Functional Characterization of Plant Patatin-like Phospholipase A III in Transgenic *Arabidopsis thaliana* Producing 18 Carbon Hydroxy Fatty Acids

by

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Abstract

Hydroxy fatty acid (HFA)-enriched oils are valuable in many oleo-chemical industries. The major natural source of HFA, however, is castor (*Ricinus communis*) seed, which is poisonous to human beings and animals. Although over-expressing a castor *OLEATE 12-HYDROXYLASE* (*RcFAH12*) in *Arabiodpsis thaliana* has been shown to lead to the synthesis of HFAs, a high proportion remained within the membrane phospholipid, phosphatidylcholine. Plant phospholipases A (PLAs) can catalyze the release of fatty acyl chains from the *sn*-1 and/or *sn*-2 positions of PC. These free fatty acids are subsequently channeled into the acyl-CoA pool, which provides substrates for TAG synthesis. Thus, it seems that the release of HFA from PC is a critical step for obtaining HFA enriched seed oils. To determine whether the heterologous expression of *PATATIN-LIKE PHOSPHOLIPASE A* of class III *(pPLAIII)* from HFA-producing plants can enhance the removal of HFA from PC, two *pPLAIII* genes (*pPLAIIIβ* and *pPLAIIIδ)* from castor (*Ricinus communis*) or *Lesquerella fendleri*, naturally accumulating >60% of C20-HFA in their seed oils, were expressed in a transgenic line of Arabidopsis producing C18-HFA. In addition, over-expression lines bearing Arabidopsis orthologs, *AtpPLAIIIα* or *AtpPLAIIIδ*, respectively, were also generated as controls. Expression of *RcpPLAIIIβ* resulted in a significant reduction in the HFA content in the seed oil and in PC compared with the control, whereas expressions of other *pPLAIII* genes did not alter HFA content in the seed oil. These results suggested that pPLAIIIβ may participate in catalyzing the removal of HFA from PC in developing castor seeds. *L. fendleri pPLAIII* did not appear to participate in the release of C18-HFAs from PC although a possible mechanism of

release involving cooperation with elongation of C18 HFA in the acyl-CoA pool could not be ruled out.

Preface

I performed most of the experimental procedures for this thesis. Some of the procedures, however, were conducted by other members of the Weselake group in the Department of Agricultural, Food and Nutritional Science in University of Alberta (Edmonton, Alberta, Canada). The isolation of cDNAs encoding *PATATIN-LIKE PHOSPHOLIPASE A IIIβ* (*pPLAIIIβ*) and *pPLAIIIδ* from *Lesquerella fendleri*, and the BLAST search of the orthologs involved in this thesis were conducted by research associate Dr. Guanqun Chen. The preparation of modified pPZP-RCS1 binary vector for plant transformation and the experimental design for cloning *pPLAIII* cDNAs into the binary vector were conducted by research associate Dr. Elzbieta Miekiewska.

The identification of genes encoding phospholipase As from developing *L. fendleri* seeds was conducted by Dr. Mark Smith at National Research Council of Canada (Saskatoon, Saskatchewan, Canada). The developing *L. fendleri* seeds were provided by Dr. John Dyer of the Agricultural Research Service of the United States Department of Agriculture (Maricopa, AZ, USA). The castor (*Ricinus communi*) cDNA template was provided by Dr. Thomas McKeon at the Agricultural Research Service of the United States Department of Agriculture (Albany, CA, USA).

For the review article by Mietkiewska et al. (2014b), which is referred to in Appendix 2 of this thesis, I prepared the outline and first draft of the manuscript. Drs Elzbieta Mietkiewska and Randall Weselake edited and further improved the document.

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Specially thanks to my family for their care and support from across the Pacific Ocean. A warm thanks to my fiancé; he was always there for me through thick and thin, encouraging me never to quit. Finally, thanks to my friends for all of their support!

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ABC transporter	ATP-binding cassette transporter
ABCD	ABC transporter protein of subfamily D
ACCase	acetyl-coA carboxylase
ACBP	acyl-CoA binding protein
ACP	acyl carrier protein
cDNA	complementary DNA
CDP-choline	cytidine diphosphate-choline
CFA	conjugated fatty acid
CoA	coenzyme A
CPT	CDP-choline:diacylglycerol cholinephosphotransferase
DAG	*sn*-1,2-diacylglycerol
DGAT	diacylglycerol acyltransferase
EFA	epoxy fatty acid
ER	endoplasmic reticulum
FA	fatty acid
FAD2	fatty acid desaturase 2
FAD3	fatty acid desaturase 3

FAEE	fatty acid ethyl ester
FAE1	fatty acid elongase 1
FAH	fatty acid hydroxylase
FAME	fatty acid methyl ester
FAS	fatty acid synthase
FAT	acyl-ACP thioesterases
FAX1	fatty acid export 1
GC/MS	gas chromatography
GPAT	*sn*-glycerol-3-phosphate acyltransferase
G3P	*sn*-glycerol-3-phosphate
HFA	hydroxy fatty acid
IEM	plastidial inner envelope membrane
IMS	intermembrane space
KAS	3-ketoacyl-ACP synthases
LACS	long-chain acyl-CoA synthetase
LCAT	lecithin:cholesterol acyltransferase
LPAAT	lysophosphatidic acid acyltransferase
LPC	lysophosphatidylcholine

LPCAT	lysophosphatidylcholine acyltransferase
MCS	membrane contact site
mFA	modified fatty acid
NADH	nicotinamide adenine dinucleotide
OEM	plastidial outer envelope membrane
PA	phosphatidic acid
PAP	phosphatidic acid phosphatase
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDAT	phospholipid:diacylglycerol acyltransferase
PDCT	phosphatidylcholine:diacylglycerol cholinephosphotransferase
PDH	pyruvate dehydrogenase
PHA	polyhydroxyalkanoate
PLA	phospholipase A
PLB	phospholipase B
PLC	phospholipase C
pPLA	patatin-like phospholipase
PUFA	polyunsaturated fatty acid

ROD1	reduced oleate desaturation 1
SAD	stearoyl-ACP desaturase
TAG	triacylglycerol
TLC	thin layer chromatography
UFA	unusual fatty acid
VLCF	very-long- chain fatty acid

1 - Introduction

The use of vegetable oil derivatives as renewable resources is a sustainable and environmental friendly strategy to overcome the limitation of petrochemical resources (Dincer, 2000; Meneghetti et al., 2006). For example, castor (*Ricinus communis*) oil has been widely used in the oleo-chemical industry because it contains an ultra-high level of hydroxyl fatty acids (HFAs; Ogunniyi, 2006). Biodiesel, varnish, lubricants, cosmetics and surfactants for medicines can be generated using castor oil as the feedstock (Conceição et al., 2007; Mutlu and Meier, 2010). However, the supply of castor oil is limited by the presence of toxic components, ricin and the allergenic 2S albumins, in the castor seed and the undesirable agronomical features of the plant associated with difficulties in mechanical harvesting (Lee et al., 2015). Another natural source of HFAs, *Lesquerella fendleri* has not become a commercial oilseed crop (Dierig et al., 2011).

Given that the global market for the castor oil is in demand (Wood, 2001), studies have turned to genetic engineering of established oilseed crops to produce HFAs (Kumar et al., 2006). From the expression of the key gene for HFA synthesis, *OLEATE 12-HYDROXYLASE* (*FAH12*), to the co-expression of additional genes encoding enzymes involved in lipid synthesis, e.g. diacylglycerol acyltransferase (DGAT) and phospholipid:diacylglycerol acyltransferase (PDAT), significant progress have been made in this field, achieving the improvement of HFA accumulation in the seed oil from up to 17% to 30% in transgenic hosts, e.g. *Arabidopsis thaliana* (Lee et al., 2015). However, more studies are required to develop oilseed crops that can accumulate a comparable level of HFAs to the natural source.

When *FAH12* was expressed in *Arabidopsis thaliana* (Arabidopsis), a relatively high level of HFAs remained in the site of synthesis, phosphatidylcholine (PC). The unobstructed shuffle of HFAs from PC to the storage lipid, triacylglycerol (TAG) is important for the accumulation of HFAs in the seed oil of transgenic plants (Bates and Browse, 2012). Fatty acyl chains can be removed from the *sn*-1 and/or *sn*-2 position of PC catalyzed by plant phospholipase As (PLAs), followed by being subsequently channeled into the acyl-CoA pool to provide substrates for TAG synthesis (Chen et al., 2013). Hence, the release of HFA from PC seems to be one of the critical steps to obtain seed oils enriched in HFA.

L. fendleri is known to accumulate high level of C20 HFAs in the seed oil via the efficient removal of HFAs from PC for the elongation in the acyl-Coenzyme A (CoA) pool (Chen et al., 2016). Recently, two *PATATIN-LIKE PHOSPHOLIPASE A* genes (homologs of *pPLAIIIβ* and *pPLAIIIδ* from Arabidopsis) were identified from the developing seeds of *L. fendleri* (unpublished data from Dr. Mark Smith of National Research Council of Canada). In this thesis, these two *pPLAIII* genes were used as candidates to selectively release HFAs from PC in the Arabidopsis expressing *FAH12*. Furthermore, considering the high accumulation of HFAs in the castor oil, the orthologs of the two *L. fendleri pPLAIIIs* from castor were also investigated as potential candidates as genes encoding PLAs involved in the release of HFA from PC.

The overall goal of this thesis was to investigate the function of *pPLAIII* from *L. fendleri* or castor in transgenic Arabidopsis CL7 lines which were developed by expressing a castor *OLEATE 12- HYDROXYLASE* (*RcFAH12*) in a *fatty acid elongase 1* (*fae1*) mutant background (Lu et al., 2006). It was hypothesized that pPLAIII enzymes

from HFA-producing oilseeds may be involved in the efficient removal of HFAs from PC and that these genes could be used in a CL7 background to selectively release HFAs from the site of synthesis in PC and thus influence HFA accumulation in the seed oil (Figure 1). Complementary DNA (cDNA) encoding pPLAIII enzymes from *L. fendleri* or castor were expressed in Arabidopsis expressing castor *RcFAH12* in a seed-specific fashion. The impact of these heterologously expressed cDNAs was assessed by examining the HFA content of seed oil and PC. The results of this investigation shed light on possible processes involved in channeling HFAs from PC to TAG which in turn contribute to metabolic engineering strategies for enhancing the accumulation of HFAs in major oilseed crops which do not normally produce HFAs.

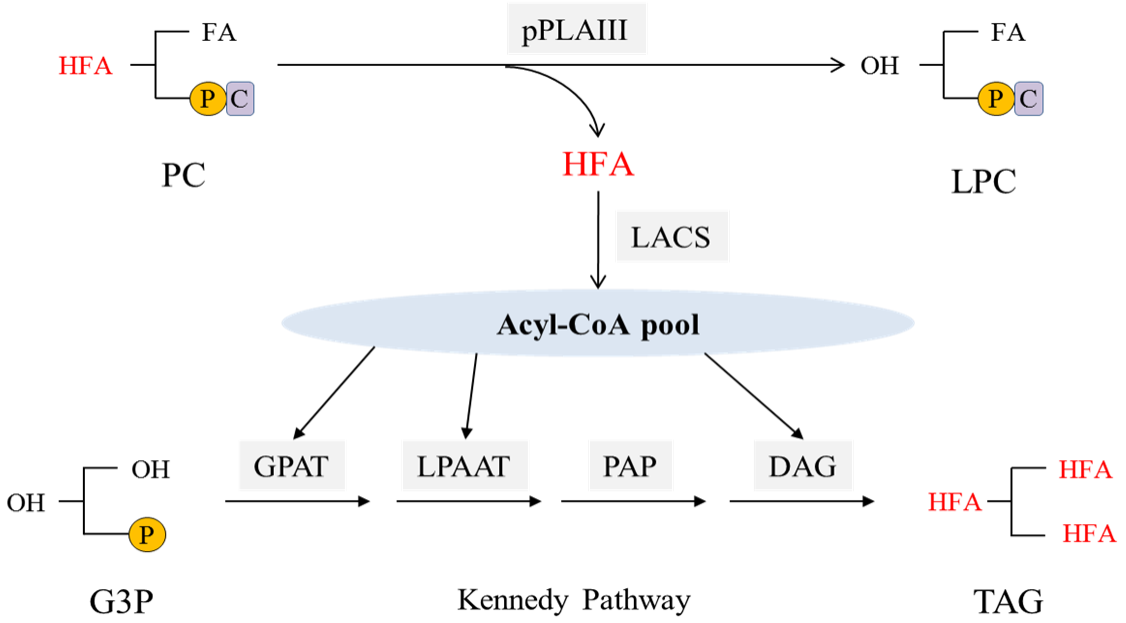


Figure 1 Hypothesis of the function of patatin-like phospholipase A III (pPLAIII) when introduced into Arabidopsis CL7 lines

This diagram is made based on the hypothesis of this thesis. pPLAIII enyzmes from hydroxy fatty acid (HFA)-producing oilseeds may be involved in the efficient liberation of HFAs from phosphatidylcholine (PC). When one of the corresponding genes is expressed in the Arabidopsis CL7 lines, i.e. a *fatty acid elongase 1* (*fae1*) mutant background bearing a castor *OLEATE 12-HYDROXYLASE* (*RcFAH12*), HFAs may be selectively removed from the site of synthesis in PC. Released HFAs may be activated to HFA-CoA, catalyzed by long-chain acyl-CoA synthetase (LACS), and may be utilized by the acyltransferases including *sn*-glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT) and diacylglycerol acyltransferase (DGAT) of the Kennedy pathway to synthesize HFA-containing triacylglycerol (TAG), the major storage lipid of seeds. The diagram of Kennedy pathway is adapted from Chapman and Ohlrogge (2012).

2 - Literature Review

The requirement for renewable resources is growing in the modern world. Conventional resources (e.g., nuclear fuels, fossil fuels) are considered to be un-renewable resources. Stocks of these resources are finite because of their extremely slow generation rates (Twidell and Weir, 2015). The development of renewable resources can relieve conventional energy supplies, contribute to sustainability and solve environmental problems (Dincer, 2000).

Biomass is one of the most common renewable resources (McKendry, 2002) with three major uses: heat/power production, vehicle fuels and chemical feedstock (Saxena et al., 2009). As members of energy crops, oil crops (e.g., oilseed rape [*Brassica napus*], castor [*Ricinus communis*] and sunflower [*Helianthus annuus]*) provide good sources for biomass. Their high energy density along with relative simple growing and extracting technologies make oil crops valuable resources (Demirbaş, 2001; Sims et al., 2006)

Elevating the seed yield or oil content of crops is an important way to feed the demanding market. In fact, for the Canadian canola industry alone, a 50 to 70 percent of absolute increase in the seed oil production was estimated to meet the requirement for the market in the following seven to ten years (Weselake et al., 2009). Another approach to improve the value of an oilseed crop is to manipulate the fatty acid composition of its seed oil to render it suitable for industrial applications. Palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1Δ^{9cis}; hereafter 18:1), linoleic acid (18:2$\Delta^{9cis,12cis}$; hereafter 18:2) and α- linolenic acid (18:3$\Delta^{9cis,12cis,15cis}$; hereafter α-18:3) are considered to be the five most common fatty acids in plant seed oils. On the other hand, unusual fatty acids (UFAs), such as hydroxy fatty acids (HFAs), epoxy fatty acids (EFAs) or conjugated

fatty acids (CFAs), are known to accumulate in the seed oils of various plant species. The properties of the UFAs make them useful in industrial applications (Kumar et al., 2006). The development of additional seed oils enriched in HFAs could increase the supply of renewable feedstock for developing industrial materials that may otherwise be derived from petrochemical sources.

Among the UFAs, HFAs are known to have great value. Their hydroxyl groups provide them unique properties for applications in the oleo-chemical industry (Ogunniyi, 2006). In this literature review, industrial applications of castor oil and HFAs and their production in transgenic plants are discussed.

2.1 Industrial applications of hydroxy fatty acids

HFAs contain a hydroxyl group (-OH) in their carbon chains. Ricinoleic acid (12-OH 18:1Δ^{9cis}; hereafter 18:1-OH) and lesquerolic acid (14-OH 20:1Δ^{11cis}, hereafter 20:1-OH) are two major HFAs found in nature. Castor oil is composed of 90% (w/w) ricinoleic acid and 10% non-hydroxylated fatty acids including oleic acid and linoleic acid (Conceição et al., 2007). The predominant triacylglycerol (TAG) species esterified with ricinoleic acid have all three positions on the glycerol backbone esterifed with HFA (i.e., tri-ricinoleoyl-glycerol) (Burgal et al., 2008).

Potential applications of vegetable oil in the industry, such as the production of lubricants, could be limited by their low oxidation stabilities associated with the degree of unsaturation. The autoxidation of oils is initiated by the generation of free radicals. The resultant peroxyl radical could abstract a hydrogen atom to produce a hydroperoxide,

which is the critical step of the autoxidation of the vegetable oil (Fox and Stachowiak, 2007). Since the formation of hydroperoxide can be prevented by hydroxyls, castor oil and its derivatives are very stable (Ogunniyi, 2006).

HFA enriched castor oil has been used in industry for a long time (Snapp et al., 2014; Lee et al., 2015). The use of castor oil and HFAs as chemical feedstock and biodiesel is discussed below.

2.1.1 Chemical feedstock

HFAs exhibit high solubility in alcohols and show up to 7-fold higher viscosity than other vegetable oils. Together with other properties, these fatty acids are suitable to be used as raw materials for chemical industries (Meneghetti et al., 2006). In general, the carboxyl groups of fatty acids are amenable to a wide range of esterifications. In the case of HFAs, the presence of the hydroxyl group imparts additional reactivity to facilitate additional chemical modifications. The versatility of the ricinoleoyl moiety for various chemical modifications is described below.

Firstly, castor oil is considered to be a non-drying oil with a single point of desaturation in the ricinoleoyl moiety. However, dehydration of the hydroxyl can introduce additional unsaturation to obtain a semi-drying or drying-oil (Ogunniyi, 2006). Varnishes, alkyds and resins made of dehydrated castor oils have outstanding qualities, including rapid drying, flexibility, high water and chemical resistance, and metal-adhesion characteristics. Hydrated castor oil is not only non-yellowing, with remarkable colour retention characteristics, but it also imparts high resistance to water and alkaline conditions (Mutlu and Meier, 2010).

Secondly, polymers can be formed through cross-linking reactions involving the hydroxyl functionalities of ricinoleic acid. Specifically, isocyanate, melamine, anhydride or t-butyl acetoacetate can be used to transform castor oil into useful polymers (Trevino and Trumbo, 2002).

Thirdly, under alkaline conditions of high-temperature pyrolysis, products with shorter chain length can be obtained through splitting of ricinoleic acid at the hydroxyl position (Ogunniyi, 2006). Pyrolysis yields two important raw materials, heptaldehyde 5 (C7) and undecenoic acid 6 (C11), for the production of cosmetics, including talcum powders, soaps, shampoo and perfume formulations. Additionally, solvents for rubber, plastics and resins can be provided by the heptaldehyde 5 (C7) while bactericides and fungicides can be made from the undecenoic acid 6 (C11; Mutlu and Meier, 2010). Products generated from alkaline splitting exhibit various industrial applications. They can be applied to the productions of alkyd resins, lubricants, plasticizers, candles, cosmetics, surfactants for medicines and hydraulic fluids (Mutlu and Meier, 2010).

In addition to the reactivity of the hydroxyl and carboxyl groups, the double bond of ricinoleic acid also adds value because it can be hydrogenated, epoxidated or vulcanized (Ogunniyi, 2006).

2.1.2 Biodiesel

The use of vegetable oil derivatives as alternatives to diesel fuels is the trend of the modern economic world. This strategy is both environmental friendly and economically viable. Although replacing conventional diesels with the castor oil is limited by the high viscosity of the crude oil, methods have been developed to improve this. For instance, castor oil can be trans-esterified with a short chain alcohol to reduce viscosity. The resultant fatty acid methyl esters (FAMEs) and ethyl esters (FAEEs) from reactions in the presence of methanol and ethanol, respectively, are good source for biodiesel applications (Meneghetti et al., 2006). Since the transesterification of castor oil can be conducted at room temperature, the cost of the castor oil-based biodiesel is lower than biodiesels generated from other oils which require higher temperatures for FAME or FAEE production (Conceição et al., 2007).

2.2 Natural sources of hydroxy fatty acids

Among higher plants, more than 12 genera of 10 families can produce ricinoleic acid (van de Loo et al., 1995). The conventional major natural source of HFA is the castor oil plant of family Eurphorbiacae (Ogunniyi, 2006). As indicated earlier, castor contains nearly 90% ricinoleic acids in its seed oil (Conceição et al., 2007). Nowadays, the plants are mainly grown in tropical and subtropical areas. The largest producer of castor oil is India, accounting for 70% of the world supply, followed by China, Brazil and Thailand (Mutlu and Meier, 2010).

In addition, *Lesquerella fendleri*, a member of the Brassicaceae family, has the potential to be a HFA-producing agronomic crop in North American, since its seed accumulates 55-60% of lesquerolic acid (20:1-OH) in the oil (Chen et al., 2011a; Lee et al., 2015). Other species of the Lesquerella genus, including *Lesquerella kathyrn* and *Lesquerella auriculata* mainly accumulate densipolic acid (12-OH 18:2$\Delta^{9cis,15cis}$, hereafter 18:2-OH) and auricolic acid (14-OH 20:2$\Delta 11^{cis,17cis}$, hereafter 20:2-OH), respectively (Reed et al., 1997).

Additionally, HFAs can also be found in non-plant sources. For example, a pathogenic fungal ergot, *Claviceps purpurea*, accumulates a substantial amount of ricinoleic acid in its sclerotia (Morris et al., 1966; Billault et al., 2004; Meesapyodsuk and Qiu, 2008).

2.3 Anticipated impact of developing oilseed crops enriched in hydroxy fatty acids

The annual global production of castor oil was 645,000 tons in 2014, which was relatively low compared to the soybean oil production at more than 42 million tons in 2013 (McKeon et al., 2016). Moreover, other limitations exist. Although castor has a very high ricinoleic acid content in its oil, there are limitations of using this plant as the major source of hydroxy oil. Firstly, castor seeds contain the extremely toxic protein ricin and the allergenic 2S albumins, which cause safety problems (Balint et al., 1974; Holic et al., 2012). Secondly, castor plants are not suitable for large-scale growth due to difficulties associated with mechanical harvesting (Holic et al., 2012). Finally, economic and

political instabilities in castor-producing regions of the world can potentially impact the supply, price and quality of castor oil (Holic et al., 2012; Lee et al., 2015).

As previously mentioned, *L. fendleri* accumulates approximately 60% of lesquerolic acid (20:1-OH) in its seed oil. *L. fendleri* oil has slightly different physicochemical properties compared to castor oil which are associated with lesquerolic acid being two-carbons longer in length than ricinoleic acid. However, despite the potential advantages of *L. fendleri*, it has yet to become a commercial oilseed crop. For acceptance for large scale growth, a number of issues need to be addressed relating to potential pests or diseases, suitable soil types, irrigation methods and herbicide options (Dierig et al., 2011).

Modifying established temperate oil crops to produce HFAs seems to be a feasible strategy to overcome limitations associated with natural sources of oils containing HFAs. It is, however, proving to be a challenge to obtain a substantial level of HFAs in the seed oils of these engineered crops (Kumar et al., 2006).

To develop oil crops enriched in HFAs, an in-depth knowledge of at least two areas is required: 1) the mechanism of the storage lipid biosynthesis in plants; 2) the biosynthesis of HFAs in organisms that naturally accumulate these fatty acids. These processes and the enzymes involved are discussed in the remainder of the literature review.

2.4 Storage lipid biosynthesis in plants

In most plants, the predominant storage lipid is TAG, the synthesis of which can be divided into three steps: 1) *de novo* fatty acid synthesis in plastids; 2) the export of nascent fatty acids from the plastid to the endoplasmic reticulum (ER) membrane and 3) the TAG assembly in the ER membrane.

2.4.1 *de novo* fatty acid synthesis

In plant cells, fatty acids are synthesized *de novo* in plastids (Chapman and Ohlrogge, 2012; Figure 2.1). Acetyl-Coenzyme A (CoA), which is mainly derived from pyruvate by the reaction catalyzed by plastidial pyruvate dehydrogenase (PDH), is the initial substrate for the plastidial fatty acid synthesis. Fatty acid synthesis involves the catalytic action of acetyl-coA carboxylase (ACCase) and the fatty acid synthase (FAS) complex (Ohlrogge and Browse, 1995; Ohlrogge and Jaworski, 1997).

Two types of ACCase exist in most plants. The plastidial heteromeric ACCase initiates the first committed step of fatty acid synthesis by converting acetyl-CoA into malonyl-CoA. Plastidial heteromeric ACCase action generates malonyl-CoA for use by the FAS complex. In contrast, the cytosolic homomeric ACCase is involved in the embryo development and the generation of very long chain fatty acids (VLCFAs) (Sasaki and Nagano, 2004).

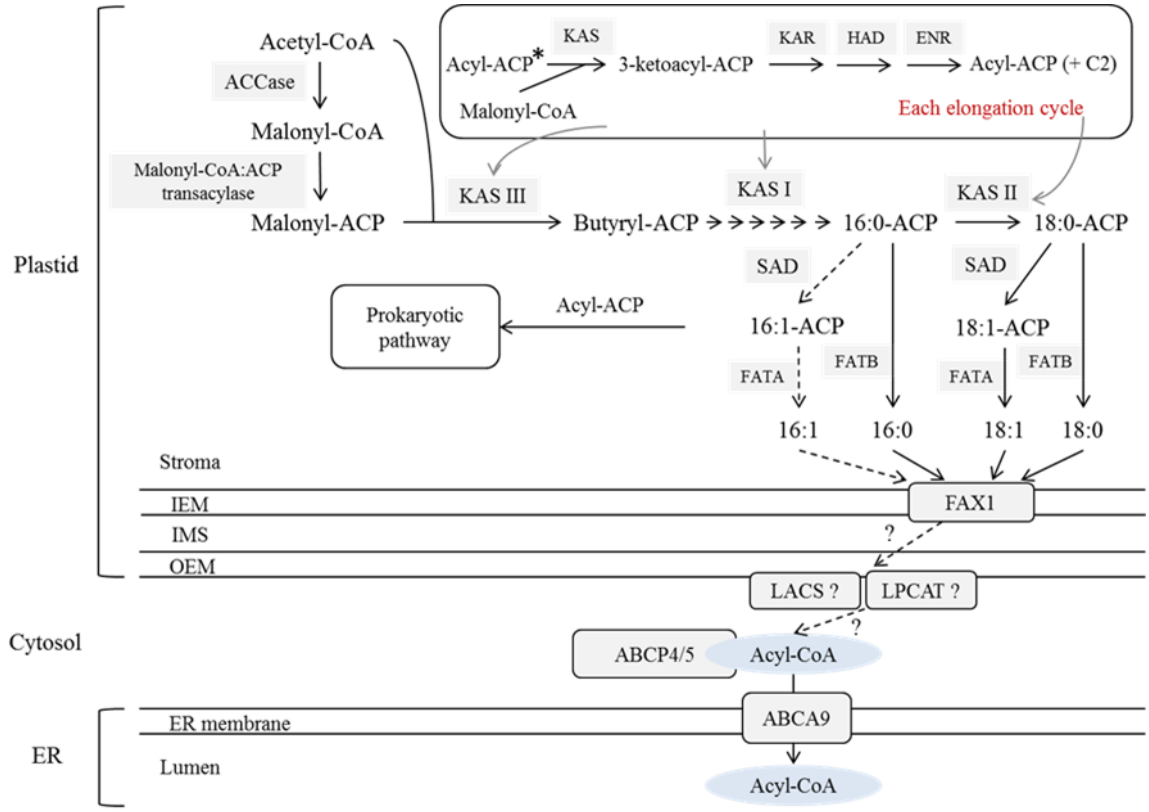


Figure 2.1 *de novo* fatty acid synthesis and the export from the plastid

Acetyl-CoA carboxylase (ACCase) catalyzes the first step of *de novo* fatty acid synthesis, converting acetyl-CoA into malonyl-CoA which is subsequently converted into malonyl-acyl carrier protein (ACP), catalyzed by malonyl-CoA:ACP transacylase. Acyl-ACP is elongated in successive cycles that requires ketoacyl-ACP synthases (KAS), ketoacyl-ACP reductase (KAR), hydroxyacyl-ACP dehydrase (HAD) and enoyl-ACP reduatase (ENR) for condensation, reduction, dehydration and reduction. KAS III catalyzes the condensation of acetyl-CoA* and manlonyl-ACP to synthesize 3-ketobutyryl (4:0)-ACP in the first cycle; KAS I and KAS II catalyze the condensation in cycles from 4:0-ACP to 16:0-ACP and 16:0-ACP to 18:0-ACP, respectively, using malonyl-CoAs as acyl-donors. Stearoyl (18:0)-ACP desaturase (SAD) catalyzye conversion of 18:0-ACP to 18:1-ACP. Acyl-ACP thioesterases A (FAT A) and FAT B catalyze hydrolysis of unsaturated and saturated acyl-ACPs, respectively, to release free fatty acids (c.f. Chapman and Ohlrogge, 2012). Free FAs are subsequently transported through the inner envelop membrane (IME) via fatty acid export 1 (FAX1) and an unknown mechanism through the intermembrane space (IMS) and outer envelope membrane (OEM). Fatty acids may be esterified to coenzyme A (CoA) by the catalytic action of long-chain acyl-CoA synthetase (LACS) or be exchanged between phosphatidylcholine (PC) and acyl-CoA catalyzed by lysophosphatidylcholine acyltransferase (LPCAT). The resultant acyl-CoA may be transported by acyl-CoA binding proteins (ABCPs) through the cytosol and channeled into endoplasmic reticulum (ER) membrane via ATP-binding cassette (ABC) transporter (c.f. Block and Jouhet, 2015).

Plant type II FAS, a dissociable complex with multiple subunits, is involved in the generation of 16- to 18- carbon fatty acids (Li-Beisson et al., 2010). After the malonyl moiety of malonyl-CoA is transferred by the catalytic action of malonyl-CoA:acyl carrier protein (ACP) transacylase, the resultant malonyl-ACP is condensed with acetyl-CoA to form butyryl (4:0)-ACP. In subsequent successive extensions, a 2-carbon unit (donated by malonyl-CoA) is added to each cycle (Ohlrogge and Browse, 1995). Each cycle consists of condensation, reduction of the 3-keto group, dehydration and reduction of the double bond. The enzymes, required to catalyze these reactions are 3-ketoacyl-ACP synthases (KAS), 3-ketoacyl-ACP reductase (KAR), 3-hydroxyacyl-ACP dehydrase (HAD) and enoyl-ACP reduatase (ENR).

KAS III catalyzes the initial condensation of acetyl-CoA and malonyl-ACP, while KAS I and KAS II catalyze the condensations within elongations from 4:0 to 16:0 and 16:0 to 18:0, respectively. Furthermore, a proportion of 18:0-ACP can be further modified by the catalytic action of a stearoyl (18:0)-ACP desaturase (SAD) to generate 18:1-ACP (Ohlrogge and Jaworski, 1997; Li-Beisson et al., 2010). In some cases, a double bond can also be introduced to the 16:0-ACP to form 16:1-ACP (Jaworski and Cahoon 2003).

When reaching 16 or 18 carbons in length, there are two different pathways for use of the newly synthesized acyl-ACPs. The acyl-ACPs can serve as acyl-donors in plastidial lipid synthesis, namely the “prokaryotic pathway” (Harwood, 1996; Jessen et al., 2015) or, alternatively, the acyl chains can be released from ACP by the catalytic action of acyl-ACP thioesterases (FATs). Two different FATs, FAT A and FAT B show substrate specificities towards unsaturated and saturated acyl chain, respectively. After

that, the nascent 16:0, 18:0 and 18:1 free fatty acids can be converted to acyl-CoA and enter the "eukaryotic pathway" to assemble glycerolipids in the ER membrane (Bates et al., 2013).

2.4.2 Export of fatty acids from the plastid

To enter the "eukaryotic pathway", the export of nascent free fatty acids from plastids is necessary (Figure 2.1). The widely accepted concept is that the plastidial fatty acids are activated to acyl-CoA before entering the lipid synthesis in the ER membrane (Koo et al., 2004). However, many details of the mechanism are unclear. After being released by FAT A/B in the plastidial inner envelope membrane (IEM), the nascent free fatty acids may then be channelled across envelop membranes, possibly by facilitated diffusion (Block and Jouhet, 2015). Recently, a membrane protein identified as fatty acid export1 (FAX1) was proposed to mediate fatty acid export through the IEM, while other proteins associated with the channel through the intermembrane space (IMS) and outer envelope membrane (OEM) remain to be characterized (Li et al., 2015).

Activation of released fatty acids to acyl-CoA was assumed to be catalyzed by long-chain acyl-CoA synthetases (LACSs) in OEM. However, inactivation of plastid envelope LACS9, which was thought to be the predominant LCAS catalyzing this process, did not alter phenotype and lipid accumulation in *Arabidopsis thaliana* (Arabidopsis; Schnurr et al., 2002). This may be because the ER-located LACS1 overlaps in function with LACS9 given that the *lacs1 lacs9* double mutants and *lacs1 lacs8 lacs9* triple mutants showed a 11% and 12% decrease in oil content, respectively (Zhao et al., 2010). Surprisingly, instead of fatty acid export from plastids to the ER, the latest research has shown that LACS4 and LACS9 have an overlapping function in trafficking

acyl chains into the plastid (Jessen et al., 2015). Subsequently, acyl-CoA binding proteins (ACBPs), such as AtACBP4 and AtACBP5 may be involved in channelling of acyl-CoAs and protecting the thioesters in the cytosol (Xiao and Chye, 2011). Furthermore, ATP-binding cassette (ABC) transporter proteins may also play roles in exporting acyl chains from plastids or importing acyl derivatives into the ER (Hurlock et al., 2014). A member of this family, the ER membrane-located AtABCA9 transporter, has been demonstrated to be involved in acyl-CoA import into the ER (Kim et al., 2013).

In addition, phosphatidylcholine (PC) may also be an intermediate to channel acyls from plastids to the ER via acyl editing. In this pathway, acyl chains are exchanged among PC, lyso-PC (LPC) and acyl-CoAs without net synthesis. The theory is supported by the fact that PC is enriched in the cytosolic leaflet of the OEM and that activities of LACS and lysophosphatidylcholine acyltransferase (LPCAT) have been found on the OEM. The forward reaction of LPCAT catalyzes the acyl-CoA-dependent acylation of LPC to produce PC and CoA while the combined forward/reverse reactions catalyzed by LPCAT may be involved in acyl-exchange between PC and the acyl-CoA pool (Chen et al., 2015). The functional importance of direct membrane contact sites (MCSs) between the plastid and the ER membrane remained to be further investigated (Block and Jouhet, 2015).

2.4.3 Triacylglycerol assembly

The assembly of TAG occurs in the ER membrane (Chapman and Ohlrogge, 2012). The basic pathway for *de novo* TAG synthesis, known as Kennedy pathway, includes four enzymatic steps (Figure 2.2). It begins with the sequential acylation of *sn*-1 and *sn*-2 position of *sn*-glycerol-3-phosphate (G3P) catalyzed by *sn*-glycerol-3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAAT), respectively. Subsequently, the phosphate group from phosphatidic acid (PA) is removed by the catalytic action of phosphatidic acid phosphatase (PAP), followed by the final step which involves acylation of the *sn*-3 position of *sn*-1, 2-diacylglycerol (DAG) catalyzed by diacylglycerol acyltransferase (DGAT) to generate TAG. The acyl substrates for this pathway are provided by the acyl-CoA pool (Weselake et al., 2009). However, over these years, growing evidences have shown that the mechanism of TAG assembly in plants is more complicated than this (Wang et al., 2012).

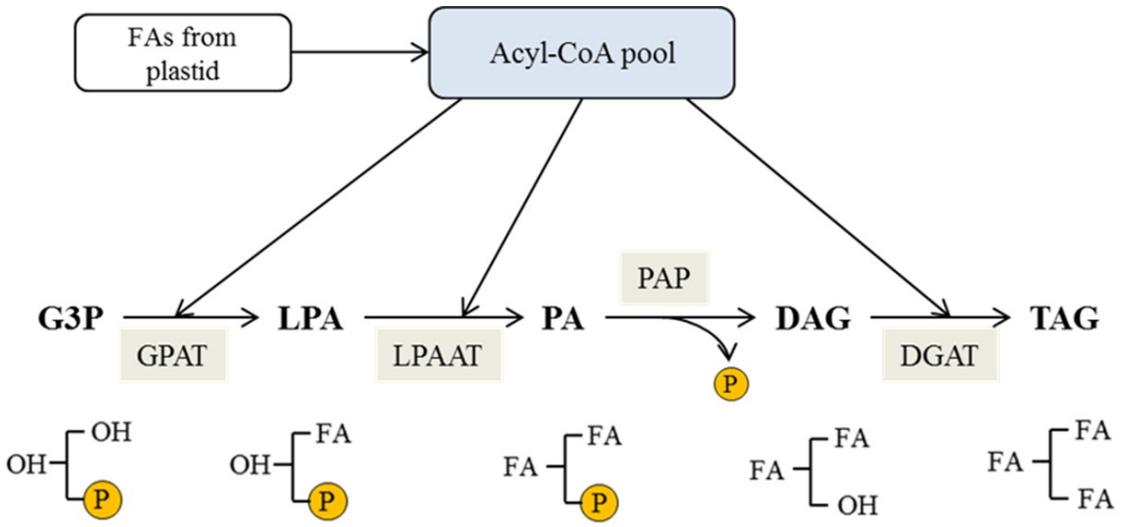


Figure 2.2 Kennedy pathway for triacylglycerol (TAG) assembly in the endoplasmic reticulum (ER) membrane

The basic pathway for *de novo* TAG synthesis, known as Kennedy pathway, includes four enzymatic steps in the ER membrane. Fatty acids (FAs) imported from *de novo* plastidial synthesis are incorporated into acyl-coenzyme (CoA) pool which provides substrates for following acylations. *Sn*-Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the acylation of the *sn-1* position of *sn*-glycerol-3-phosphate (G3P) to generate *sn-1* lysophosphatidic acid (LPA) and lysophosphatidic acid acyltransferase (LPAAT) catalyzes the acylation of the *sn*-2 position of LPA to generate phosphatidic acid (PA). Subsequently, phosphatidic acid phosphatase (PAP) catalyzes the removal of the phosphate group from PA to form *sn*-1, 2-diacylglycerol (DAG). In the last step, diacylglycerol acyltransferase (DGAT) catalyzes the acylation of the *sn*-3 position of DAG to generate TAG (c.f. Chapman and Ohlrogge, 2012).

A large proportion of fatty acids entering the "eukaryotic pathway" are 18:1, together with a relatively small amount of 18:0 and 16:0. In oleaginous plants which produce polyunsaturated fatty acids (PUFAs), such as 18:2 and α-18:3, the fatty acid composition of the seed oil is influenced by fatty acid modifications in the ER (Bates et al., 2012). Very-long-chain fatty acids (VLCFAs, fatty acids with greater than 18-carbon in length) are generated by the elongation of C18-CoAs in the cytosol. The key enzyme for this process, 3-ketoacyl-CoA synthase (KCS), is encoded by the *FATTY ACID ELONGASE 1* (*FAE1*) locus (Rossak et al., 2001; Das et al., 2002). Other modifications, such as desaturation and hydroxylation, utilize PC as substrates (Allen et al., 2015). PC can be derived from DAG synthesized *de novo* in the Kennedy pathway (Bates and Browse, 2011) or the acylation of LPC with nascent fatty acids (Wang et al., 2012). In most cases, within PC, the fatty acid desaturase 2 (FAD2) and fatty acid desaturase 3 (FAD3) catalyze the formation of 18:2 and α-18:3, respectively (Bates et al., 2012). In other species FAD2-like enzymes, including fatty acid hydroxylases (FAHs), epoxgenases, conjugases or acetylenases, catalyze the modification of 18:1 to produce UFAs (Singh et al., 2005).

There are three general processes for facilitating the movement of modified fatty acids (mFAs) within PC into TAG: 1) Acyl editing, also called "remodeling" or "retailoring"; 2) conversion of PC to DAG; and 3) direct transfer from PC to TAG (Bates et al., 2012; Figure 2.3).

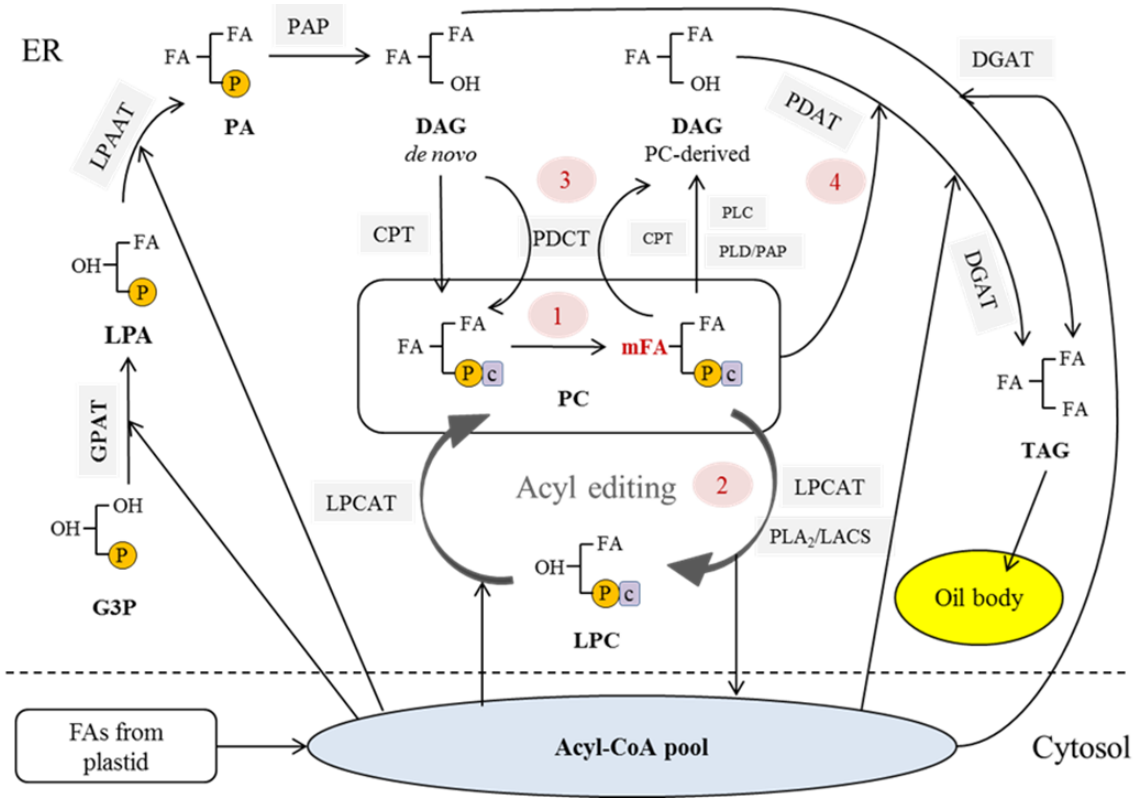


Figure 2.3 Possible pathways for modified fatty acids (mFAs) to be channelled from phosphatidylcholine (PC) into triacylglycerol (TAG)

(1) 18:1 esterified at the *sn*-2 position of the PC can be modified by fatty acid desaturase 2 (FAD2) or FAD2-like enzymes to generate modified fatty acids (mFAs). For example, ricinoleic acid (12-OH 18:1) can be synthesized by the catalytic action of oleate 12-hydroxylase (FAH12) and converted into densipolic acid (12-OH 18:2$\Delta^{9cis,15cis}$) catalyzed by fatty acid desaturase 3 (FAD3); **(2)** hydroxyl fatty acids (HFAs) can be incorporated into acyl-coenzyme A (CoA) pool through acyl editing catalyzed by lysophosphatidylcholine acyltransferase (LPCAT) or the combined reaction of phospholipase A_2 (PLA_2) and long-chain acyl-CoA synthetase (LACS). Subsequently, the HFA-CoA can be utilized by acyltransferases (*sn*-glycerol 3-phosphate acyltransferase [GPAT], lysophophatidic acid acyltransferase [LPAAT], diacylglycerol acyltransferase [DGAT]) for TAG synthesis. **(3)** *sn*-2 HFA-PC can be converted into PC-derived DAG catalyzed by phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) or CDP-choline:diacylglycerol cholinephosphotransferase (CPT) or phospholipase C (PLC) or the combined reaction of phospholipase D (PLD) and PAP. The PC-derived DAG can be utilized for TAG synthesis. **(4)** HFA can be directly transferred from the *sn*-2 position of PC to the *sn*-3 position of *sn*-1,2-DAG catalyzed by phospholipid:diacylglycerol acyltransferase (PDAT) to synthesize TAG (c.f. Allan et al., 2015). Other abbreviations: ER, endoplasmic reticulum; DAG, diacylglycerol; G3P, glycerol 3-phosphate; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PA, phosphatidic acid

Acyl editing is defined as acyl exchanges between polar lipids but without any net synthesis of polar lipids (Bates et al., 2007). It involves the hydrolysis of PC to LPC and free fatty acids, possibly catalyzed by phospholipase A_2, and the reacylation of LPC to produce PC catalyzed by LPCAT (Bates et al., 2009; Shindou and Shimizu, 2009). LPCAT1 and LPCAT2 have been shown to be responsible for the acylation of *sn*-2 position of LPC to reproduce PC in Arabidopsis (Bates et al., 2012; Wang et al., 2012). This process is referred to as the Lands cycle (Wang et al., 2012). Alternatively, the deacylation of PC can be catalyzed by the reverse reaction of LPCAT (Yurchenko et al., 2009; Wang et al., 2012). The mFAs in the acyl-CoA pool could be utilized by enzymes of Kennedy pathway (Allen et al., 2015) and for the net synthesis of PC (Bates et al., 2007).

Another pathway for mFAs to enter TAG is through the acyl exchange between PC and DAG. The DAG molecule synthesized from Kennedy pathway is called *de novo* DAG. However, the predominant DAG molecules for TAG synthesis are derived from PC, namely PC-derived DAG (Bates and Browse, 2011). It has been demonstrated that phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT), encoded by the *REDUCED OLEATE DESATURATION1* (*ROD1*), is the main enzyme that catalyzes the interconversion between PC and DAG in Arabidopsis (Lu et al., 2009). Early studies proposed that CDP-DAG:cholinephosphotransferase (CPT) may be essential for *de novo* PC synthesis and its reverse reaction may also contribute to the synthesis of PC-derived DAG (Slack et al., 1983; Slack et al., 1985; Lu et al., 2009). Moreover, phospholipase C (PLC) can catalyze the hydrolysis of the phosphorylated polar headgroup of PC to yield DAG (Carrasco and Mérida, 2007). The combined reaction of phospholipase D (PLD)

and PAP may be involved due to the fact the PUFA species decreased in TAG in the PLD-suppressed soybean (*Glycine max*) lines (Lee et al., 2011).

Alternatively, mFAs can be directly transferred from PC to DAG. Phospholipid:diacylglycerol acyltransferase (PDAT) catalyzes the transfer of an acyl chain from the *sn*-2 position of PC to the *sn*-3 position of DAG to yield TAG and LPC (Dahlqvist et al., 2000). Furthermore, the lethality of *dgat1-1 pdat1-2* double Arabidopsis mutants revealed the essential role of PDAT in TAG synthesis and its overlapping function with DGAT1 for seed development (Zhang et al., 2009). These enzymes may be specific to UFAs based on the species. For example, *Crepis palaestina* PDAT preferred vernoloyl moieties (12, 13-epoxy 18:1Δ^{9cis}) and castor PDAT preferred both ricinoleoyl and vernoloyl groups (Dahlqvist et al., 2000) whereas flax (*Linum usitatissimum* L.) PDAT selectively catalyzed the transfer of α-18:3 to α-18:3-enriched DAG (Pan et al., 2013).

Acyl fluxes for TAG assembly vary among species and tissues of plants (Allen et al., 2015). For example, cocoa (*Theobroma cacao*), which contains <2% PC-mFAs in storage lipid, utilizes the conventional Kennedy pathway as the major route to assemble TAG (Griffiths and Hardwood, 1991; Allen et al., 2015). Castor, however, appears to use a combination of acyl editing, conversion of PC to DAG and direct transfer of acyl groups from PC to TAG in channelling HFA into TAG (Bates and Browse, 2011). A similar mix of processes may be used to move PUFA from PC into TAG in temperate oilseeds such as soybean and Arabidopsis (Bates and Browse, 2011).

2.5. Plant phospholipase A

Recent studies have suggested that plant phospholipase As (PLAs) may have a role in TAG assembly and acyl editing (Li et al., 2013; Chen et al., 2015). Phospholipases are a group of enzymes that catalyze the hydrolysis of phospholipids. These enzymes can be classified based on their positional specificity towards substrates (Wang, 2001; Figure 2.4).

PLA catalyzes the hydrolysis of fatty acyl groups from phospholipids to yield lysophospholipids. Phospholipase B (PLB), which has not been characterized in plants, works on both the *sn*-1 and *sn*-2 positions of phospholipids. Phospholipase C (PLC) and phospholipase D (PLD) cleave the glycerophosphate bond and terminal phosphodiesteric bond, to yield DAG and PA, respectively (Wang, 2001; Chen et al., 2011b).

In plants, the hydrolytic products of phospholipase action directly or indirectly mediate many biochemical and physiological processes including lipid metabolism, cellular signal transduction and stress responses (Chen et al., 2011b). This section focuses on PLA, with an emphasis on patatin-like phospholipase A (pPLA) and the function of this phospholipase category in plant lipid biosynthesis.

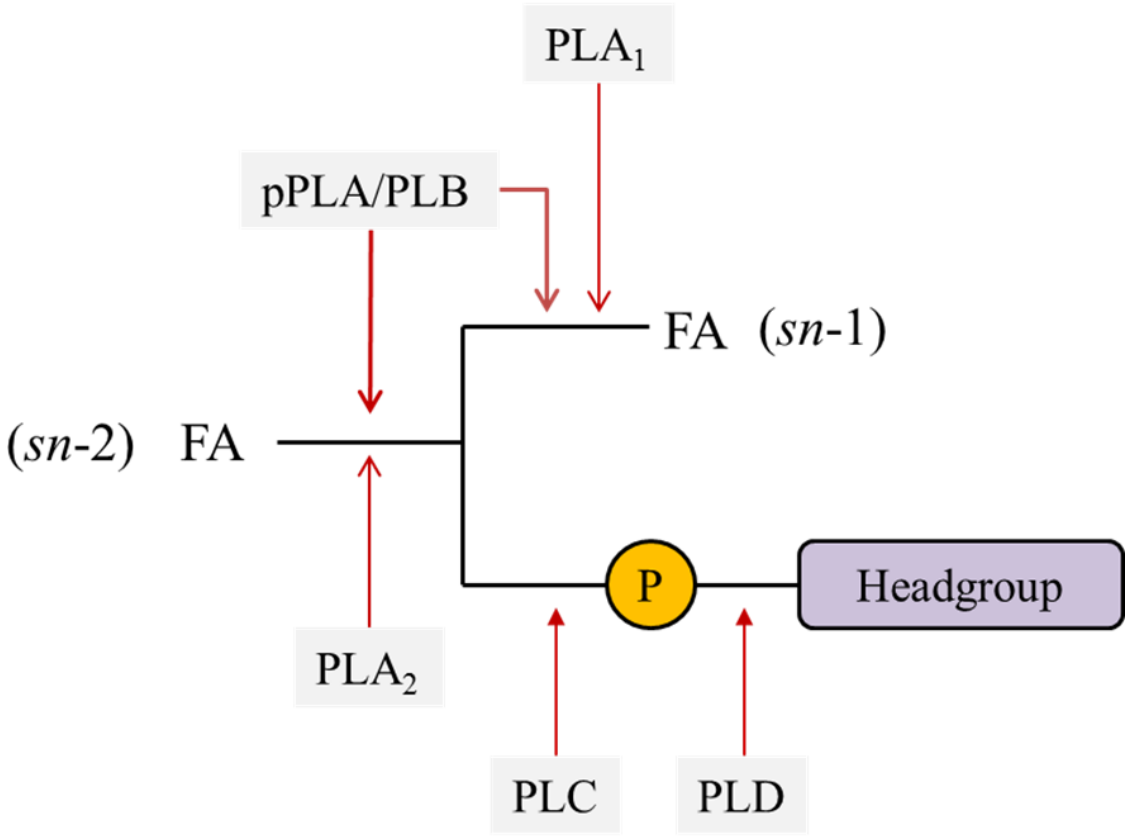


Figure 2.4 Positional specificity of phospholipases

Phospholipase A (PLA) is composed of three families: PLA_1 and PLA_2 catalyze the release of the acyl chain from the *sn*-1 and *sn*-2 position of phospholipid, respectively, while patatin-like phospholipase A (pPLA) can catalyze fatty acid liberation at both positions. PLB is able to sequentially cleave acyl chains from *sn*-1 and *sn*-2 positions. Phospholipase C (PLC) and phospholipase D (PLD) attaci the glycerophosphate bond and the terminal phosphodiesteric bond of phospholipids, respectively. This diagram is adapted from Chen et al. (2011b).

2.5.1 Classification of plant phospholipase As

There are three families of PLAs according to the bond they cleave. PLA_1 and PLA_2 attack the *sn*-1 and *sn*-2 positions of phospholipids, respectively, whereas pPLA display activity at both positions (Chen et al., 2013; Figure 2.4).

Plant PLA_1s can be further classified into five groups according to the existence of N-terminal stretches and similarities of amino sequences in the catalytic region (Figure 2.5A). These five groups are named as PLA_1-I, PLA_1-II, PLA_1-III, lecithin:cholesterol acyltransferase-like PLA_1 (LCAT-PLA_1) and PA-PLA_1 (Chen et al., 2011b). Although their cellular localizations vary, all plant PLA_1 shares some common features: 1) molecular masses ranging from 45-50kDa; 2) the presence of a conserved Gly-x-Ser-x-Gly (GxSxG) motif (but it is SxSxG in LCAT-PLA_1); 3) the existence of a catalytic triad consisting of Ser, Asp and His residues (Matos and Pham-Thi, 2009; Chen et al., 2013).

Plant secretory phospholipase A_2 (sPLA_2; Figure 2.5B) are soluble and small in molecular mass (13-18kDa). In Arabidopsis, four forms of sPLA_2 (α, β, γ, δ) have been identified and functionally expressed (Chen et al., 2011b). They all contain a conserved calcium binding-loop (YGKYCGxxxxGC) and a catalytic site DACCxxHDxC motif within which a well-conserved Histidine/Aspartate dyad (HD) has been observed (Chen et al., 2013).

The gene encoding a novel PLA known as LCAT-PLA has been shown to be highly expressed in roots and siliques of Arabidopsis. *In vitro* enzyme assays showed that LCAT-PLA exhibits activity to both the *sn*-1 and *sn*-2 position, with a preference for catalyzing the release of fatty acid from the *sn*-2 position of PC (Chen et al., 2012).

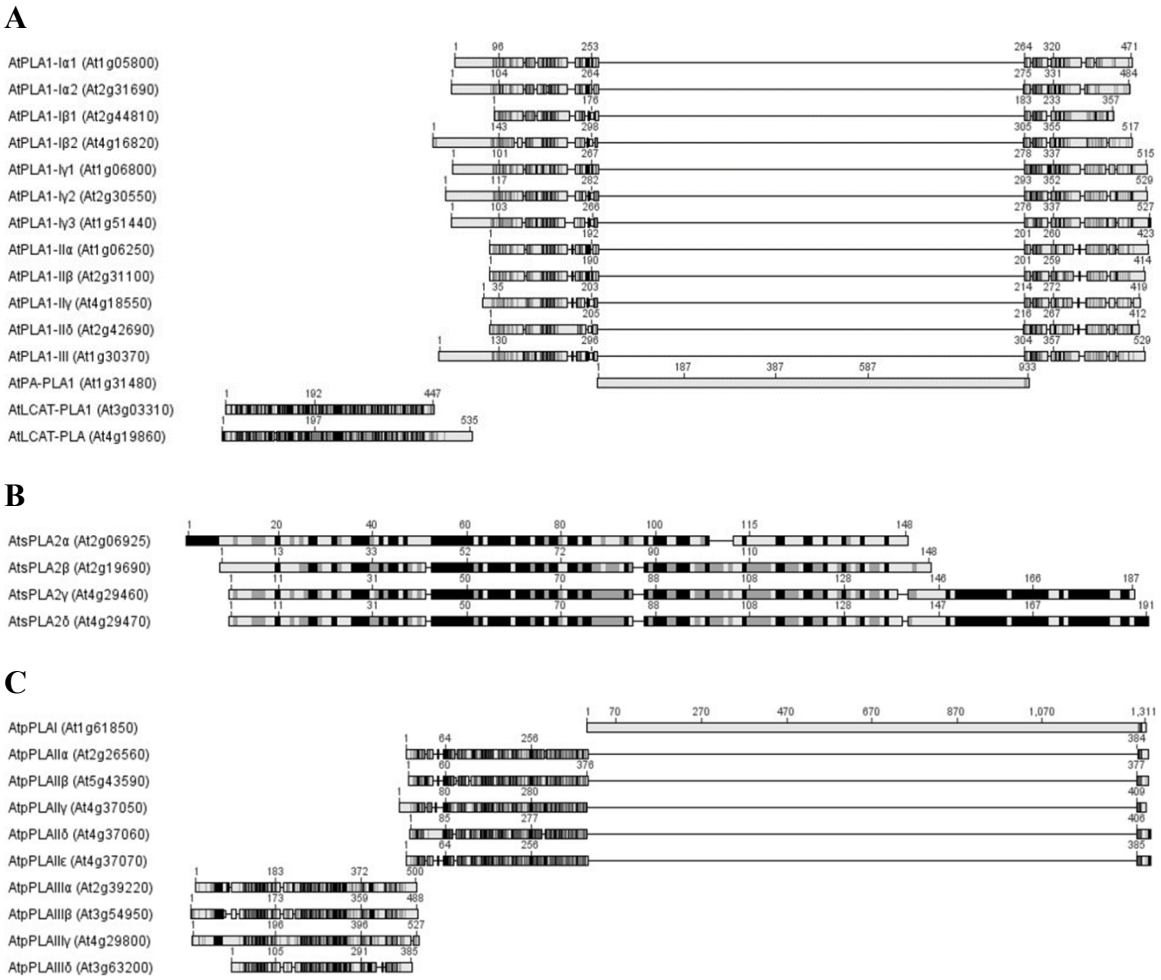


Figure 2.5 Alignment of deduced amino acid sequences of phospholipase As (PLAs) from Arabidopsis

(A) AtPLA$_1$: Sublass I (α1, α2, β1, β2, γ1, γ2, γ3), Subclass II (α, β, γ, δ), Subclass III; PA-PLA$_1$, LCAT-PLA$_1$; LCAT-PLA are aligned with LCAT-PLA$_1$ to show their homology. (B) AtPLA$_2$ (α, β, γ, δ). (C) AtPatatin-like phospholipase A (pPLA): Subclass I, Subclass II (α, β, γ, δ), Subclass III (α, β, γ, δ).

The deduced amino acid sequences were aligned using Geneious Alignment of Geneious Pro 5.4.6 software, which was based on the progressive pairwise alignment method, in the type of global alignment. The grey shadings represented consensuses of two or more sequences; the black shadings represented consensuses of all sequences aligned; the lines represented the absences of amino acids. See Appendix1 for more detailed sequence alignments.

However, AtLCAT-PLA is distant from all known PLA_2s in plants and instead shares close homology with AtLCAT-PLA_1 (Figure 2.5A; Chen et al., 2012) which is a homolog of animal lecithin:cholesterol acyltransferases (LCATs) and belongs to PLA_1 (Noiriel et al., 2004).

pPLA is another family of PLAs that catalyze the hydrolysis of fatty acids from both the *sn*-1 and *sn*-2 position of phospholipids and other glycerolipids (Chen et al., 2013). These enzymes share sequence homology with patatins (Scherer et al., 2010) which are vacuolar lipid hydrolases found in tubers of solanaceous plants (Chen et al., 2013). In Arabidopsis, there are 13 pPLAs that have been identified. Ten of these enzymes (Figure 2.5C) are grouped into three classes: pPLAI, pPLAII (α, β, γ, δ, ε) and pPLAIII (α, β, γ, δ), according to their genomic sequences, gene structure and enzymatic activities (Li and Wang, 2014).

The pPLAI, with the relatively large molecular mass of 156 kDa, is considered to be the evolutionarily oldest due to its recognizable homology to animal iPLA_2s. It contains a leucine-rich repeat domain in the C terminal region and a weak ankyrin-like domain in N terminal region (Scherer et al., 2010; Chen et al., 2013). Subclass II pPLAs are more similar to patatin than the ones of subclass III (Li and Wang et al., 2014). In the catalytic centers, the canonical sequences have been found in both the pPLAI and pPLAIIs. They are comprised of the esterase box GxSxG, a conserved aspartic residue in motif DGG/DGA, and the binding element DGGGxxG for phosphate/anion. The Ser residue in the esterase box and the Asp residue in DGG are critical in the S-D catalytic dyad (Li et al., 2011). In contrast, pPLAIIIs include a GxGxG motif instead of GxSxG (Chen et al., 2011b). The Asp residue of the DGG/DGA motif is replaced by Gly in

pPLAIIIβ and pPLAIIIγ (Li et al., 2011). The same as pPLAI, pPLAIIs have five to six introns in the catalytic domains while pPLAIIIs have only one intron (Li et al., 2011). The distinct characteristics of pPLAIIIs suggested their plant specificities (Scherer et al., 2010). The fourth subclass of pPLAs, including SDP1, SDP1-L and adipose triglyceride lipase-like (ATGL-L), show TAG lipase activities that are not found in other three subclasses (Li and Wang, 2014).

2.5.2 Enzymatic activities of patatin-like phospholipase AIIIs

pPLAIIIβ has been shown to be localized to the plasma membrane in Arabidopsis. The transcripts of this gene were detected at various developmental stages with relatively lower levels in young and developed rosettes. Among the plant organs, the expression of *pPLAIIIβ* was relatively high in roots and old leaves but low in siliques (Li et al., 2011).

In vitro enzyme assays suggested that pPLAIIIβ can catalyze the hydrolysis of both phospholipids and galactolipids. It showed both PLA_1 and PLA_2 activities towards PC, but preferentially catalyzed the hydrolysis of acyl chains from the *sn*-2 position. In addition, PA, PE, phosphatidylglycerol (PG) and phosphatidylserine (PS) also served as substrates for pPLAIIIβ (Li et al., 2011). Among the galatolipids, pPLAIIIβ preferred digalactosyldiacylglycerol (DGDG) to monoglactosyldiacylglycerol (MGDG). Furthermore, pPLAIIIβ exhibited acyl-CoA thioesterase activity but did not act upon the neutral lipids, DAG or TAG (Li et al., 2011). *In vivo* analysis indicated changes in lipid content and composition which accompanied alterations of *pPLAIIIβ* in Arabidopsis. Specifically, compared with the wild type, young rosettes of *pPLAIIIβ* knockout mutants (*pPLAIIIβ*-KO) accumulated 20% less total fatty acids while those from *pPLAIIIβ*-ove-rexpressing lines (*pPLAIIIβ*-OE) accumulated 15% more fatty acids. *pPLAIIIβ*-OE also

contained a 15% higher level of lysophospholipids than the wild type. Total lipids, including phospholipids and galactolipids, accumulated at 15% higher levels in *pPLAIIIβ*-OE, but were substantially lower in *pPLAIIIβ*-KO than in the wild type (Li et al., 2011).

pPLAIIIα is highly expressed in siliques (Li et al., 2011) but mostly in seed coats and peripheral endosperm which are not the main oil storage tissues in Arabidopsis seeds (Li et al., 2013). Rice (*Oryza sativa*) *pPLAIIIα*-KO had no significant changes in lipid compositions, whereas *pPLAIIIα*-OE displayed substantial reduction in PA content and slight decrease in the contents of PC, PG, MGDG and DGDG in leaves relative to wild-type rice (Liu et al., 2015).

Both pPLAIIIα and pPLAIIIβ have been reported to affect the morphology of plants (Li et al., 2011; Liu et al., 2015). *pPLAIIIβ*-OE Arabidopsis plants exhibited shorter primary roots, root hairs, leaves, petioles and hypocotyls, while *pPLAIIIβ*-KO had the opposite phenotype. Likewise, pPLAIIIα-OE rice plants had shorter stems, roots, leaves, seeds and panicles, whereas pPLAIIIα-KO produced longer seeds and panicles (Liu et al., 2015). Decreased cellulose contents and mechanical strengths were observed in both pPLAIIIβ-OE Arabidopsis (Li et al., 2011) and pPLAIIIα-OE rice (Liu et al., 2015).

In contrast to other pPLAIIIs, pPLAIIIδ contains the conserved Asp-Gly-Gly motif in its esterase box. The majority of pPLAIIIδ are associated with plasma membrane whereas others are associated with intracellular membranes. In addition, *pPLAIIIδ* was found to be highly expressed in developing embryos, the major seed oil storage tissues in

Arabidopsis (Li et al., 2013). Similar to pPLAIIIβ, pPLAIIIδ can catalyze the release acyl groups from both the *sn*-1 and *sn*-2 positions of PC and preferentially catalyze the cleavage of fatty acid from the *sn*-2 position. Both *in vivo* and *in vitro* studies indicated that acyl-CoA can also serve as a substrate for the enzyme (Li et al., 2013).

Among all four pPLAIIIs, only pPLAIIIδ has been demonstrated to play a role in TAG biosynthesis in developing Arabidopsis seeds. Over-expression of *pPLAIIIδ* in Arabidopsis significantly up-regulated the expression level of several genes involved in the glycerolipid biosynthesis (Li et al., 2013). Arabidopsis mutants deficient in *pPLAIIIδ* (*pPLAIIIδ*-KO) and over-expressing *pPLAIIIδ* (*pPLAIIIδ*-OE) showed 33% and 40.5% seed oil content, respectively, whereas the wild type exhibited 35.5% seed oil content. The expression of *pPLAIIIδ* in *pPLAIIIδ*-KO (*pPLAIIIδ*-COM) restored the seed oil content to the wild type level (Li et al., 2013).

Since pPLAIIIδ may efficiently catalyze the release of 18:1 from PC for eventual use in the acyl-CoA pool for elongation, *pPLAIIIδ*-OE produced C20 and C22 fatty acids at the expense of C18 fatty acids. In addition, alteration of *pPLAIIIδ* expression influenced the level of total acyl-CoA due to the thioesterase activity of the gene product.

The thioester pool was higher in pPLAIIIδ-KO and lower in pPLAIIIδ-OE compared to the wild type (Li et al., 2013). Side effects of constitutive over-expression of pPLAIIIδ in Arabidopsis and *Camelina sativa* (Camelina) were compromised plant growth (Li et al., 2014) and reduced seed yield (Li et al., 2013). Epidermal cells of cotyledon and hypocotyls, seed pods, leaves and stems were shorter (Li et al., 2014) and seed yield was 50-60% lower than the wild type (Li et al., 2013; 2014). However, these

effects could be relieved when *pPLAIIIδ* was over-expressed under the control of a seed specific promoter (Li et al., 2013; 2014).

2.6 Biosynthesis of hydroxy fatty acids in organisms that naturally accumulate these fatty acids

2.6.1. Biosynthesis of hydroxy fatty acids in plants

In castor seed, the synthesis of TAG enriched in 18:1-OH occurs in the developing endosperm (Brown et al., 2012), while germinating cotyledons can also synthesize oil with similar TAG species in the mature seed (He et al., 2006).

As shown in Figure 2.6, the 18:1 12-fatty acid hydroxylase (RcFAH12) is responsible for converting 18:1 to 18:1-OH esterified to the *sn*-2 position of PC in the ER (Brown et al., 2012). RcFAH12 shares approximately 67% sequence homology with the Arabidopsis FAD2 (Lin et al., 1998) and both enzymes have similar mechanisms that remove the pro-R hydrogen (i.e., to distinguish from pro-S hydrogen in prochirality) from C-12 of the oleoyl moiety (Broadwater et al., 2002). In the hydroxylation, the hydroxyl is directly introduced to the C-12 of oleoyl moiety (Lin et al., 1998) with the presence of molecular oxygen and NADH (van de Loo et al., 1995). Electrons for the hydroxylation are channeled from NADH through the cytochrome b5 to the hydroxylase (Smith et al., 1992), which has been confirmed based on the dramatic decrease in HFA accumulation observed in *RcFAH12*-expressing Arabidopsis lines where NADH:cytochrome b5 reductase was down-regulated (Kumar et al., 2006).

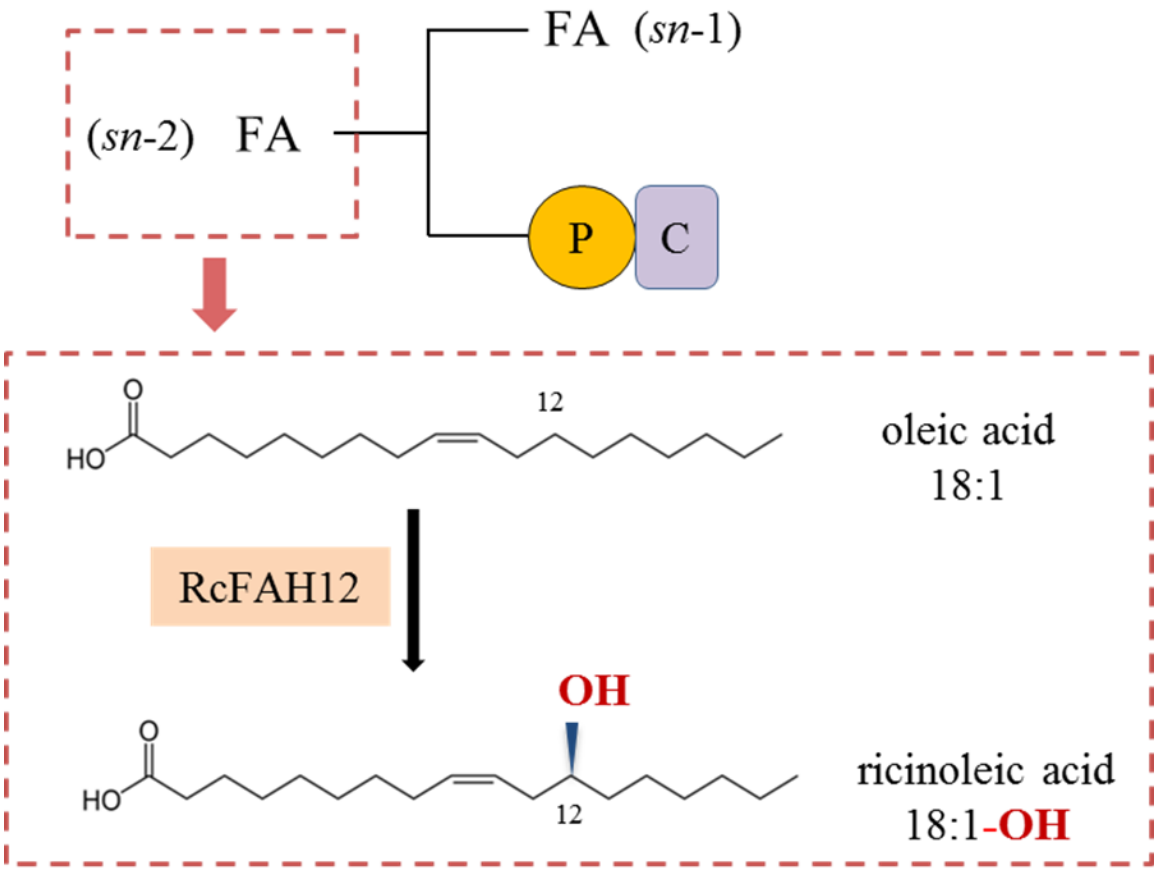


Figure 2.6 Biosynthesis of hydroxy fatty acid (HFA) in castor seed

The biosynthesis of hydroxy fatty acid occurs on the *sn*-2 position of phosphatidylcholine (PC), where the castor oleate 12-hydroxylase (RcFAH12) catalyzes the introduction of a a hydroxyl to carbon 12 of oleic acid ($18{:}1\Delta^{9cis}$) to generate ricinoleic acid (12-OH $18{:}1\Delta^{9cis}$ or 18:1-OH; Lin et al. (1998).

The sequential acylation of three positions of TAG in castor may involve activities of *RcGPAT9*, *RcLPAAT2*, *RcDGAT1* and *RcDGAT2* according to their high expression in the developing castor endosperm (He et al., 2004a; Kroon et al., 2006; Brown et al., 2012). Expression of *RcLPAAT2* promoted the accumulation of ricinoleic acid at the *sn-2* position of TAG in transgenic *L. fendleri* (Chen et al., 2016). Both DGAT1 and DGAT2 are involved in the final step of TAG assembly (Mckeon and He, 2015). More specifically, both of the isoenzymes displayed expression patterns corresponding to the oil synthesis in the developing castor endosperm (He et al., 2004a; Kroon et al., 2006). RcDGAT1 exhibited a stronger preference for diricinoleoylgylcerols over other diacylglycerols (He et al., 2004b) and its activity to catalyze the acylation of diricinoleoylglycerol was two-fold higher than AtDGAT1 *in vitro* (Mckeon and He, 2015). In addition to oil synthesis, RcDGAT1 may be involved in maintaining the membrane function of vegetative tissues though regulating the balance of acyl-CoA and DAG molecules (Chen et al., 2007). Likewise, RcDGAT2 also showed strong preference for diricinoleoylglycerol and was able to utilize ricinoleoyl-CoA as an acyl-donor (Kroon et al., 2006; Burgal et al., 2008). In addition, co-expression of *RcFAH12* with *RcDGAT2* led to an increase in HFA content in the seed oil of transgenic Arabidopsis (Burgal et al., 2008).

As shown in Figure 2.3, the channeling of HFAs from PC to TAG may include acyl editing, interconversion between PC and DAG and the direct transfer of HFAs from PC to TAG (Bates and Browse, 2011). In acyl editing, $RcPLA_2\alpha$ may participate in the selective release of HFAs from PC since its activity toward HFA-containing PC was higher than $AtPLA_2\alpha$ and its expression affected HFA accumulation in the seed oil

(Bayon et al., 2015). The expression of *RcPDCT* or *RcPDAT1A* (or *PDAT1-2*) led to increased HFA content in the seed oil of Arabidopsis expressing *RcFAH12* (van Erp et al., 2011; Kim et al., 2011; Hu et al., 2012), thus demonstrating the existence of the other two routes for channeling HFAs into TAG from PC.

While RcFAH12 strictly catalyzes the production of ricinoleoyl moieties, the FAH from *L. fendleri* (LfFAH12) is a bi-functional enzyme which can catalyze Δ^{12} hydroxlylation and Δ^{12} desaturation to produce 12-OH 18:1 and 18:2, respectively (Broun et al., 1998; Chen et al., 2011a). The synthesized C18 HFAs are liberated from the *sn*-2 position of PC and activated to HFA-CoA followed by rapid elongations (Chen et al., 2016). A condensing enzyme, LfKCS3 has been identified from the embryo of *L. fendleri* which was shown to specifically catalyze the elongation of C18 HFAs to C20 HFAs (Moon et al., 2001). Unlike the situation where triricinoleoylglycerol is the predominant TAG species in castor oil, most HFAs in *L. fendleri* seed oil are located at the *sn*-1 and *sn*-3 positions of TAG (Chen et al., 2011a), possibly because of the selectivity of LfLPAAT towards common fatty acids (Chen et al., 2016).

2.6.2 Biosynthesis of hydroxy fatty acids in *Claviceps purpurea*

The multifunctional 18:1 FAH12 was identified and characterized from the fungal source, *C. purpurea* (Meesapyodsuk and Qiu, 2008). CpFAH12 belongs to the 12-hydroxylase family and uses the common mechanism for hydroxylations (Meesapyodsuck and Qiu, 2008; Kim and Oh, 2013; Meesapyodsuk et al., 2015). The enzyme not only shows hydroxylation and desaturation activities to the C-12 of 18:1 and palmitoleic acid ($16:1\Delta^{9cis}$), but also, to a minimum extent, catalyzes desaturations at the ω-3 position of 18:2 and 12-OH 18:1 (Meesapyodsuk and Qiu, 2008).

Both *CpDGAT1* and *CpDGAT2* have been shown to encode functional DGAT enzymes for TAG synthesis in *C. purpurea* (Mavraganis et al., 2010; Meesapyodsuk et al., 2015). *In vitro* assays indicated that CpDGAT2 preferentially utilized ricinoleoyl-CoA as an acyl donor but its specificity to diricinoleoylglycerol as the accepter has not been reported (Mavraganis et al., 2010). Nevertheless, CpDGAT1 has been demonstrated to be more active in TAG synthesis than CpDGAT2 when produced recombinantly in yeast (*Saccharomyces cerevisiae*), and co-production of CpDGAT1 with CpFAH12 resulted high accumulation of ricinoleic acids in *Pichia pastoris* (Meesapyodsuk et al., 2015).

2.7 Metabolic engineering to produce oils enriched in hydroxy fatty acids

In this section, progress on the metabolic engineering of oleaginous plants to produce oils enriched in HFAs is discussed. Additional metabolic factors, or 'bottlenecks', which appear to limit the accumulation of HFAs in transgenic plants are also discussed.

2.7.1 Expression of *OLEATE 12-HYDROXYLASES* in transgenic plants

The expression of the *RcFAH12* under the control of the constitutive cauliflower mosaic virus 35S promoter in transgenic tobacco (*Nicotiana tabacum)* led to the accumulation of detectable level (~0.1%) of 12-OH18:1 in the seed oil (van de Loo et al., 1995). This was recognized as a milestone despite the low yield of HFA. Since then, the RcFAH12 has been widely used as the key enzyme for genetic engineering of plants to produce HFAs in their seed oils.

The expression of *RcFAH12*, under the control of a seed-specific *Brassica napus* napin promoter, in transgenic Arabidopsis resulted in much higher percentage (17%) of

HFA in the seed oil. A number of HFAs, however, were produced including ricinoleic acid, lesquerolic acid, densipolic acid and traces of auricolic acid (Broun and Somerville, 1997).

Similarly, transgenic *Camelina sativa* (Camelina) expressing *RcFAH12* accumulated 12-15% total HFAs which was composed of four different species of HFAs (Lu and Kang, 2008). To obtain seed oil enriched in a specific HFA, various Arabidopsis mutants deficient in the single or combined enzymatic activities of FAD2, FAD3, or elongation *(*FAE1) have been used a backgrounds for *RcFAH12* expression (Lee et al., 2015). With seed specific expression of *RcFAH12* in a *fad2*/*fae1* background, C18 HFAs (8.7% ricinoleic acid and 10.5% densipolic acid) accumulated. In a *fad3* background, only monounsaturated HFAs (16.2% ricinoleic acid and 2.5% of lesquerolic acid) accumulated. In a *fad3*/*fae1* background, 7% ricinoleic acid accumulated. Expression of *RcFAH12* in a wild type background, however, resulted in the accumulation of all four HFAs (9.6% in total; Smith et al., 2003). Lu et al. (2006) selected two transgenic *RcFAH12*-expressing lines lacking elongase (*fae1*), designated CL7 and CL37, which contained about 17% HFA content in the seed oils. Thereafter, many studies for characterizing additional genes involved in HFA accumulation in seed oils utilized the CL7 and/or CL37 mutant lines (Lee et al., 2015).

Hydroxylases from other sources have been examined as well. Transgenic Arabidopsis and *B. napus* bearing the bifunctional hydroxylase (LfFAH12) from *L. fendleri* accumulated about 16% and 10% of HFAs in their seed oils, respectively (Broun et al., 1998). The hydroxylase from *Lesquerella linheimeri* (*LlinFAH12*) was not considered to be bi-functional and expression of the cDNA in an Arabidopsis *fad2/fae1*

mutant background resulted in up to 18% HFA in the seed oil (Dauk et al., 2007). The novel hydroxylase from the fungus *C. purpurea* (CpFAH) has also been introduced into wild type Arabidopsis and the *fad2*/*fae1* mutant background. The resulting seed oils contained about 20% and 25% HFA, respectively (Meesapyodsuk and Qiu, 2008).

2.7.2 Co-expression of *OLEATE-12-HYDROXYLASE* with genes encoding enzymes involved in triacylglycerol assembly in transgenic plants

Heterologous expression of cDNAs encoding only hydroxylases in transgenic plants failed to accumulate desirable levels of the expected fatty acids (van Erp et al., 2011). Arabidopsis wild-type and the various mutant background lines, with varying levels of oleic acid, showed limited influence on the production of HFAs (Smith et al., 2003). However, the introduction of additional enzymes involved in TAG synthesis into those backgrounds, to some extent, elevated the accumulation of HFAs in the seed oil transgenic plants (Burgal et al., 2008; Kim et al., 2011; van Erp et al., 2011; Hu et al., 2012; c.f. Figure 2.3).

In plants, DGAT catalyzes the acyl-CoA-dependent acyltation of the *sn*-3 position of DAG to produce TAG (Weselake et al., 2009). Seed-specific over-expression of *AtDGAT1* in Arabidopsis has been shown to increase seed oil content and seed weight (Jako et al., 2001). Furthermore, suppression of *DGAT1* expression in tobacco (*Nicotiana tabacum*) and *B. napus* reduced seed yield and oil deposition (Zhang et al., 2005; Lock et al., 2009). The DGAT2 isoenzyme, however, shares no significant sequence identity with DGAT1 and appears to associate with different regions of the ER membrane (Lung and Weselake, 2006). When RcDGAT2 was expressed in Arabidopsis lines CL7 or CL37 (Lu et al., 2006), HFA levels in the seed oil increased from 17% to 30%. When compared

with CL7 or CL37 lines, the *RcFAH12*/*RcDGAT2* double transgenic lines contained a significantly higher amount of castor oil-like TAG molecular species and less molecular species of TAG which were devoid of HFA (Burgal et al., 2008). In addition, seed oil content and seed weight were increased in the co-expression transgenic lines (Burgal et al., 2008; Bates et al., 2014). In addition, germination and growth of the seedlings were normal.

As indicated previously, PDAT catalyzes the acyl-CoA-independent acylation of DAG to produce TAG using acyl groups from the *sn*-2 position of PC (Dahlqvist et al., 2000). Transcript levels of *PDAT* have has been found to be higher in developing seeds of castor than those of Arabidopsis and soybean (Li et al., 2010). Three isoforms of PDAT have been found in castor while one of them, termed RcPDAT1A (van Erp et al., 2011) or RcPDAT1-2 (Kim et al., 2011), was highly expressed during seed development (Lee et al., 2015). Expression of the cDNA of this PDAT in CL37 Arabidopsis resulted in HFA contents increasing from 17% to 25% (Kim et al., 2011) or 27% (van Erp et al., 2011) in the seed oils. The HFA content of PC decreased from about 11% in CL37 to about 7% in *RcPDAT1A*/*RcFAH12* lines (van Erp et al., 2011). In addition, the fatty acid composition of the HFA-containing TAG species became more castor oil-like; the proportion of diricinoleoylglycerol and triricinoleoylglycerol increased whereas TAG molecules containing a single HFA or no HFA decreased in prevalence (van Erp et al., 2011; Kim et al., 2011). According to Kim et al. (2011), co-expression of *RcDGAT2* in *RcPDAT1-2*/*RcFAH12* did not appear to further increase HFA accumulation, which may due to the enzymatic competition between the two specific acyltransferases. In contrast, van Erp et al. (2011) found about a 20% increase in the amount of HFAs per seed and

suggested that the improvement in HFA accumulations was masked by the substantial elevation of total fatty acids.

PDCT (encoded by *ROD1*) catalyzes the transfer of the phosphocholine headgroup of PC enriched in mFAs to 18:1-enriched DAG (produced *de novo* in the Kennedy pathway) to produce mFA-enriched DAG and 18:1-enriched PC (Lu et al., 2009). It has been shown that the conversion of *de novo* DAG into PC is reduced by about 70% in the Arabidopsis expressing *RcFAH12* (Bates and Browse, 2011). When the activity of PDCT was deficient (*rod1*), expression of *RcFAH12* resulted in half of the HFA level (4~5%) of the wild type host (10%, indicating the necessity of PDCT for the efficient HFA accumulation in seed oils (Hu et al., 2012). Over-expression of the castor *PDCT* (*RcROD1*) in wild type Arabidopsis, bearing *RcFAH12*, doubled the HFA level to 10% of total fatty acids (Hu et al., 2012). As expected, the accumulation of HFAs in the *sn*-2 position of TAG was enhanced in *RcROD1*/*RcFAH12* mutants, possibly because of the improved efficiency in converting HFA-PC into HFA-DAG (Hu et al., 2012). When the CL37 and *RcDGAT2*-CL7 lines were used as background, expression of *RcROD1* further elevated the HFA level to 23% and 28%, respectively (Hu et al., 2012). In addition, the reduction in seed oil content was restored by PDCT action in the *RcFAH12*-expressing lines (Hu et al., 2012).

2.7.3 Other approaches to increase hydroxy fatty acid content in transgenic plants and yeast

Apart from co-expression of *FAH12* with additional genes involved in TAG synthesis in transgenic hosts, other approaches have also been used to improve the HFA contents in seed oils (Snapp et al., 2014; van Erp et al., 2015).

Condensing enzyme from *L. fendleri* (LfKCS3) has been shown to specifically catalyze the elongation of C18 HFA-CoA to C20 HFA-CoA (Moon et al., 2001). When *LfKCS3* was co-expressed with *RcFAH12* in transgenic *C. sativa*, the HFA content of the seed oil increased from about 14% (with *RcFAH12* expression alone) to about 20% (Snapp et al., 2014). In addition, the HFA content of PC dropped to 1.5%, from 5% (*RcFAH12* expression alone), in the co-expression lines. The added expression of *LfKCS3* also led to an increase in the accumulation of C20 HFAs from 1.4% to 8.1%. The authors noted that efficient elongation of C18 HFAs in acyl-CoA pool may facilitate their removal from PC and prevent them from re-entering PC via acyl editing. Normal seed germination and oil content were also restored with the expression of *LfKCS3* (Snapp et al., 2014).

Enzymatic competition between foreign enzymes and their endogenous counterparts may also limit the accumulation of HFAs in seed oils (Van Erp et al., 2015). Although competition between RcFAH12 and AtFAD2 did not appear to affect the synthesis of ricinoleoyl moieties (Smith et al., 2003), van Erp et al. (2015) identified acyltransferase competition at the level of TAG synthesis. When *AtDGAT1* was replaced with *RcDGAT2,* HFAs accumulation in seeds showed a 17% of improvement. Furthermore, expression of *RcPDAT1A* in this background elevated HFA levels from 28%

to 31.4% in the seed oils (van Erp et al., 2015). However, because of the essential overlapping function of *AtDGAT1* and *AtPDAT*, down-regulation of *AtPDAT1*in *Atdgat1-2 RcDGAT2 RcPDAT1A* CL7 mutants reduced seed germination rates and failed to increase the HFA accumulations (Zhang et al., 2009; van Erp et al., 2015).

Promising results have also been obtained using a microbial system to produce HFAs. Ricinoleic acids accumulated to nearly 53% of total fatty acids in *Schizosaccharomyces pombe* (yeast) expressing *CpFAH12* (Holic et al., 2012). Co-expression of *CpFAH12* and *CpDGAT1* in the *fad2 Pichia pastoris* (yeast) mutant resulted in approximately 56% ricinoleic acid in the oil, mostly in the form of free fatty acids, three days after the induction of expression (Meesapyodsuk et al., 2015).

2.7.4 Phospholipase As and their potential role use in releasing hydroxy fatty acids from phosphatidylcholine

Despite numerous recent advances in boosting the HFA content of oils in transgenic plants, metabolic engineers still have a long way to go in engineering high levels of HFAs comparable with natural sources of HFAs such as castor. Castor, with ultra-high HFA content has a specific and efficient mechanism to release HFAs from PC and make them available for TAG biosynthesis (Bates and Browse, 2012). Indeed, ricinoleic acid only constitutes about 5% of the fatty acids in PC during embryo development (Ståhl et al., 1995; Thomæus et al., 2001), while the seed oil contains 90% of ricinoleic acid and nearly 70% of TAG is in the form of triricinoleoylglycerol (Lin et al., 2003). Another natural HFA plant source, *L. fendleri*, also has an efficient mechanism to liberate HFAs from PC to acyl-CoA pool for elongation (Chen et al., 2016).

The microbial system *P. pastoris* (*CpFAH12/CpDGAT1/fad2*) produced over 50% ricinoleic acids, mainly in the free fatty acid form, while retained less than one percent in phospholipids, implying an effective mechanism to release ricinoleic acid from PC that exist in yeast (Meesapyodsuk et al., 2015). In plant systems, PLA_2 action may be involved in releasing HFAs from PC (Allen et al., 2015). Recently, Bayon et al. (2015) isolated the most highly expressed *PLA*, *RcPLA$_2$α*, from the developing castor endosperm and demonstrated that this enzyme selectively released HFA from PC *in vitro*. Interestingly, they found a dramatic decrease in HFAs accumulation in both PC and TAG in seed oils of CL7 Arabidopsis lines expressing *RcPLA$_2$α*. These results suggested that RcPLA$_2$α may specifically catalyze the release of HFA from HFA-PC but the released HFAs may have undergone β-oxidation in the peroxisome and thus were not incorporated into TAG (Bayon et al., 2015).

pPLAIII enzymes have been shown to be involved in phospholipid hydrolysis in plants (Li et al, 2011; 2013; Liu et al., 2015). *LfpPLAIIIβ* and *LfpPLAIIIδ* are the only two *pPLA* members identified, so far, from developing *L. fendleri* seeds (data unpublished). Their homologs from castor, 28327.m000363 (hereafter termed as *RcpPLAIIIβ*) and 29647.m002082 (hereafter termed as *RcpPLAIIIδ*) have been shown to be expressed in developing castor endosperm (Brown et al., 2012). In this thesis, pPLAIIIβs and pPLAIIIδs from castor and *L. fendleri*, are evaluated as possible molecular tools for the release of HFAs from PC in transgenic Arabidopsis CL7.

3 - Materials and Methods

3.1 Cloning of *pPLAIIIs* cDNAs from Arabidopsis (*Arabidopsis thaliana*), castor (R*icinus communis*) and *Lesquerella fendleri.*

3.1.1 Sequence identification of *pPLAIIIs* from *L. fendleri* and castor

L. fendleri pPLAIIIβ and *pPLAIIIδ* sequences were identified from an Expressed Sequence Tags database provided by Dr. Mark Smith of National Research Council of Canada (Saskatoon, Saskatchewan, Canada). Castor *pPLAIIIβ* and *pPLAIIIδ* sequences were identified by the *Ricinus communis* Sequence Blast Search (http://blast.jcvi.org/er-blast/index.cgi?project=rca1) using Arabidopsis *pPLAIIIβ* and *pPLAIIIδ* (Accession number AT2G39220 and AT3G63200) as probes, and two gene sequences (Gene identifier: 28327.m000363 and 29647.m002082) were found in the data base (Brown et al., 2012). Above sequence identification were conducted by Dr. Guanqun Chen (Department of Agricultural, Food and Nutritional Science, University of Alberta, AB, Canada).

3.1.2 Amplification of *pPLAIIIs* from Arabidopsis, castor and *L. fendleri* cDNAs

Arabidopsis total RNA was extracted from developing siliques of wild-type Columbia-0 (Col-0) Arabidopsis grown in greenhouse (Department of Agricultural, Food and Nutritional Science, University of Alberta, AB, Canada) using the protocol of RNeasy Mini Kit (Qiagen). Extracted RNA was run on an agarose gel (1% [w/v] of agarose, 0.01% (v/v) of 10,000× SYBR safe DNA gel stain [in DMSO] in TAE buffer; Thermo Fisher Scientific) under 120 volts (V) to examine its integrity. The synthesis of single-stranded cDNA was performed based on the protocol of the QuantiTect Reverse Transcription Kit (Qiagen). The castor cDNA template was obtained from Dr. Thomas McKeon of the

Agricultural Research Service of United States Department of Agriculture in Albany, CA, USA.

The *pPLAIIIs* were obtained by polymerase chain reaction (PCR) amplifications, in which 1 µl each of cDNA template was added to a master mix containing 10 µl of 5× *Phusion* HF buffer, 1 µl of dNTPs (10mM; 2.5mM each), 2.5 µl each of the forward and reverse primers (10µM), 0.5 µl of *Phusion* DNA polymerase and 32.5 µl of water. Buffers and *Phusion* DNA polymerase were provided by New England Biolabs, dNTPs were obtained from Invitrogen, and primers (Table 2.1) were synthesized by Integrated DNA Technologies (IDT). The PCR amplification systems were: 98℃ for 30 s, 35 cycles of 98℃ for 8 s, 63℃ (*AtpPLAIIIδ* and *RcpPLAIIIδ*)/65℃ (*AtpPLAIIIα*)/66℃ (*RcpPLAIIIβ*) for 20 s, and 72℃ for 1 min; and 72℃ for 10min. Melting temperatures were calculated using the NEB T_m Calculator. PCR reactions were run on 1% agarose gel under 120 volts for 25 min and the DNA bands were isolated based on the protocol of QIAquick Gel Extraction Kit (Qiagen).

LfpPLAIIIβ and *LfpPLAIIIδ* were cloned from *L. fendleri* cDNA using rapid amplification of cDNA ends (RACE)-PCR. This was conducted by Dr. Guanqun Chen (Department of Agricultural, Food and Nutritional Science, University of Alberta, AB, Canada). Developing *L. fendleri* siliques were obtained from Dr. John Dyer of the Agricultural Research Service of the United States Department of Agriculture in Maricopa, AZ, USA.

Table 2.1 Primers for amplification of *pPLAIII* cDNAs

Gene	Forward primer	Reverse primer
AtpPLAIIIα	5'-ATGTTAACTACGATGCAAAGAGTACAC-3'	5'-TCAAAACATACAATCAATATCCTTGAA-3'
AtpPLAIIIδ	5'-ATGGAGATGGATCTCAGCAAG-3'	5'-TTAACGGCCGTCAGCG-3'
RcpPLAIIIβ	5'-ATGGCTAGCGATCAATCTTTAGA-3'	5'-CTAGGTGGGTTTAGAAGCAGCT-3'
RcpPLAIIIδ	5'-ATGGAGCTTAGTAAGGTAACACTTGAG-3'	5'-CTAACGGCCGTTGGATAGTG-3'
LfpPLAIIIβ	5'-ATCATGGATAGAGTACGCAATAAGCC-3'	5'-TCATCGTTCTCTTGCAGTAACACC-3'
LfpPLAIIIδ	5'-ATTATGGATATTGATCTCAGTAAGGTTACTCTT-3'	5'-TTAACGGCCGTCAGCTAGTGG-3'

3.1.3 Cloning *pPLAIIIs* cDNAs into pCR^{TM}2.1-TOPO vectors (TA cloning)

A deoxyadenosine (A) was added to the 3´ ends of PCR products in reactions (72℃, 20 min) composed of 15 µl of each purified PCR products, 5 µl of 10× PCR buffer minus Mg^{2+}, 1 µl of dATP (10 mM), 1.5 µl of $MgCl_2$ (50 mM), 0.2 µl of *Taq* DNA polymerase and 27.3 µl of water. The PCR products containing A-overhangs were purified using QIAquick PCR purification Kit (Qiagen) and cloned into pCR^{TM}2.1-TOPO vectors (Invitrogen).

The pCR^{TM}2.1-TOPO constructs were transformed into One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen) and the presence of expected DNAs in the colonies were confirmed by PCR with paired gene specific primers. Positive colonies were inoculated into 5 ml of Luria-Betani (LB) media (1% [w/v] tryptone or peptone, 0.5% [w/v] yeast extract, 0.5% NaCl, 0.1% [v/v] 1N NaCl in water) for 16 h (220 rpm, 37℃) and plasmids were isolated using the GeneJET^{TM} Plasmid Minprep Kit (Thermo Fisher Scientific), and sequences of the cloned *pPLAIIIs* were confirmed by Economy DNA sequencing with M13 forward primer and M13 reverse primer provided by the TOPO TA Cloning Kit (Invitrogen) and gene internal primers (Molecular Biology Facility, University of Alberta, AB, Canada).

3.2 Construction of plasmids containing *pPLAIIIs* for over-expression in Arabidopsis CL7

For over-expression in Arabidopsis CL7, *pPLAIIIs* were cloned into modified pPZP-RCS1 binary vectors (Goderis et al., 2002) under the control of seed-specific napin

promoter (Mietkiewska et al., 2014a). The first step was to use auxiliary vector pAUX3131 (Goderis et al., 2002) to assemble expression cassettes containing napin promoter, *pPLA* genes, and NOS terminator (Mietkiewska et al., 2014a). Subsequently, the assembled expression cassettes were transferred into the binary vector systems for expression.

3.2.1 Assembly of expression cassettes of *pPLAIIIs*

Modified pAUX3131 auxiliary vectors containing napin promoter and NOS terminator that were constructed previously were used for the assembly of expression cassettes.

The napin promoter, *pPLAIIIs* were amplified by PCR with primers that contained certain restriction sites for digestions and ligations. For PCR amplifications, 1 µl of each DNA templates was added to a master mix containing 5 µl of 10× *Pfx* Amplification Buffer, 1.5 µl of dNTPs (10 mM, 2.5 mM each), 1 µl of $MgSO_4$ (50 mM), 1.5 µl each of the forward and reverse primers (10µM, Table 2.2), 0.4 µl of Platinum *Pfx* DNA Polymerase and 38.1 µl of water. The PCR amplification system for *AtpPLAIIIα* was: 94℃ for 2 min, 35 cycles of 94℃ for 15 s, 55℃ for 30 s, and 68℃ for 2 min; and 68℃ for 7 min. Systems for napin promoter and *AtpPLAIIIδ* were 94℃ for 2 min, 35 cycles of 94℃ for 15 s, 59℃ for 30 s, and 68℃ for 1 min 30s; and 68℃ for 7min. The system for *RcpPLAIIIβ* were 94℃ for 2 min, 35 cycles of 94℃ for 15 s, 55℃ for 30 s, and 68℃ for 1 min 30s; and 68℃ for 7min. The system for *RcpPLAIIIδ* was 94℃ for 2 min, 35 cycles of 94℃ for 15 s, 59℃ for 30 s, and 68℃ for 1 min 10s; and 68℃ for 7 min. Restriction site-added DNA fragments were purified using the protocol of QIAquick Gel Extraction Kit (Qiagen).

Table 2.2 Primers for addition of restriction sites to *pPLAIIIs*

Gene	Direction	Restriction	Sequence (restriction sites underlined)
Napin	Forward	EcoRI	5'-TATAGAATTCAAGCTTTCTTCATCGGTGATTG-3'
	Reverse	BamHI	5'-ATATGGATCCGTCCGTGTATGTTTTTAATCTTGTTTG-3'
AtpPLAIIIα	Forward	SalI	5'-TATAGTCGACATGTTAACTACGATGCAAAGAGTACAC-3'
	Reverse	Acc65I	5'-ATATGGTACCTCAAAACATACAATCAATATCCTTGAA-3'
AtpPLAIIIδ	Forward	BamHI	5'-TATAGGATCCATGGAGATGGATCTCAGCAAG-3'
	Reverse	NcoI	5'-ATATCCATGGTTAACGGCCGTCAGCGA-3'
RcpPLAIIIβ	Forward	SalI	5'-TATAGTCGACATGGCTAGCGATCAATCTTTAGA-3'
	Reverse	Acc65I	5'-ATATGGTACCCTAGGTGGGTTTAGAAGCAGCT-3'
RcpPLAIIIδ	Forward	SalI	5'-TATAGTCGACATGGAGCTTAGTAAGGTAACACTTGAG-3'
	Reverse	NcoI	5'-ATATCCATGGCTAACGGCCGTTGGATAGTG-3'
LfpPLAIIIβ	Forward	SalI	5'-TATAGTCGACATGCATAGAGTACGCAATAAG-3'
	Reverse	NcoI	5'-ATATCCATGGTCATCGTTCTCTTGCAGTAAC-3'
LfpPLAIIIδ	Forward	XhoI	5'-ATATCTCGAGATGGATATTGATCTCAGTAAGG-3'
	Reverse	XbaI	5'-TATATCTAGATTAACGGCCGTCAGCTAGT-3'

The purified DNA fragments were cloned into expression cassettes within the pAUX3131 backbone by restriction digestions and ligations as following: 1) The purified *AtpPLAIIIα* was digested with SalI/Acc65I, and ligated into SalI/Acc65I-digested modified pAUX3131 vector; 2) the napin promoter and the *AtpPLAIIIδ* were digested with EcoRI/BamHI and BamHI/NcoI, respectively, and ligated together into EcoRI/NcoI-digested vector; 3) *RcpPLAIIIβ* was digested with SalI/Acc65I and ligated into

SalI/Acc65I-digested vector; 4) *RcpPLAIIIδ* was digested with SalI/NcoI and ligated into SalI/NcoI-digested vector; 5) *LfpPLAIIIβ* and NOS were digested with SalI/NcoI and NcoI/NotI, respectively, and ligated into SalI/NotI digested vector; and 6) *LfpPLAIIIδ* was digested with XhoI/XbaI, and ligated into XhoI/XbaI digested vector.

Restriction digestions were performed based on the protocols from New England Biolabs (NEB) and the ligations were performed using the protocol of T4 DNA ligase (Invitrogen).

3.2.2 Cloning expression cassettes of *pPLAIIIβs* and *pPLAIIIδs* into binary vector systems

A binary vector modified from pPZP-RCS1 (Goderis et al., 2002) that contained a kanamycin-resistant gene, *NPTII*, under the control of nos promoter was used for the Agrobacterium-mediated plant transformation. The preparation of the modified pPZP-RCS1 binary vector was conducted by Dr. Elzbieta Miekiewska (Department of Agricultural, Food and Nutritional Science, University of Alberta, AB, Canada). The expression cassettes were cut from the modified pAUX3131 vectors by homing endonuclease I-SceI together with the backbone being digested with HF-*Sca*I in the same reactions. Homing endonuclease sites were utilized in the vectors because they are extremely uncommon in natural sequences and suitable for unidirectional cloning (Goderis et al., 2002). Digestion products were separated on 1% agarose gel and the I-SceI-digested bands were purified QIAquick Gel Extraction Kit (Qiagen). The NptII-contained pPZP-RCS1 was digested with endonuclease I-SceI, purified using QIAquick Gel Extraction Kit (Qiagen) and treated with FastAP Thermosensitive alkaline phosphatase (Thermo Fisher Scientific) to prevent self-ligations. Subsequently, the I-

SceI-digested expression cassettes (Napin/pPLA/NOS) were transferred into I-SceI-digested binary vectors to make plasmids *pPZP-RCS1-AtpPLAIIIα*, *pPZP-RCS1-AtpPLAIIIδ*, *pPZP-RCS1-RcpPLAIIIβ*, *pPZP-RCS1-RcpPLAIIIδ*, *pPZP-RCS1-LfpPLAIIIβ*, and *pPZP-RCS1-LfpPLAIIIδ*.

3.3 Transformation of *pPZP-RCS1*-derived plasmids into Agrobacterium

Electrocompetent *Agrobacterium tumefaciens* cells, GV3101 strain, were prepared based on the following protocol (Clough and Bent., 1998). Agrobacterium cells were inoculated into 5 ml of LB media containing gentamycin (25 µg/ml) and rifampicin (50 µg/ml) and grown 16 h at (28℃, 220 rpm). One millilitre was transferred to 10 ml of fresh LB media and allowed to grow another 16h (28℃, 220 rpm). Eight hundred microliters were transferred to 50 ml of fresh LB media and allowed to grow until the OD_{600} reached 0.5, which takes 7 h. The cells were pelleted by centrifugation (3,000 g, 15 min at 4℃). The pellet was washed in 40 ml of ice-cold water twice and 40 ml of ice-cold 10% glycerol twice. Finally, the cells were resuspended in 0.5 ml of ice-cold 10% glycerol and dispensed into 40 µl of each Eppendorf tube, frozen in liquid nitrogen and stored at -80℃ until use.

Thawed Agrobacterium cells (40 µl) were mixed with 10 ng of each pPZP-RCS1-derived plasmid (*pPZP-RCS1-AtpPLAIIIα*, *pPZP-RCS1-AtpPLAIIIδ*, *pPZP-RCS1-RcpPLAIIIβ*, *pPZP-RCS1-RcpPLAIIIδ*, *pPZP-RCS1-LfpPLAIIIβ* and *pPZP-RCS1-LfpPLAIIIδ*) and transferred to Gene Pulser Cuvettes (Bio-Rad). Plasmids were transformed into Agrobacterium cells by electroporation (1.8 volts, 1 s) in the MicroPulser™

Electroporator (Bio-Rad). The transformed cells were grown for 3-5 h (28℃, 220 rpm) in 1 ml of LB media containing spectinomycin (50μg/ml), gentamycin (25 μg/ml) and rifampicin (50 μg/ml). Agrobacterium cultures (10 μl) were grown on LB agar media plates at 30℃ for 2 days. The presence of pPZP-RCS1-derived plasmids in Agrobacterium cells were confirmed by PCR with gene specific primers.

3.4 Transformation of Arabidopsis with floral dip method

Arabidopsis CL7 (T_0) was vernalized in dark (4℃) for 2 days and allowed to grow in a growth cabinet under 22℃ with 18-h-photoperiod (300 umol/m2/s % light intensity). Plant transformation was performed by the Agrobacterium-mediated floral dip method (Clough and Bent., 1998). Agrobacterium cells (transformed by pPZP-RCS1-derived plasmids) were grown 24 h (28℃, 220 rpm) in LB media containing spectinomycin (50 μg/ml), gentamycin (25 μg/ml) and rifampicin (50 μg/ml). Agrobacterium cells were pelleted by centrifugation (3000 rpm, 30 min) and resuspended 500 ml of fresh-prepared 5% sucrose solution containing 0.05% (v/v) silweet-L77. For transformations, plants were inverted and dipped into the suspensions for about 1 min. Dipped plants were covered by plastid domes for 24 h to maintain the humidity. Seeds (T_1) were harvested for the selection of positive transformation after siliques were mature and dry.

3.5 Selection of transgenic Arabidopsis and homozygotes

T_1 seeds were surface-sterilized by rinsing in 70% (v/v) ethanol for 30 s and in 30% (v/v) bleach containing Triton X-100 (3 drops in 100 ml) for 20-25 min, followed by washed with sterile water four times. Sterile seeds were spread onto selection plates which

contained half Murashige and Skoog (MS) medium (0.21% [w/v] MS basal salts, 1% [w/v] sucrose, 0.03% [w/v] MES, 0.8% [w/v] phytagar, 20 mg/ml kanamycin, 99.6 µg/ml timentin, pH was adjusted to 5.7-5.8 by KOH). Meanwhile, Arabidopsis CL7 seeds were surface-sterilized and planted on a MS selection plate without kanamycin. Seeds were vernalized in dark (4°C) for 2 days and grown under growth light for 7-10 days. Kanamycin-resistant plants (transformants) that had well-established roots and green leaves were identified as T_1 plants. Selected T_1 plants and CL7 plants that had two true leaves were transplanted into soil and grown individually (covered with plastic wrap) in a growth cabinet until mature to harvest T_2 seeds and CL7 seeds.

T_2 seeds of single T_1 plants were surfaced-sterilized and screened on selection plates using the same protocol as above. Lines with the ratio of kanamycin-resistant and non-resistant plants (approximately 3:1) were assumed to contain single loci of transgenes. Kanamycin-resistant plants from these lines were considered to be T_2 plants. Selected T_2 plants were transplanted into soil and grown individually in a growth cabinet together with CL7 plants until mature to harvest T_3 seeds and CL7 seeds. In this step, the plants without transgenes (null) were screened out.

T_3 seeds of each single plant were screened on kanamycin-selection plates to select homozygotes and heterozygotes. Lines that had all kanamycin-resistant seeds were considered to be homozygous T_2 plants and the seeds were T_3 homozygous seeds. In contrast, lines with kanamycin resistant to non-resistant ratio close to 3:1 were considered to be heterozygous (Bent, 2006).

3.6 DNA extraction from transgenic lines

Leaves were harvested from growing T_1 plants, frozen in liquid nitrogen and stored at -80°C. DNA extraction was performed using the following protocol which was modified from Edwards et al. (1991). Frozen leaf tissues were grounded in liquid nitrogen and incubated at 60°C for 10 min with 750 µl of extraction buffer composed of 100 mM Tris-HCl (pH 8.0), 500 mM NaCl, 50 mM EDTA, 1% SDS and 10 mM β-mercaptoethanol, followed by mixed with 250 µl of 5 M potassium acetate. The mixtures were chilled on ice for 20 min and centrifuged for 10 min (14000 rpm, 4°C). Subsequently, the supernatant was transferred to a fresh Eppendorf tube, mixed with 500 µl of isopropanol and incubated at -20C for 20 min. Genomic DNA was then pelleted by centrifugation for 10 min (14000 rpm, 4°C) and washed with 500 µl of 70% ethanol. The dried pellet was then resuspended in 100 µl of water. PCR amplification was performed to confirm the presence of transgenes in T_1 plants.

3.7 Lipid analysis of T_2 and T_3 Arabidopsis seeds

3.7.1 Determination of Arabidopsis total lipid content and fatty acid composition

About 10 mg of dried T_2, T_3 and CL7 seeds were weighed and placed in Teflon-lined screw capped glass tubes which were pre-rinsed with hexane to remove residual lipids and dried. Internal standard, C17:0 TAG (100 ng), was added to each tube and dried under nitrogen gas. For transmethylation, 2 ml of 3N methanolic HCl were added to the mixture followed by incubation for 16 h at 80℃. After cooling on ice for 5 min, 2 ml of 0.9% NaCl were added to the reactions. The fatty acid methyl esters (FAMEs) were

extracted twice by adding 2 ml of hexane, vortexing for 30 s, spinning down at 2,000 g for 4 min, and transferring the hexane phases (upper layer) to a fresh glass tube. Pooled extracts were dried completely under nitrogen gas at 37℃ and dissolved in 1 ml of iso-octane containing C21:0 methyl ester (0.1 mg/ml) and analyzed by gas chromatography (GC)-mass spectrometry (MS).

In GC (Agilent Technologies 7890A GC system), a split/splitless inlet was used and the injection volume was 1 μl in the ten-to-one split mode; FAMEs separation was performed in a DB-23 capillary column (Agilent Technologies: 30 m × 250 μm × 0.25 μm) with helium as carrier gas (1.2 ml/min), of which the temperature program was: 165℃ for 4 min, 165-180℃ for 5 min, and 180-230℃ for 5 min. Ingredients were detected by mass spectrometry (Agilent Technology 5977A Mass Selective Detector) and peaks were identified with the software NIST MS Search 2.0.

3.7.2 Determination of the fatty acid composition of triacylglycerol and phosphatidylcholine from mature seed of Arabidopsis

About 50 mg of dried T_2, T_3 and CL7 seeds were weighed and place in hexane-rinsed teflon-lined screw capped glass tubes. Seeds with 3 ml of chloroform/methanol (2:1, v/v) were homogenized in a blender and then an additional 3 ml of chloroform/methanol (2:1, v/v) were added followed by vigorous vortexing for 1 min. Subsequently, one-fourth total volume of 0.9% NaCl (2 ml) was added to the mixture and vortexed for 20 s to extract lipids. The chloroform phase (lower layer) was transferred to a fresh glass tube. Four millilitres of chloroform were added to the mixture for the second extraction in chloroform/methanol (2:1, v/v). Lipids were recovered from chloroform by drying

completely under nitrogen and dissolving in 80 µl of chloroform (Mietkiewska et al., 2014a).

Total extracted lipids were separated on a pre-heated (80℃, ≥1 h) thin layer chromatography (TLC) plate, in two different solvent systems. The TLC was developed in chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:2, v/v/v/v/v) until the solvent front was half the height of the plate. After drying in the fume hood, the plate was further developed in hexane/ether/acetic acid (65:35:2) until the solvent line was about 1 cm from the edge of the plate (Mietkiewska et al., 2014a). TAG and phospholipid (PL) bands were visualized by spraying with 0.05% primuline (w/v) in acetone/water (80:20, v/v). Direct methylation was performed on TAG and PC scraped from TLC, by the incubation with 2 ml of 3N methanolic HCl at 80C for 1 h. Two millilitres of 0.9% NaCl were added to the reaction and the FAMEs were extracted with 4 ml of hexane twice and dried under nitrogen. FAMEs produced from TAG and PC were dissolved in 6 ml and 100 µl of iso-octane containing C21:0 methyl ester (0.1 mg/ml) and analyzed by GC/MS (mentioned in 3.7.1).

4 - Results

4.1 Phylogenetic characterization of pPLAIIIs

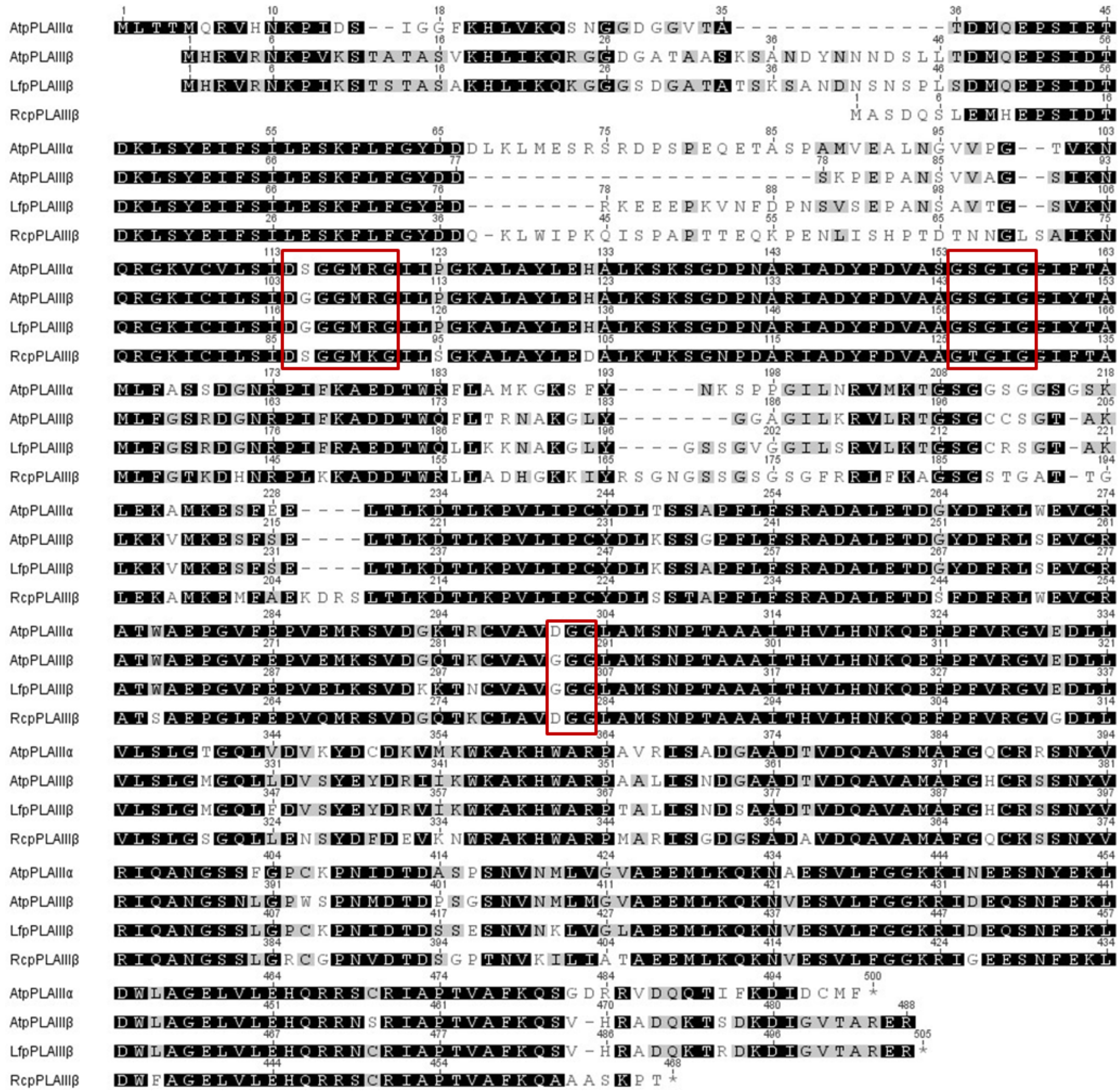
Deduced amino acid sequences of pPLAIIIs from castor, *L. fendleri* and Arabidopsis were aligned to compare the sequence similarities (Figure 4.1). A phylogenetic tree was also generated to show the evolutionary relationship of deduced amino acid sequences of pPLAIIIs from different plant species (Figure 4.2).

Li et al. (2011) have identified four *pPLAIII* (α, β, γ, δ) genes in Arabidopsis, which is a member of the Brassicaceae family. Although *L. fendleri* also belongs to this family, it accumulates ~60% of HFAs in the seed oil (Chen et al., 2011a). Only two *pPLAIII* genes have been found to be expressed in developing *L. fendleri* seeds. They were named *LfpPLAIIIβ* and *LfpPLAIIIδ* after the closest Arabidopsis homologs. The deduced amino acid sequence of LfpPLAIIIβ shares 87% identity with AtpPLAIIIβ, while LfpPLAIIIδ shares 89% identity with AtpPLAIIIδ (Figure 4.1). As shown in the phylogenetic tree in Figure 4.2, both LfpPLAIIIβ and LfpPLAIIIδ belong to the pPLAIII family.

In castor bean, three candidate *pPLAIIIs* (Gene ID: 28327.m000363, 28359.m000278 and 29647.m002082 from *Ricinus communis* sequence Blast Search) have been found to be expressed in the castor developing endosperm (Brown et al., 2012; Bayon et al., 2015). The deduced amino acid sequence of 28327.m000363 is closely related to both AtpPLAIIIα and AtpPLAIIIβ (Figure 4.2), sharing 71% identities with both ApPLAIIIα and AtpPLAIIIβ, respectively (Figure 4.1A). It was named RcpPLAIIIβ to be consistent with its *L. fendleri* homolog. The deduced amino acid sequence of

29647.m002082 shares 73% homology with AtpPLAIIIδ, and was designated as RcpPLAIIIδ (Figure 4.1). Both RcpPLAIIIβ and RcpPLAIIIδ are from the pPLAIII family, based on the phylogenetic tree (Figure 4.2).

A



B

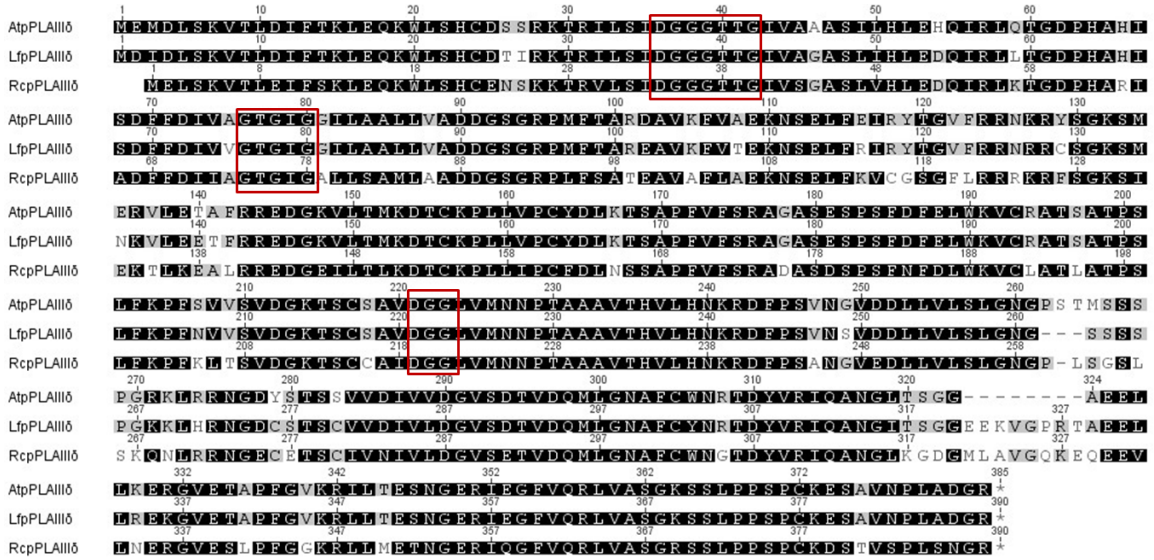


Figure 4.1 Alignment of deduced amino acid sequences of *pPLAIIIs* from Arabidopsis, *L. fendleri* and castor

(A) Amino acid sequence of AtpPLAIIIα, AtpPLAIIIβ, LfpPLAIIIβ and RcpPLAIIIβ; (B) Amino acid sequence of AtpPLAIIIδ, LfpPLAIIIδ and RcpPLAIIIδ.

Deduced amino acid sequences were translated from the coding DNA sequences (CDSs) and aligned using Geneious Alignment of Geneious Pro 5.4.6 software, which was based on the progressive pairwise alignment method, in the type of global alignment. The grey and black shadings represented consensuses of two or more sequences, which showed the pPLAIIIs from *L. fendleri* and castor shared high similarities with the Arabidopsis homologs. The catalytic center was marked, including the phosphate or anion binding element (DGGGxxG), esterase box (GxGxG) and the catalytic dyad-containing motif (DGG or DGA or GGG).

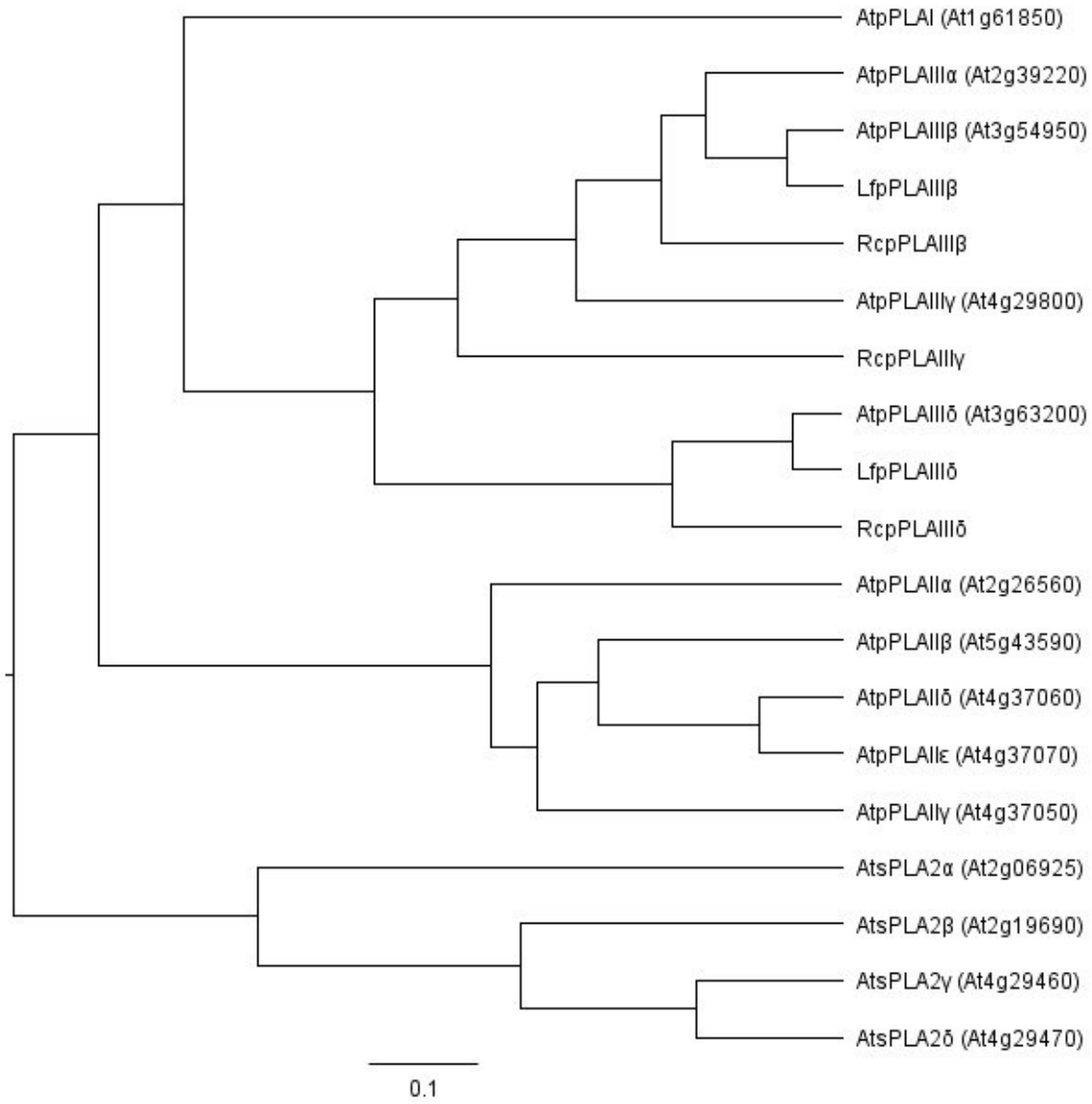


Figure 4.2 Phylogenetic relationship of pPLAIII family in Arabidopsis (At), *L. fendleri* (Lf) and castor (Rc)

The phylogenetic analysis was done using the Geneious Tree Builder of Geneious Pro 5.4.6 software. The clustering method was UPGMA (Unweighted Pair Group Method with Arithmetic Mean); the genetic distance model used for the estimation of the branch length was "Jukes Cantor". In the tree, the tips represent the sampled sequences, the internal nodes represent the putative ancestors and the horizontal branch lengths are proportional to divergence (i.e. substitutions per site).

4.2 Expression of *L. fendleri pPLAIIIs* in T_2 transgenic CL7 seeds resulted in no changes in hydroxy fatty acid content or seed oil content.

As mentioned previously, an efficient mechanism for the liberation of freshly synthesized ricinoleic acid from PC to enter acyl-CoA for elongation exists in *L. fendleri* but little is known about this process (Snapp et al., 2014).

To determine whether pPLAs are involved in selectively releasing ricinoleic acids from PC in *L. fendleri*, the two *pPLA* isolated from *L. fendleri*, *LfpPLAIIIβ* and *LfpPLAIIIδ* were expressed under the seed-specific napin promoter in Arabidopsis CL7 lines to generate LfpPLAIIIβ-CL7 and LfpPLAIIIδ-CL7 transgenic Arabidopsis lines. The CL7 line was previously developed by expressing *RcFAH12* in a *fae1* Arabidopsis mutant, which accumulated up to 17% of HFAs, including ricinoleic acids and densipolic acids (Lu et al., 2006).

T_1 transgenic plants were identified by their kanamycin resistances based on the presence of kanamycin-resistant gene *NPTII* in the plasmid for plant transformations. Thirty-two T_1 lines expressing each transgene were grown to harvest mature T_2 seeds. Samples of T_2 seeds from 20 independent T_1 lines were dried in a desiccator for two weeks and subjected to seed oil and HFA content analyses (Figure 4.3).

A column scatter graph was generated to visualize HFA contents of individual lines and the scattering of values within each group (i.e., control lines or lines expressing the same transgene). Bulk analyses on T_2 seeds indicated that T_1 LfpPLAIIIβ-CL7 and LfpPLAIIIδ-CL7 lines contained approximately 16.3% and 16.7% of HFAs in the seed oil, which was not significantly different from the 15.6% HFAs in control CL7. The range of HFA contents in CL7 seeds were from 7.8% to 19.9% (mostly from 12.9% to

18.6%). All LfpPLAIIIβ-CL7 lines produced HFA contents within the range of CL7 HFA content, except a single line which displayed nearly 24% HFA content in the seed oil (Figure 4.3A).

The proportions of other fatty acid in LfpPLAIIIβ-CL7 and LfpPLAIIIδ-CL7 seeds were also similar to control CL7 lines, despite the slight decrease in 18:1 in LfpPLAIIIβ-CL7 and 18:2 in LfpPLAIIIδ-CL7 seeds (Table 4.1). In addition, the seed oil content of T_1 LfpPLAIIIβ-CL7 and LfpPLAIIIδ-CL7 lines were not changed relative to control CL7 lines (Figure 4.3B).

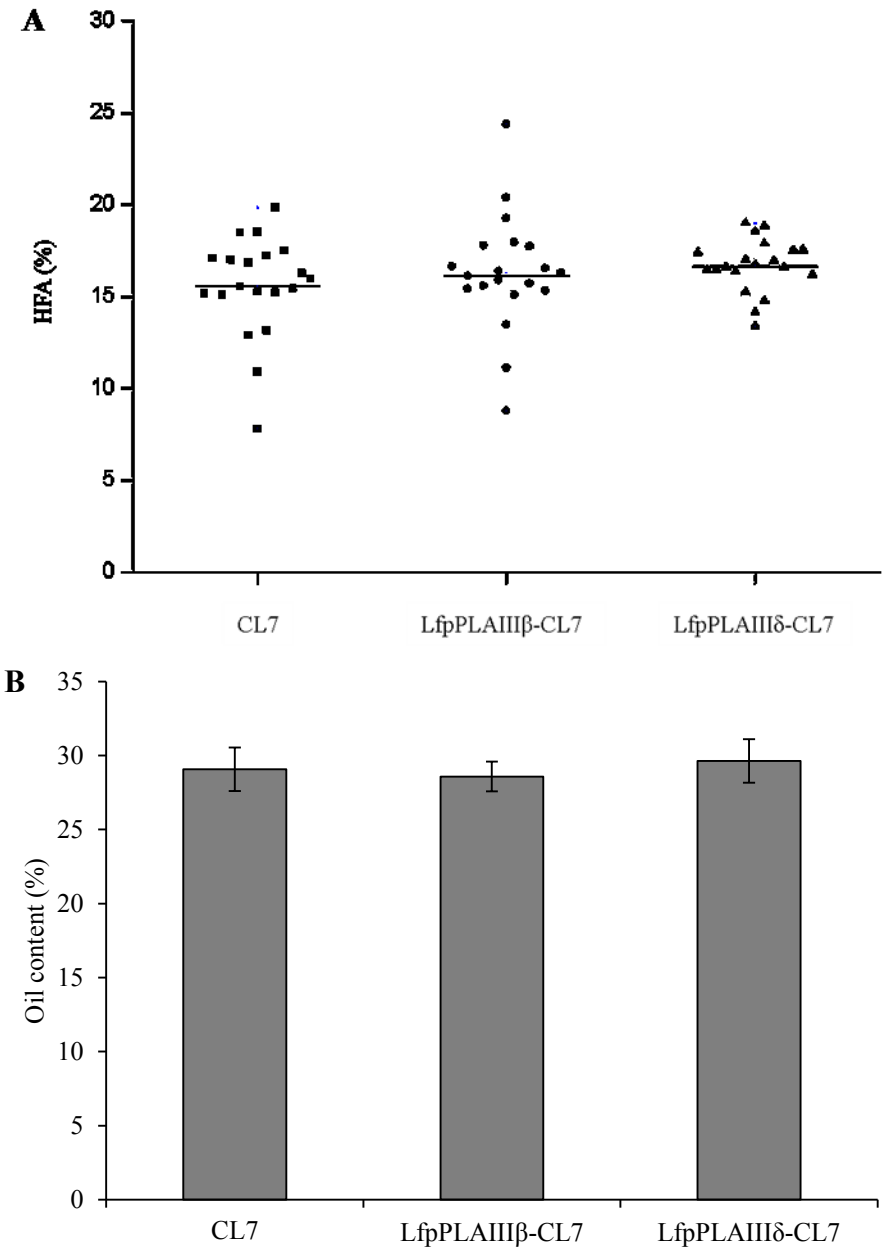


Figure 4.3 Hydroxy fatt acid (HFA) content of the seed oil (A) and total seed oil content (B) in T_2 seeds of T_1 LfpPLAIIIβ-CL7 and LfpPLAIIIδ-CL7 transgenic lines

(A) HFA content as a percentage of total fatty acids based on weight. Each point represents the HFA content in seed samples of an individual T_1 transgenic plant (n=2); horizontal bars represent the mean of HFA content of 20 independent lines expressing the same transgene. (B) Oil content is percentage of seed weight. Each column represents the mean of 20 independent lines (technical two replications each line) expressing the same transgene. Error bars represent standard deviation of samples (SD). Data were analyzed by Two-tailed *t* test.

Table 4.1 Fatty acid composition of the seed oil of T_1 LfpPLAIIIβ-CL7 and LfpPLAIIIδ-CL7 lines (T_2 seeds)

Data are mean (SD) of seed samples of 20 independent lines (n=2) expressing each transgene. The fatty acid composition was percentage of each fatty acid of total fatty acids in weight base and analyzed by two-tailed *t* test. (▲/▼) indicates the values greater/lower than CL7 control at α=0.05 level.

Line	Fatty acid composition (%)				
	16:0	16:1	18:0	18:1	18:2
CL7	10.43 (0.60)	0.33 (0.07)	5.12 (0.33)	36.67 (1.43)	20.46 (0.90)
LfpPLAIIIβ-CL7	10.53 (0.52)	0.33 (0.05)	5.18 (0.26)	35.69 (1.36)▼	20.63 (1.26)
LfpPLAIIIδ-CL7	10.36 (0.57)	0.33 (0.07)	5.14 (0.25)	36.45 (1.18)	19.88 (0.71)▼

Line	Fatty acid composition (%)			
	18:3	20:0	20:1	22:0
CL7	9.75 (1.33)	1.03 (0.07)	0.32 (0.03)	0.31 (0.03)
LfpPLAIIIβ-CL7	9.66 (1.22)	1.05 (0.04)	0.32 (0.02)	0.31 (0.02)
LfpPLAIIIδ-CL7	9.50 (0.66)	1.02 (0.05)	0.32 (0.02)	0.30 (0.02)

Line	Fatty acid composition (%)		Sum of HFA
	18:1-OH	18:2-OH	
CL7	12.49 (2.21)	3.10 (0.63)	15.59 (2.69)
LfpPLAIIIβ-CL7	13.24 (2.61)	3.07 (0.64)	16.31 (3.19)
LfpPLAIIIδ-CL7	13.50 (1.28)	3.20 (0.30)	16.70 (1.42)

4.3 Expression of castor *pPLAIIIβ* or *pPLAIIIδ* in CL7 seeds

To further investigate the possible role of pPLAs in the removal of HFAs from PC in Arabidopsis expressing *RcFAH12* (CL7 lines), *pPLAs* candidates from castor were also investigated. Similar to *L. fendleri*, castor also has efficient processes in place to release HFAs from PC making the fatty acids available for TAG biosynthesis (Bates and Browse et al., 2011; 2012). Hence, the focus of the current study was on *RcpPLAIIIβ* and *RcpPLAIIIδ*, the two castor orthologs *of L. fendleri pPLAIIIs*.

RcpPLAIIIβ or *RcpPLAIIIδ* were expressed under the control of a seed-specific napin promoter in CL7 to develop RcpPLAIIIβ-CL7 or RcpPLAIIIδ-CL7 transgenic lines. After that, T_1 transgenic plants were identified based on kanamycin-resistance.

Thirty-two T_1 lines expressing each transgene were grown to harvest mature T_2 seeds, and T_2 seeds from 20 independent T_1 lines were subjected for oil analyses (Figure 4.4).

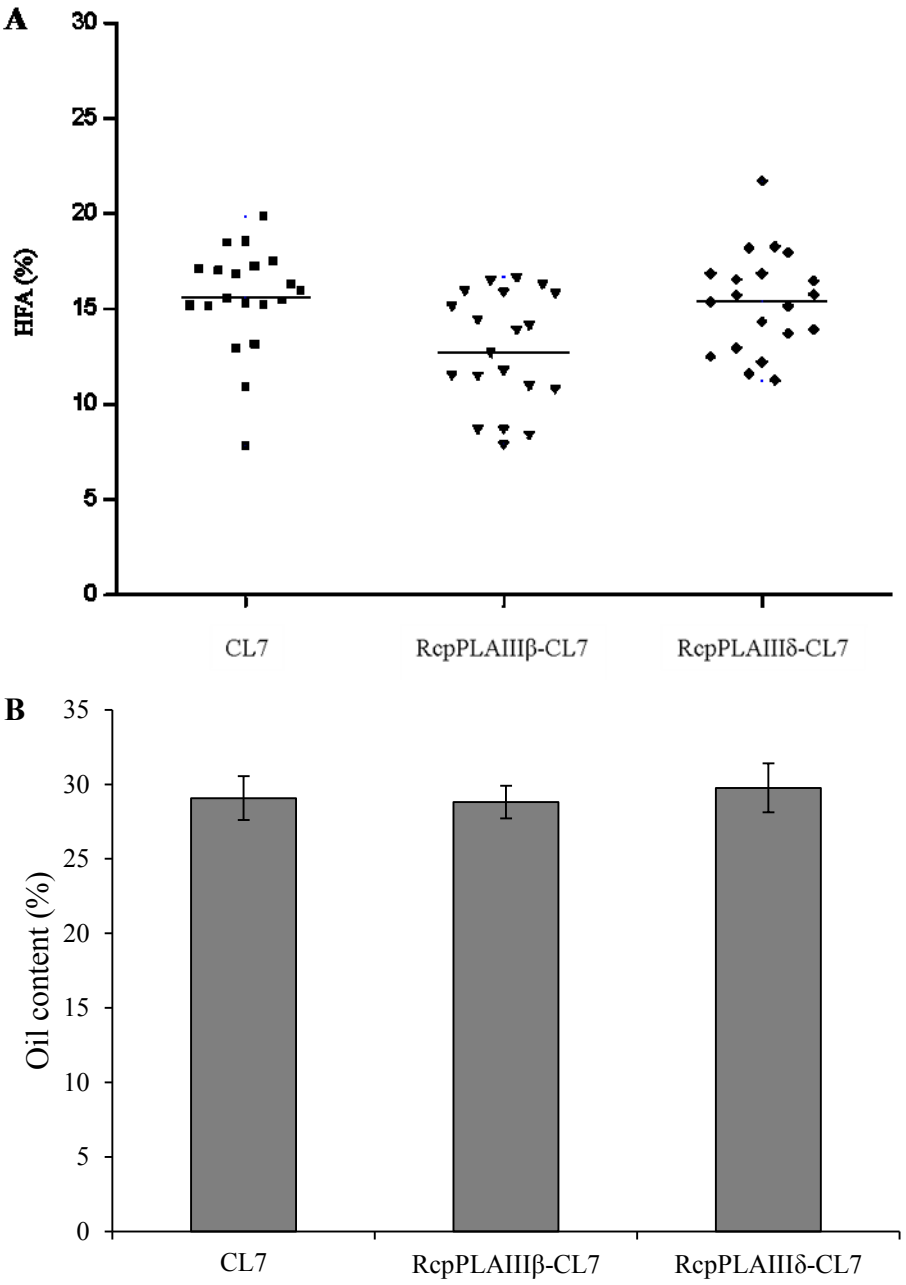


Figure 4.4 Hydroxy fatty acid (HFA) content of the oil (A) and total oil content (B) in T_2 seeds of T_1 RcpPLAIIIβ-CL7 and RcpPLAIIIβ-CL7 transgenic lines

(A) HFA content as a percentage of total fatty acids based on weight. Each point represents the HFA content in T_2 seed samples from an individual T_1 transgenic plant (n=2); horizontal bars represent the mean of HFA content of 20 independent lines expressing the same transgene. (B) Oil content is percentage of dry seed weight. Each column represents the mean of 20 independent lines (technical two replications each line) expressing the same transgene. Error bars represent standard deviation of samples (SD). Data were analyzed by Two-tailed *t* test.

4.3.1 Expression of *RcpPLAIIIβ* resulted in a significant decrease in hydroxy fatty acid content in CL7

On average, RcpPLAIIIβ-CL7 transgenic lines produced approximately 12.9% HFA content in the T_2 seed, which was approximately 17% lower, on a relative basis, than the control CL7 lines (Figure 4.4A). Although HFA contents among T_1 RcpPLAIIIβ-CL7 lines showed a relatively large variation, more than half of the examined lines contained significantly lower HFA content than the mean of HFA content in CL7 in the seed oil. The significance of HFA reduction was confirmed based on statistical analysis (Table 4.2).

To ascertain whether the decrease in HFAs was indeed caused by the expression of *RcpPLAIIIβ*, selected lines were selected to be grown to homozygosity for further oil analyses. More specifically, seven T_1 lines containing a single insertion of transgene, *RcpPLAIIIβ*, were identified based on the 3:1 ratio of T_2 seed segregations of kanamycin-resistance and non-resistance. Among them, two lines had similar HFA content, while the other five lines (designated RcpPLAIIIβ-CL7 Line 1 to 5) showed significantly lower HFA content compared with CL7. Seeds of RcpPLAIII-CL7 lines 1 to 5 were planted on kanamycin selection plates to screen out segregants that lacked the *RcpPLAIIIβ* transgene. After that, 36 transgenic plants from each line were grown individually in soil along with CL7, followed by the identification of homozygotes and heterozygotes based on T_3 seed segregations of kanamycin resistance.

Seeds of 10-11 independent homozygous plants from each line (53 plants in total), and of 26 independent CL7 plants were subjected to oil analysis.

Table 4.2 Fatty acid composition of the oil ofT_2 seeds of RcpPLAIIIβ-CL7 and RcpPLAIIIδ-CL7 lines

Data are mean (SD) from seeds of 20 independent CL7 control lines or lines expressing each transgene (two technical replications each line). The fatty acid composition was calculated as each fatty acid of total fatty acids in weight basis based on the relative peak area and analyzed by two-tailed T-test. (▲/▼) indicates the values greater/lower than CL7 control at α=0.05 level; (▲▲/▼▼) indicates the values greater/lower than CL7 control at α=0.01 level.

Line	Fatty acid composition (%)				
	16:0	16:1	18:0	18:1	18:2
CL7	10.43 (0.60)	0.33 (0.07)	5.12 (0.33)	36.67 (1.43)	20.46 (0.90)
RcpPLAIIIβ-CL7	10.44 (0.55)	0.30 (0.05)	4.75 (0.31)▼▼	37.81 (1.44)▲	21.22 (1.19)▲
RcpPLAIIIδ-CL7	10.52 (0.64)	0.32 (0.06)	5.13 (0.24)	36.72 (1.40)	20.74 (1.15)

Line	Fatty acid composition (%)			
	18:3	20:0	20:1	22:0
CL7	9.75 (1.33)	1.03 (0.07)	0.32 (0.03)	0.31 (0.03)
RcpPLAIIIβ-CL7	10.92 (1.22)▲▲	1.00 (0.05)	0.33 (0.03)	0.30 (0.02)
RcpPLAIIIδ-CL7	9.59 (1.01)	1.01 (0.05)	0.31 (0.03)	0.30 (0.02)

Line	Fatty acid composition (%)		Sum of HFA
	18:1-OH	18:2-OH	
CL7	12.49 (2.21)	3.10 (0.63)	15.59 (2.69)
RcpPLAIIIβ-CL7	10.55 (2.44)▼	2.38 (0.55)▼▼	12.93 (2.91)▼▼
RcpPLAIIIδ-CL7	12.45 (2.03)	2.92 (0.75)	15.37 (2.57)

In T_3 homozygous seeds, HFA content was reduced by 13% (RcpPLAIIIβ-CL7 line 5) to 47% (line 2) relative to CL7, with a few plants from line 2 and line 3 accumulating only 5 - 6% HFA (Figure 4.5). The mean of HFA content in samples from CL7 was 13.9% (lowest 8.7%; highest 17.2%), while most of the samples ranged from 11.6% to 17.6%. By contrast, the mean of HFA content in samples of RcpPLAIII-CL7 lines was 10.2%, which was significantly lower than for CL7. In particular, line 2 displayed an average HFA content of 7.4% (lowest 5.0%; highest 9.1%) and line 3 showed an average of 8.6% HFA content (lowest 6.7%; highest 9.9%) in the seed oil. In these two lines, almost all the samples contained lower HFA contents than the majority of samples from CL7 (Figure 4.5). RcpPLAIIIβ-CL7 line 2 and line 3 displayed the most substantial reduction in HFA content among the five lines relative to CL7 (47% and 38%, respectively, on a relative basis). In addition, samples from line 4 showed an average of 11.5% HFA content in the seed oil (lowest 9.8%; highest 13.3%), which was also significantly lower (decreased by 17%) than for CL7. The HFA reductions in line 1 and line 4 were not as obvious as other lines due to the variation HFAs accumulated to 12.0% of fatty acids (lowest 9.8%; highest 14.5%) in line 1 and 12.1% (lowest 9.3%; highest 15.2%) in line 5. The observed reductions in HFA contents in these two lines were indicated by statistical analysis (Table 4.3).

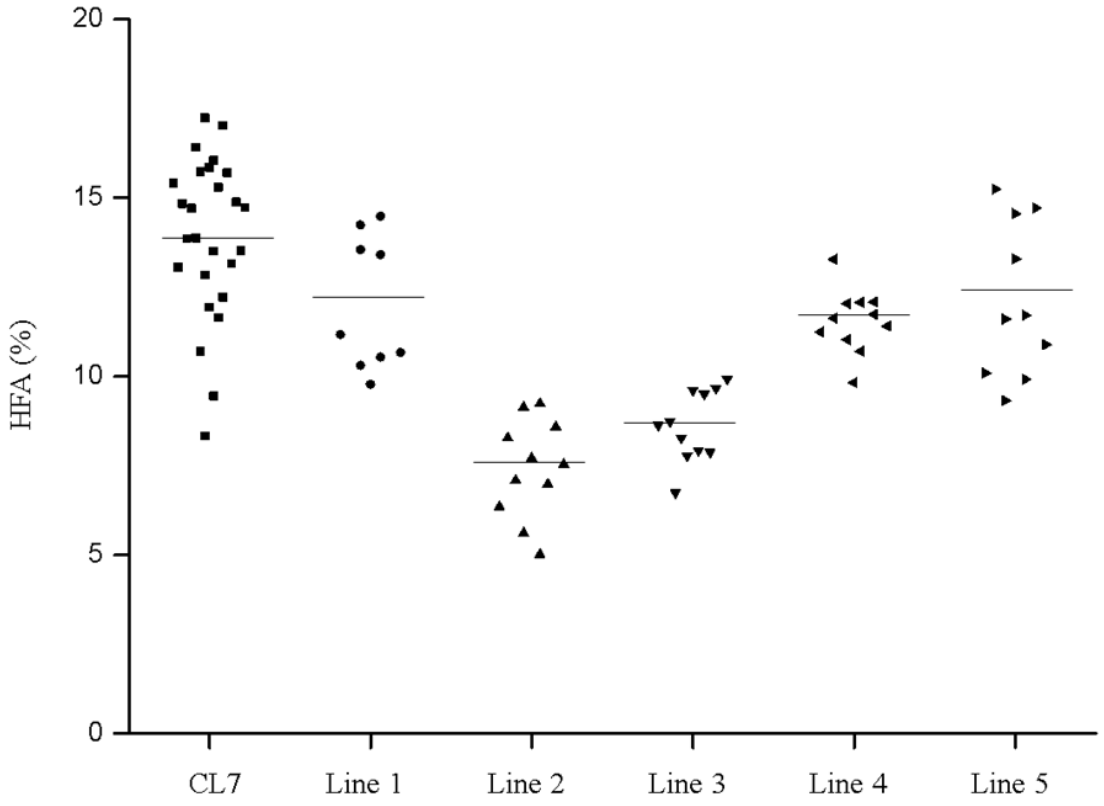


Figure 4.5 Hydroxy fatty acid (HFA) content of the oil in T_3 seeds from T_2 RcpPLAIIIβ-CL7 homozygous lines

HFA content is percentage of total fatty acids on a weight basis. Each point represents the HFA content in seed samples of an individual T_2 transgenic plant (n=3); horizontal bars represent the mean of HFA content of homozygous plants derived from the same parent T_1 line with a single transgenic insertion. Data were analyzed by Two-tailed *t* test.

Table 4.3 Fatty acid composition of the oil of T_3 seeds of RcpPLAIIIβ-CL7 homozygous lines

CL7 data are mean (SD) from seeds of 26 independent CL7 lines. *RcpPLAIIIδ* data are mean (SD) from T_3 homozygous seeds of independent T_2 lines with single transgenic inserts. Ten plants from line 1, 11 plants from line2, 11 plants from line 3, 11 plants from line 4 and 10 plants from line 5 (three technical replications for each line). All plants were grown in the same growth cabinet at the same time. The fatty acid composition was calculated as each fatty acid of total fatty acids in weight basis based on the relative peak areas in GC/MS. Data were analyzed by Two-tailed t test. (▲/▼) and (▲▲/▼▼) indicated the value was higher/lower than CL7 at the 0.05 or the 0.01 level. Differences between transgenic samples but not CL7 were not shown.

Line	Fatty acid composition (%)				
	16:0	16:1	18:0	18:1	18:2
CL7	11.32 (0.20)	0.37 (0.04)	5.50 (0.29)	35.84 (0.61)	19.96 (1.18)
Line 1	11.24 (0.17)	0.41 (0.06)	5.39 (0.29)	35.90 (0.67)	21.21 (1.05)▲
Line 2	11.20 (0.16)	0.39 (0.03)	5.25 (0.12)▼	34.98 (0.99)▼	24.35 (1.28)▲
Line 3	11.30 (0.10)	0.38 (0.01)	5.36 (0.09)▼	35.67 (0.68)	23.27 (0.59)▲
Line 4	11.09 (0.09)▼	0.38 (0.05)	5.28 (0.17)▼	35.51 (0.58)	21.6 (0.52)▲
Line 5	11.25 (0.19)	0.39 (0.02)	5.40 (0.25)	35.87 (0.95)	20.94 (1.09)▲

Line	Fatty acid composition (%)			
	18:3	20:0	20:1	22:0
CL7	11.16 (1.22)	1.19 (0.06)	0.39 (0.02)	0.37 (0.01)
Line 1	11.84 (1.14)	1.23 (0.05)	0.40 (0.01)▲	0.38 (0.20)
Line 2	14.47 (0.77)▲	1.19 (0.03)	0.40 (0.03)	0.37 (0.01)
Line 3	13.44 (0.55)▲	1.21 (0.03)	0.39 (0.01)	0.37 (0.01)
Line 4	12.63 (0.66)▲	1.20 (0.04)	0.41 (0.01)▲	0.37 (0.01)
Line 5	12.00 (0.96)▲	1.24 (0.04)▲	0.40 (0.02)▲	0.39 (0.01)▲

Line	Fatty acid composition (%)		Sum of HFA
	18:1-OH	18:2-OH	
CL7	10.63 (1.68)	3.28 (0.56)	13.91 (2.20)
Line 1	9.38 (1.32)▼	2.62 (0.46)▼▼	12.01 (1.76)▼
Line 2	5.87 (0.99)▼	1.53 (0.33)▼	7.40 (1.31)▼
Line 3	6.86 (0.71)▼	1.74 (0.25)▼	8.61 (0.95)▼
Line 4	9.01 (0.65)▼	2.54 (0.25)▼	11.54 (0.85)▼
Line 5	9.33 (1.58)	2.80 (0.49)▼	12.13 (2.06)▼

Other fatty acid compositions were changed in RcpPLAIIIβ-CL7 compared with CL7. Fatty acid compositions of the oils were similar in T_2 seeds and T_3 seeds (Table 4.2; 4.3). In RcpPLAIIIβ-CL7 lines, 18:2 increased by approximately 5% (line 5) to 22% (line 2), while 18:3 was enhanced by 8% (line 5) to 30% (line 2), except that samples from line 1 did not displayed significant increases. Correspondingly, a slight but significant decrease of 2% in 18:1 was observed in line 2 which accumulated the lowest HFA levels (Table 4.3).

In summary, the expression of *RcpPLAIIIβ* resulted in a significant reduction in HFA contents compared to control CL7. HFA reductions were observed in both T_2 (Figure 4.4A) and T_3 seeds (Figure 4.5), while the reduction in the T_3 seeds, especially samples from line 2 and 3, was larger than in the T_2 seeds.

4.3.2 Expression of *RcpPLAIIIδ* in Arabidopsis CL7 did not alter the fatty acid composition of the seed oil or seed oil content

Similar to the *L. fendleri* ortholog *LfpPLAIIIδ*, the expression of *RcpPLAIIIδ* did not result in any changes in HFA content in T_2 seeds relative to control CL7 lines (approximately 15.4% versus 15.6%; Figure 4.4A). Likewise, neither the composition of other fatty acids (Table 4.2) nor the total seed oil content was changed by the *RcpPLAIIIδ* expression (Figure 4.4B).

4.3.3 Expression of *RcpPLAIIIβ* in Arabidopsis CL7 led to a decrease in hydroxy fatty acids in both triacylglycerol and phosphatidylcholine

The fatty acid composition of TAG and PC were determined in Arabidopsis CL7 expressing *RcpPLAIII β* in order to gain insight into reason for the significant decrease in HFA content of the seed oil of these transgenic lines. Total lipids were extracted from four samples from RcpPLAIIIβ-CL7 line 2, followed by the separation of TAG and PC. Fatty acid compositions were determined in both lipid classes. The HFA content of TAG in RcpPLAIIIβ-CL7- line 2 was 4.5% compared to 14.6% in CL7 which represented a relative decrease in HFA of 69% (Figure 4.6). In contrast, the HFA content of PC in RcpPLAIIIβ-CL7- line 2 was 3% compared to 8% in CL7 which represented a relative decrease in HFA content of over 60% (Figure 4.6).

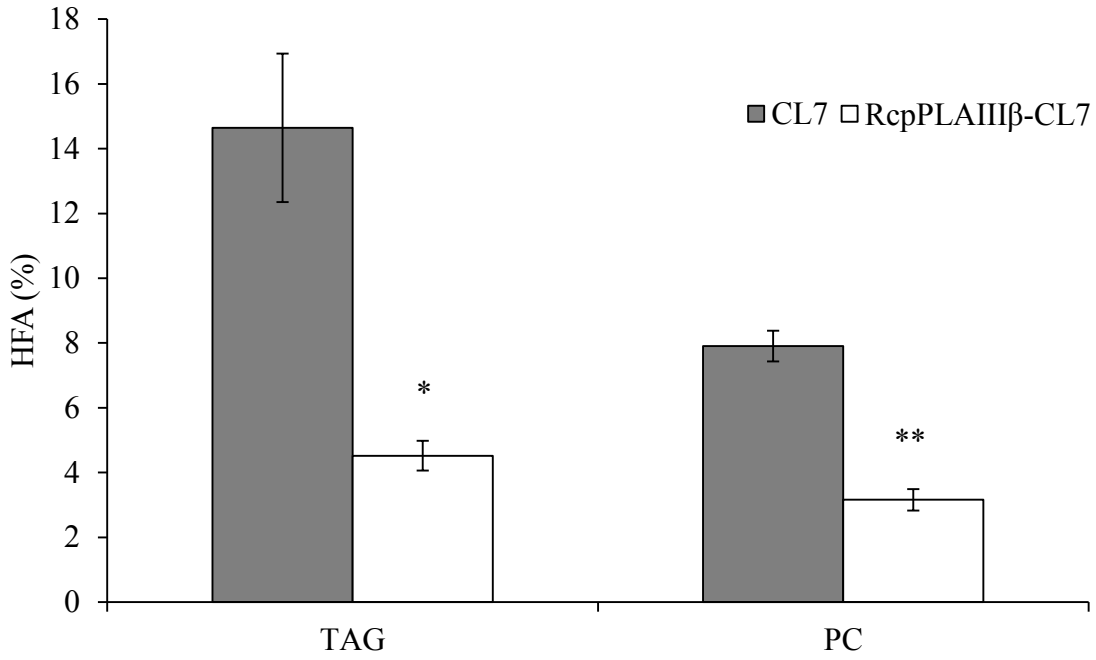


Figure 4.6 Hydroxy fatty acid (HFA) content of triacylglycerol (TAG) and phosphatidylcholine (PC) from T_3 RcpPLAIIIβ-CL7 mature seeds

Data are mean (SD) of HFA content of total fatty acids (w/w) in four independent plants from RcpPLAIIIβ-CL7 Line 2 or CL7 (three technical replications for each plant). Data were analyzed by Two-tailed *t* test. */** indicates significant differences in α=0.05/α=0.01 level.

4.3.4 Expression of *RcpPLAIIIβ* in Arabidopsis CL7 did not lead to changes in the oil content of T_3 homozygous seed

As previously shown in Figure 4.4B, T_2 RcpPLAIIIβ-CL7 seeds did not exhibit any changes in seed oil content. Seed oil content was also determined for T_3 RcpPLAIIIβ-CL7 homozygous seeds. Even though the five RcpPLAIIIβ-CL7 lines produced different levels of HFAs, they consistently exhibited approximately 24% seed oil content (Figure 4.7).

A scatter plot of seed oil content versus HFA content was generated to investigate any possible correlations between these two factors. Although the HFA contents of samples from RcpPLAIIβ-CL7 ranged from 5% to 15%, the seed oil content was not proportional to the HFA level (data not shown).

In conclusion, the expression of *RcpPLAIIIβ* led to a significant decrease in HFA accumulation in CL7 seeds, but the alteration in fatty acid composition had no effect on the level of seed oil accumulation.

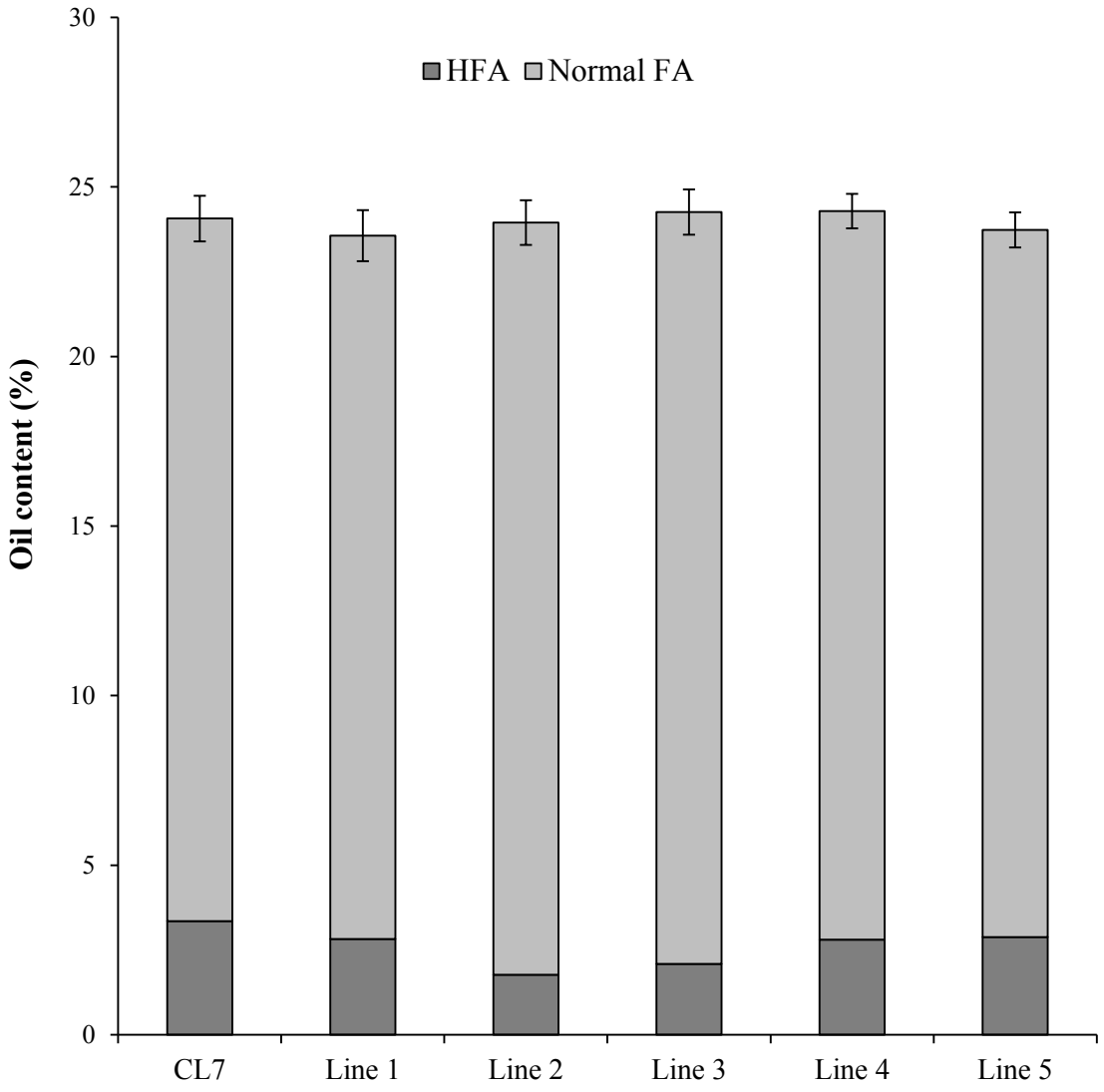


Figure 4.7 Oil content in T_3 RcpPLAIIIβ-CL7 seeds

Each column represents the mean (SD) of seed oil content of 10 or 11 independent plants (three technical replications each plant) from the same parent. The proportions of hydroxy fatty acids (HFAs) and normally occurring fatty acids are indicated. Data were analyzed with Two-tailed t test. No significant changes were noticed compared with the CL7 controls. FA, fatty acid.

4.4 Over-expression of Arabidopsis *PLAIIIα* in Arabidopsis CL7 did not change the hydroxyl fatty acid or content of the seed oil

The Arabidopsis orthologs of *RcpPLAIIIβ* or *RcpPLAIIIδ* were over-expressed in CL7 (designated AtpPLAIIIα-CL7 and AtpPLAIIIδ-CL7, respectively) to determine if pPLAIII from a non-HFA-producing species was capable of removing HFA from PC in Arabidopsis CL7. T_1 over-expression plants bearing *AtpPLAIIIα* or *AtpPLAIIIδ*, respectively, were identified based on kanamycin-resistance. Thirty-two T_1 lines over-expressing each gene were grown to harvest mature T_2 seeds, and T_2 seeds from 20 independent T_1 lines were subjected to oil analysis.

Over-expression of *AtpPLAIIIα* in Arabidopsis CL7 did not lead to significant changes in total HFA contents compared with the CL7 control (Figure 4.8A; Table 4.4). There was, however, a slight change with 18:2-OH, decreasing from 3.1% to 2.6%. In general, the fatty acid composition of the oil from AtpPLAIIIα-CL7 lines was similar to CL7 (Figure 4.8A). The average seed oil content of the AtpPLAIIIα-CL7 lines, however, decreased to about 27.5% from 29.1% in CL7 (Figure 4.8B). The over-expression of endogenous Arabidopsis *AtpPLAIIIδ* in CL7 had no effects on either fatty acid composition or seed oil content (Figure 4.8; Table 4.4).

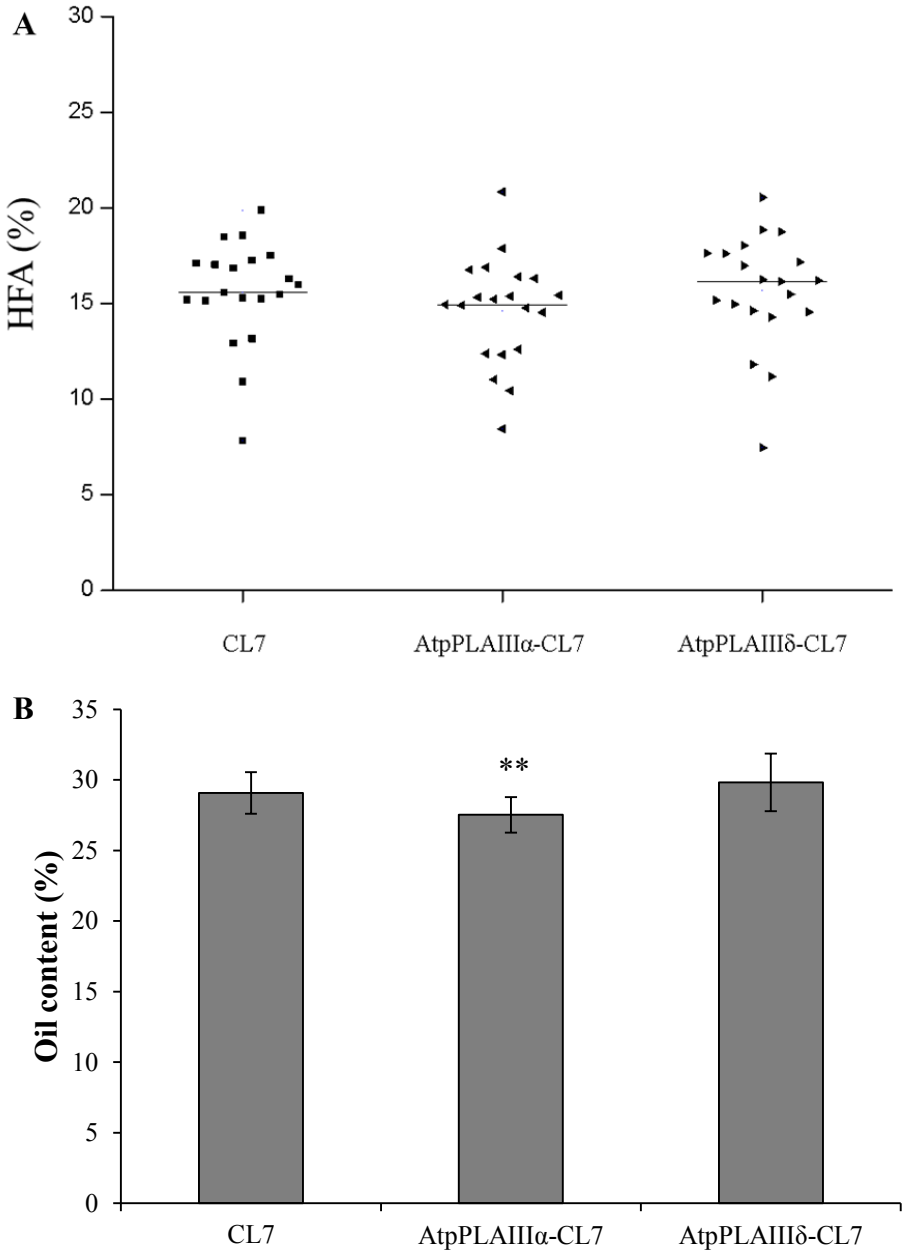


Figure 4.8 Hydroxy fatty acid (HFA) content of the oil (A) and total oil content (B) in T_2 seeds of AtpPLAIIIα-CL7 and AtpPLAIIIδ-CL7 transgenic lines

(A) HFA content is the percentage of total fatty acids based on weight. Each data point represents the HFA content in seed samples of an individual T_1 transgenic plant (n=2); horizontal bars represent the mean of HFA content of 20 independent lines expressing the same transgene. (B) Oil content is percentage of dry seed weight. Each column represents the mean of 20 independent lines (technical two replications each line) expressing the same transgene. Error bars represent standard deviation of samples (SD). Data were analyzed by Two-tailed *t* test.

Table 4.4 Fatty acid composition of the oil of T_2 seeds of AtpPLAIIIα-CL7 and AtpPLAIIIδ-CL7 transgenic lines

Data are mean (SD) from seeds of 20 independent CL7 control lines or lines expressing each transgene (two technical replications each line). The fatty acid composition was calculated as each fatty acid of total fatty acids in weight basis based on the relative peak area and analyzed by two-tailed T-test. (▲/▼) indicates the values greater/lower than CL7 control at α=0.05 level; (▲▲/▼▼) indicates the values greater/lower than CL7 control at α=0.01 level.

Line	Fatty acid composition (%)				
	16:0	16:1	18:0	18:1	18:2
CL7	10.43 (0.60)	0.33 (0.07)	5.12 (0.33)	36.67 (1.43)	20.46 (0.90)
AtPLAIIIα-CL7	10.63 (0.59)	0.33 (0.07)	4.95 (0.26)	37.11 (1.46)	20.59 (1.20)
AtpPLAIIIδ-CL7	10.47 (0.57)	0.33 (0.05)	5.18 (0.21)	36.10 (1.69)	20.82 (1.42)

Line	Fatty acid composition (%)			
	18:3	20:0	20:1	22:0
CL7	9.75 (1.33)	1.03 (0.07)	0.32 (0.03)	0.31 (0.03)
AtpPLAIIIα-CL7	10.04 (1.25)	1.06 (0.04)	0.34 (0.03)▲	0.32 (0.02)
AtpPLAIIIδ-CL7	9.76 (1.24)	1.03 (0.06)	0.32 (0.03)	0.30 (0.02)

Line	Fatty acid composition (%)		Sum of HFA
	18:1-OH	18:2-OH	
CL7	12.49 (2.21)	3.10 (0.63)	15.59 (2.69)
AtpPLAIIIα-CL7	12.07 (2.25)	2.57 (0.62)▼	14.64 (2.74)
AtpPLAIIIδ-CL7	12.78 (2.39)	2.90 (0.62)	15.68 (2.90)

5 - Discussion

Castor oil, with 90% ricinoleic acid, has proven extremely useful in the oleo-chemical industry. The hydroxyl in the ricinoleic acid stabilizes the oil and provides unique properties for use of this as a chemical feedstock (Ogunniyi, 2006). The supply of castor oil, however, is limited by the presence of toxic components, the undesirable agronomical features of the crop and political instabilities within the exporting countries (Holic et al., 2012). Another source of HFAs, *L. fendleri*, accumulates approximately 60% lesquerolic acid in its seed oil, but has yet to become a commercial oilseed crop (Dierig et al., 2011). Thus, modifying established temperate oil crops to produce HFAs seems to be a feasible but challenging strategy to overcome limitations associated with the natural sources of hydroxyl oils (Kumar et al., 2006). To date, moderate successes have been achieved in the metabolic engineering of temperate oleaginous plants to produce hydroxy oils since some of the key genes governing the synthesis of HFAs have been identified (Lee et al., 2015). In a transgenic host, however, the inefficient utilization of the HFAs by any part of the TAG synthetic pathway could produce bottlenecks for the desirable accumulation of HFAs in the seed oil (Bates and Browse, 2012).

This thesis mainly focused on examining the potential role of PLAIII in releasing HFAs from their synthesis site in PC. *RcpPLAIIIβ* was shown lead to a significant reduction in the HFA content of both TAG and PC when expressed in Arabidopsis expressing *RcFAH12* (CL7 lines). The discussion will include possible strategies for utilizing *RcpPLAIIIβ* as a molecular engineering tool to boost the accumulation of HFAs in seed oils of transgenic oil-forming species.

5.1 RcpPLAIIIβ catalyzes the removal of hydroxyl fatty acids from their site of synthesis in phosphatidylcholine

As indicated in the Literature Review, RcFAH12 catalyzes the hydroxylation of oleoyl moieties esterified to the *sn*-2 position of PC to generate ricinoleoyl moieties (Lin et al., 1998). Transgenic Arabidopsis expressing *RcFAH12* (CL7 and CL37 lines) contains twice as much HFA in PC as castor suggesting that removal of HFA from PC represents a bottleneck for the production of HFAs in Arabidopsis CL7 and CL37 (van Erp et al., 2011).

RcpPLAIIIβ belongs to the pPLA family (Figure 4.2) and is one of the 13 *PLAs* expressed in developing castor endosperm (Bayon et al., 2015). Plant pPLAs catalyze the liberation of fatty acids at both the *sn*-1 and *sn*-2 positions of phospholipids and other glycerolipids (Chen et al., 2013). When *RcpPLAIIIβ* was expressed in the CL7, the HFA content of PC was reduced to 3% from 8% in CL7 (Figure 4.6). In addition, 18:2 and 18:3 levels were elevated while the 18:1 level tended to decrease. It has been proposed that the buildup of HFAs in PC may inhibit activities of FAD2-like enzymes in Arabidopsis, ending up with the decreases in 18:2 and 18:3 and an increase in 18:1 (Thomæus et al., 2001; Smith et al., 2003). Therefore, *RcpPLAIIIβ* may encode a PLA that selectively catalyzes the liberation of HFAs from the site of synthesis and may partially alleviate the FAD2 suppression. *RcPLA$_2$α*, another *PLA* expressed in the developing castor endosperm, has recently been reported to cause similar results when expressed in Arabidopsis CL37 lines (Bayon et al., 2015). Collectively, the results of the current study and that of the study conducted by Bayon et al. (2015) suggest that castor utilizes a PLA-mediated acyl editing process to remove HFAs from PC in support of

hydroxy-TAG synthesis and more than one PLA appears to be involved in this pathway. Castor seed produces an extremely high level of HFAs in the seed oil and nearly 70% of TAG molecular species are in the form of tririncinoleoylglycerol (Lin et al., 2003). This requires not only the efficient release of HFAs from PC but efficient incorporation of HFAs into the three positions of the glycerol backbone of TAG (Bates and Browse, 2012). Arabidopsis, however, appears to lack the processes required for incorporation of HFAs into TAG (Bayon et al., 2015). Arabidopsis uses PC-derived DAG as major source of acyl chains for TAG production (Bates and Browse, 2011). In Arabidopsis CL37, half of the TAG molecules are mono-HFA-TAG, with 70% of HFAs located in the *sn*-2 position. The majority of these TAG molecules are synthesized from the PC-derived mono(*sn*-2)-HFA-DAG molecules (van Erp et al., 2011; Bates and Browse, 2011). The selective removal of HFAs from the *sn*-2 position of PC resulted in a decrease in mono(*sn*-2)-HFA-DAG for TAG synthesis. Meanwhile, the released HFAs failed to be efficiently incorporated to TAG synthesis. Thus, both the inability to channel released free HFA into TAG (route 1) and the reduced HFA-enriched DAG (route 2) may explain why HFA content was substantially reduced in the RcpPLAIIIβ-CL7 lines compared with Arabidopsis CL7 (Figure 4.5; see the model in figure 5.1).

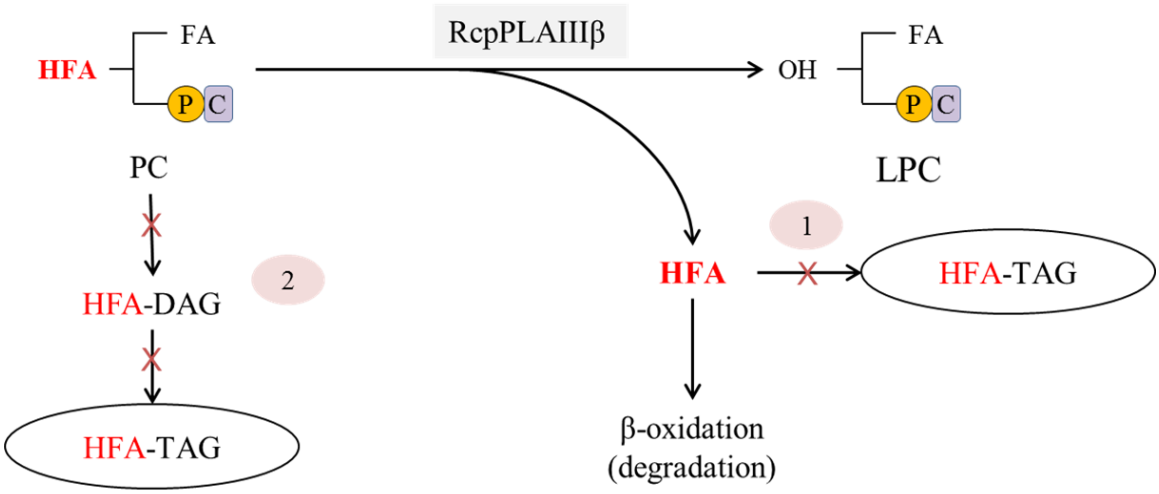


Figure 5.1 The hypothesized model to explain the reduction in hydroxy fatty acid (HFA) in the seed oil of Arabidopsis RcpPLAIII-CL7 lines

RcpPLAIIIβ may catalyze the hydrolysis of HFA-containing phosphatidylcholine (PC) to release lysophoshatidylcholine (LPC) and free HFA. (1) Released HFAs may not be efficiently utilized for the triacylglycerol (TAG) assembly and be degraded through β-oxidation (Bayon et al., 2015). (2) The major acyl flux for TAG synthesis in Arabidopsis is through "PC to PC-derived DAG to TAG" (Bates and Browse, 2011). The hydrolysis of HFA-containing PC may led to a decrease in PC-derived mono(*sn*-2)-HFA-DAG molecules and thus the decrease in HFA-containing TAG.

After being removed from PC through PLA action, released HFAs may have been degraded via β-oxidation in the peroxisome (Bayon et al., 2015). Peroxisomal β-oxidation represents fatty acid catabolism that sequentially cleaves the acyl-CoA at the β-carbon to generate acetyl-CoA, which is essential for the production of carbon skeletons and cellular energy for the germinating seed and growing seedling (Baker et al., 2006; Li et al., 2016). In Arabidopsis, the peroxisomal membrane-localized ABCD1 transporter catalyzes the hydrolysis of acyl-CoA and transports the free fatty acid into the lumen (de Marcos Lousa et al, 2013; Li et al., 2016). Subsequently, the free fatty acids are reactivated to acyl-CoAs catalyzed by LACS6 and/or LACS7 and degraded by β-oxidation (Shockey et al., 2002; de Marcos Lousa et al., 2013). The hydrolytic activity of ABCD1 is stimulated by acyl-CoA. An ABCD1-independent pathway, however, may exist to facilitate the entry of free fatty acid into the peroxisome for activation by LACS6/7 (de Marcos Lousa et al., 2013). The selective degradation of ricinoleic acid via β-oxidation has been demonstrated by the analysis of the polyhydroxyalkanoate (PHA) synthesized from the intermediates of the β-oxidation (Moire et al., 2004).

As mentioned previously, pPLAs can utilize a wider range of substrates relative to sPLA$_2$s (Liu et al., 2015). pPLAIIIβ and pPLAIIIδ can catalyze the hydrolysis of both phospholipids and acyl-CoAs (Li et al., 2013), and show preference for the *sn*-2 position over the *sn*-1 position in the PC (Chen et al., 2011b; Li et al., 2011; 2013). *In vitro* assays of the substrate selectivity and specificity of RcpPLAIIIβ for different molecular species of PC may provide for additional information on the effectiveness of this enzyme in catalyzing the release of HFA from PC. Indeed, recombinant RcPLA$_2$α (produced in yeast) has been shown to be more effective in releasing HFA from PC than AtPLA$_2$α

(Bayon et al., 2015). Furthermore, the possibility that HFA-CoA were not available for TAG synthesis because they were hydrolyzed via the catalytic action of RcpPLAIIIβ cannot be ruled out. In this scenario, free HFAs may have been channeled into peroxisome for degradation via the ABCD1-independent pathway (de Marcos Lousa et al., 2013) Thus, it may also be worthwhile to investigate the activity of recombinant RcpPLAIIIβ towards HFA-CoA using i*n vitro* assays.

5.2 Potential strategies involving *RcpPLAIIIβ* as a molecular tool to boost the hydroxyl fatty acid accumulation in the seed oil of Arabidopsis CL7

Although HFAs could be removed from PC through the catalytic action of RcpPLAIIIβ, the transgenic Arabidopsis CL7 host lacked a process to facilitate the incorporation of liberated HFA into TAG. Possible obstacles could be the absence of specific LACS that can efficiently catalyze the formation of HFA-CoA, acyltransferases in the Kennedy pathway with low specificity for HFA-CoAs, or both. In this section, potential strategies to facilitate the incorporation of HFAs into TAG will be discussed.

5.2.1 Strategies involving long-chain acyl-CoA synthetase

In the RcpPLAIIIβ-CL7 lines, the released HFAs may not have been effectively activated to HFA-CoAs and thus directly entered the peroxisome via an ABCD1-independent route (de Marcos Lousa et al., 2013) for degradation. The analysis of the acyl-CoA species during the seed development in RcpPLAIIIβ-CL7 lines may give an indication if HFA-CoAs were synthesized. Alternatively, synthesized HFA-CoAs may have been targeted to peroxisomal β-oxidation rather than TAG synthesis. HFA-CoA is not the predominant acyl-CoA species in the developing castor endosperm (Brown et al., 2012), which

suggests there may be a discrete acyl-CoA pool specific for the TAG synthesis in the cell where HFA-CoA could be rapidly utilized (Brown et al., 2012).

A possible approach to increase the HFA content of TAG in RcpPLAIIIβ-CL7 lines might involve expressing a cDNA that encodes a LACS enzyme which specifically activates HFA to HFA-CoA. The question is which LACS is associated with this pathway in castor. In the case of the RcPLA$_2$α-CL37 lines developed by Bayon et al. (2015), three different castor *LACSs* (orthologs of *AtLACS1*, *AtLACS4* or *AtLACS8*) were evaluated for this ability, but none of them could promote the HFA accumulation. Thus, it appears that the burden of the HFA-CoA synthesis falls upon other LACS(s) in the castor. Four other *LACS* were found to be expressed in the developing castor endosperm (Brown et al., 2012). A gene (old name: *RcACS2*) encoding the homolog of LACS6 from Arabidopsis has been known to have 60% higher activity towards HFAs than other fatty acids *in vitro*, but it was likely to be the peroxisomal LACS (He et al., 2007). Another castor peroxisomal LACS-like enzyme showed low activity to HFAs (He et al., 2007; Brown et al., 2012). Furthermore, the *LACS* gene encoding a ortholog of *LACS2* from Arabidopsis was relatively poorly expressed in the developing castor endosperm (Brown et al., 2012) and the LACS2 has been reported to be associated with the cutin synthesis rather than lipid synthesis in Arabidopsis (Schnurr et al., 2004).

The most highly expressed *LACS* in the developing castor endosperm, which encoded an ortholog of *LACS9* from Arabidopsis, seemed to be possible candidate (Brown et al., 2012). Despite that the fact that LACS9 may overlap in function with LACS4 in the import of fatty acids into plastid in Arabidopsis, LACS9 also appears to be involved in the TAG metabolism (Jessen et al., 2015). The double mutants *lacs1 lacs9*

and *lacs4 lacs9* displayed 10% and 27% reduction of TAG levels in the seed, respectively, compared to the wild type Arabidopsis (Zhao et al., 2010; Jessen et al., 2015). Therefore, it may be worthwhile expressing *RcLACS9* in RcpPLAIIIβ-CL7 lines.

5.2.2 Strategies involving acyltransferases of the Kennedy pathway

In the event that HFA-CoA is effectively formed from free HFA in RcpPLAIIIβ-CL7 lines, it is possible that the acyltransferases of the Kennedy pathway may not effectively utilize HFA-CoA in Arabidopsis. Some of the acyltransferases that are important in TAG synthesis in castor have been investigated (Kroon et al., 2006; Burgal, 2008; Mckeon and He, 2015; Chen et al., 2016). As we know, Arabidopsis utilizes PC-derived DAG as a main source of acyl chains for TAG synthesis. The expression of *RcFAH12* reduced this flux by approximately 70% with no increase in the synthesis of TAG from *de novo* DAG (Bates and Browse, 2011). In RcpPLAIIIβ-CL7 lines, the efficient utilization of HFA-CoA by acyltransferases with increased specificity for HFA-CoA may assist in the effective incorporation of HFA into TAG. Furthermore, the rapid incorporation of HFAs into TAG may also facilitate the flux of free HFAs into the acyl-CoA pool, and thus alleviate the obstacle caused by the nonspecific LACS activity.

The acyl-CoA dependent Kennedy pathway involves activities of GPAT, LPAAT and DGAT to catalyze the sequential acylation of three positions of the glycerol backbone (Chapman and Ohlrogge, 2012; see Figure 2.2 from Literature Review). DGAT catalyzes the final step of TAG synthesis and in some species this enzyme activity may have a substantial effect on the flow of carbon into seed oil (Weselake et al., 2009). In developing castor seed, TAG formation involves the catalytic action of RcDGAT1 and RcDGAT2 (Mckeon and He, 2015). RcDGAT2 may be a useful molecular tool to

increase HFA content in the seed oil of RcpPLAIIIβ-CL7 lines since this DGAT isoenzyme effectively uses diricinoleoylgylcerols or ricinoleoyl-CoAs as substrates (Kroon et al., 2006; Burgal et al., 2008). If *RcDGAT2* is expressed in RcpPLAIIIβ-CL7 lines, activated HFAs released from PC may be utilized by RcDGAT2, preventing their catabolism. Furthermore, RcDGAT2 may selectively utilize HFA-containing DAG for the TAG synthesis (Burgal et al., 2008; Bates and Browse, 2011). Recently, it has also been shown that recombinant RcDGAT1 utilizes dricinoleoylglycerol more effectively that AtDGAT1 (McKeon and He, 2015). Therefore, co-expression of *RcDGAT1* and *RcDGAT2* in RcpPLAIIIβ-CL7 lines may also prove effective in promoting an increase in the HFA content of TAG.

LPAAT catalyzes the acyl-CoA-dependent acylation of LPA to produce PA (see Figure 2.2). Thus, RcLPAAT2 might be useful for catalyzing the formation of PA containing HFA. When *RcLPAAT2* was expressed in the *L. fendleri*, ricinoleic acid incorporation at the *sn*-2 position of TAG increased from 2% to 17%, and the tri-HFA-TAG levels increased from 5% to almost 14% (Chen et al., 2016). The absence of *de novo* di-HFA-DAG in CL37 may be due to endogenous LPAATs which exclude HFA-LPA as an HFA acceptor (Bates and Browse, 2011). Expressing the *RcLPAAT2* may promote the utilization of HFA-LPA to generate *de novo* di-HFA-DAG in RcpPLAIIIβ-CL7 lines. However, if *RcLPAAT2* is expressed in RcpPLAIIIβ-CL7 lines, the *de novo* di-HFA-DAG may still be rapidly turned over to generate a futile cycle (Bates et al., 2014). It is also possible that the increased di-HFA-DAG would hinder the flux from *de novo* DAG to PC, thus creating a new bottleneck. Therefore, the co-expression of *RcDGAT2* with *RcLPAAT2* in RcpPLAIIIβ-CL7 may be necessary to overcome a

potential bottleneck which channels *de novo* di-HFA-DAG into a futile cycle. In addition, the PA generated by the catalytic action of *RcLPAAT2* would have to be acceptable by the resident PAP enzyme, which catalyzes the dephosphorylation of PA in the Kennedy pathway (see Figure 2.2). It may even be necessary to consider introducing an RcPAP with demonstrated specificity for catalyzing the liberation of phosphate from *de novo* di-HFA-phosphatidic acid.

In the Kennedy pathway, GPAT catalyzes the acyl-CoA-dependent acylation of G3P to produce LPA (see Figure 2.2). The activity of GPAT may be worthwhile to modify as well. It has been shown that *RcGPAT9* had the highest expression amongst *RcGPATs* (Brown et al., 2012). Recently, GPAT9 was shown to be GPAT involved in the Kennedy pathway leading to intracellular TAG in Arabidopsis (Shockey et al., 2016). Unlike AtGPAT9, several other Arabidopsis GPATs were previously shown to acylate the *sn*-2 position of G3P to generate *sn*-2 LPA (Chen et al., 2011c). In addition, these GPATs had a phosphatase activity which resulted in the production of *sn*-2 monoacylglycerol in support of surface lipid polyester synthesis. If RcGPAT9 is shown to have increased specificity for HFA-CoA, it may also be useful in combination with RcLPAAT2 and RcDGATs to further boost the content of HFA in the TAG of RcpPLAIIIβ-CL7 lines.

It is possible that multiple interventions may be required to bring about a high level of HFA content in the TAG of RcpPLAIIIβ-CL7 lines. This may require the combined heterologous expression of forms of *LACS*, *GPAT9, LPAAT2*, *PAP* and *DGAT* encoding enzymes with demonstrated enhanced specificity and selectivity for substrates containing HFAs.

5.3 The orthologs of *RcpPLAIIIβ* from *L. fendleri* and Arabidopsis may encode phopsholipases which are not effective in catalyzing the release of hydroxy fatty acids from the *sn*-2 position of phosphatidylcholine

In the current study, six *pPLAIII* genes were screened for their impacts on HFA accumulation in Arabidopsis expressing *RcFAH12*. *RcpPLAIIIβ* was the only *pPLAIII* gene which resulted in of HFAs from PC when expressed in Arabidopsis CL7. In contrast to RcpPLAIIIβ which could efficiently catalyze the hydrolysis of PC to release HFAs, two pPLAIII enzymes from the *L. fendleri*, LfpPLAIIIβ and LfpPLAIIIδ, did not affect HFA accumulation when the encoding cDNAs were expressed in the Arabidopsis CL7. It is possible that pPLAIIIs do not play a role in the selective release of HFAs from *sn*-2 position of PC in *L. fendleri*. The slight decrease in C18 normal fatty acids in LfpPLAIIIβ –CL7 or LfpPLAIIIδ-CL7 lines may simply be due to the increased pPLAIII activities, since the AtpPLAIIIs have been reported to promote the accumulation of C20 fatty acids at the expense of C18 fatty acids in Arabidopsis (Li et al., 2011; 2013).

A notable difference from castor, which only produces ricinoleic acid, is that *L. fendleri* is known to produce C20 HFAs through elongation of C18 HFAs in the form of acyl-CoA catalyzed by LfKCS3 (Moon et al., 2001). The expression of *LfKCS3* in Camelina led to both an increase in total HFA content and accumulation of C20 HFAs, together with less HFAs retained in PC (Snapp et al., 2014). It is interesting that even though no PLAs specific to HFAs were introduced to the transgenic host, HFAs were removed from PC with high efficiency. The phenomenon implied that the efficient channeling of HFAs from the PC to acyl-CoA pool in *L. fendleri* may be associated acyl editing acting in concert with acyl-CoA modification. Thus, it cannot be ruled out that

LfpPLAIIIs are responsible for the selective removal of HFAs from the synthesis site in their natural source where cooperation with LfKCS3 is required (Figure 5.2).

It may be useful to conduct substrate specificity and selectivity studies with recombinant LfpPLAIIIs in order to gain more insight into the enzyme's ability to utilize *sn*-2 HFA-PC. In addition, expression of *LfKCS3* may promote the HFA accumulation in the RcpPLAIIIβ-CL7 lines, but that intervention would potentially result in the seed oil containing C18 C20 HFAs, limiting its industrial usefulness compared to the oil containing a single type of HFA.

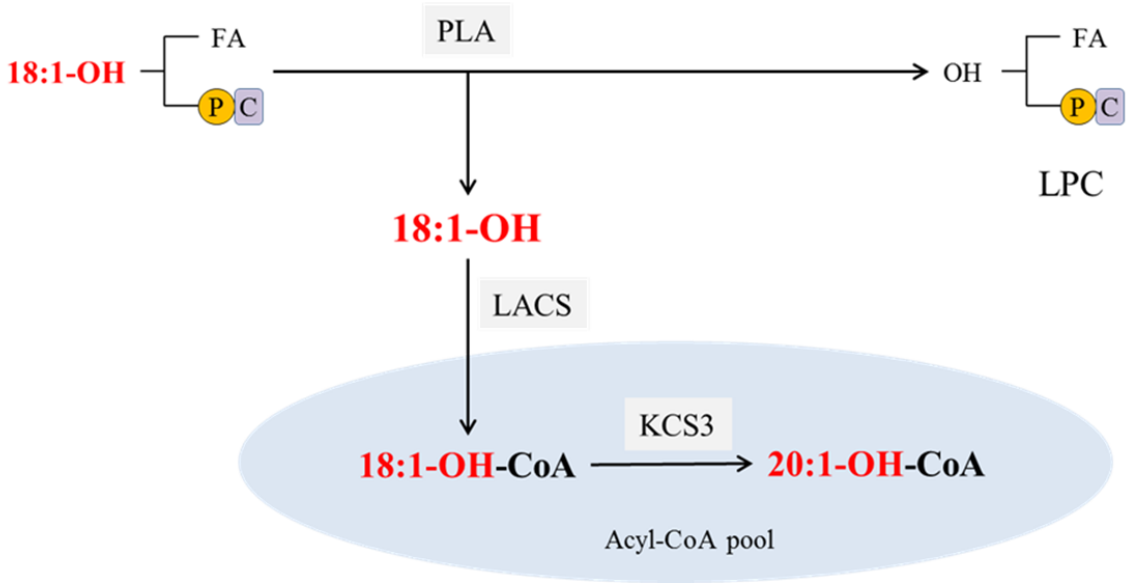


Figure 5.2 The hypothesized cooperation of phospholipase A (PLA) and acyl-Coenzyme A (CoA) modification in *L. fendleri*

In *L.fendleri*, phospholipase A (PLA) may catalyze the removal of ricinoleic acid (12-OH 18:1Δ^{9cis}; or18:1-OH) from the *sn*-2 position of phosphatidylcholine (PC). A long-chain acyl-CoA synthetase (LACS) may catalyze the activation of 18:1-OH to enter the acyl-CoA pool, wherein 18:1-OH-CoA has been shown to be elongated into lesqueroloylyl (14-OH 20:1Δ^{11cis})-CoA (Moon et al., 2001). The elongation of 18:1-OH in the acyl-CoA pool may be required to facilitate the release of 18:1-OH from PC.

AtpPLAIIIα, the ortholog of *RcpPLAIIIβ*, also did not affect the HFA content of the seed oil when over-expressed in CL7 (Figure 4.8A). The HFA content did not change despite of slight decrease in 18:2-OH content (from 3.1% to 2.6%; Table 4.4). The seed oil content of CL7 over-expressing *AtpPLAIIIα*, however, was compromised (Figure 4.8B). This observation suggested that AtpPLAIIIα may play a role in oil synthesis in Arabidopsis which may be associated with the release of other fatty acids from PC.

Expression of *RcFAH12* resulted in a seed oil content reduction in Arabidopsis, which may be due to the post-translational down-regulation of *de novo* fatty acid synthesis induced by the inefficient glycerolipid synthesis (Bates et al., 2014). The over-expression of endogenous *pPLAIIIδ* elevated seed oil accumulation in wild-type Arabidopsis or Camelina (Li et al., 2013; 2014). However, the expression of *AtpPLAIIIδ, LfpPLAIIIδ* or *RcpPLAIIIδ* failed to prevent the reduction in seed oil content (Figure 4.3B; 4.4B; 4.8B). These results also implied that these pPLAIIIδ may be non-specific to HFA-containing substrates and are not able to relieve the turnover of HFA-containing DAG that may be responsible for the impaired *de novo* fatty acid synthesis (Bates et al., 2014).

5.4 Closing comments

It is clear that our ability to produce HFAs in the seed oils of temperate species, such as Arabidopsis and Camelina, is dependent on our knowledge of HFA accumulation in the species (e.g., castor*, L. fendleri*) that naturally produce these UFAs. This thesis project demonstrated that RcpPLAIIIβ from castor was involved in catalyzing the release of HFA from PC in the transgenic Arabidopsis expressing *RcFAH12*. This result also demonstrated that *pPLAIIIβ* may operate along with *RcPLA$_2$α* (Bayon et al., 2015) in catalyzing the release of HFA from PC in developing castor seeds. The highest possible levels of HFA accumulation in the seed oil of Arabidopsis RcpPLAIIIβ-CL7 may come from an additional combined heterologous expression of *LACS*, *GPAT9*, *LPAAT2*, *PAP* and *DGAT* encoding enzymes with demonstrated high specificity and selectivity for substrates containing HFAs. Because RcDGAT2 have been proven to promote the accumulation of HFAs in the seed oil when introduced into Arabidopsis CL7 lines (Burgal et al., 2008), heterologous expression of cDNA encoding RcDGAT2 in RcpPLAIIIβ-CL7 lines could be selected for the first trial.

It should also be noted that PC is the synthesis site for other UFAs, such as CFAs and EFAs (Mietkiewska et al., 2014b). Therefore, the knowledge gained about promoting HFA accumulation in temperate oil crops could potentially be applied in the enrichment of other unusual fatty acids in these crops. This study has shown how pPLAIII can be used as a molecular tool to alleviate one of the bottlenecks in the accumulation of unusual fatty acids in the seed oil of a temperate oil-producing species

6 - Conclusions and Future Directions

In this thesis, cDNAs of two *pPLAIII* genes, *pPLAIIIβ* and *pPLAIIIδ,* have been cloned from *L. fendleri* and castor, the two natural sources of HFAs. Their orthologs, *AtpPLAIIIα* and *AtpPLAIIIδ* were cloned from Arabidopsis cDNA as well. Each of these *pPLAIII* cDNAs were expressed under the control of a seed-specific napin promoter in a transgenic line of Arabidopsis producing C18-HFA (CL7; Lu et al., 2006).

Expression of castor *RcpPLAIIIβ* led to a significant decrease in HFA content in the seed oil (Figure 4.5). Lipid class analysis revealed that the reduction in HFA content occurred not only in the storage lipid TAG but the site of HFA synthesis PC (Figure 4.6). This study demonstrated that, RcpPLAIIIβ from castor could catalyze the release of HFA from PC in the transgenic Arabidopsis bearing RcFAH12. Expression of *RcpPLAIIIδ*, however, resulted in no effects on the HFA content of the seed oil (Figure 4.4).

Expression of orthologs of castor *RcpPLAIIIβ* or *RcpPLAIIIδ* from *L. fendleri* (*LfpPLAIIIβ* or *LfpPLAIIIδ*, respectively) or Arabidopsis (*AtpPLAIIIα* or *AtpPLAIIIδ*, respectively) did not impact the HFA content of the seed oils (Figure 4.3; Figure 4.8). *LfpPLAIIIβ* and *LfpPLAIIIδ* are the only two members of *pPLAs* identified from the developing *L. fendleri* seeds. This study suggested that pPLA from *L. fendleri* and the orthologs from Arabidopsis were not effective in in catalyzing the release of HFA from PC in the transgenic Arabidopsis expressing RcFAH12.

Additional investigations are required to develop further insight into the use of pPLA in engineering increased HFA content in Arabidopsis CL7. Substrate specificity and selectivity studies with recombinant RcpPLAIIIβ, LfpPLAIIIβ or LfpPLAIIIδ,

respectively, could be conducted to determine if these enzymes can utilize *sn-2* HFA-PC as substrate. Strategies involving the expression of cDNAs encoding enzymes with enhanced specificity for substrates containing HFAs in Arabidopsis RcpPLAIIIβ- CL7 should be considered as means of boosting the HFA content of the seed oil. A LACS, with enhanced specificity for activating HFA, may be useful in making HFA-CoA available for the Kennedy pathway. In addition, Kennedy pathway acyltransferases and PAP, which are specific for substrates containing HFA, could be introduced into RcpPLAIIIβ-CL7 Arabidopsis. Furthermore, *LfKCS3* could be co-expressed with *LfpPLAIIIβ* or *LfpPLAIIIδ* to examine the possible cooperation between *LfpPLAIIIs* and acyl-CoA modifications. Lastly, transcriptome analysis could be conducted to gain insight into possible changes in gene expression that potentially lead to changes in HFA contents which accompany the co-expression of *RcFAH12* and *RcpPLAIIIβ* in Arabidopsis. For example, if genes encoding enzymes involved in β-oxidation exhibit increased expression during seed development, this would support the hypothesis that HFA released from PC by RcpPLAIIIβ is targeted for degradation.

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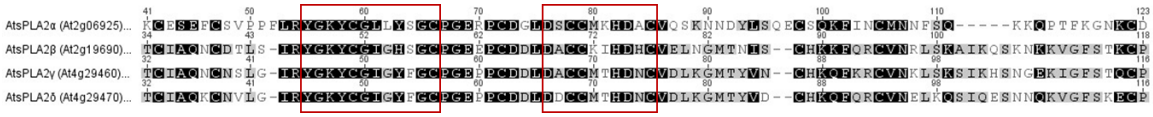
Appendix 1 Amino Acid Sequences of Arabidopsis phospholipase As (PLAs)

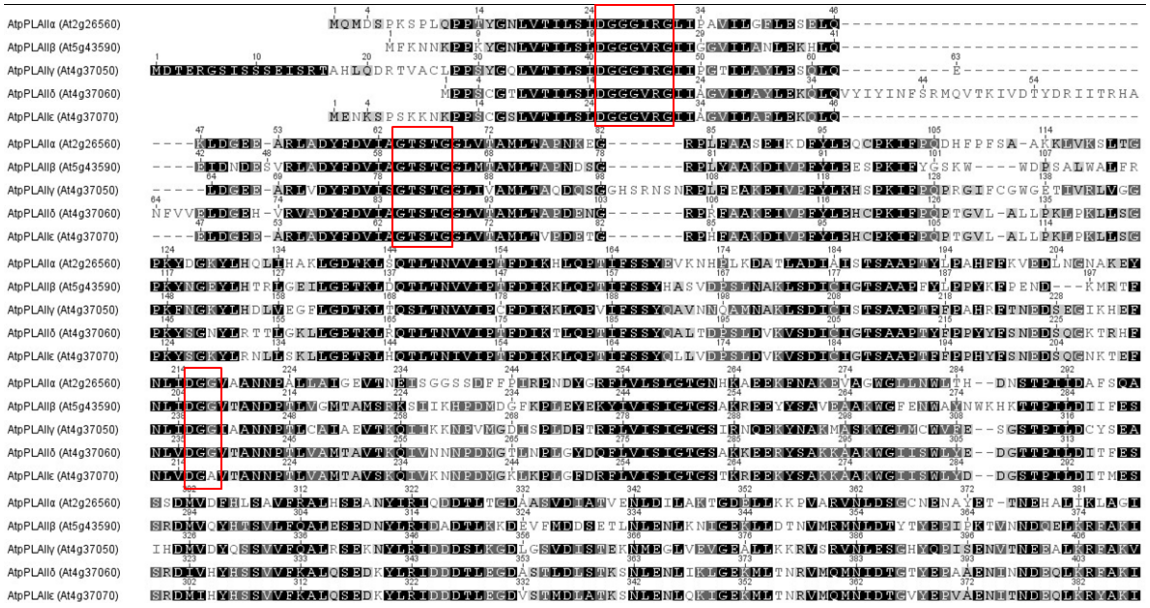
(A) Partial deduced amino acid sequences of subclass I, II and III of PLA_1. The red box indicated the conserved Gly-x-Ser-x-Gly (GxSxG) motif

(B) Partial deduced amino acid sequences of LCAT-PLA_1 and LCAT-PLA. The red box indicated the SxSxG motifs.

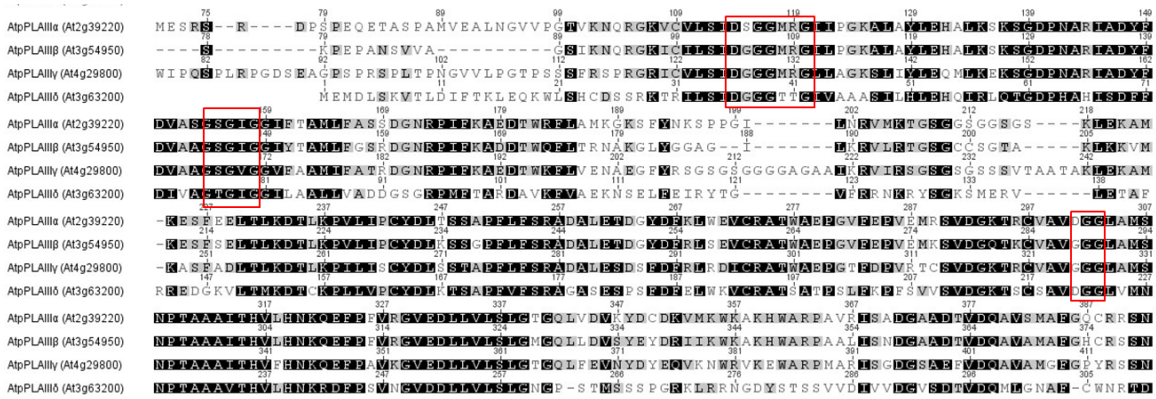
(C) Partial deduced amino acid sequences of small PLA_2s. The first red box indicated the alcium binding-loop (YGKYCGxxxxGC); the second box indicated the catalytic site DACCxxHDxC motif with a conserved HD dyad.



(D) Partial deduced amino acid sequences of subclass II of patatin-like phospholipase A (pPLA). The first red box indicated the phosphate/anion binding element (DGGGxxG); the second box indicated the esterase box (GxSxG); and the third box indicated the motif DGG/DGA



(E) Partial educed amino acid sequences of subclass III of patatin-like phospholipase A (pPLA). The first red box indicated the phosphate/anion binding element (DGGGxxG); the second box indicated the esterase box (GxSxG or GxGxG); and the third box indicated the motif DGG/GGG



Appendix 2 Contribution to a Review Article

I was involved in the preparation of a review article which dealt with engineering production of C18 conjugated fatty acids in developing seeds of oil crops (Mietkiewska et al., 2014b). Preparing the draft of this review article was my very first assignment in the Master's program. I collected publications, prepared the outline, wrote the first draft of the manuscript and assisted Drs. Mietkiewska and Weselake in editing and further improving the document. Although it was a challenging process, it was a great experience for a new Master's student who just arrived in Canada and used English as her third language. I am grateful to Dr. Weselake for getting me involved in the preparation of this review article.