). Moreover, only up to 19% tri-HFA-TAG was detected in the seeds of these transgenic Arabidopsis plants, whereas castor oil contains more than 70% of HFA in the form of tri-HFA-TAG (Lin et al., 2003). Since the overexpression of *RcDGAT2* or *RcPDAT1A* can increase the level of tri-HFA-TAG (van Erp et al., 2011), and Arabidopsis may mostly utilize PtdCho-derived DAG for TAG synthesis, it was proposed that insufficient PtdCho-derived DAG might limit RA accumulation in Arabidopsis (Bates, 2016). Maybe there are some other enzymes that play a role in PtdCho-derived HFA-DAG biosynthesis. Moreover, PtdCho plays an important role in maintaining membrane integrity and functionality. Since RA is likely deleterious to cell membranes, it is important to remove RA from PtdCho or transfer it to other lipids.

Phospholipase D (PLD) is a major family of enzymes that hydrolyzes membrane phospholipids in plants. It can convert PtdCho to phosphatidic acid (PtdOH), which is then converted to DAG by PAP. There are 12 PLD members in Arabidopsis (Qin and Wang, 2002). Different PLD have distinguishable preferences for specific lipids, but most PLD prefer to hydrolyze PtdCho rather than other phospholipids. Meanwhile, different PLD also have distinguishable preferences for PtdCho species

with different fatty acids, resulting in specific fatty acid accumulation in different species (Wang, 2000). In soybean seeds, PLDα suppression increased PtdCho unsaturation while decreasing the unsaturation of TAG molecular species, which indicates a positive role for PLDα in the conversion of PtdCho into TAG (Lee et al., 2011; Zhang et al., 2019). When two Arabidopsis phospholipase genes *PLDζ1* and *PLDζ2* were coexpressed in *Camelina sativa* (camelina), the steady-state pool concentrations of DAG and PtdCho were altered and DAG accumulation was enhanced in transgenic lines (Yang et al., 2017). However, the functions of PLD in RA accumulation are yet to be identified.

In the present study, the Castor Bean Genome Database was examined to identify all PLD homologous genes in castor. Expression pattern analysis showed that *RcPLD9*, which is a homolog of Arabidopsis *PLDζ2*, uniquely has high expression in developing seeds but not in other tissues. The expression of *RcPLD9* in the Arabidopsis CL7 line (van Erp et al., 2011; Lu et al., 2006), which expresses *RcFAH12* in a *fatty acid elongase 1* background, resulted in a lower RA content in the polar lipid fraction and mono-HFA-TAG fraction, but a higher RA content in the di-HFA-TAG fraction in seed oil. Since the production of di-HFA-TAG likely requires the involvement of HFA-DAG, the results of this study indicate that RcPLD9 contributed to the chanelling of HFA from PtdCho to DAG, and the assembly of di-HFA-TAG in developing seeds.

Materials and methods

Plant material

Castor seeds were collected from plants cultivated at the Yunnan Academy of Agricultural Sciences in Kunming, China. Mature female flowers were individually pollinated and tagged according to the number of days after pollination (DAP). Capsules were harvested at 19, 33, and 47 DAP as previously described by Chen et al. (2007). Samples were also collected from fully expanded young leaves, male flowers and female flowers on mature plants for RNA extraction and quantitative real-time

polymerase chain reaction (PCR) analysis. All tissue samples were frozen immediately in liquid nitrogen and stored at -80°C for RNA extraction.

Identification of *PLD* genes from castor bean

BLAST searches and sequence analysis were performed as described by Cagliari et al. (2010). A tBLASTx search was performed against the Castor Bean Genome Database (http://castorbean.jcvi.org) using known Arabidopsis phospholipase genes from NCBI (http://www.ncbi.nlm.nih.gov/protein/) as queries (Chen et al., 2011) to identify all castor *PLD* genes. Deduced amino acid sequences from the coding sequences of the identified genes were aligned using Molecular Evolutionary Genetics Analysis (MEGA 7) (Kumar et al., 2016). *In silico* characterization of protein domains and characteristic signatures of enzyme classes were performed using available data from the literature. Based on the complete protein sequences, dendrograms were drawn using MEGA 7 software (Kumar et al., 2016).

Analysis of gene expression patterns using quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA was extracted from flash frozen mature leaves, early flowers, and the whole seeds including the embryo and endosperm, using RNAiso reagent (Takara, Dalian, China). Primers were designed with Primer Express Software (version 3.0; Applied Biosystems, Foster City, CA, USA) to produce amplifications of between 100 and 250 base pairs (bp) of *RcPLD* genes (Table 1). The castor bean gene encoding *actin* (AY360221) (Chen et al., 2007) was used as an internal control to normalize the relative amount of mRNA for all samples. Relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ comparative threshold cycle (Ct) method. Three technical repetitions were performed for each of the three biological replicates.

Cloning of castor bean *PLD9* genes and their phylogenetic analysis

cDNA derived from castor seed total RNA was used to amplify the open reading frame (ORF) of *PLD9* using the forward primer, 5'-ATGTCAACAGCGAACGAGC-3' and reverse primer 5'-CTAATGGAATACATGAGGGGAT-3'. The amplicon was sequenced and the amino acid sequence was deduced. Homologous sequences of PLDζ2 from plants were identified by BLASTp searches using AtPLDζ2 as a query against public databases at the National Center for Biotechnology Information. Thirteen highly homologous sequences of PLDζ2 from plants were chosen for phylogenetic analysis. Multiple alignment of amino acid sequences was performed using the ClustalW multiple alignment method. An unrooted phylogenetic tree was generated from the alignment and displayed using MEGA7.

Vector construction and expression of *RcPLD9* in the Arabidopsis CL7 line

The Arabidopsis CL7 line (Lu et al., 2006), which stably expresses the *RcFAH12* gene and produces hydroxyl fatty acids (kindly provided by Prof. John Browse from Washington State University USA) was used as the parental plant. *Agrobacterium tumefaciens* strain GV3101 and the binary vector pCambia 2300-napin (Wang et al., 2006) were kindly provided by Prof. Bangquan Huang from Hubei University, China for Arabidopsis transformation. To construct the expression vector using the one-step ISO assembly strategy (Gibson, 2011), the coding region of the *RcPLD9* gene and the binary vector pCambia 2300-napin were amplified using Phusion® High-Fidelity DNA Polymerase (NEB, cat. M0530S) with primer pairs Napin-promoter-PLD9\Napin-nos-PLD9 5′-AAAACATACACGAACCCGGGATGTCAACAGCGAACGAGCC-3′\5′-CAAATGTTTGAACGCTGCAGCTAATGGAATACATGAGGGG-3′ and PLD9-Napin-promoter\ PLD9-Napin-NOS 5′-GGCTCGTTCGCTGTTGACATCCCGGGTTCGTGTATGTTTT-3′\5′-CCCCTCATGTATTCCATTAGCTGCAGCGTTCAAACATTTG-3′, respectively. The amplicons were combined to yield the seed-specific plant expression vector napin-RcPLD9. The resulting plasmids were transformed into *A. tumefaciens* GV3101 via the freeze-thaw method. The resulting *Agrobacterium* strains were used to transform

Arabidopsis CL7 using the floral dip method (Clough and Bent, 1998). Plants transformed with an empty vector pCambia 2300-napin were used as controls. T1 seeds of transgenic plants were selected on half-strength Murashige and Skoog (MS) agar plates supplemented with 50 μg/mL of kanamycin. Plants were grown in growth chambers under long-day conditions (16-h light/8-h dark) at 22°C. The presence of the target genes was confirmed using the Direct Plant Tissue PCR kit (Transgene Biotech, Beijing) with T2 young leaf tissues as the template and the following primers: Napin sense primer 5′-AACTCATCCGCTTCACTCTTTA-3′ and RcPLD9 antisense primer 5′-ATGAGTATCAAAAGGGCGTCT-3′. T3 seeds were collected and used for total lipid and fatty acid analyses.

Seed oil content and fatty acid composition analysis

Oil content and fatty acid composition of Arabidopsis seeds were determined as previously described (Tian et al., 2019). Arabidopsis seeds were dried to a constant weight in desiccators. Approximately 10 mg of seeds per replicate was weighed for fatty acid composition analysis by gas chromatography (GC). One hundred micrograms of triheptadecanoin (C17:0 TAG) were used as the TAG internal standard. Seeds were subjected to treatment with 2 mL of 3 N methanolic HCl and incubated at 80 °C for 16 h. Following transmethylation, 2 mL of aqueous 0.9% NaCl was added, and fatty acid methyl esters were recovered by three sequential extractions with 2 mL of hexane. Fatty acid methyl esters were then analyzed by GC (PerkinElmer Clarus 680) with flame ionization detection on a 30m × 0.25μm × 0.32mm (inner diameter) Elite-225 column (PerkinElmer). The following temperature program was applied: 150 °C, hold for 3 min; 10 °C/min to 180 °C, hold 9 min; 5 °C/min to 210 °C, hold for 8 min. The total lipid content was determined by multiplying the peak-area ratio of the total fatty acid and the internal standard by the initial internal standard amount.

Thin layer chromatography (TLC) analysis

Lipid extraction and separation were performed as previously described (Pan et al., 2013). Briefly, approximately 10 mg of seeds were heated in 2 mL of isopropanol with 0.01% butylated hydroxytoluene at 85 °C for 10 min to inactivate lipases and then homogenized with a Superfine Homogenizer (FLUKO, Germany). Chloroform: methanol (2:1, v/v) was added for extraction. After re-extraction twice, the combined chloroform phase (lower phase) was washed with 0.9% (w/v) NaCl to remove proteins and carbohydrates. The chloroform phase was dried under a nitrogen stream and resuspended in 30 μL of chloroform. The extracted lipids were spotted onto silica G60 thin layer chromatography (TLC) plates (Merck) with the solvent system of hexane/diethyl ether/acetic acid (140:60:2, v/v). The TAG bands were identified based on the number of OH groups in castor oil (TAG, 1OH-TAG, 2OH-TAG, and 3OH-TAG). The TAG bands and polar lipids were visualized by lightly staining with iodine and were scraped off the TLC plates. They were then converted to fatty acid methyl esters and analyzed by GC, as described above.

Results

Eleven *PLD* genes were identified in the castor bean

Eleven putative PLD genes were identified in the castor bean genomic database based on homology to Arabidopsis PLD genes, and they were designated sequentially from *RcPLD1* to *RcPLD11*(Table 1). Dendrogram analysis, based on the putative amino acid sequences of RcPLD proteins and AtPLD proteins, showed that the 11 RcPLD proteins were classified into six categories (*α*, *β*, *γ*, *δ*, *ε*, and *ζ*) corresponding to the AtPLD proteins (Figure 1). In detail, three PLDα homologs (RcPLD6, 7, and 11), two PLDβ homologs (RcPLD4 and 5*)*, three PLDδ homologs (RcPLD1, 2, and 3), one PLDε homolog (RcPLD8), and two PLDζ homologs (RcPLD9 and 10), but no PLDγ homolog, were identified in the castor bean genome.

RcPLD9 is specifically expressed in developing seeds

PLD family members are involved in a wide range of cellular processes. To determine PLDwhich PLD are specifically expressed in castor developing seeds, expression pattern analysis of all RcPLD genes was performed by qRT-PCR. The highest levels of expression were observed for *RcPLD6, 8*, 9, and *11* in later developmental stages (S3) of seeds where the RA content was almost the highest. In addition, *RcPLD6* and *RcPLD8* were also expressed at high levels in the leaves. *RcPLD7* showed high expression levels in the early stages of developing seeds (S1) but presented a gradual decrease during seed maturation. Other *RcPLD* genes, including *RcPLD1, 2, 3, 4, 5, 10, and 11*, had lower expression in developing seeds eventhough their expression was comparably higher in leaves and flowers (Figure 2). Since it was only expressed in developing seeds, the *RcPLD9* gene was further investigated in the present study.

RcPLD9 is a homolog of AtPLDζ2

The *RcPLD9* gene was isolated by reverse transcription PCR (RT-PCR) based on the genomic sequence from the Castor Bean Genome Database. The cloned cDNA sequence was 3670 bp, which contained an ORF of 3288 bp encoding a protein of 1095 amino acids (Accession number MN939396). A number of undetermined bases and a missing fragment were noticed for sequence 30128.m008869 compared to the ORF sequence of *RcPLD9* (Figure 3A), which indicated that there was an error in the Castor Bean Genome Database resulting from sequencing or sequence assembly. The putative protein sequence of RcPLD9 contained the PX and PH domains in C-terminus, but not the C2 domain, and showed higher identity with AtPLDζ2 (69.9%), which indicting RcPLD9 is a homolog of AtPLDζ2 (Figure 3B). Thirteen plant PLDζ2 homologs were identified using the BLASTp algorithm with amino acid sequences of AtPLDζ2. Then sequence similarities between RcPLD9 and the identified plant PLDζ2s were analysed. PLDζ2 of *Theobroma cacao* (TcPLDζ2), *Herrania umbratica* (HuPLDζ2), *Durio zibethinus* (DzPLDζ2), and *Gossypium hirsutum* (GhPLDζ2) were much closer

homologs of RcPLD9 than AtPLDζ2 and other PLDζ2 from Brassicaceae plants (Figure 3C).

Seed-specific expression of *RcPLD9* leads to a reduction in oil content and HFA levels in CL7 seeds

To investigate the functionality of RcPLD9, we expressed the ORFs of *RcPLD9* in the Arabidopsis line CL7 under the regulation of the seed-specific napin promoter. A total of 10 T1 transgenic lines were selected by kanamycin resistance and PCR screening, using gene-specific primers for detection of the castor bean *PLD9* transgene. HFA was analyzed in T3 seeds from T2 transgenic plants of eight lines. Introduction of *RcPLD9* into CL7 transgenic plants significantly decreased the amount of hydroxyl fatty acid, with an average of 7.69 ± 0.20% (average ± SE) in the bulk of segregate T3 seeds compared with 9.52 ± 0.13% (average ± SE) in the parental CL7 control line transformed with an empty vector. The decrease in hydroxyl fatty acid ranged from 8.71% (line 4) to 6.92% (line 5) in *RcPLD9* transgenic plants compared to 9.52% hydroxyl fatty acid in CL7 (Figure 4). Fatty acid composition and oil content was analyzed in T3 seeds of three individuals from three independent lines (D1-1, D3-1 and D8-1) (Table 2). The total oil content was significantly decreased in *RcPLD9* transgenic plants, with a range from 20.89% (D3-1) to 22.09% (D1-1), compared to the average of 26.65% in CL7. The fatty acid compositions of the seed samples are shown in Table 2. In *RcPLD9* transgenic plants, RA and 18:2n-6-OH fatty acid content decreased by up to 24.2% (D8-1) and 23.8% (D1-1), respectively. Total HFA content was decreased by up to 23.5% (D8-1), relative to CL7 controls. Simultaneously, 18:2n-6 18:3n-3was increased by 16.5% (D1-1), 17.0% (D8-1), and 18.3% (D3-1), and 18:3n-3 was increased by 34.9% (D1-1), 37.1% (D3-1), and 44.5% (D8-1), whereas 18:1n-9 was decreased by 20.2% (D1-1), 22.7% (D8-1), and 23.8% (D3-1).

***RcPLD9* expression decreased the HFA level in polar lipids and increased the di-**

HFA-TAG level of CL7 seeds

Total lipids were extracted from the seeds of **the** CL7- RcPLD9 and CL7 control line, and HFA-TAG was separated using TLC. TAG1(mono-HFA-TAG), TAG2 (di-HFA-TAG) and TAG3 (tri-HFA-TAG) species, having the same mobility as the 1OH-TAG, 2OH-TAG and 3OH-TAG of castor bean seeds, respectively, were detected in TLC plates (Figure 5A). Generally, HFA was more abundantly detected in TAG1 and TAG2 than in TAG3, in both CL7-RcPLD9 and control CL7 plants. *RcPLD9* expression in Arabidopsis, resulted in reduced HFA levels in TAG1 and increased HFA levels in TAG2, but there was no significant difference in TAG3 compared to the control lines. In CL7- RcPLD9 plants, the proportion of HFA decreased to 64.2% from 74.3% of CL7 seeds in TAG1 and increased to 34.4% from 24.3% of CL7 seeds in TAG2 (Figure 5B).

Furthermore, total lipid fractions were separated into polar lipids and neutral lipids (Figure 5). TLC spots containing total polar lipids of seeds from CL7-RcPLD9 and CL7 control plants were analyzed for fatty acid composition. RA in polar lipids of CL7-RcPLD9 was significantly reduced compared to the CL7 control line. The CL7-RcPLD9 plants contained 6.2% RA in polar lipids, while the RA content of the parental line was 10.0%. Simultaneously, RcPLD9 significantly decreased the levels of 18:1n-9 and 18:3n-3, and increased the 18:2n-6 and 16:0 levels in polar lipids (Figure 5C).

Discussion

In seeds, TAG is synthesized through two pathways, the acyl-CoA dependent Kennedy pathway and the acyl-CoA independent pathway. DAG is a key substrate for TAG biosynthesis in both pathways. DAG can be generated by the Kennedy pathway (de novo DAG) or be derived from PtdCho (PtdCho-derived DAG). PtdCho plays important roles in unusual fatty acid synthesis and plays a central role in TAG accumulation in plant seeds by recycling or incorporation of the newly synthesized fatty acids in TAG acyl editing. Previous studies have indicated that the efficient flux of HFA through PtdCho represents the major bottleneck of high levels of HFA

accumulation in heterologous transgenic seeds (Bates *et al*., 2014). In transgenic Arabidopsis, a major pathway for HFA-TAG biosynthesis was reported through PtdCho-DAG interconversion. Except for RcPDCT and RcPLC (Hu et al., 2012; Aryal and Lu, 2018), PLD has the potential to contribute to the PtdCho-derived HFA-DAG pool. PLD can convert PtdCho to PtdOH, and then PtdOH is catalyzed by phosphatidic acid phosphatase to produce the PtdCho-derived DAG.

PLD play important roles in diverse cellular functions in plants owing to their molecular and biochemical heterogeneity (Chen et al., 2011). Among different plant species, the number of PLD genes varies greatly. There are 32, 19, 18, 17, 12 and 11 PLD genes in *Brassica napus*, *Gossypium arboretum*, *Populus tremula*, *Oryza sativa*, *Arabidopsis thaliana*, and *Vitis vinifera*, respectively (Elias et al., 2002; Li et al., 2007; Liu et al., 2010; Tang et al., 2016; Lu et al., 2019). In this study, we identified 11 PLD homologs from the castor bean genome (Table 1). Unlike Arabidopsis and other plant species, we found only five subfamilies, namely α, β, δ, ε, and ζ, through homology BLAST and no PLDγ subfamily homologs were identified for *RcPLD* genes (Figure 1). Maybe the deficiency of *PLDγ* genes is because *PLDβ* and *PLDγ* genes perform a similar function or the reported reference genome of castor bean is incomplete. Among the 11 *RcPLD* genes, only *RcPLD9* presented high levels of transcript accumulation in developing seeds, especially at the S3 stage, whereas relatively low levels of transcript accumulation was evidenced in leaves and flowers (Figure 2), which is consistent with the RA accumulation pattern in castor bean, suggesting that RcPLD9 probably plays a major role in ricinolate metabolism in castor bean. Although *RcPLD6* and *RcPLD8* presented high levels at the S3 stage, they also presented high levels in leaves and flowers. Except for *RcPLD2*, the expression patterns of RcPLD genes were consistent with the results of RNA-seq transcriptome analysis (Brown et al., 2012). Phylogenetic and protein sequence analysis indicated that RcPLD9 is a homolog of AtPLDζ2. Although RcPLD10 clustered into the same subfamily as RcPLD9, its transcript accumulation was very low in developing seeds, and relatively high levels of transcript accumulation were detected in leaves and male flowers (Figure 1 and 2).

A previous study indicated that AtPLDζ can enhance the PtdCho-derived DAG pathway of TAG synthesis in camelina (Yang et al., 2017). In the present study, when *RcPLD9* was heterologously expressed in the Arabidopsis CL7 line, the 18:1n-9-OH decreased 37.9% in polar lipids of transgenic lines (Figure 5C), suggesting that RcPLD9 could efficiently convert HFA-PtdCho to neutral lipids. In the TAG of transgenic lines, the increased TAG2 and decreased TAG1 (Figure 5B) provided a reasonable explanation for the activation of RcPLD9. It was proposed that the expression of *RcPLD9* increased the PtdCho-derived mono-HFA-DAG pool. In camelina seeds, the increased AtPLDζ activity may enhance DGAT over PDAT activity for TAG synthesis, resulting in increased PUFA concentration and increased elongation of fatty acids (Yang et al., 2017). In our study, RcPLD9 activity may enhance PDAT activity for TAG production using PtdCho-derived HFA-DAG and HFA-PtdCho as substrates, resulting in increased TAG2, and correspondingly decreased TAG1.

In castor oil, the release of HFA from PtdCho in castor endosperm is thought to allow conversion to the CoA thioester and incorporation into TAG by three acyl transfer reactions catalyzed sequentially by GPAT, LPAAT, and DGAT (Bafor et al., 1991; Bayon et al., 2015), which is probably an important pathway for TAG3 accumulation in castor TAG. However, expression of *RcPLD9* did not increase the content of TAG3 in the current study(Figure 5B). A recent study reported that coexpression of three castor acyltransferase enzymes in the Kennedy pathway produced 19% TAG3 and concentrated 44% of seed HFA moieties into this one TAG species. RA was more abundant than any other fatty acid in these seeds, which had three-fold more HFA by weight than that in seeds following simple hydroxylase expression (Lunn et al., 2019). It may be explained that RcPLD9 co-evolved with other essential genes in the lipid metabolic pathway of castor bean, especially genes using effective RA-CoA for TAG synthesis in the Kennedy pathway such as DGAT, LPAAT, and GPAT.

On the other hand, HFA can inhibit the FAD2 and FAD3 desaturases that catalyze the conversion of 18:1n-9 to 18:2n-6 and 18:3n-3, respectively (Broun and Somerville, 1997; Bayon et al., 2015). With the decrease in HFA in polar lipids of

transgenic lines, the activity of FAD2 and FAD3 increased, resulting in an increase in 18:2n-6 and 18:3n-3 in total fatty acids (Bayon et al., 2015). In the present study, the decrease in HFA, 18:1n-9 and 18:3n-3, and the increase of 18:2n-6 in polar lipids also indicated a probable relationship between the level of HFA and the activity of FAD2 or FAD3. Although *RcPLD9* expression resulted in increased TAG2 accumulation in TAG, due to the reduced HFA content in polar lipids, the overall HFA content in RcPLD9-expressing seeds was lower than that in the control in the current study (Figure 4). This may be due to substrate competition between RcFAH12 and AtFAD2 or AtFAD3. PLD prefer to hydrolyze PtdCho, the substrate of the PDAT pathway and DAG-PtdCho conversion toward TAG biosynthesis, which probably was the reason that the oil content decreased in RcPLD9-expressing seeds. Meanwhile, the product of the PLD catalyzed reaction is PtdOH. PtdOH is an important intermediary in glycerolipid metabolism or functions as a cellular mediator involved in a wide range of metabolic, cellular and physiological processes in plants (including fatty acid and membrane lipid synthesis, and lipid transport) (Wang *et al*., 2014). Although RcPLD9 plays a role in ricinolate metabolism and HFA synthesis, the exact mechanism may be complex and remains to be determined.

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Conflict of Interest The authors declare that there is no conflict of interest.

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Figure Legends

Fig.1. Phylogenetic analysis of phospholipase D genes from Arabidopsis and the identified genes encoding PLD from castor. The dendrogram was constructed by the neighbor-joining method, using the MEGA7 program, after 1000 bootstrap replications, with pairwise deletion. Scale bar indicates genetic distance. Arabidopsis enzyme queries are designed by their respective gene names and the corresponding Genome Database accession numbers are as below, AtPLDα1 (AT3G15730), AtPLDα2 (AT1G52570), AtPLDα3 (AT5G25370), AtPLDβ1 (AT2G42010), AtPLDβ2 (AT4G00240), AtPLDγ1 (AT4G11850), AtPLDγ2 (AT4G11830), AtPLDγ3 (AT4G11840), AtPLDδ (AT4G35790), AtPLDε (AT1G55180), AtPLDζ1 (AT3G16785), AtPLDζ2 (AT3G05630).

Fig.2. Relative expression patterns of castor PLD genes. Relative expression of PLD genes were detected during castor seed development (stages S1–S3), as well as in leaf (L), male flower (MF), and female flower (FF). Expression levels were normalized with respect to the housekeeping control genes. Each data point represents the mean of three experimental replicates.

Fig. 3. Sequence and phylogenetic analysis of *RcPLD9* gene.

A. Nucleotide sequence comparison between *RcPLD9* and the sequence in the Castor Bean Genome Database. B. Deduced amino acid sequence alignment of RcPLD9 and AtPLDζ2. The lines and letters above the sequences indicate functional domains. C. Evolutionary dendrogram showing RcPLD9 together with PLDζ2 homologs from other species. The dendrogram was constructed by the neighbor-joining method, using the MEGA7 program, after 1000 bootstrap replications, with pairwise deletion. Bootstrap values higher than 70% are given at the respective nodes. Scale bar indicates genetic distance. Al, *Arabidopsis lyrata*; At, *Arabidopsis thaliana*; Br, *Brassica rapa*; Bn, *Brassica napus*; Cs, *Camelina sativa*; Cr, *Capsella rubella*; Dz,

574 *Durio zibethinus*; Es, *Eutrema salsugineum*; Gh, *Gossypium hirsutum*; Hu, *Herrania*
575 *umbratica*; Rs, *Raphanus sativus*; Th, *Tarenaya hassleriana*; Tc, *Theobroma cacao.*

576

577 **Fig. 4.** Ricinolate acid content of seeds from Arabidopsis lines co-expressing castor
578 bean *RcFAH12* and *RcPLD9*. Each data point represents the average RA content of
579 three individual T3 seeds of a T2 individual progeny plant derived from T1
580 transgenic plants. The data represent the averages of three independent
581 measurements ± SE.

582

583 **Fig. 5.** Molecular species composition of HFA containing TAG and fatty acid
584 composition of polar lipids of CL7 and CL7-RcPLD9 seeds. A. Total lipid separation
585 by TLC: TAG, normal TAG; TAG1, 1OH-TAG; TAG2, 2OH-TAG; TAG3, 3OH-
586 TAG; PL, polar lipids. B. mol % of HFA in total fatty acids of TAG molecular
587 species from CL7 and CL7-RcPLD9 seeds. C. mol % of fatty acid species in polar
588 lipids of CL7 and CL7-RcPLD9 seeds. HFA represent the sum of ricinoleate (18:1n-
589 9-OH) and densipolate (18:2n-6-OH). The data represent the averages of three
590 individual T3 lines, viz.1-1, 3-1 and 8-1 ± SE.

591 Table 1. Summary of castor PLD genes searched for in the Castor Bean Genome
592 Database and primer sequences for qRT-PCR.

Acronym	**Castor bean ID**	**Forward primer (5'- 3')** **Reverse primer (5'- 3')**
RcPLD1	30170.m014290	AGCGTGTTCGCCGTTGTT GCGTTCGCTTTTGTGTTCTTT
RcPLD2	29784.m000369	CCCGGAAGTTTTTCAAGCATT TGCTGTTCCAACAACCTTTTGT
RcPLD3	28725.m000311	CTTCCCATATGAATCAACAAACTGA CATACTCATCATCAACTATCATCCCTTT
RcPLD4	30190.m011102	ATAGTGGACGATGAGTATGTA GTATGCTGAGGCTGATATG
RcPLD5	30174.m008942	TTACTTTGGGAGGGTTGATTCTTC AATCTGCGTACTTTGGCTATGCT
RcPLD6	29848.m004631	GCATCTGTTCAGGCAATA AATCAGGTTCAGGTATCTCT
RcPLD7	29841.m002847	GAGCCTATCAACCACATCACTTGT AATGACATACGGAAACCATGGAT
RcPLD8	28694.m000682	TGGTACAGAAAAGGCTAGTCCTAGAGAT TTGAGGCTGTAGCAGTGTTTCTTC
RcPLD9	30128.m008869	TCGCTGTCAGATTATCAGAAGTGTT GAAATGCTGTGCTTTCTCAATGA
RcPLD10	29726.m004097	TCGATGTTGTCGTCGTTTTTCT TCGTGGCCGTAGGTAATTCAT
RcPLD11	28320.m001141	AGAAGGAACTAAAGTTGGCAGAACTAG GCACAGTAAATGTGGAAAGATTCG

593

Table 2. Fatty acid composition and oil content (mol%) of T3 seeds coexpressing castor *RcPLD9* with castor *RcFAH12* (CL7 background)

Line	Fatty Acid Composition								Sum of HFA	Oil
	16:0	18:0	18:1n-9	18:2n-6	18:3n-3	20:1n-11	18:1n-9-OH	18:2n-6-OH		
CL7	11.44±0.16[a]	4.54±0.17[a]	33.33±0.53[a]	25.87±0.28[a]	11.92±0.31[a]	1.13±0.05[a]	9.41±0.21[a]	2.39±0.05[a]	11.8±0.23[a]	26.65±0.25[a]
D1-1	12.85±0.09[b]	3.77±0.06[b]	26.59±0.11[b]	30.14±0.47[b]	16.08±0.34[b]	1.06±0.07[b]	7.72±0.18[b]	1.82±0.02[b]	9.55±0.16[b]	22.09±0.24[b]
D3-1	13.21±0.24[b]	3.58±0.16[b]	25.39±0.33[b]	30.6±0.40[b]	16.34±0.47[b]	1.03±0.04[b]	7.86±0.19[b]	1.98±0.10[b]	9.84±0.26[b]	20.89±0.36[b]
D8-1	13.14±0.15[b]	3.53±0.05[b]	25.76±0.28[b]	30.28±0.27[b]	17.23±0.35[b]	1.03±0.08[b]	7.13±0.38[b]	1.90±0.10[b]	9.03±0.30[b]	21.69±0.26[b]

Values followed by different letters in the same column indicate significant differences ($P < 0.05$).

All data are the averages of three replicates ± SE.

Figure 1

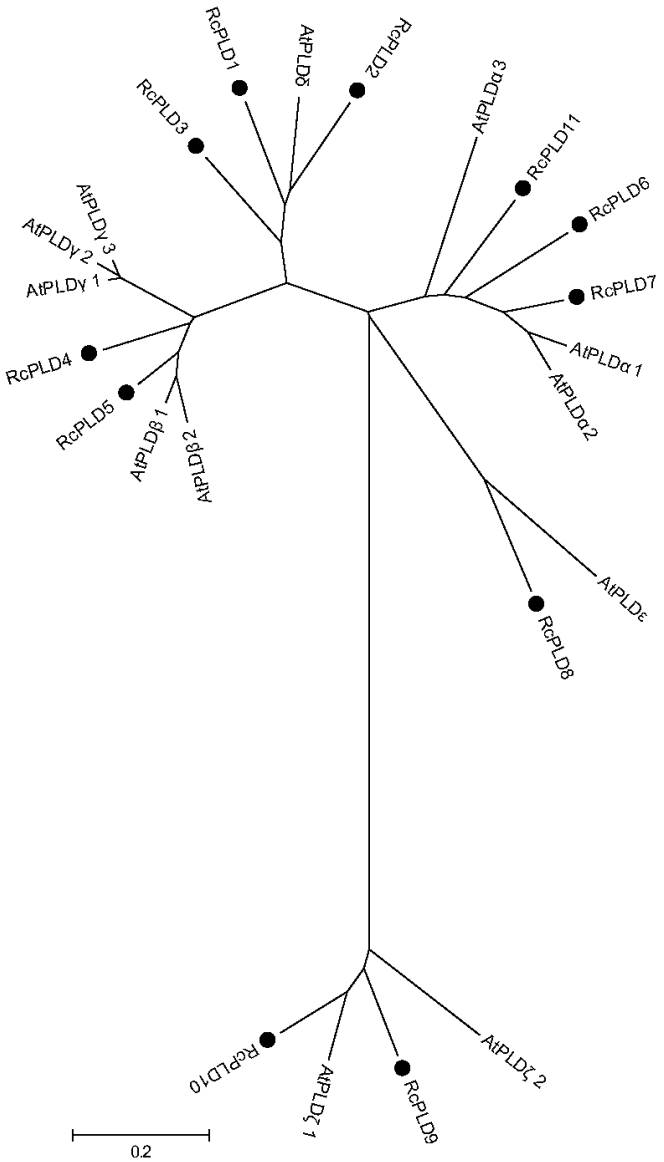

RcPLD1
AtPLDδ
RcPLD2
AtPLDα 3
RcPLD11
RcPLD6
RcPLD7
AtPLDα 1
AtPLDα 2
AtPLDε
RcPLD8
RcPLD3
AtPLDγ 3
AtPLDγ 2
AtPLDγ 1
RcPLD4
RcPLD5
AtPLDβ 1
AtPLDβ 2
RcPLD10
AtPLDζ 1
RcPLD9
AtPLDζ 2
0.2

Figure 2

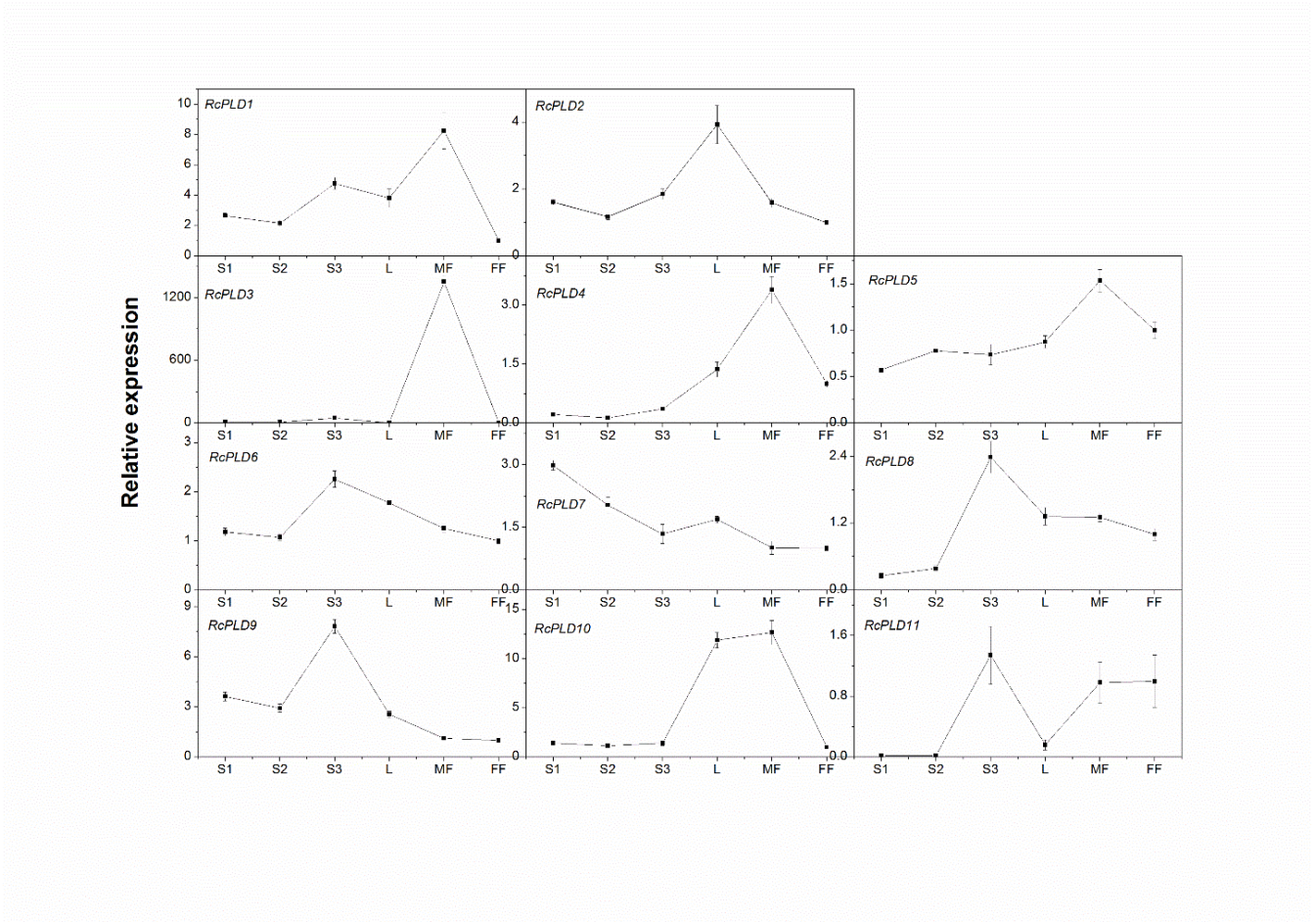

Relative expression
RcPLD1
RcPLD2
RcPLD3
RcPLD4
RcPLD5
RcPLD6
RcPLD7
RcPLD8
RcPLD9
RcPLD10
RcPLD11
S1
S2
S3
L
MF
FF

Figure 3

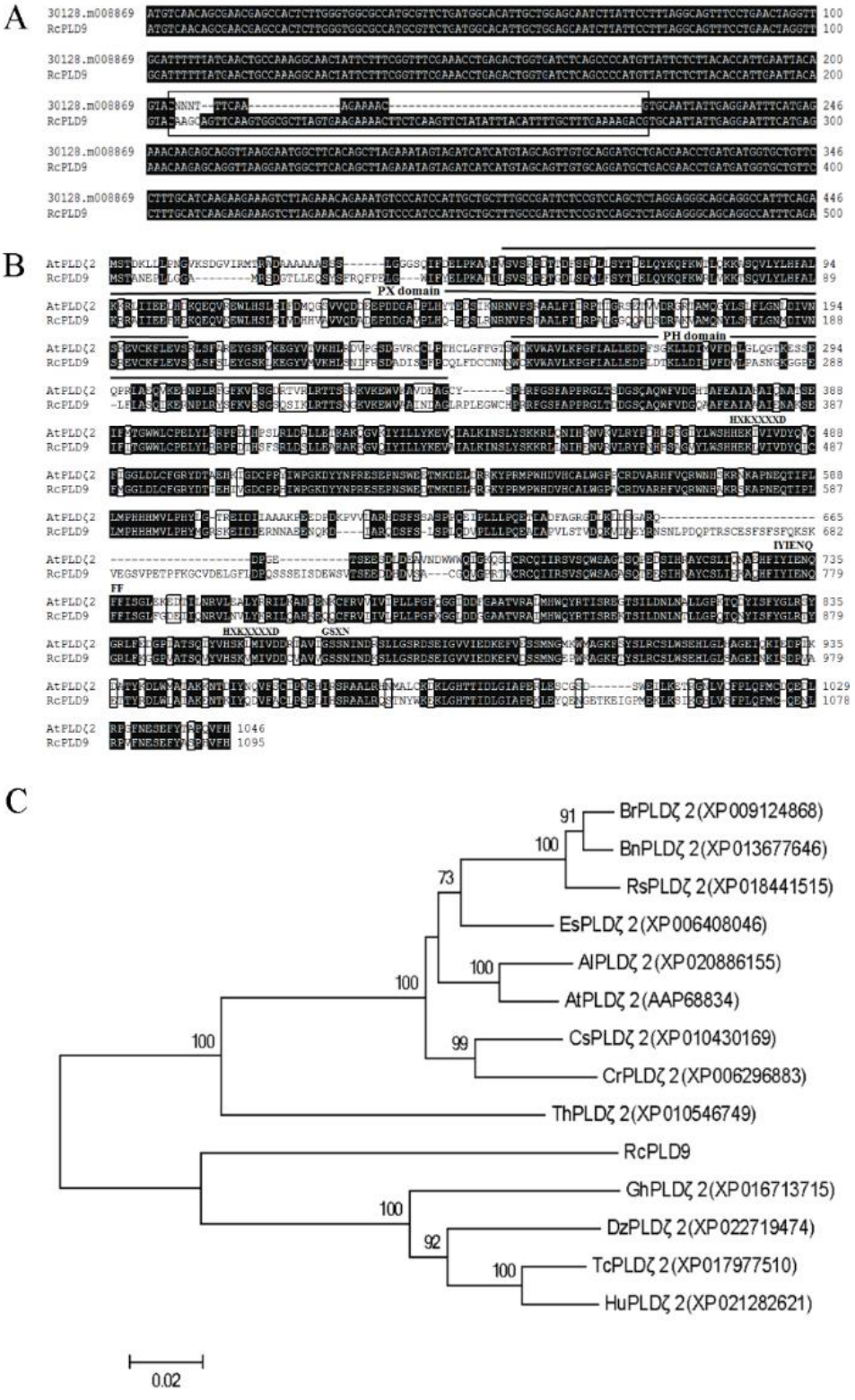

A
30128.m008869
RcPLD9
B
AtPLDζ2
RcPLD9
PX domain
PH domain
HXKXXXXD
IVIENQ
FF
HXKXXXXD
GSXN
C
BrPLDζ 2(XP009124868)
BnPLDζ 2(XP013677646)
RsPLDζ 2(XP018441515)
EsPLDζ 2(XP006408046)
AlPLDζ 2(XP020886155)
AtPLDζ 2(AAP68834)
CsPLDζ 2(XP010430169)
CrPLDζ 2(XP006296883)
ThPLDζ 2(XP010546749)
RcPLD9
GhPLDζ 2(XP016713715)
DzPLDζ 2(XP022719474)
TcPLDζ 2(XP017977510)
HuPLDζ 2(XP021282621)
91
100
73
100
100
99
100
100
92
100
0.02

Figure 4

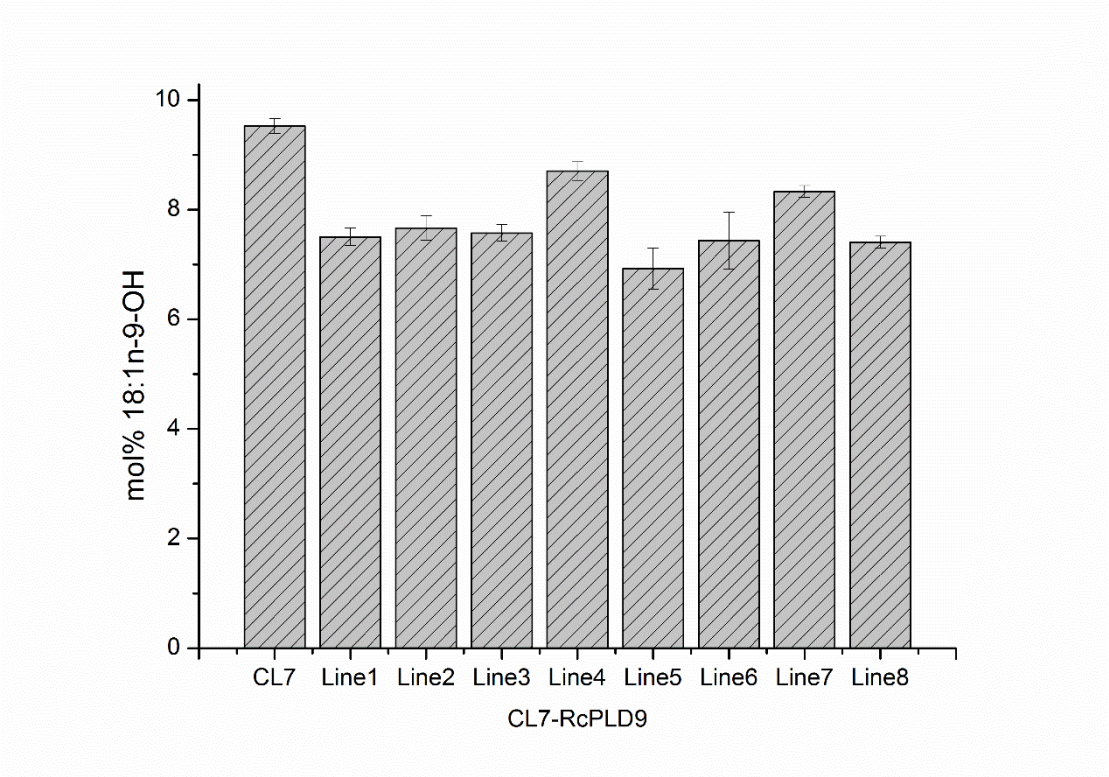
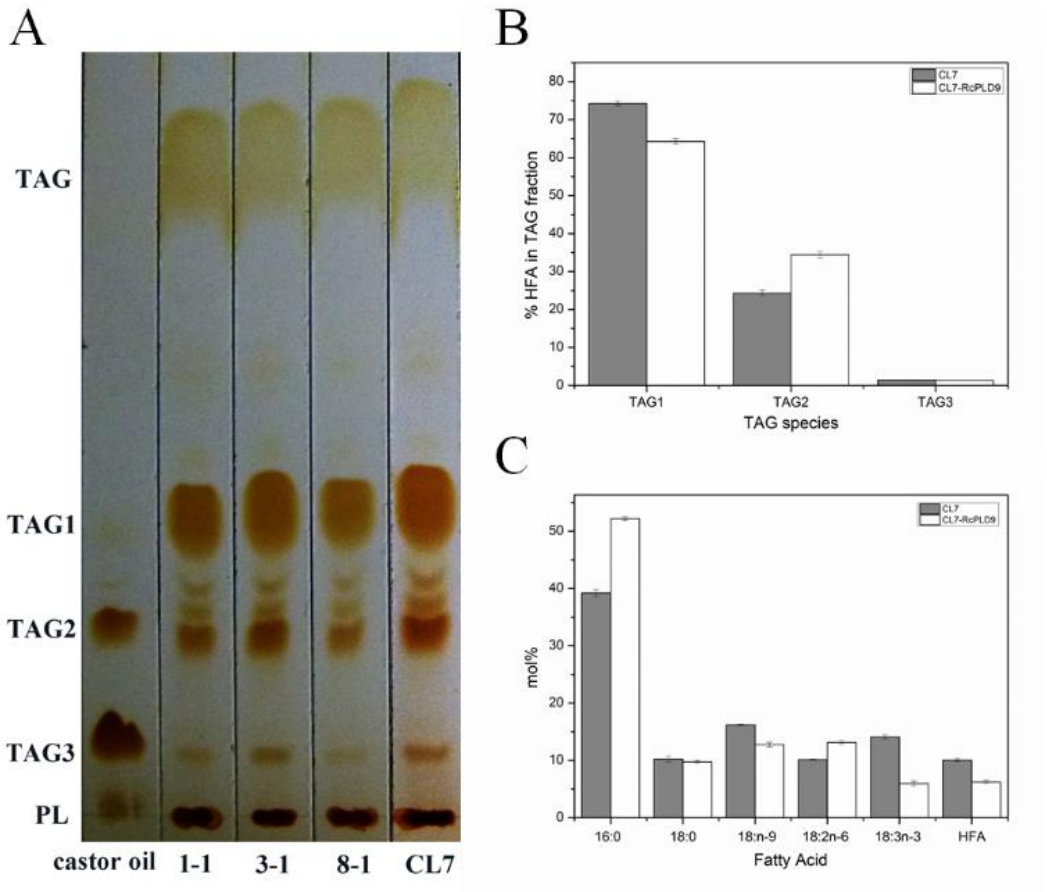

mol% 18:1n-9-OH
0
2
4
6
8
10
CL7
Line1
Line2
Line3
Line4
Line5
Line6
Line7
Line8
CL7-RcPLD9

Figure 5


A
TAG
TAG1
TAG2
TAG3
PL
castor oil
1-1
3-1
8-1
CL7
B
CL7
CL7-RcPLD9
% HFA in TAG fraction
TAG1
TAG2
TAG3
TAG species
C
CL7
CL7-RcPLD9
mol%
16:0
18:0
18:n-9
18:2n-6
18:3n-3
HFA
Fatty Acid