THE ROLES OF PHOSPHOLIPASE C IN OIL BIOSYNTHESIS IN OILSEEDS

by

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DEDICATION

To my father Dambar Prasad Aryal and my mother Tulasa Devi Aryal.

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NOMENCLATURE

ACCase:	acetyl CoA carboxylase
ACL:	ATP citrate lyase
ACP:	acyl carrier protein
ACS:	acetyl CoA synthase
ADP:	adenosine diphosphate
ATP:	adenosine triphosphate
CoA:	co-enzyme A
CoA:	coenzyme A
CPT:	CDP-choline:DAG cholinephosphotranferase
DAG:	diacylglycerol
DGAT:	acyl-CoA:DAG acyltransferase
ER:	endoplasmic reticulum
FAD:	oleate and linoleate desaturases
FAE1:	Fatty acid elongase 1
G3P:	glycerol-3-phosphate
GPAT:	acyl-CoA:G3P acyltransferase
HFA:	hydroxy fatty acids
LACS:	long-chain acyl-CoA synthetase
LPA:	lysophosphatidic acid
LPAT:	acyl-CoA:LPA acyltransferase
LPC:	lysophosphatidylcholine

NOMENCLATURE CONTINUED

LPCAT:	acyl-CoA:LPC acyltransferase
NADH:	nicotinamide adenine dinucleotide
NPC:	non-specific phospholipase C
PAP:	phosphatidic acid phosphatase
PC:	phosphatidylcholine
PC-PLC:	phosphatidycholine specific phospholipase c
PDAT:	PC:DAG acyltransferase
PDCT:	PC:DAG cholinephosphotransferase
PI:	phosphatidylinositol
PI-PLC:	phosphatidylinositol specific phospholipase c
PLA:	Phospholipase A
PLC:	Phospholipase C
PLD:	Phospholipase D
TAG:	triacylglycerol

ABSTRACT

Research on the biosynthesis pathways of storage oil (triacylglycerol, or TAG) in plants has gained momentum for the last couple of decades. Despite significant achievements, a complete understanding of such pathways is still lacking. Also, the production of industrially important unusual oils such as hydroxy fatty acids (HFA) at the commercial level is limited in both native and transgenic plants. In this study, I have examined the roles of Phospholipase C in the TAG biosynthesis.

To test roles of non-specific PLCs (NPCs) in TAG synthesis, FA composition was analyzed in mature seeds of T-DNA lines of 6 Arabidopsis *npc* T-DNA mutants. Among them, *npc1*, *npc4* and *npc6* showed increases in oleic acid (18:1) and decreases in lineoleic acid (18:2) and linolenic acid (18:3). The 18:1 was further increased in the *npc1/npc6* double mutant compared to *npc1* and *npc6*. These changes in FA profile suggest the role of NPCs in TAG biosynthesis.

Given the possible roles of NPCs in TAG synthesis in the seeds of Arabidopsis, I searched for suitable PLC from Castor (RcPLC) for further investigation and to transform to the 7-1 line of *Camelina sativa* expressing a fatty acid hydroxylase (RcFAH12). From previously published Castor transcriptome, *RcPLCL1* was the phospholipase C-like gene that was primarily expressed in the endosperm. Exploration of the RcPLCL1 protein using bioinformatics tools placed it to the PLC-like phosphodiesterase family which has not been characterized yet. Based on the sequence analyses, the protein contains the X domain for the PLC activity on phosphatidylinositols (PI), but it lacks the Y and C2 domains. Enzyme assays using heterologous expression in yeast showed that the protein had both PC- and PI-PLC activities. Based on the genomic sequence analysis, 8 genes were found in Arabidopsis that were predicted to encode PLC-like phosphodiesterases. The seeds of 5 T-DNA knockout lines were analyzed for fatty acid composition, which showed decreased linolenic acid (18:3) and increased oleic acid (18:1). The FA composition change suggested the involvement of these proteins in oil biosynthesis in Arabidopsis seeds. Upon transformation of RcPLCL1 into the 7-1 line, HFA content in the seeds was increased to as high as 24% from 15%. Concurrently the amount of HFA decreased in the membrane lipid phosphatidylcholine (PC). However, the Arabidopsis homologue, AtPLCL1, did not increase the HFA in seeds of 7-1. These results suggest that RcPLCL1 is involved in HFA accumulation.

The HFA-producing 7-1 plants were tested in the field. Results from two-year experiments indicated that both wild type and 7-1 demonstrated better performance for traits like oil content and yield. However, the HFA level in 7-1 was significantly decreased in field-grown plants than greenhouse plants.

CHAPTER ONE

INTRODUCTION, GOALS AND OBJECTIVES

Biosynthesis of Oil in Developing Seeds of Oil-seed Plants

Living cells generate energy from catabolic reactions. Proteins, carbohydrates, and oils are broken down to generate ATP. ATP is then used for other anabolic processes in the cell (Banks and Vernon 1970). Of the different energy sources, triacylglycerols (TAG) are the highest energy yielder; complete oxidation of TAGs produce twice as much energy as is produced by the catabolic reactions of proteins and carbohydrates (Theodoulou and Eastmond 2012). Seeds store lipids for their use in post-germination growth. Storage lipids in most of the plant-seeds are in the form of TAG, except for a few plants such as the desert shrub, jojoba (*Simmondsia chinensis*), which has wax ester in its seed as a storage lipid (Li et al. 2008). These TAGs are hydrolyzed to free fatty acids which are further processed downstream for ATP generation and other cellular uses.

The storage lipids of plant seeds are also used by humans in various applications including edible oils and for industrial uses. They can provide an alternative source of energy to traditional petroleum fuels. The energy generated from plant seed oil is environmentally friendly and sustainable, though current production methods often face difficulties in efficiency that make the relative cost of production higher than the well-established petroleum energy (Gressel 2008). Hence, improving the synthesis of desirable TAG compositions and quantities in the seeds of oil-seed plants is an active area of research and has attracted plant-biotechnologists and environmentalists. However, a

complete understanding of the oil-biosynthesis process in developing seeds has not been achieved, especially with regards to the genetic regulatory components (Bates 2016).

For biosynthesis of TAGs, cells compartmentalize the work into three divisions (Meyer et al. 2012). The first compartment in the plastids has been described as exerting a "push" effect as carbon is fixed into organized fatty acid structures that are pushed out of the organelle for oil synthesis. The second compartment is comprised of the transmembrane proteins of the endoplasmic reticulum, which are described as having a "pull" effect. TAGs are formed in this process from the fatty acids that are exported from plastids. The third compartment in the cytosol is the formation of oil bodies or oleosomes that function as storage reserves of the TAGs. These organelles are composed of TAGs, a phospholipid monolayer, and associated protective enzymes that stabilize the stored TAGs (Tzen et al. 1993)

Fatty Acid Synthesis in Plastids

Synthesis of Acetyl-CoA

Acetyl-CoA can be synthesized in plastids via four different pathways (Rawsthorne 2002). The first one is an ATP-dependent reaction in which Acetyl CoA is activated from free acetate by Acetyl CoA synthase (ACS). Antisense experimentation on ACS has revealed that this is not the major pathway for synthesis of Acetyl-CoA (Nikolau et al. 2000). The second possible route for Acetyl-CoA synthesis is from pyruvate. The pyruvate required for this reaction is obtained from either cytosolic glycolysis or plastidial glycolysis. The pyruvate dehydrogenase complex (PDC) converts pyruvate to Acetyl-CoA. The reaction also produces NADH (Rawsthorne 2002). A

plastidial carnitine acetyl transferase reaction is the third possible route. This reaction transfers acetate from acetyl carnitine to CoA (Masterson and Wood 2000). However, this pathway has not been completely accepted as a function *in planta* by researchers yet. The fourth possible route is the synthesis from citrate. In this reaction, the enzyme ATP citrate lyase (ACL) lyses an acetyl group from citrate and attaches it to the CoA. This reaction requires ATP to drive it, converting it to ADP. However, the localization of the ACL enzyme is still not completely clear (Rangasamy and Ratledge 2000; Fatland, Nikolau, and Wurtele 2005).

Utilization of Acetyl-CoA by Acetyl-CoA carboxylase (ACCase)

Acetyl CoA carboxylase (ACCase) catalyzes the formation of malonyl CoA from acetyl CoA utilizing ATP as an energy source. Two types of ACCases are found in plants. Type I is analogous to yeast and mammalian ACCase, while type II is analogous to the prokaryote ACCase. In higher plants, type I ACCase is mostly extra plastidial (cytosolic). Several experiments have provided evidence that type II ACCase regulates the synthesis of fatty acids in the plastid of higher plants except for *Graminiae spp.*, which utilizes type I enzymes (Sasaki, Konishi, and Nagano 1995).

More studies are required to conclude if ACCase also regulates the carbon flux and fatty acid synthesis for storage oil. Inability to utilize unusual fatty acids in the endoplasmic reticulum has been shown to post-translationally regulate ACCase in plastids (Snapp et al. 2014). The mechanism of this reaction is still unknown. But studies indicate that ACCase has a role in regulating carbon flux and fatty acid synthesis.

Elongation of Malonyl CoA to Fatty Acids

The enzyme complex fatty acid synthase (FAS) elongates malonyl-CoA into fatty acids (Voelker et al. 1992). Two carbons are added to the elongating FA at a time (Bao, Pollard, and Ohlrogge 1998). The process continues until 16:00 acyl carrier proteins (16:00 ACP) or 18:00 ACPs are produced. Acyl-ACPs are then hydrolyzed by the enzymes theoesterases. Fatty Acid theoesterases (FAT) can be divided into two classes; FATA and FATB. FATA hydrolyzes 18:1 ACP while FATB hydrolyzes saturated fatty acids, ACPs such as 16:0 or 18:0 ACP. The free fatty acids are then exported to the cytosol. The complete mechanism for fatty acid transport from plastid to cytosol is unknown, though the plastid transmembrane protein fatty acid export 1 (FAX1) is known to play a critical role in model plants (Li et al. 2015).

ATP and Reducing Power for Fatty Acid Synthesis in Plastid

The addition of carbon to the growing acyl chain during fatty acid synthesis requires energy generated from the dephosphorylation of ATP and reduction of NADPH (Slabas and Fawcett 1992). In photosynthetic tissues, the light-dependent reactions of photosynthesis provide the ATP and NADPH. However, for heterotrophic tissues, ATP and NADPH should be imported or made within the plastid by carbohydrate metabolism (Rawsthorne 2002).

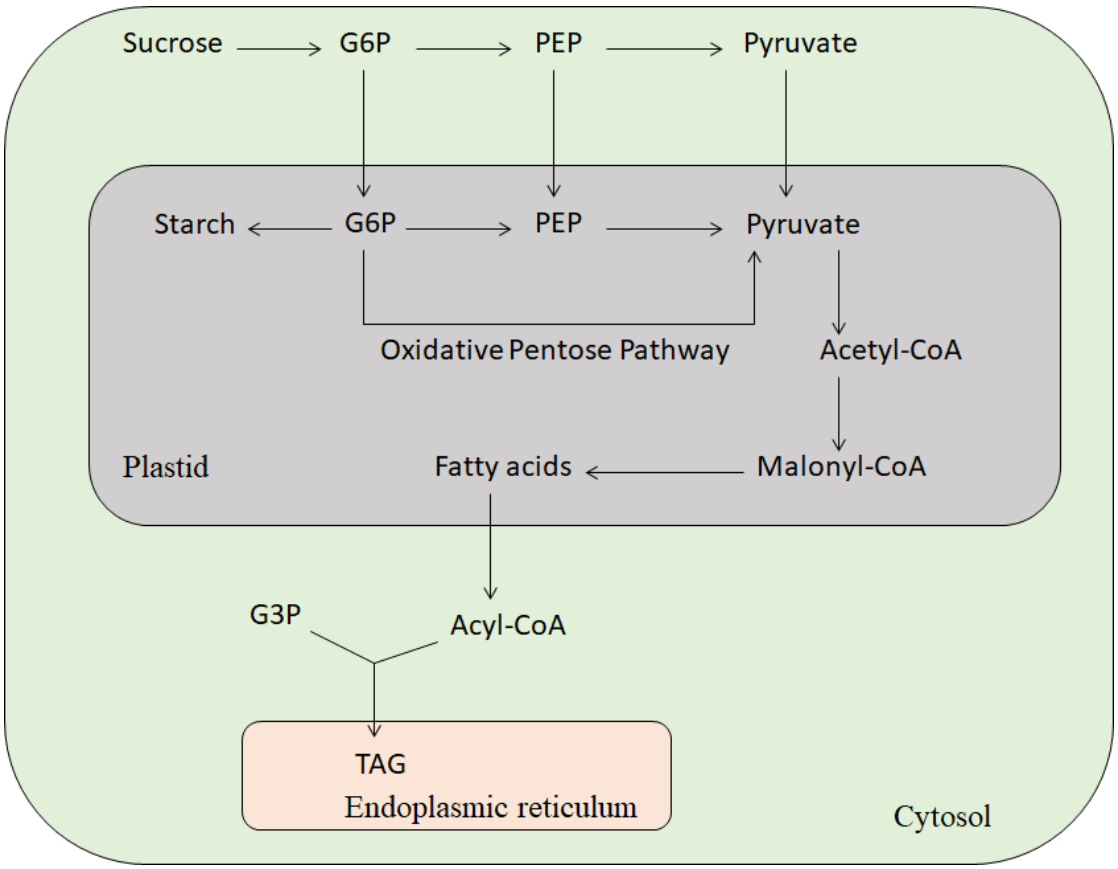


Fig 1. Current understanding on partition of carbons for the TAG biosynthesis in a plant cell. G6P, Glucose 6 Phosphate; PEP, Phosphoenol Pyruvate; G3P, Glucose 3 Phosphate; TAG, Triacylglycerol.

TAG Formation in Endoplasmic Reticulum

Fatty acids are formed in plastids and exported out into the cytosol. The dominant group of fatty acid exported from plastid to cytosol depends on the plant species. In Arabidopsis, 18:1 is the major fatty acid exported from plastid. The enzyme acyl-CoA synthetase (ACS) forms the Acyl- CoA pool (Schnurr, Shockey, and Browse 2004). Some modifications such as elongation by the enzyme fatty acid elongation (FAE) take place in the cytosol. The acylated fatty acids then enter the endoplasmic reticulum and

get incorporated into the G3P backbone to form triacylglycerols. G3P are reduced in the cytosol from the reduction of dihydroxyacetone phosphate (DHAP) via glycerol 3 dehydrogenase (G3Pdh) pathway (Venugopal et al. 2009). Two routes for the formation of TAGs in the ER have been proposed (Zhang, Fan, et al. 2009). The first one is sequential acylation of the G3P backbone. The second route is PC-derived or acyl-editing synthesis of TAG. The second route provides the opportunity to modify the fatty acids and incorporate modified fatty acids into the G3P backbone to form TAG. The TAG formed through the second route contains modified fatty acids while the sequential acylation mainly contains the de-novo fatty acids which are primarily exported out from plastid to cytoplasm. Several enzymes play crucial role in the biosynthesis of TAG in ER through either route. The sequential acylation is quite well known. PC-derived synthesis still requires advancement for a very clear understanding.

Sequential Acylation

The enzyme Glycerol phosphate acyl transferase(GPAT) starts the acylation of G3P backbone. G3P is acylated at its first position. Acyl-CoA and Sn-glycerol 3 phosphate react to give 1-acyl-sn-glycerol 3 phosphate and coenzyme A (Zheng et al. 2003). Then the 1-acyl-sn-glycerol 3phosphate is acylated at sn 2 position. This reaction is catalyzed by the enzyme Lysophosphatitade acyl transferase (LPAAT). Acyl CoA reacts with 1-acyl-sn-glycerol 3phosphate to give 1,2-diacyl-sn-glycerol 3 phosphate and coenzyme A (Maisonneuve et al. 2009). Phosphatadic acid Phosphatase (PAP) then cleaves the phosphate group from sn 3 position and forms diacylglycerol (Bates, Stymne, and Ohlrogge 2013). A water molecule is used to convert phosphate monoester to alcohol

and phosphate. The resulted diacylglycerol either goes to the phosphatidycholine pool for the modification of fatty acids esterified to the glycerol backbone or it takes a fatty acid from acyl-CoA pool at its sn3 position and forms triacylglycerol. The enzyme diacylglycerol acyl transferase (DGAT) catalyzes the reaction in which acyl-CoA and 1,2-diacyl-sn-glycerol react to give triacyl-sn-glycerol and coenzyme A (Jako et al. 2001).

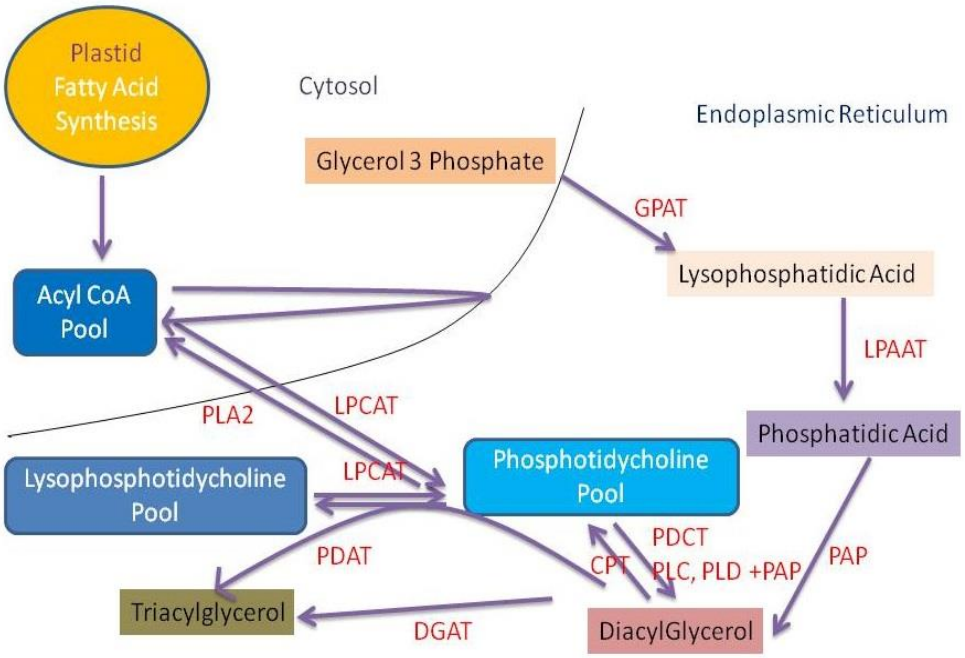


Fig 2. Current understanding of the synthesis of oil in the developing seed of oil seed plants. GPAT, Glycerol Phospho acyltransferase; LPAAT, Lysophosphatitade acyl transferase; PAP, Phosphatadic acid phosphatase; PLA2, Phospholipase A2; LPCAT, Lysophosphatidic acid acyl transferase; CPT, Choline phosphotransferase, PDCT, Phosphatidycholine choline transferase; PLC, Phospholipase C; PLD, Phospholipase D; PDAT, Phosphatidycholine acyltransferase; DGAT, Diacylglycerol acyltransferase.

PC Derived TAG Synthesis

The de-novo DAG enters the PC pool. A modification such as desaturation or an addition of an unusual group of the fatty acid takes place in the PC pool. The modified fatty acids are then exported out of the PC either as free fatty acid or in the form of DAG. The DAG then incorporates the acylated fatty acid to the sn 3 position and forms TAG. The TAG thus formed contains modified fatty acids in it.

The exact mechanism of how de novo fatty acids enter the PC pool, get modified, and come out of it is not yet completely understood. Many enzymes have been proposed to play important roles in this central TAG synthesis hub in the endoplasmic reticulum. Choline phosphotransferase (CPT) forms the phosphatidylcholine from de novo DAG. 1,2, diacyl-sn-glycerol and CDP choline reacts to form phosphatidylcholine and choline monophosphate and releases a hydrogen ion (Vogel and Browse 1996).

Phosphatidylcholine choline transferase (PDCT) is another major enzyme for the formation of phosphatidylcholine. The enzyme catalyzes the reaction in which head groups are exchanged between the de novo DAG and phosphatidylcholine. Thus the phosphocholine group is transferred to the de novo DAG, converting it to PCs and PCs with modified fatty acid form DAG (Lu et al. 2009). The DAG thus formed incorporates acylated fatty acid to its sn3 position by the enzyme DGAT and forms TAGs. Free fatty acids are released from sn2 position of PC by the enzyme lysophosphatidic acid acyl transferase (LPCAT). The enzyme has both forward and reverse activity. The forward activity adds acylated fatty acid to the sn2 position of lysophosphatidylcholine, forming a phosphatidic acid. The reverse reaction releases the fatty acid from the sn2 position of

phosphatidylcholine and forms lysophosphatidylcholine (Wang et al. 2012). Two thirds of 18:1, which is the primary FA exported out of plastids, flux in and out of PC are found to be controlled by the enzymes PDCT and LPCAT in Arabidopsis (Lu et al. 2009; Bates et al. 2012a). Apart from these two enzymes, phospholipases such as phospholipase A (PLA), phospholipase C (PLC) and phospholipase D (PLD) are proposed to play roles in the flux of FA out from PC. PLA releases free fatty acids from PC which later enters the sequential acylation or the Kennedy pathway for TAG formation; PLC cleaves the headgroup PC and release DAG; PLD cleaves the choline from head group and then the enzyme phosphatidic acid phosphatase (PAP) cleaves the phosphate and thus release the DAG (Jaworski and Cahoon 2003a). More studies are required to validate the roles of phospholipases in FA flux out of PC.

Phosphatidylcholine acyltransferase (PDAT) is another enzyme that utilizes the acylated fatty acid released from sn2 position of PC and incorporates into the sn3 position of the DAG. In this reaction, phosphatidylcholine and 1-2-diacyl-sn-glycerol forms triacyl-sn-glycerol and 2-lyso-phosphatidylcholine (Stahl et al. 2004). DGAT1 and PDAT1 enzymes in Arabidopsis seems to have overlapping functions. The DGAT1 mutant line *dgat1-1* demonstrated 20-40% decrease in oil from mature seeds. The double mutant *dgat1-1 pdat1-1* was lethal. However, the RNAi effect on PDAT1-1 in *dgat1-1* plants demonstrated decrease in oil by 70% (Zhang, Fan, et al. 2009) .

Long chain fatty acid CoA ligase (LACS) converts long chain fatty acids into long chain fatty acyl CoA. It catalyzes the reaction in which long chain fatty acid and

Coenzyme A reacts to form long chain acyl-CoA and diphosphate (Zhao et al. 2010). ATP is changed to AMP during the reaction.

Storage of TAG in seeds

The storage lipids or the triacylglycerols are stored in the cytoplasm of cotyledons or endosperm cells in organelles called oleosomes or oil bodies (De Chirico et al. 2018). To prevent the fusing of triglycerides from adjacent oil bodies, oleosomes are protected by many specific proteins called oleosins (Siloto et al. 2006). Accumulation of oleosin proteins determine the size of oil bodies (Siloto et al. 2006). The storage lipids are later metabolized to form sucrose, which is mobile extracellularly and can be transported in plant vascular tissue. This process requires several enzymes and takes place in different cellular compartments: oleosomes, glyoxysomes, mitochondria, and the cytosol.

Unknown steps in TAG biosynthesis pathway

The pathway for TAG biosynthesis in seeds of oilseeds is still developing and requires extensive researches for a complete understanding. The genes involved in several steps such as export of FAs from plastid to cytosol and FA flux in and out of PC still require more studies for their identification and confirmations. The cell biology of the TAG synthesis is not clear. There are several questions to be answered such as; if PC is the carrier of fatty acids between organelles and if endoplasmic reticulum and oil body are connected and how the connection affects the storage of TAGs. Similarly, the feedback effect on carbon flux in plastid from the endoplasmic reticulum has been proposed but the question how the ER-plastid connection works is an unsolved question

(Bates et al. 2014). The question over the control of downstream transcriptomes is also unknown; if there is involvement of transcription factors and microRNAs. The greater question is how the oil composition varies across the plant species.

Camelina sativa, a potential plant for producing transgenic oils

Camelina sativa, common name false flax, is a plant from the family *Brassicaceae*. It is a cultivated oilseed crop mainly grown in Europe and North America. The plant has a long history of domestication, originating in approximately 600 BCE in Germany. The plant was less significant in the Middle Ages but continued to evolve as a weed, gaining the name false flax (Budin, Breene, and Putnam 1995). At present, the plant is spread all over the world. The plant grows in sub-tropical and tropical regions, though it seems to be better acclimated and more commonly adapted to cold climate regions. The plant grows about 30-120cm in height and a single seed weight varies from 0.7 to 1.6 mg (Gehringer et al. 2006). The height and branching vary largely among the accessions from various parts of the world. The plant has attracted the biotechnology industries in recent years for its ability to easily incorporate genes from different plants. The plant is also cooperative for mutations of several endogenous genes. *Camelina* is a low input crop and can grow on marginal lands unsuitable for other crops production. It can tolerate cold and drought climate conditions. The complete growth and maturation duration is comparatively low (about three and a half months) compared to other oil seed crops. These features make the growth and production of *Camelina* as a crop agronomically feasible. The seed oil content varies from 30 to 40 mol percentage

depending on the growth conditions. *Camelina* seed oil contains high amounts(30-40%) of omega-3 fatty acids (Fig 1).

The seed oil has garnered interest in the plant for the production of jet fuel and biodiesel. The greenhouse gas emissions from *Camelina* generated jet fuel is much lower than that from traditional non-renewable energy sources (de Jong et al. 2017). In Europe and America, *Camelina* is perceived by advocates as a next generation oilseed crop. However, several agronomic characteristics, especially yield-related traits, are required to be improved in the plant.

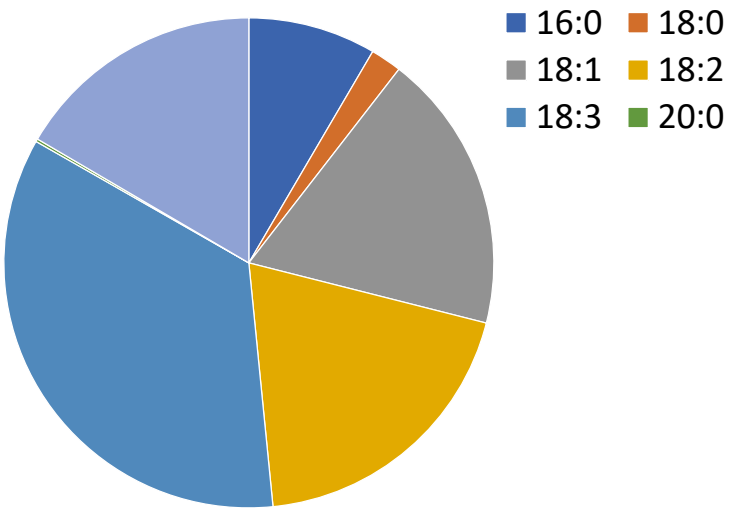


Fig 1. Pie diagram for fatty acid composition in the oil of camelina seed.

Hydroxy Fatty Acid and its Production in Transgenic Plants

Storage oil in the seeds of oilseed plants has a diverse composition. The ecology for the distribution of fatty acids for the oil from different plants is still unclear.

Characteristics of the oil are defined by the fatty acids esterified to the glycerol backbone. Many unusual fatty acids are present in the oil of different plant species.

Commercial-level production of these oils from their native plants has several limitations, resulting in a large interest for the possibility of their production in transgenic crop plants with preferential agronomic characteristics. However, several factors have limited the production to the level that native plants accumulate unusual fatty acids.

Hydroxy fatty acids (HFAs) have a peculiar importance among the unusual fatty acids found in the storage lipids of seeds from the few plant species that natively produce them. The hydroxylated fatty acids in oil increase its scope in various industrial applications such as lubricants, soaps, dye etc.(Burgal et al. 2008a). So far, 14 plant species from 10 families are reported to produce hydroxy fatty acids, of which Castor (*Ricinus communis*) and *Physaria spp.* are the two most significant (Broun, Boddupalli, and Somerville 1998). However, both plants have several drawbacks which limit the commercial production of oil containing hydroxyl fatty acids (Snapp and Lu 2013b). The castor plant is mostly limited to the tropical production regions. Because it is both highly allergenic, and produces a highly toxic protein called ricin, commercial cultivation has been banned in the United States and other developed countries, though it is still often found as an invasive species in tropical regions such as Florida and Hawaii (Kajikawa et al. 2016). Physaria spp. are natively distributed in the Southwestern United States region. The agronomic characteristics of these plants are poorly characterized and still need more investigation for domestication and use as commercial crops (Hayes, Kleiman, and

Phillips 1995). Hence there is a demand that HFAs are produced in the transgenic oilseed crop such as Camelina.

When a Fatty Acid Hydroxylase (FAH12) enzyme from castor was put into Arabidopsis and Camelina, about 15% of the HFA was accumulated in the seed oil (Lu et al. 2006); (Lu and Kang 2008b). The accumulation is far less than the 90% found in the native plant. Several attempts have been made since then to increase the HFA in Arabidopsis and Camelina. Co-expression of RcDAGT2 and RcFAH12 in Arabidopsis plants produced as high as 30% of HFA in seed TAG (Burgal et al. 2008a). The parent plants were *fae1* mutant, which lacked the production of 20:1 FA (Lu et al. 2006). In another attempt, co-expression of RcFAH12 and RcPDAT1A increased the HFA up to 27.5% (van Erp, Bates, Shockey, et al. 2011). In Camelina, the co-expression of RcFAH12 and ketoacyl CoA synthase from Physaria (PfKCS3) increased the HFA amount to 22% (Snapp et al. 2014). These improvements still could not accumulate the HFA level even close to that of the native plant.

Several obstacles within the transgenic plant at the genetic level have been proposed to explain this failure. A significant one concerns the enzymes involved in the unusual fatty acid metabolism. Foreign genes are not always well tolerated in the host plants they are transferred to. In addition, the co-evolution of the enzymes for different plant species makes it more complex than simply transferring single genes, such as hydroxylases, from a native HFA accumulator to a desired host plant such as *Camelina*. The competition of foreign genes with endogenous genes for substrate, and differences in affinity also decrease the working potential of the foreign gene. Coevolution of TAG

synthesis genes for particular plant species producing specific FA containing TAGs has been a major obstacle in selecting a combination of genes from native unusual FA synthesizers and accumulators to be transferred to the preferred host plants to produce agronomically feasible unusual FA (van Erp, Bates, Shockey, et al. 2011). The isozyme competition effects the accumulation of targeted fatty acids in the plant. When AtDGAt1 was mutated in CL37-RcFAH-RcDGAT2 plants, the HFA was increased by 17% (van Erp et al. 2015a). The other bottleneck in this bio-engineering is the inefficient pull of HFA produced in PC for incorporation into TAG. Co-expression of PDCT with RcFAH12 increased the HFA in the oil of Arabidopsis. The PDCT mutant plants accumulated half the amount of HFA than in the parent (Hu, Ren, and Lu 2012). Roles of phospholipases also cannot be undermined for efficient removal. Gene-stacking of several genes also could be a way to accumulate efficient HFA in TAG. After the transcriptome analysis of Castor and Physaria developing seeds, more genes are eyed as potential candidates for increasing HFA in transgenic plants (Kim and Chen 2015; Brown et al. 2012; Horn et al. 2016a).

Plant Phospholipases and their Biochemistry in TAG Synthesis

Phospholipids, a polar subtype of glycerolipids, are glycerol-3 phosphate molecules with non-polar hydrophobic fatty acids esterified at sn-1 and sn-2 positions, covalently bonded to hydrophilic polar heads esterified to the phosphoryl group. The polar portion can include nitrogenous bases, glycerol, or inositol units that are esterified to the phosphoryl group. The divergent polarities of phospholipid components cause them to be amphiphilic in nature. A primary function of plant phospholipids is to serve as

membranes, particularly as primary cell and organelle membranes. Additionally, they serve as precursors of second messengers, associated with the fatty acid signaling pathway and for the formation of storage lipids (Xue, Chen, and Mei 2009). Phospholipids are enzymatically cleaved by phospholipases as part of cellular maintenance and downstream processing.

Phospholipases

Phospholipases are enzymes that catalyze the hydrolysis of phospholipids into fatty acids and other lipophilic molecules. Based on the mechanism of their hydrolytic reaction of the phospholipid ester bond, they are classified into four major groups(Fig 2); phospholipase A (PLA), phospholipase B (PLB), Phospholipase C (PLC) and Phospholipase D (PLD) (Wang, Ryu, and Wang 2012).

Phospholipase A cleaves the ester bond at either the sn-1 or the sn-2 position (Matos and Pham-Thi 2009), while phospholipase B cleaves the ester bond at both sn-1 and sn-2 position (Zhang, Zhang, et al. 2009). Phospholipase C cleavages target the glycerophosphate bond (Chen et al. 2011). Similarly, phospholipase D cleaves the phosphodiester bond on the head group and releases the hydrophilic head group and phosphatidic acid (PA) (Hong et al. 2016a). Each of these phospholipases have specific role in the cellular function (Fig 3). Each phospholipase group is further classified into subgroups.

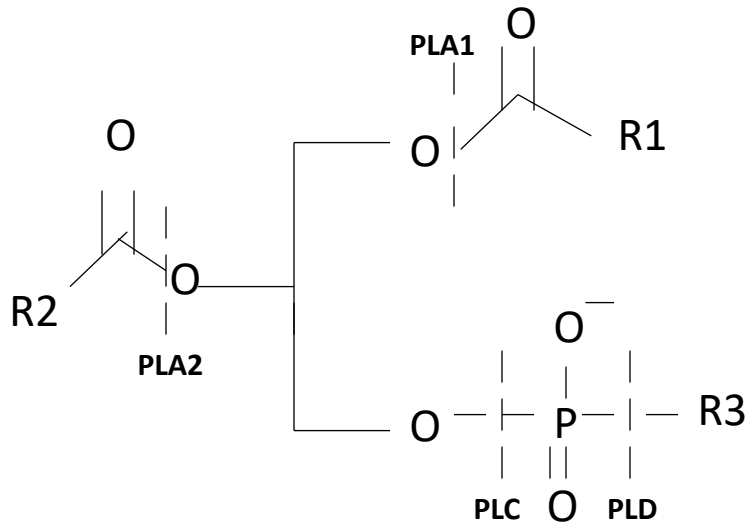


Fig 2. Cleavage sites for different phospholipases on phospholipid. PLA1, Phospholipase A1; PLA2, Phospholipase A 2; PLC, Phospholipase C; PLD, Phospholipase D; R, Fatty acyl group.

Phospholipase A

Based on the cleavage position of phospholipids, phospholipase A is divided into two groups: phospholipase A1 (PLA1) and phospholipase A2 (PLA2). PLA1 cleavages the ester bond at sn-1 position while PLA2 cleavages the ester bond at sn-2 position (Wang, Ryu, and Wang 2012). Depending on the sequence information and substrate, PLA is divided into four groups: phosphatidylcholine(PC) hydrolyzing PLA1 (PC-PLA1), Phosphatidic acid (PA) hydrolyzing PLA1 (PA-PLA1), low molecular weight secretory PLA2 (sPLA2) and patatin-like PLA (pPLA) (Wang, Ryu, and Wang 2012).

Based on sequence similarities, PLA1 is further divided into 3 groups (I , II, and III) (Ryu 2004). So far, fourteen isoforms of PLA1 have been reported from *Arabidopsis* genomic analyses (Chen, Greer, and Weselake 2013). Interestingly, one homologue of PA-PLA appears to be conserved in bovine testicles as well as the plants *Arabidopsis*,

rice and tomato. Similarly, four sPLA2 and ten pPLAs isoforms have been identified in *Arabidopsis* (Chen et al. 2011).

Based on sequence analysis, class I PLAs are localized in the chloroplast, while class II PLAs are present in the cytosol, and class III PLAs are in mitochondria. These PLAs are shown to play critical roles in plant physiology. For instance, class II proteins are reported to have a key role in the degradation of membrane lipids during senescence. Additionally, chloroplastic AtPLA1-Iβ1 releases linoleic acid, leading to the synthesis of the stress-response and development related hormone jasmonic acid (JA) (Ryu 2004) (Ishiguro et al. 2001).

pPLAIIIα and β are actively being explored for their role in triacylglycerol (TAG) biosynthesis in the endoplasmic reticulum. The possibility of their use in accumulating transgenic oil in the seeds of crop plants such as *Camelina sativa* is an area of active research. PLAIII- β has been shown to hydrolyze PC to free fatty acids in vitro, indicating its role in the acyl-editing of the PC pool (; Li et al. 2011).

Phospholipase D

PLD catalyzes the hydrolysis of head groups of phospholipids. Twelve PLDs are identified in *Arabidopsis*. These PLDs are grouped into four groups; PLDα, PLDβ, PLDγ, PLDδ, PLDε, and PLDζ based on their molecular and enzymatic characterization (Li, Hong, and Wang 2009)

PLDα, PLDβ, PLDγ, PLDδ and PLDε contain the C2 domain and require calcium for activation. PLDα hydrolyzes PC, PE and PG. PLDβ and PLDγ are difficult

to distinguish from each other by in-vitro characterization. PLDδ prefers PE over PC as a substrate. PLDζ differs from all other PLDs as it does not have a C2 domain, and hence does not require calcium for activation. Instead it requires PIP2. The protein has N-terminal phox homology (PX) and pleckstrin homology (PH) domains (Li, Hong, and Wang 2009). These plant PLDs are shown to be actively working in different biotic and abiotic stresses, plant growth and development, and signaling processes (Wang, Ryu, and Wang 2012).

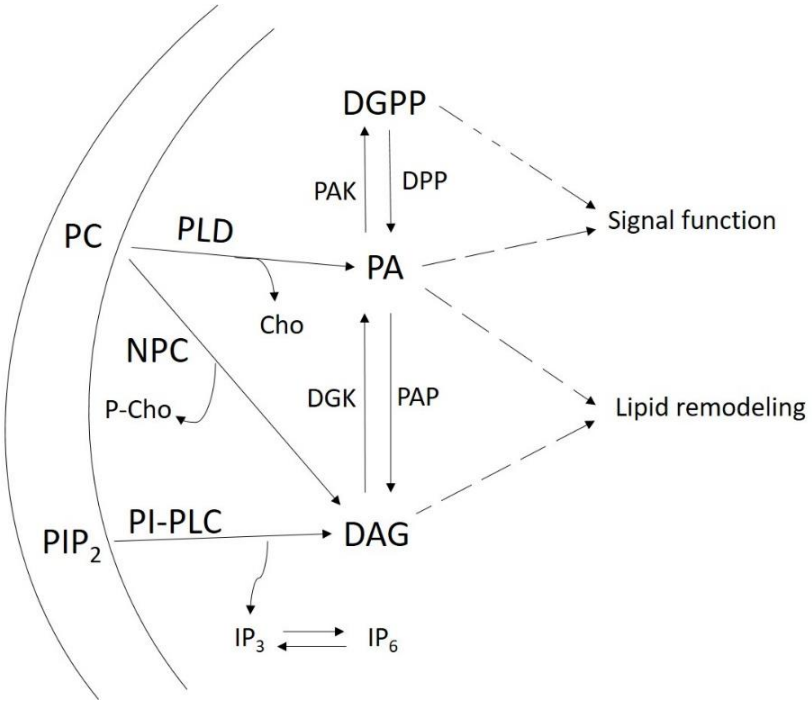


Fig 3. The function of different phospholipases in plant cell signaling and lipid remodeling. PC, Phosphatidylcholine; PIP_2, Phosphatidylinositol 4,5 bisphosphate; PLD, Phospholipase D; NPC, Nonspecific phospholipase; PI-PLC, Phosphoinositol specific phospholipase C; Cho, Choline; P-Cho, Phosphocholine; IP_3, Inositol triphosphate; IP_6, Inositol hexaphosphate, PA, Phosphatidic acid; DAG, Diacylglycerol. DGPP, Diacylglycerol pyrophosphate; PAK, p21 activated kinases; DGK, Diacylglycerol kinases; DPP, Dipeptyl peptidase; PAP, Phosphatidic acid phosphatase.

Phospholipase C

Plants have three types of PLCs based on their substrate specificity, of which two are well-studied: phosphoinositide specific PLC (PI-PLC) and non-specific PLC (NPC), the latter of which hydrolyzes common phospholipids such as phosphatidylcholine, phosphoethanolamine and phosphatidic acid. The third type of PLC, which is often considered as a PI-PLC, glycosylphosphatidylinositol (GPI) PLC (GPI-PLC), releases the GPI-anchored membrane proteins (Wang 2001). However, the GPI-anchored proteins are also cleaved by the PLD and other phosphodiesterases (Lehto and Sharom 1998; Zenewicz et al. 2005; Klöppel et al. 2009)

Phosphoinositide Specific PLC (PI-PLC)

PI-PLC hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP2) to generate 1,4,5 triphosphate (IP3) and diacylglycerol (DAG) (Abd-El-Haliem et al. 2016). In *Arabidopsis*, nine PI-PLCs have been reported; AtPLC1 to AtPLC9 (Di Fino et al. 2017). Of these, AtPLC8 and AtPLC9 have a large deletions in their catalytic site and hence lack enzymatic activity (Wang 2004). Several PI-PLC from several other plants such as rice, corn, tobacco also have been reported.

PI-PLCs can further be divided into six groups: PI-PLC (β, γ, δ, ε, η and ζ) based on the domain structures present. In plants, only the ζ subgroup of PI-PLCs has been identified so far (Hong et al. 2016a). These PI-PLCs contain “XY” catalytic domains and a C2 domain. The XY domain consists of an X and Y region that forms a TIM-barrel structure of alternating α and β sheets (Cocco et al. 2015). Plant PI-PLCs have not been

extensively studied with structural and mutational analyses. His311 & His356 on the XY domain have been shown to be essential for catalytic activity in mammalian PLC δ1 (Moroz et al. 2017). These two residues are conserved across the plant PI-PLCs reported so far. The C2 domain is also conserved as well and interacts with XY motif and also targets PI-PLCs to the plasma membrane (Singh et al. 2015).

Calcium is required for plant PI-PLCs for hydrolytic activity. AtPLCs 1-5 have been shown to require Ca2+ for enzymatic activity. AtPLC2 and AtPLC5 require much higher calcium concentration than AtPLC1, 4 and 5 (Nakamura et al. 2004).

Plant PI-PLCs have been shown to act very actively to generate second messengers in response to biotic and abiotic stresses (Singh et al. 2015). Except for AtPLC2, all other eight AtPLCs have shown to be upregulated in response to environmental stresses. The level of expression however varies for different tissues. Expression level of PI-PLCs in response to stresses has been studied in several other plants as well. For example, SIPLC 1,2,4, and 5 were upregulated when tomato plants were inoculated with *C. fulvum* (Vossen et al. 2010). However, SIPLC 3 and SIPLC6 did not exhibit any change in expression. PI-PLCs also plays a role in plant development.

Non-Specific Phospholipase C (NPC)

NPCs are structurally and biochemically different from PI-PLCs. They do not contain any motif from PI-PLCs. These are approximately 60 kDa proteins. Plant NPCs have high sequence similarity compared to bacterial PC-PLCs. Three domains are conserved among plant and bacterial NPCs (Pokotylo et al. 2013). Plant NPCs are less studied compared to PI-PLCs. However, bacterial PC-PLCs are well characterized.

Bacterial NPCs

Various NPCs or PC-PLCs are identified in bacteria. The first PC-PLC was discovered in *Clostridium perfringens* (Macfarlane and Knight 1941). The enzyme identified was toxic to the host cells. Since then PC-PLC activity has been observed in several gram positive and gram negative bacteria. Bacterial PC-PLCs falls under two groups. The first group is toxic and is produced in gram positive bacteria. The second group is non-toxic and mainly produced in gram negative bacteria (Vasil et al. 1990); (Titball 1993).

Toxic-PC-PLCs require zinc for activation and are inactivated by *EDTA or* o-*phenanthroline metal chelators*. Some toxic PC-PLCs are also shown to require calcium and magnesium for activation (Ikezawa 1999). These are single polypeptide proteins of molecular weight 28-34 kDa. Analysis of amino acid sequences show these enzymes are secretory. Although these enzymes are named as PC-PLCs, they have a wide range of specificity towards PC, PE and PA (Titball 1993). Toxic PC-PLCs have two domains: N-terminal domain and C terminal domain (α toxin) (Bateman and Sandford 1999). Removal of α toxin domain from the protein largely reduced the toxicity. However, PC-specific hydrolysis was still retained. Thus, N-terminal domain has PC-specific activity (Titball et al. 1991) .

The other group of bacterial NPCs mainly produced by gram negative bacteria are non-toxic. These proteins have higher molecular weight than toxic NPCs. The activity of some of the identified non-toxic NPCs, such as PlcHR2 from *Pseudomonas species*, are independent of zinc and Ca2+ (Pokotylo et al. 2013,).

It clearly shows that toxic and non-toxic NPCs are independently evolved. Non-toxic NPCs more closely resemble plant NPCs indicating their common evolution history (Nakamura et al. 2004).

Mammalian PC-PLCs

Based on sequence, mammalian or animal PC-PLCs are clearly distinct from other PC-PLCs across the living organisms. So far, no PC-PLCs haven been cloned and characterized from animal cells. However there is enough evidence that PC-hydrolyzing PLCs play a role in generating secondary messengers in animal cells which are required for cell metabolism and signaling (Pokotylo et al. 2013).

Plant NPCs

Previously, Plant NPCs were taken as a separate set of enzymes from known plant phospholipase families. These enzymes do not contain any of the motifs present in other plant phospholipases involved in signaling. Plant NPC aligned to PLC from *M. tuberculosis* revealed three conserved regions that are completely different from known phosphodiesterases in plants (Nakamura et al. 2004). Mutational characterization of the motifs has not been done so far in the plant NPCs. Four domains that are conserved in gram-negative bacteria PC-PLC and plant NPCs may play an active role in PC hydrolysis of phospholipids (Fig 4A). The N-terminal region of NPC1, NPC2 and NPC6 contains a signal peptide while the signal peptide is missing in NPC3, NPC4 and NPC5. The signal peptide is followed by ENRSFDxxxG motif at the beginning of phosphoesterase domain. ENRSFDxxxG motif is then followed by three other invariable motifs: TxPNR, DExxGxxDHV, and GxRVPxxxxxP. 50-100 amino acid residuals at the c-terminal

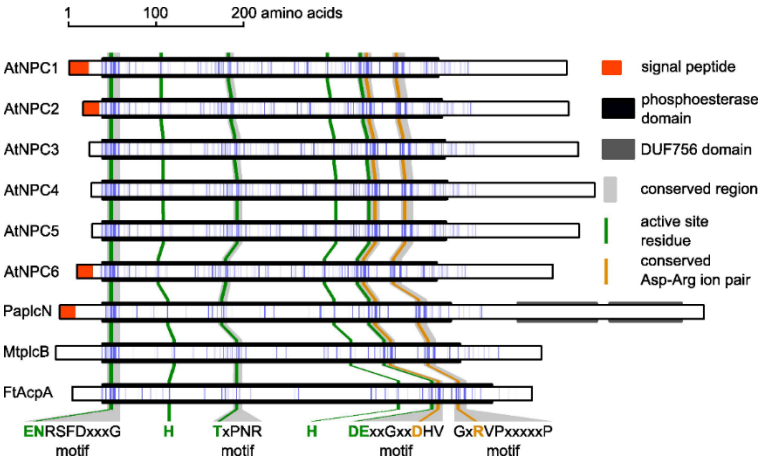
regions are the most divergent among plant NPCs. These divergent residuals may define the localization, tissue specificity and substrate specificity of these NPCs (Pokotylo et al. 2013).

Six NPCs are identified in Arabidopsis, namely NPC1 (At1g07230), NPC2 (At2g26870), NPC3 (At3g03520) , NPC4 (At3g03530), NPC5(At3g03540) and NPC6 (At3g48610) (Pejchar et al. 2015). These NPCs have been studied for their roles in different stress responses and plant growth and development. These NPCs are differentially expressed in different Arabidopsis tissues (Wang, Ryu, and Wang 2012).

NPC1, NPC3, NPC4 and NPC5 are functionally characterized while NPC2 and NPC6 are yet to be characterized. All these proteins are differentially expressed in different tissues (Fig 4B). NPC1 has been characterized to demonstrate that it releases DAG from PC (Krčková et al. 2015). The enzyme is expressed across all the tissues but the expression in siliques is more prominent. NPC1 is shown to predominately localize at endoplasmic reticulum based on sequence analysis. The enzyme is predicted to play roles in thermotolerance. NPC3 uses lysophosphatidic acid as a substrate. NPC3 is mainly expressed in the roots (Wimalasekera et al. 2010). It is activated for BL signaling, auxin and cytokinin signaling. NPC4 acts on both PC and PE to release DAG. The enzyme is expressed in siliques, roots, leaves, stem and flowers. However, the expression in leaves and roots dominates the expression in other tissues. NPC4 has been shown to be expressed during phosphate deficiency (Nakamura et al. 2009). It is also activated during abiotic stress events such as salinity and hormonal stresses. NPC5 acts on PC and PE but its activity is 40-fold less than that of NPC4 (Gaude et al. 2008). The enzyme is

expressed during inflorescence development and is localized in the cytosol. It can also be activated via phosphate deficiency (Pokotylo et al. 2013).

A



B

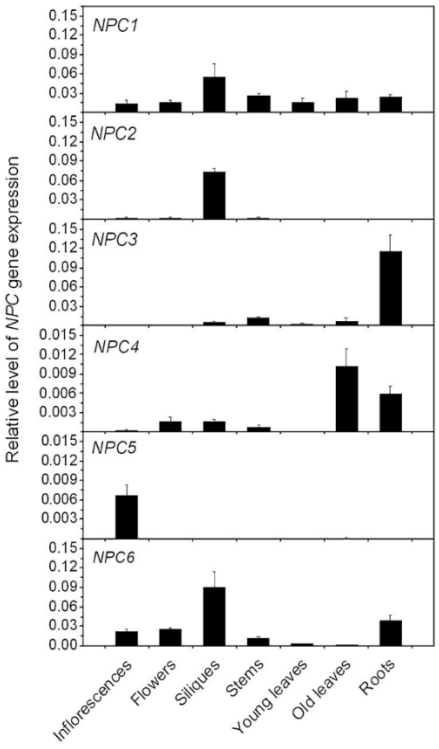


Fig 4. A. Conserved domains for AtNPCs (Poktylo et al., 2012). B. Expression of NPCs in different tissues of Arabidopsis (Peters et al., 2010)

PLC like Phosphodiesterases

A separate set of phospholipase-like-proteins were identified based on sequence analysis. The proteins are also much smaller than PI-PLCs. These proteins are not yet functionally characterized. These proteins do not contain the motifs that are conserved in NPCs. The phylogenetic analysis places these proteins as evolutionarily distinct from NPC and PI-PLC. These proteins are widely distributed in the plant kingdom which indicates their probable roles in different cellular processes.

Goal and Objectives

Overall Goal

The overall goal of this study is to expand the current understanding of TAG biosynthesis in oilseeds and to increase the amount of HFA in transgenic Camelina. I focused on the pathway of PC to DAG conversion during oil biosynthesis, which is still not completely understood and is considered a major bottleneck in the accumulation of unusual fatty acids in the TAG of transgenic seeds. I aimed to examine the role of PLCs in the release of DAGs from PC, and to investigate their contribution to HFA accumulation by selecting the most appropriate PLC from Castor and transforming into a RcFAH12-expressing Camelina line. To achieve my goal, I set the following objectives:

1. Examine the Role of NPCs in TAG Biosynthesis in Arabidopsis

NPCs are previously shown to hydrolyze PC. To investigate if the NPCs have a role in TAG biosynthesis pathway in Arabidopsis developing seeds, I analyzed the T-DNA lines for 6 NPCs; NPC1 to 6. Double mutants *npc1/npc6* and *npc1/rod1* were created to inspect the cumulative effect of these NPCs enzymes.

2. Examine the Role of PLC-like (PLCL) Enzymes in TAG Biosynthesis in Arabidopsis and Functional Analysis of RcPLCL1 and AtPLCL1.

To increase HFA accumulation in transgenic Camelina, I searched for the phospholipase Cs that are expressed in Castor. My first choice was RcNPCs since these enzymes convert PC into DAG. RcNPC3 and RcNPC6 were expressed in the castor. Particularly, RcNPC6 was expressed in the developing endosperm, where oil is synthesized and stored in castor. However, RcNPC6 was predominantly expressed in

other tissues such as leaves and male flowers. Interestingly, RcPLC likes were the primarily expressed phospholipase C in the endosperm. Out of 6 phospholipase C expressed, five of them were RcPLC likes (RcPLCLs). The highest expression level was for RcPLCL1. Hence, I chose this as the candidate gene. Meanwhile, I also examined the roles of AtPLC likes in TAG biosynthesis in Arabidopsis. Eight genes are found to encode PLC-like phosphodiesterases from the genomic sequence of Arabidopsis. I analyzed the FA composition from T-DNA lines for 5 closely related genes. The enzyme activities of RcPLCL1 and AtPLCL1 were tested against PC and PI using heterologous expression in yeast.

3. Coexpression of RcPLCL1 with RcFAH12 in Camelina

Inefficient release of HFA from PC is a major bottleneck for accumulating a high amount of HFA in transgenic Camelina. After analyzing phospholipases in castor plants (Objective 2), I selected RcPLCL1 as my candidate gene. I co-expressed RcPLCL1 with RcFAH12 to test whether it could release HFA-containing DAG from PC and thus increase HFA in the oil.

4. Test the Performance of HFA Producing Plants in the Field

An experiment was carried out for two years at the Arthur H. Post Research farm (8431 Huffine Ln, Bozeman, MT 59718) of Montana State University to measure the different traits of a wild type (WT) and a transgenic line (7-1) that produce Hydroxy Fatty Acids (HFAs) in the seed oil of Camelina.

CHAPTER TWO

THE ROLE OF NON-SPECIFIC PHOSPHOLIPASE C IN TAG BIOSYNTHESIS IN SEEDS OF ARABIDOPSIS

Abstract

To examine the role of NPCs in TAG biosynthesis pathway in the seeds of Arabidopsis, I ordered the T-DNA lines for 6 AtNPCs; NPC1 to NPC6, from Arabidopsis Biological Research Centre (ABRC). These plants were confirmed for their homozygosity and were grown side by side with the wild type. FA composition in the oil of the mature seeds was then analyzed to investigate the role of these enzymes in TAG biosynthesis. Mature seeds from *npc1, npc4* and *npc6* showed different FA composition compared to the wild type; *npc2, npc3* and *npc5* did not show any difference. NPC1, NPC2, NPC4 and NPC6 are expressed in developing seeds. No change in FA composition was found in the npc2 seeds, however 18:1 increased and 18:2 and 18:3 decreased in *npc1, npc4* and *npc6* seeds. The *npc1/npc6* double mutant further increased the 18:1. The *npc1 pdct* double mutant was created, which resulted in further increased 18:1 to the *pdct* seed. The decrease of polyunsaturated FA (18:2 and 18:3) and increase in monounsaturated FA (18:1) suggests the role of NPC1, NPC4 and NPC6 in TAG biosynthesis in developing seeds of Arabidopsis.

Introduction

Production of desired composition of storage lipids in the seeds of crop plants has attracted many researchers. However, a complete understanding of storage lipid biosynthesis in developing seeds is not yet completely achieved. Several genes have been discovered and several others have been proposed to play an active role in the pathway. Two pathways, Kennedy pathway or sequential acylation of Glycerol 3 phosphate (G3P) and acyl-editing, are proposed for the accumulation of oil in the developing seeds. Of the two pathways proposed, the acyl-editing pathway is more complex than sequential acylation pathway. Flux of FAs in and out of the PC pool, which is the hub for modification of fatty acids, is still a complicated subject. Formation of PC from CDP-choline by the enzyme Choline phosphotransferase (CPT) was elucidated 55 years ago (Young and Lynen 1969). However, this great discovery could not solve the mystery alone. After discovery of Phosphatidycholine diacylglycerol cholinephosphotransferase (PDCT), a better understanding of FA flux in and out of PC was obtained. PDCT catalyzes the exchange of head groups between de novo DAG and PC(Lu et al. 2009). This exchange provides the entry of de novo fatty acids to the PC pool and also provides the exit of modified fatty acids, which are acylated to DAG, out from the PC pool. Another enzyme, lysophosphatidycholine transferase (LPCAT), has forward and reverse reactions between lysophosphatidycholine (LPC) and PC. The forward reaction incorporates free fatty acyl to LPC and forms PC while the reverse reaction releases the free fatty acid from the sn 2 position of PC and forms LPC (Bates et al. 2012b). The

PDCT and LPCAT enzymes are found to control two third flux of 18:1 FA in and out of PC in Arabidopsis.

Recently, phospholipases have also been studied for their roles in releasing DAGs from PC. Phospholipase A (PLA) releases free fatty acids from PC which then is incorporated into a glycerol backbone via Kennedy pathway for the formation of TAG. PLC and PLD release DAG from PC which contains modified fatty acids. These DAGs then incorporate free acyl FA into sn 3 position and form TAG. AtPLDζ1 and 2 have been shown to increase the TAG by 3% in total percentage and also alter the FA composition when overexpressed in Camelina (Yang et al. 2017). Non-specific phospholipase Cs have been shown to release DAG from PC in-vitro. Six non-specific phospholipase Cs are shown to be present in Arabidopsis. These NPCs are shown to act on phosphatidycholine from previous research. These genes have been studied to show their effects in several developmental phases of the plants. However, their role in oil biosynthesis has not been experimentally verified yet (Bates 2016). Based on their expression in developing seed and substrate specificity toward phosphatidycholine, I selected NPC1(At1g07230), NPC3(At3g03520), NPC4(At3g03530) and NPC6(At3g48610) as the candidate genes to study for their role in TAG synthesis. NPC2 is well-expressed in the siliques, however I did not see any effect of the mutation of this gene on FA composition of mature seeds from the preliminary analysis. Hence, I decided to not include *npc2* for the further study. I expected that disruption of these PLCs would decrease desaturated or modified fatty acids in the oil and thus increase 18:1, which is the main FA for Arabidopsis seeds to be exported out of plastid.

Materials and Methods

Plant Growth

Plants were grown in the "Plant Growth Chamber" at Montana State University. Four 10-day old seedlings were transplanted into new 3x3-inch pots from high density grown pots. The growth chamber temperature was maintained at 22^{o}C; 377 uMol of light was given to the plants. 16-8 hours of day-night circadian cycle was maintained. The plants were watered once a day.

Test for Mutation

T-DNA lines were obtained from Arabidopsis Biological Resource Center (ABRC) (Table 1). Mutations of genes for these lines were verified from the genomic DNA by using three primers. DNA from the leaves was extracted from labeled plants by the cetyl trimethylammonium bromide (CTAB) method. Leaf samples were crushed and 300 ul of 2x CTAB was added. Solutions were then heated at 65^{0}C for 1 hour. They were then cooled to room temperature. 300 ul of chloroform was added to the cooled tubes and centrifuged briefly. The upper aqueous layer was then transferred to a new tube. 300 ul of isopropanol was added and mixed briefly. It was then centrifuged for 5 minutes at maximum speeds. A pellet of DNA formed at the bottom of the tube. The upper layer was then discarded, and the pellet was washed twice with ethanol and dissolved in 100 ul of TE buffer. The DNA was then used for the PCR using three primers: left and right genomic primers and left border primer for T-DNA. PCR products were then analyzed by running on agarose gel. Wild type without any T-DNA insertion produced a larger DNA

band. Heterozygous lines produced two bands: one larger and the other smaller. Homozygous mutated lines produced only smaller DNA bands.

Measurement of Fatty Acid Composition and Oil Content

Fatty acid composition was measured by creating fatty acid methyl esters and then running on gas chromatography. Thirty to 50 mature and dry seeds were heated in 1 ml of 2.5% H_2SO_4 (v/v) in methanol at 80^0C for 90 minutes. Oil was then extracted with 200 ul of Hexane and 1.5 ml 0.9% (v/v) NaCl. The mixture was vortexed briefly then centrifuged. The upper aqueous layer was transferred to autoinjector vials. For Agilent 6890 GC fitted with a 30 M x 0.25-mm DB-23 column, 1 ul of sample was injected. A GC program of 190^0C for 2 minutes followed by an increase of 8^0C per minute to 230^0C and maintenance for 6 minutes was used. For the measurement of total oil content, 5 ul of 10 mg/ml 17:00 FA (standard) was added to 20 mature seeds prior to heating.

Separation of TAG Molecules by Thin Layer Chromatography

Total lipids were extracted from mature seeds and two-dimensional thin layer chromatography was run for the lipids extracted. Lipids were extracted according to (van Erp, Bates, Shockey, et al. 2011)). Isopropanol with 0.01% butylated hydroxyl toluene (BHT) was heated and 100 ul of isopropanol with BHT was added to the tubes containing 30 to 50 mature dry seeds. Seeds were crushed finely in solution with polytron dispersing and mixing technology (PT-10-35). Tubes were well capped and heated at 85^0C for 15 minutes in a water bath. Phase separation was then induced to the cooled samples by adding 1.6 ml H_2O, 2ml chloroform and 2 ml of 0.9% NaCl. Samples were then spun down for 10 minutes at 250 rpm. The chloroform layer in the bottom of the tube was

transferred to a new tube and dried with liquid nitrogen. Dried samples were resuspended in 0.25 ml of chloroform and stored at -20^{0}C until further used. Thin-layer chromatography was then run to separate the TAG molecules. 50 ul of lipids were run in the 2-dimensional silica gel (TLC silica gel 60, 20*20 cm, Analytical Chromatography Germany). The solvent system of Hexane: diethyl ether: formic acid :: 70:30:1 was used. The silica gel was stained with 0.005% (w/v) primulin in 80% (v/v) acetone solution. Air dried gel was then viewed under UV light. Separated TAG molecules were then scrapped and fatty acid composition was measured by creating fatty acid methyl esters.

Reverse Transcriptase PCR

Semi-quantitative reverse transcriptase PCR was performed to test the expression level of genes. Total RNA was extracted from developing siliques of plant lines. Trizol-chloroform extraction method was used for total RNA extraction. Ten DAF siliques were harvested and directly stored in liquid nitrogen. 50-100 mg of silique sample was grinded in presence of liquid nitrogen. 1 ml of trizol was added to the grinded sample. Trizol-sample solution was incubated at room temperature for 5 minutes for complete dissociation of nucleoprotein complex. 0.2 ml of chloroform was then added and stayed at room temperature for 2-3 minutes. The samples were then centrifuged for 15 minutes at 12000 xg at 4^{0}C. The mixture was separated into a lower red phenol-chloroform, an interphase, and an upper aqueous layer. The aqueous layer was transferred to a new tube and 0.5 ml of isopropanol was added. The sample was gently mixed and incubated at room temperature for 10 minutes. It was then centrifuged at 12000 xg for 10 minutes. A white colored pellet was visible at the bottom of the tube. The pellet was washed by

adding 1 ml of 75% ethanol. The pellet was air dried and dissolved in 50 ul of nuclease free water. cDNA was made using oligo dT primers (FastScript cDNA synthesis kit).

Table 1. T-DNA lines for Non-specific phospholipases and the primers used for confirmation of the homozygous mutant.

Gene name	**Locus**	**T-DNA germplasm**	**Primers**
NPC1	At1g07230	SAIL_548_H09 /CS874750	FP: GGAGAATCTCGGATACGGAAG RP: GCAACGTGAAGAAAGATCTCG
NPC2	At2g26870	SALK_115455C	FP: AGAACATGCACACCTTTGACC RP: CACAAAGAGAGCGCCAGTAAG
NPC3	At3g03520	SALK_078975C	FP: AGTTGGACACATGCAAAAACC RP: ACCGGGAAATTGATAGAGTGG
NPC4	At3g03530	SALK_046713C	FP: AATTCCACCCACACACAAGAG RP: CTACGAGGCATTGAGATCGAG
NPC5	At3g03540	SAIL_293G10	FP: GGGACAACAGTGATACCCATG RP: TAACCATCGTTCATTAACCGG
NPC6	At3g48610	SALK_077041C	FP: ATGTGATTTGGTTTTTGCAGC RP: ACCGGTGGTTTTCTTCAATTC

Results and Discussion

AtNPCs are Different from other Phospholipases in Arabidopsis.

From their similarity to the bacterial PC-PLCs, 6NPCs were identified in Arabidopsis, namely NPC1(At1g07230), NPC2 (At2g26870), NPC3 (At3g03520) , NPC4 (At3g03530), NPC5(At3g03540) and NPC6 (At3g48610) (Pejchar et al. 2015). They are 515 to 540 aa length and molecular weight ranges from 55 to 60 Kda. Localization of these enzymes was predicted using Target P program. NPC1, 2 and 6 have putative secretory signal peptide while NPC3, 4 and 5 do not contain these signal peptides. When a neighbor-joining tree was drawn for different NPCs present in the Arabidopsis, NPCs formed a distinct group from PI-PLCs and PLC-likes (Fig 1)

NPC1, NPC3, NPC4 and NPC5 are functionally characterized while NPC2 and NPC6 are yet to be characterized. These enzymes are differentially expressed in different Arabidopsis tissues (Wang, Ryu, and Wang 2012). They also have been studied for their role in different stress responses and plant growth and development (Table 2). NPC1 has been characterized to demonstrate that it releases DAG from PC (Krčková et al. 2015). The enzyme is expressed across all the tissues but the expression in siliques is more prominent. NPC1 is shown to predominately localize at the endoplasmic reticulum based on sequence analysis. The enzyme is predicted to play a role in thermotolerance. NPC3 uses lysophosphatidic acid as a substrate. NPC3 is mainly expressed in the roots (Wimalasekera et al. 2010). It is activated for BL signaling, Auxin and cytokinin signaling. NPC4 acts on both PC and PE to release DAG. The gene is expressed in siliques, roots, leaves, stems, and flowers. But the expression in leaves and roots

predominates the expression in other tissues. NPC4 has been shown to be active under phosphate deficiency (Nakamura et al. 2009). It is also activated against abiotic stress such as salt stress and hormonal stresses. NPC5 acts on PC and PE but its activity is 40 times less than that of NPC4 (Gaude et al. 2008). The enzyme is expressed during inflorescence and is localized at the cytosol. It is also activated under phosphate deficiency (Pokotylo et al. 2013).

Table 2. List of Non-Specific Phospholipase C present in Arabidopsis genome. Localization was Predicted using TargetP Program (http://www.cbs.dtu.dk/services/TargetP) Secretory,contains signal peptidefor the secretory pathway. Substrate Specificity and Predominant Tissue Expression is obtained from literature review.

Gene name	At locus	aa length	Molecular weight (Kda)	Localization	Substrate Specificity (Experimentaly verified)	Predominant tissue expression
NPC1	At1g07230	533	60.0	Secretory	PC	Siliques
NPC2	At2g26870	514	57.6	Secretory	-	Siliques,roots
NPC3	At3g03520	523	59.1	Others	LPA	Leaves, roots
NPC4	At3g03530	538	60.7	Others	PC,PE	Old leaves, roots
NPC5	At3g03540	521	59.0	Others	PC,PE	Inflorescences
NPC6	At3g48610	520	57.9	Secretory	-	Siliques, roots

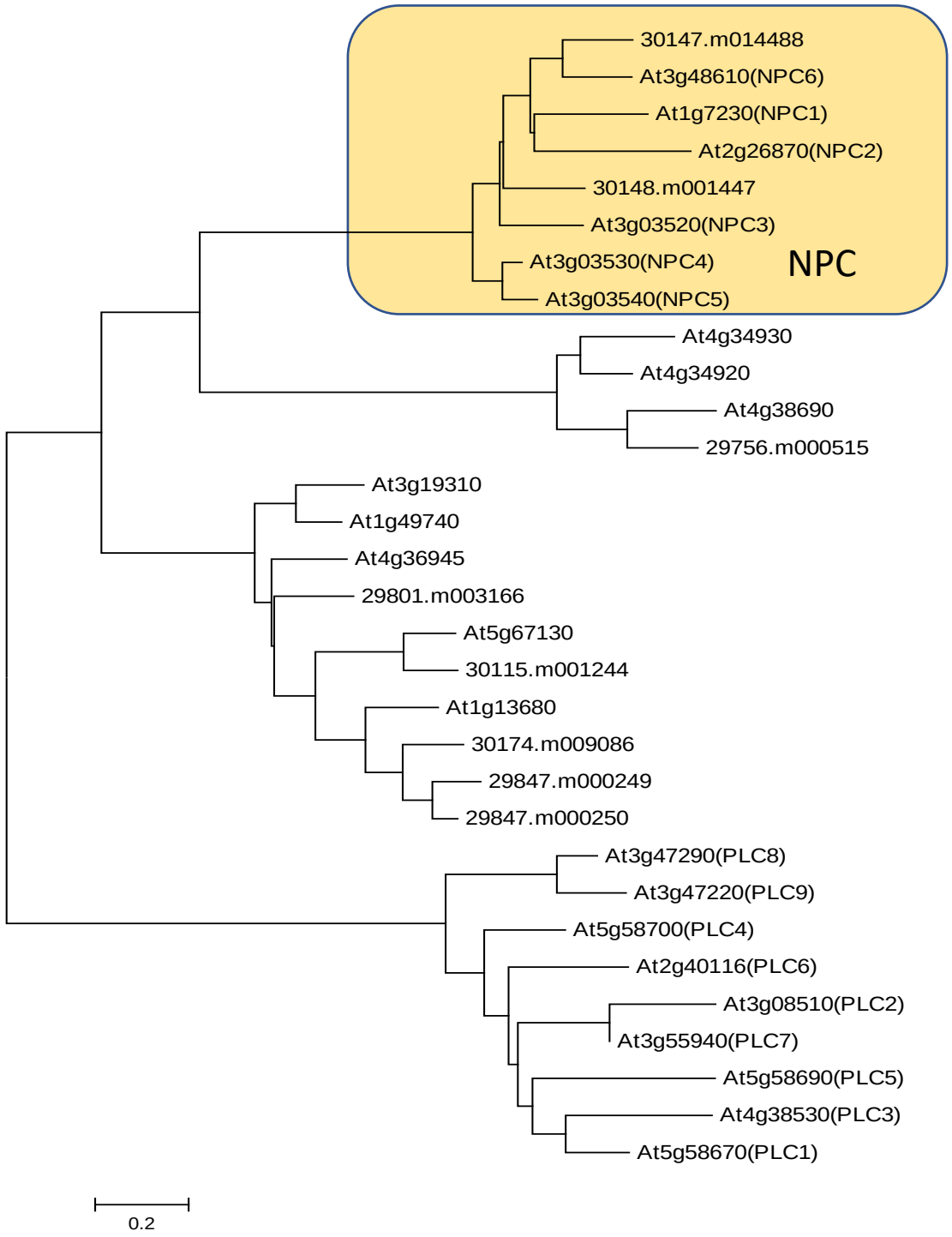


Fig 1. Neighbor-joining phylogenetic tree for different phospholipase Cs in *Arabidopsis thaliana*.

Mutation of NPCs Alters FA Composition in Seed Oil

Fatty acid composition was analyzed from the mature seeds of single mutants using Gas Chromatography. Changes in FA profile were observed for *npc1, npc4* and *npc6*; but *npc2, npc3* and *npc5* did not show any difference in FA profile compared to the wild type. NPC3 is not expressed in the siliques/seeds, which is the site for the synthesis and storage of TAGs, it also did not show change in FA compared to wild type in my preliminary GC run. Hence, I included the mutant *npc3* in my experiment as a control along with the wildtype.

For *npc1*, *npc4* and *npc 6*, there was a significant increase in 18:1 compared to wild type (Fig 3 and Table 3). Wild type seed oil constituted of 14.2% of 18:1 while *npc*1, 4 and 6 contained 16.9, 17.6 and 16.4 % respectively. A decrease in 18:2FA was observed for those plant lines. The increase monounsaturated (18:1) FA, which is the primary FA exported out from the plastids in the developing seeds of Arabidopsis, and the decrease in polyunsaturated (18:2 FA) suggests the role of AtNPCs in TAG synthesis. Polyunsaturated FAs are made in the PC by the fatty acid desaturases (FAD2 and FAD3) which are then released from there for the incorporation into TAG. Though the change in FA composition is not large, these results in the NPC mutants suggest that NPCs may play roles in FA flux out from PC by formation of DAG.

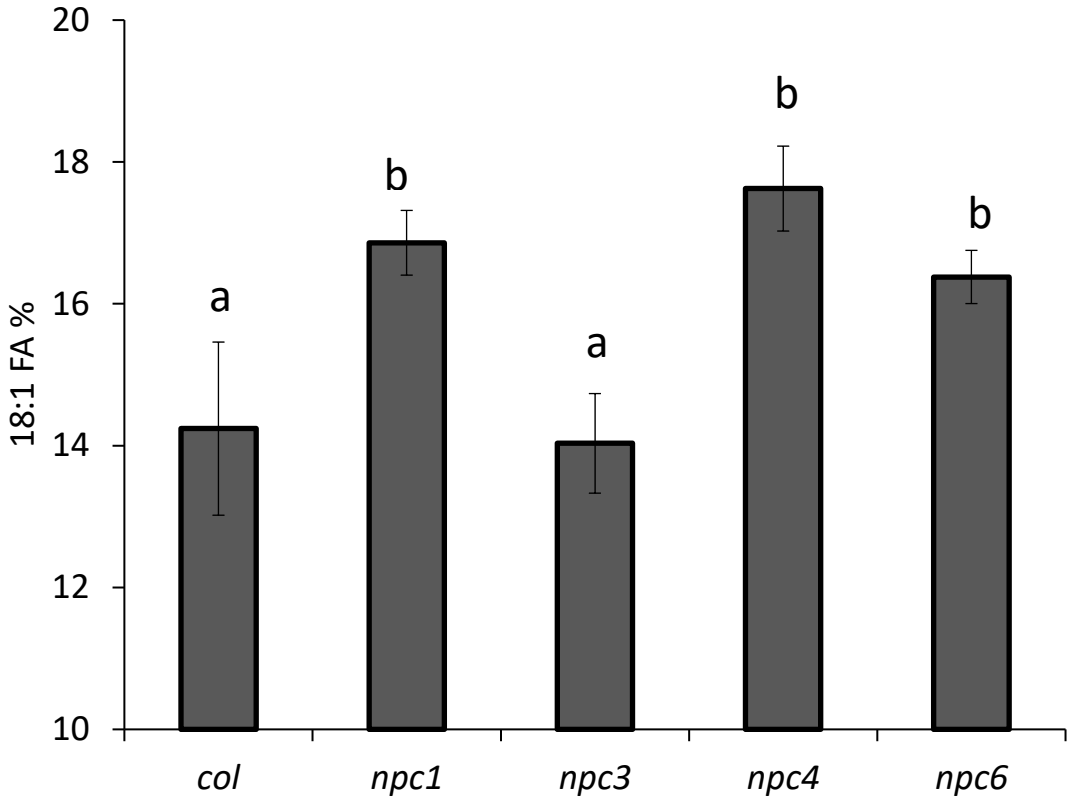


Fig 3. 18:1 levels in the mature seeds of Arabidopsis lines. Col is wild type and other lines are mutated for respective genes. The data represents average ± SD for 3 technical replicates and 7 biological replicates. Significantly different data are grouped as "a" and "b". $P<0.01$ for 2 tail T-Test.

The Double Mutant npc1/6 Contains Further Increased 18:1

The change in FA composition for single mutants was significant, however, it was not large enough to conclude their roles. Hence, I created a double mutant by crossing *npc1* and *npc6*. Both of these single mutants showed significant increase in 18:1 in their seed oil compared to that of wild type and these genes are also evolutionarily close to each other among NPC1, 4 and 6. For *npc1/npc6* double mutant, 18:1 was further

increased compared to the single mutants *npc1* and *6* (Fig 4 and Table 3). This cumulative increase in 18:1 indicated the complementation of NPC1 and NPC6 and further supports my hypothesis.

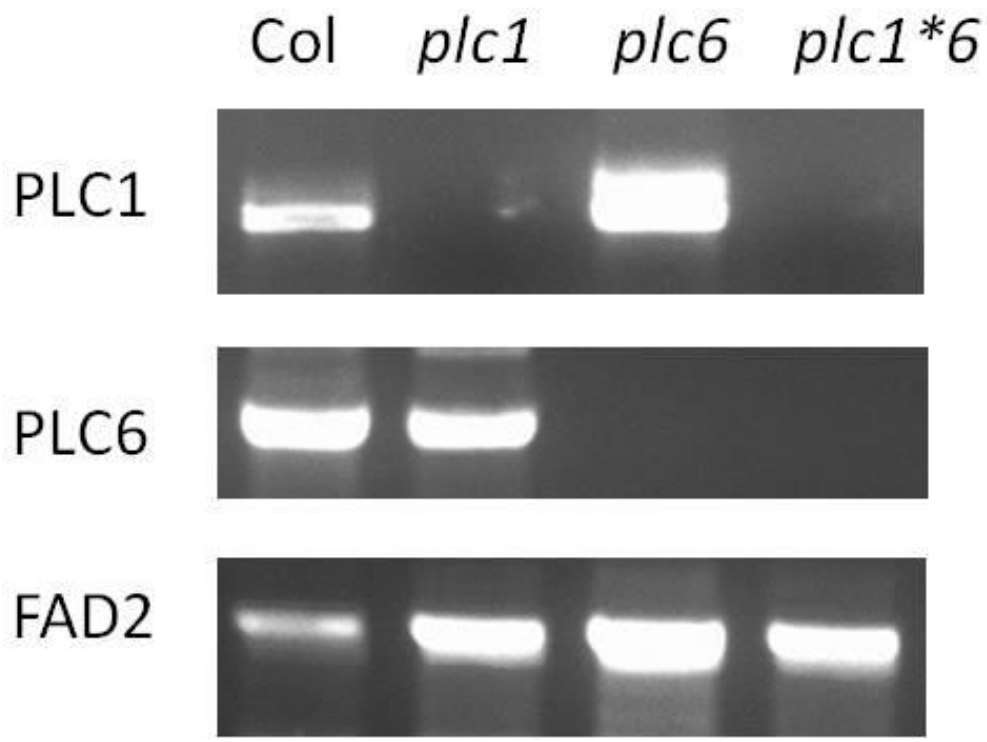


Fig 2. RT PCR for different PLCs from the mutant plant lines.

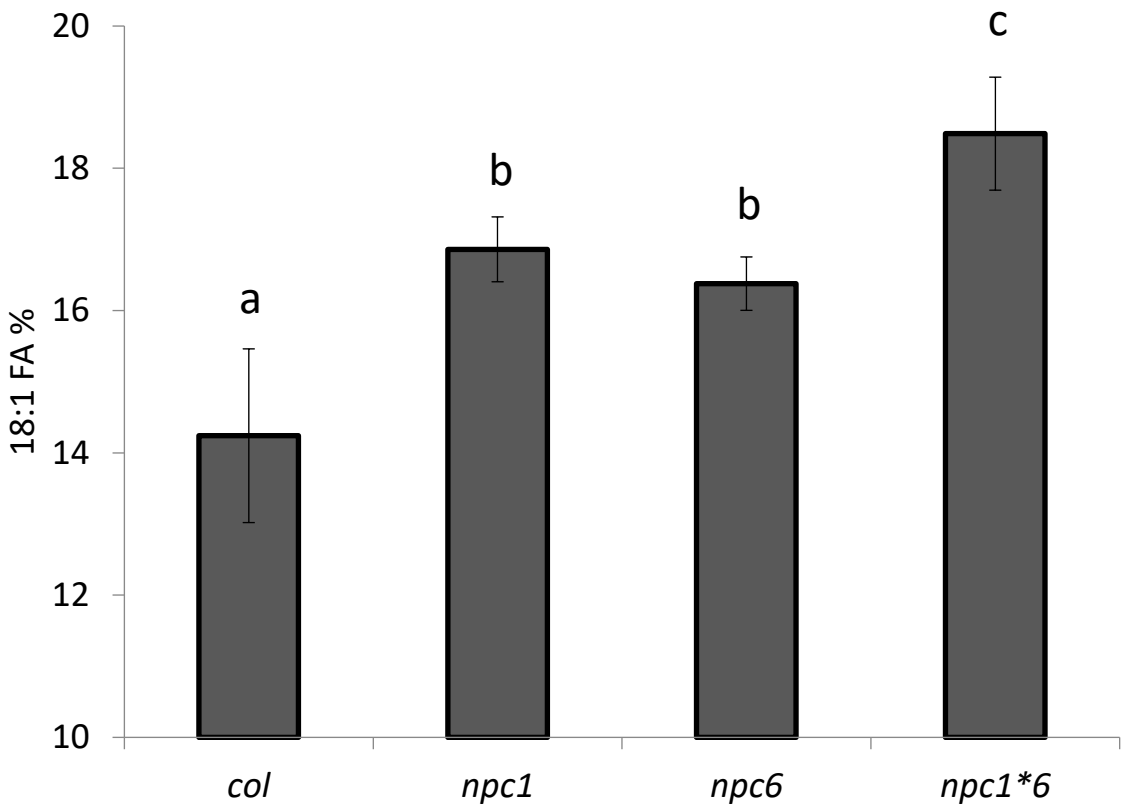


Fig 4. The 18:1 levels in the mature seeds of Arabidopsis lines. Col is wild type and other lines are mutated for respective genes. The data represents average ± SD for 3 technical replicates and 7 biological replicates. Significantly different data are grouped as "a","b" and "c". $P<0.01$ for 2 tail T-Test.

Table 3. FA composition in the mature seeds of Arabidopsis lines. Col is wild type and other lines are mutated for respective genes. The data represent average ± SD for 3 technical replicates and 7 biological replicates.

Line	FA Composition						
	16:0	18:0	18:1	18:2	18:3	20:0	20:1
			wt % of total				
col	9.7±0.5	4.0±0.3	14.2±1.2	31.3±1.2	17.8±0.6	3.1±0.3	19.8±1.1
npc1	9.0±0.6	4.0±0.4	16.9±0.5*	28.7±1.5*	18.4±0.4	2.9±0.4	20.6±0.4
npc3	9.9±0.7	4.3±0.6	14.0±0.7	30.1±0.6	18.3±0.7	2.9±0.1	20.4±1.0
npc4	8.9±0.3	4.0±0.3	17.6±0.6 *	28.4±0.4*	17.9±0.6	2.7±0.1	20.5±0.3
npc6	9.5±0.8	4.4±0.6	16.4±0.4*	28.0±1.1*	18.2±0.4	3.0±0.2	20.5±0.4
npc1/6	8.1±0.2	3.6±0.1	18.5±0.8**	28.2±0.3*	17.9±0.3	2.6±0.1	21.1±0.4

The Double Mutant *npc1/pdct* Contains Further Increased 18:1

PDCT was previously shown to have a major role in releasing modified DAG from PC; *npc1* plants were crossed with *pdct* plants to create a double mutant. The double cross was grown side by side with *npc1*, *pdct* and wild type (WT). FA profile was then measured from the mature seeds (Fig 5, Table 4); *npc1/pdct* showed increased 18:1 compared to *pdct.* In the *pdct* line, seed-oil contained 32.9% of 18:1 FA while *pdct / npc1*

contained 35.9% of 18:1 FA. Similarly, the polyunsaturated FA, 18:2 , was decreased for *pdct/npc1*. The *pdct* was previously shown to have increased 18:1FA; the further increment after crossing with *npc1* suggests that PLCs also have role in releasing polyunsaturated FA from PC, apart from PDCT.

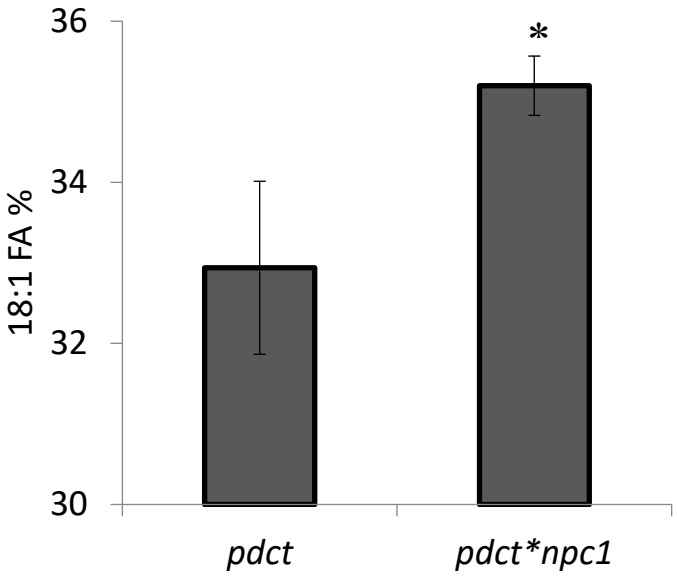


Fig 5. 18:1 levels in the mature seeds of Arabidopsis lines; *pdct* is mutated for PDCT and *pdct/npc1* is mutated for both PDCT and PLC1. The data represent average ± SD for 3 technical replicates and 7 biological replicates. *P<0.01 for 2 tail T-Test.

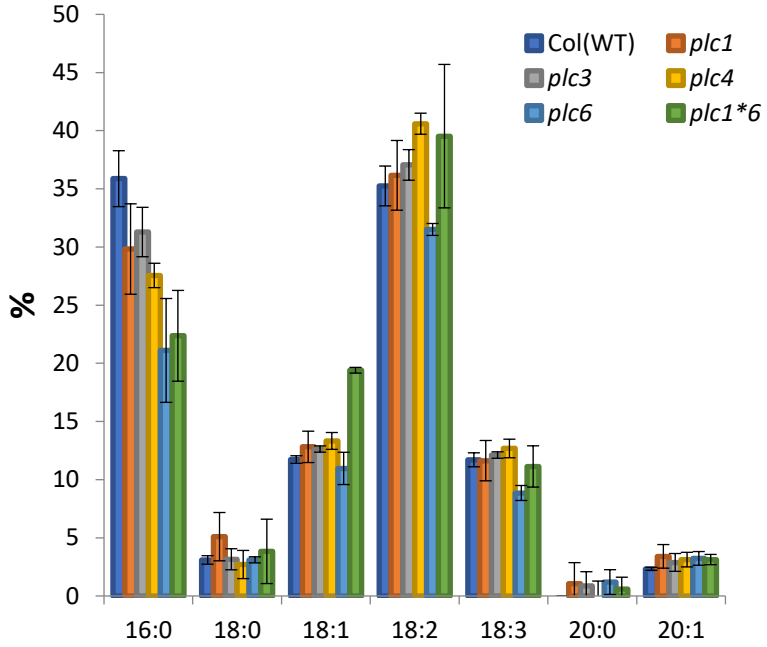
Table 4. FA composition in the mature seeds of Arabidopsis lines. Col is wild type and other lines are mutated for respective genes. The data represent average ± SD for 3 technical replicates and 7 biological replicates. *P<0.05

Line	FA Composition						
	16:0	18:0	18:1	18:2	18:3	20:0	20:1
			wt % of total				
rod1	9.7±0.3	3.7±0.1	32.9±1.1	15.7±0.9	14.3±0.5	2.7±0.1	21.0±0.3
rod1/npc1	9.9±0.6	4.4±0.6	35.2±0.4*	13.2±0.6*	13.7±0.4	2.9±0.1	20.8±0.2

Stereochemical Analysis of Total Lipids

TAG and PC were separated for the total lipids extracted from mature seeds. FA composition was analyzed in these two fractions. For PC fraction, 16:0 was decreased for all mutated lines. There was not much change in other FAs except for an increase in 18:1 and 18:2 for *npc1/npc6 lines* (Fig 5A). When TAG fraction was run in GC and FA composition was analyzed, 18:1 FA was increased for *npc1*, *4, 6* and *npc1/6.* 18:2 was decreased for these lines (Fig 5B). Other FAs were not changed significantly. For *npc3,* FA composition did not change compared to the wild type.

A



B

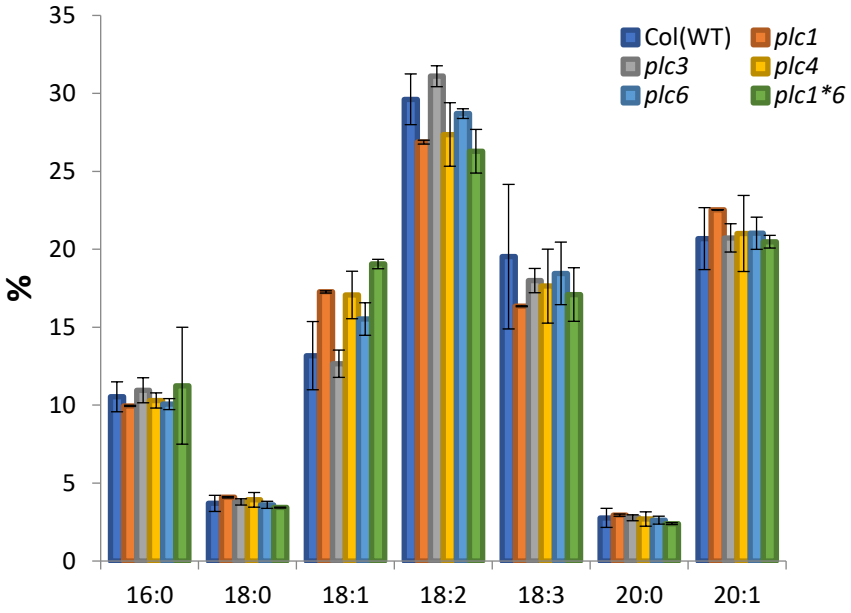


Fig 5. Distribution of FA in A) PC fraction B) TAG fraction of the mature seeds of Arabidopsis lines. The data represents average ± SD for 3 technical and 3 biological replicates.

Mutation of NPCs did not Affect the Total Oil Accumulation

Total oil content per seed was measured for WT, *npc1*, *npc3*, *npc4*, *npc6* and *npc1/6* lines. Total oil was extracted and measured using heptadeaconoic acid (17:0). The oil content did not change significantly among those single and double mutant lines (Fig 6).

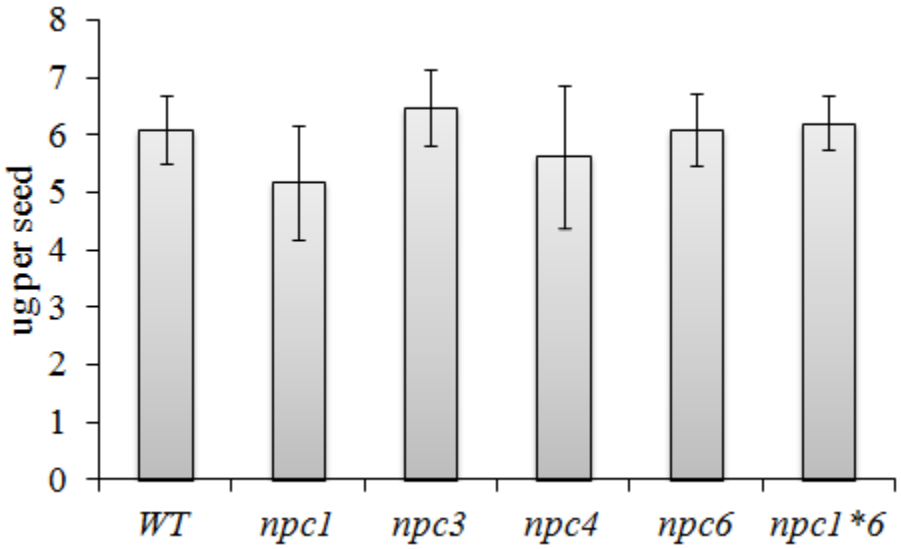


Fig 6. Oil content per seed of the wild type and NPC mutants Arabidopsis lines. Data represents average ± SD for 6 biological replicates.

Conclusion

Plant NPCs were previously studied to show their roles in various plant metabolisms such as stress and plant hormone signaling. The functional analysis had also shown that these enzymes have activity on various substrates including PC. However, their role in the TAG biosynthesis pathway was only predicted earlier but not completely

demonstrated. By analyzing the oil from the mutants, I have shown here that these NPC1, NPC4 and NPC6 have roles in TAG biosynthesis. Decreases in the amount of polyunsaturated fatty acid and increases in monounsaturated FA ie 18:1, which is a de-novo FA that the plastid produces, indicate that NPCs release modified DAG from PC. Further, an increase in 18:1 in *npc1/6* cross suggests that these NPCs are working complementarily with each other. The mutation of NPCs expressed in the seeds together might produce a much clearer observation.

CHAPTER THREE

ROLES OF PLC-LIKE ENZYMES IN TAG BIOSYNTHESIS IN ARABIDOPSIS AND FUNCTIONAL ANALYSIS OF RCPLC1 AND ATPLCL1

Abstract

After examining the expression of PLCs in castor from a previous study (Brown et al. 2012), I selected RcPLCL1 as a candidate for further investigation. PLC likes have not been yet characterized and their roles in TAG biosynthesis pathway are completely unknown. For the substrate-specificity characterization of PLC-likes, RcPLCL1 and AtPLCL1 were put into the pYES2 vector and then transformed into the yeast. The enzyme assay from microsomal extract showed that RcPLCL1 and AtPLCL1 have both PC and PI hydrolyzing activities. To investigate the role of PLC-like phosphodiesterases in oil biosynthesis pathways in the developing seeds of Arabidopsis, I ordered T-DNA lines for 5 closely related PLC-likes. The confirmed homozygous lines were grown side by side with wildtype (col). FA composition was measured for the oil of the mature seeds. I observed the difference in amount of saturated and desaturated fatty acids compared to wild type. 18:1 was increased in the mutants while 18:3 was decreased. This difference might reveal the role of PLC-likes in releasing DAG from the PC pool.

Introduction

Despite the major breakthroughs in the research of TAG biosynthesis in the developing seeds of oilseeds, the pathway still lacks a complete understanding (Bates,

Stymne, and Ohlrogge 2013). Of the several inconclusive steps, the flux of FA in and out of PC, which is considered a bottleneck in acyl-editing pathway still requires to be studied for a clear understanding. This step is also proposed as a major hurdle in accumulating higher amount of unusual fatty acids, such as HFA which are formed in PC, in transgenic plants such as Camelina (Snapp and Lu 2013b). The enzymes PDCT and LPCAT are demonstrated to direct the two third of the 18:1 FA in and out of PC in Arabidopsis (Lu et al. 2009; Bates et al. 2012a). Apart from these two enzymes, phospholipases such as PLCs and PLDs are also proposed to play roles in releasing DAG from PC.

From my study in chapter one, I observed that AtNPCs, which were previously shown to have an PC-hydrolyzing activity, have roles in TAG biosynthesis in Arabidopsis. As my goal is to increase the HFA in transgenic Camelina, I looked at the expression of PLCs in castor plant. After analyzing the roles of AtNPCs in TAG synthesis (chapter 1), my first choice was RcNPCs. RcNPC3 and RcNPC6 were expressed in the different tissues of castor, of which RcNPC6 was expressed in the developing endosperm, which was our focus as the oil is synthesized and stored in the endosperm for castor plant. However, RcNPC6 was predominantly expressed in other tissues; leaves and male flowers, indicating its minor role in TAG biosynthesis pathway in endosperm. Interestingly, PLC-likes were the primarily expressed phospholipase c in the endosperm. Out of 6 phospholipase c expressed, five of them were PLC-like. The highest expression value was for RcPLCL1, which is a novel protein, and has not yet been characterized. Hence, I analyzed the invitro activity of the RcPLCL1 and AtPLCL1

towards PC and PI using yeast expression system. I also examined the roles of AtPLC likes in TAG biosynthesis in Arabidopsis. Eight PLC-like phosphodiesterases proteins are found to be present in the genome of the Arabidopsis. I analyzed the FA composition from T-DNA lines for 5 closely related genes.

Materials and Methods

Enzyme Activity Assay

Genes for *RcPLC* and *AtPLC* (At5g67130) were amplified from cDNAs of castor endosperm and Arabidopsis respectively. The primers for *RcPLC* were GCGAATTCATGTTTGCGTGCTTCGCGGACTACTGTA and CGCCTCGAGTCATAATAAAGATGCCA and the primers for *AtPLC* were GAATTCATGTCGGCGTGCATCAATGGC and TAGTAGAAATATCAGCAACGG. The PCR products were cloned into the pGEMT-Easy vector (Promega, Madison, WI, USA) and sequenced for the confirmation of complete and correct sequences. *AtPLC1*(PI-PLC) and *NPC1* (PC-PLC) were also amplified from Arabidopsis cDNA and put into PGEMT-Easy vector, which were used as control in the experiment. Then all the four genes were cloned into the PYES2 expression vector at the ECor1 and XhoI sites and transformed into InvSc1 expression yeast cells by lithium acetate method (Kang, Snapp, and Lu 2011). The yeast culture of 300 ml was grown by shaking at 30°C for 24 hours in SD-Ura medium containing 2% galactose. Protein purification and enzyme assay was performed according to (Lu et al. 2009). Cells were harvested by centrifugation at 2000 xg for 10 minutes. The pellet was washed with sterile water. Washed cells were then resuspended in ice-cold glucose-Tris-EDTA (GTE) buffer [20% glycerol, 50 mM

Tris-HC1 (pH 7.4), 1 mM EDTA]. Freeze and thaw method was used for releasing the membrane proteins. Solution was frozen using liquid nitrogen for 3 minutes and then thawed rapidly at 42°C. The process was repeated for 3 times. The solution was then centrifuged at 2000 xg for 10 minutes and then the supernatant was collected which was used for further enzyme assay. The microsomes thus obtained were quantified by running a protein gel and by measuring with the nanodrop at 280 nm wavelength. 5-10 ug of protein was used for the enzyme assay. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (di16:0 PC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoinositol (di16:0 PI) were used as the substrates. Substrates were dried under nitrogen gas and resuspended in reaction buffer [final concentrations: 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS)/NaOH (pH 7.5), 20 mM MgCl2, 0.45% Triton X-100]. Microsomes suspended in GTE buffer was added and reaction volume was made to 200 ul using reaction buffer. The reaction was performed at 15°C for 1 hour. The reaction was then stopped by adding 1 ml of chloroform:ethanol :: 2:1 and 1.5 ml of 0.9% NaCl. The mixture was then centrifuged at 2000 xg for 5 minutes and the upper layer was dried with nitrogen gas and dissolved in 100 µl of chloroform. The solution was then run in two-dimensional thin layer chromatography. Hexane: ethyl ether: formic acid (70:30:1) were used as solvents. The origin (PC/PI) and the DAG bands were recovered and analyzed using gas chromatography by creating FAMEs.

Test for Mutation

TDNA lines were obtained from Arabidopsis Biological Resource Center (ABRC). Mutations of genes for these lines were verified from the genomic DNA by

using three primers; left and right genomic primers and left border primer for TDNA (Table 2). Wild type without any TDNA insertion produced a larger DNA band. Confirmed homozygous mutated lines were then analyzed for their FA composition in the seed oil.

Measurement of fatty acid composition and oil content

Fatty acid composition was measured by creating fatty acid methyl esters and then running on gas chromatography. Thirty to 50 mature and dry seeds were heated in 1 ml of 2.5% H_2SO_4 (v/v) in methanol at 80^0C for 90 minutes. Oil was then extracted with 200 ul of Hexane and 1.5 ml 0.9% (v/v) NaCl. The mixture was vortexed briefly to mix and centrifuged. The upper aqueous layer was transferred to autoinjector vials. For agilent 6890 GC fitted with a 30 M* o.25-mm DB-23 column, 1 ul of sample was injected. A GC program of 190^0C for 2 minutes followed by an increase of 8^0C per minute to 230^0C and maintenance for 6 minutes was used. For the measurement of total oil content, 5 ul of 10 mg/ml 17:00 FA (standard) was added to 20 mature seeds prior to heating.

Results and Discussion

Functional Analysis of PLC likes from Castor and Arabidopsis

Phospholipase C (PLC) is a major membrane phospholipid hydrolyzing enzyme. In plants, the PLC class has been divided into two groups: a well-studied phosphatidylinositol-specific PLCs (PI-PLCs) and a recently identified phosphatidylcholine-PLCs (PC-PLCs). Their reactions yield diacylglycerols (DAG) and phosphoinositol or phosphocholine head-groups, respectively (Wang 2001). Previous

studies identified eight genes encoding putative phospholipase C (RcPLC) in the castor plant, out of which six of them were expressed in the developing endosperm (Table 1). From sequence analysis, five of them were categorized as members of PLC-like phosphodiesterase family and one as PC-hydrolyzing phospholipase C. I named those PLC-likes as RcPLCL1 to RcPLCL6. I chose one RcPLCL1 (30015.m001244) that was shown to be highly expressed especially in castor endosperms where HFA-containing oil is stored (Brown et al. 2012; Brown et al. 2011). I hypothesized that this RcPLCL1 may play important roles in oil synthesis in castor seeds and may facilitate accumulation of HFAs. Other putative PLCs were primarily expressed in leaves and/or male flowers, but very low or no expression in developing endosperm of castor (Brown et al. 2012).

When a phylogenetic tree was made using the software, MEGA, PLC-likes are found to be widely distributed among the plant kingdom (Fig 1), which predicts that these proteins have their long and independent evolutionary history. In *Arabidopsis*, 8 sequences were predicted from the genome that may be grouped under the PLC-like phosphodiesterase family (Table 2). None of these proteins are characterized yet. They are 36 to 46 kDa in molecular weight. Most of these proteins contain signal peptide for secretary pathway. When the phylogenesis of these proteins were compared to the PI-PLCs and NPCs in the Arabidopsis, they formed a clear distinct group from 6 NPCs and 9 PI-PLCs proteins reported (Fig 2).

The NCBI conserved domain search put the gene into a PI-PLCc-GDPD_SF superfamily. Analyzing the protein sequence using Prosite at ExPasy

(https://prosite.expasy.org/), PLC-like proteins showed an X domain with two Histidine active sites. However, it did not predict Y and C2 domain of PI-PLCs (Fig 3).

When the molecular functions for At5g67130 was searched for in SCOP hierarchy, Superfamily HMM server (Wilson et al. 2007), phosphoric diester hydrolase activity, phospholipase activity, phosphoric ester hydrolase activity, phospholipid binding, phosphatidylinositol-4,5-biphosphate binding were predicted (Table 3). This suggests that these new sets of phospholipases might have a wide range of molecular functions.

To determine its biochemical activity, I cloned the *RcPLC* and the homologue *AtPLC* cDNA into a PYES2 vector and expressed in the yeast cells. The previously characterized AtPLC1 (PI-PLC) and NPC1 (PC-PLC) were used as the control. Microsomal preparations were incubated with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (di16:0 PC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (di16:0 PI) substrates. As shown in Fig. 4, DAG was detected in reactions of both RcPLC and AtPLC with both PI and PC substrates. The DAG bands were analysed with GC for the fatty acid composition. Fatty acid composition of DAGs was the same as those in the PI or PC substrates. These results suggested that the RcPLC and AtPLC possessed both PI-PLC and PC-PLC phospholipid hydrolyzing activities.

Table 1. List of Phospholipase C expressed in the developing endosperm of *Ricinus communis*. [a], [b], [c] Informations are from Brown et al. 2012. [d] Predicted using TargetP Program (http://www.cbs.dtu.dk/services/TargetP) Secretory, contains signal peptide for the secretory pathway.

Gene name	**Gene ID**	**Arabidopsis homolog**	**Amino acid identity (%)**	**Endosperm Stage II/III**	**Endosperm Stage IV/V**	**Germinating seed**	**Leaf**	**Male flowers**
RcPLC-Like 1	30115.m001244	At5g67130	69	49.50	38.33	43.21	31.74	24.53
RcPLC-Like 2	29801.m003166	At1g49740	71	9.56	24.59	5.31	6.26	23.92
RcPLC-Like 3	29847.m000250	At1g13680	63	2.55	1.34	1.00	74.04	-
RcPLC-Like 4	30174.m009086	At1g13680	64	1.10	1.63		-	244.03
RcPLC-Like 5	29847.m000249	At1g13680	62	0.58		-	1.50	-
RcPLC-like 6	29756.m000515	At4g48690	64	-	-	12.43	64.21	15.78
RcNPC3	30148.m001447	At3g03530	63	-	-	-	-	0.83
RcNPC6	30147.m014488	At3g48610	83	14.63	8.31	8.31	45.04	65.71

Table 2. List of PLC-like genes from Arabidopsis that are members of "uncharacterized"/" sub-family not named" sub family of proteins. [a] Predicted using PANTHER classification system (http://pantherdb.org/tools/hmmScoreForm.jsp?). [b] Predicted using TargetP Program (http://www.cbs.dtu.dk/services/TargetP) Secretory, contains signal peptide for the secretory pathway.

Gene id	**Gene name[a]**	**Characterization[a]**	**aa length**	**Molecular weight (kDa)**	**Localization[b]**
RcPLC (30115.m001244)	RcPLCL1	Uncharacterized	420	45.97	Secretory
AT5G67130	AtPLCL1	Uncharacterized	427	46.6	Secretory
AT3G19310	AtPLCL2	Uncharacterized	414	45.86	Secretory
AT1G13680	AtPLCL3	Uncharacterized	347	38.79	Secretory
AT1G49740	AtPLCL4	Uncharacterized	360	39.83	Secretory
AT4G36945	AtPLCL5	Uncharacterized	409	44.33	Secretory
AT4G34930	AtPLCL6	Uncharacterized	392	44.34	Chloroplast
AT4G38690	AtPLCL7	Uncharacterized	319	36.29	Other
AT4G34920	AtPLCL8	Uncharacterized	319	36.40	Other

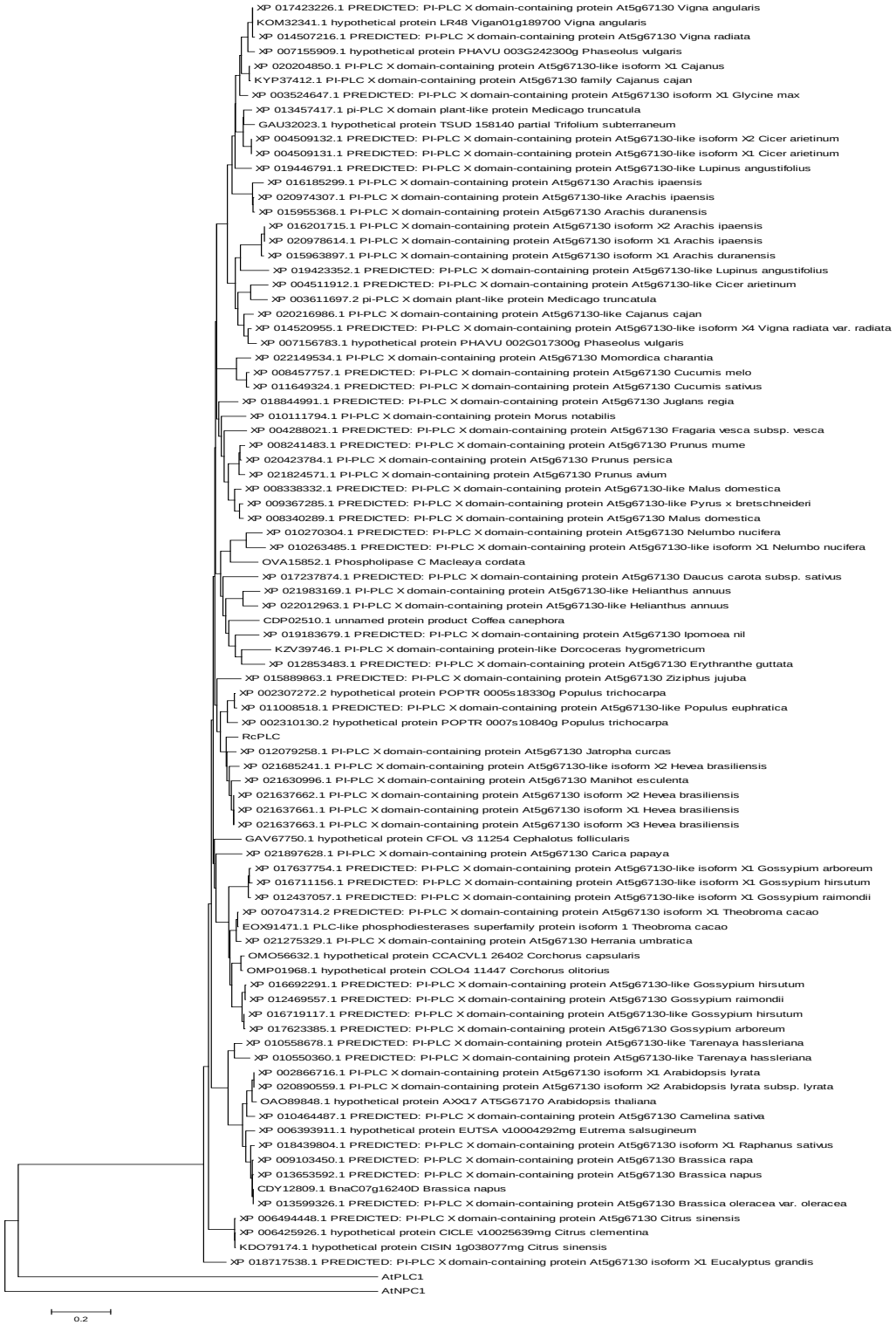


Fig 1. Phylogenetic tree for PLC-like phosphodiesterases from across different plants. The sequences were obtained from NCBI.

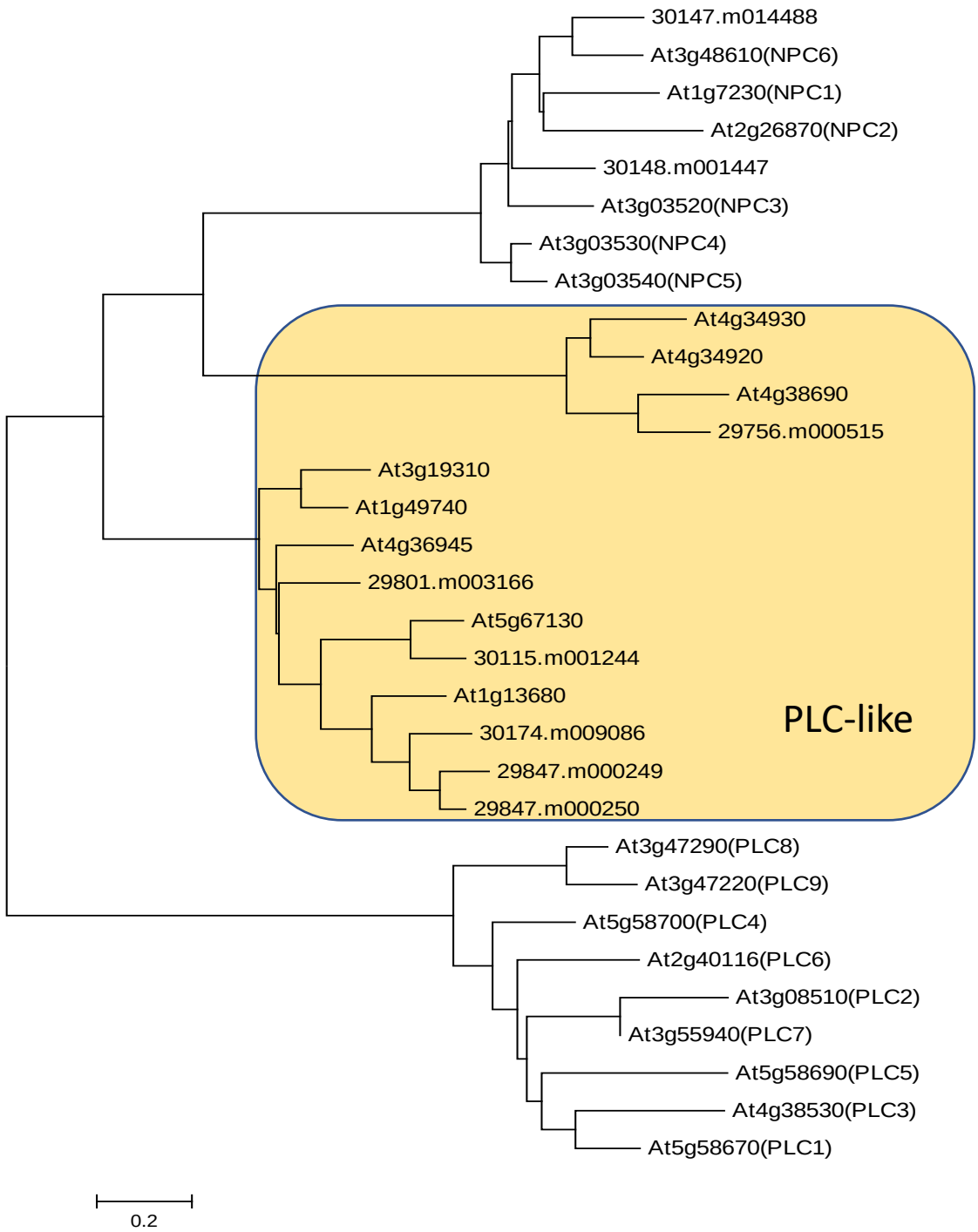


Fig 2. Neighbor-joining tree for putative PLCs in castor and their in Arabidopsis.

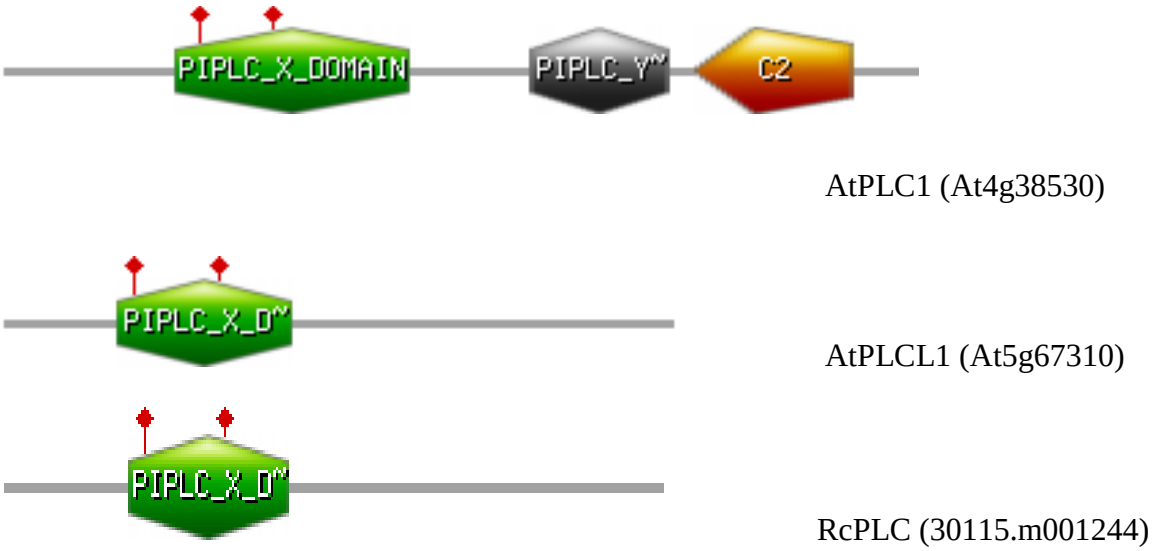


Fig 3. Domain annotation of PI-PLC and PLC-like phosphodiesterase proteins. The prediction is drawn using ExPasy, Bioinformatics and Recourse Portal.

Table 2. Molecular function for the protein At5g67130. Prediction is generated by SCOP hierarchy, Superfamily HMM server and genome assignments server (http://supfam.org/SUPERFAMILY/cgi-bin/allcombs.cgi?genome=ATL;sf=51695;;password=;subdomain=n).

Molecular Function	IC (bits)	H-Score
Phosphoric diester hydrolase activity	1.864	149.3
phospholipase activity	1.966	144.4
phosphoric ester hydrolase activity	1.494	72.24
phospholipid binding	1.435	6.43
phosphatidylinositol-4,5-biphosphate binding	2.029	6.43

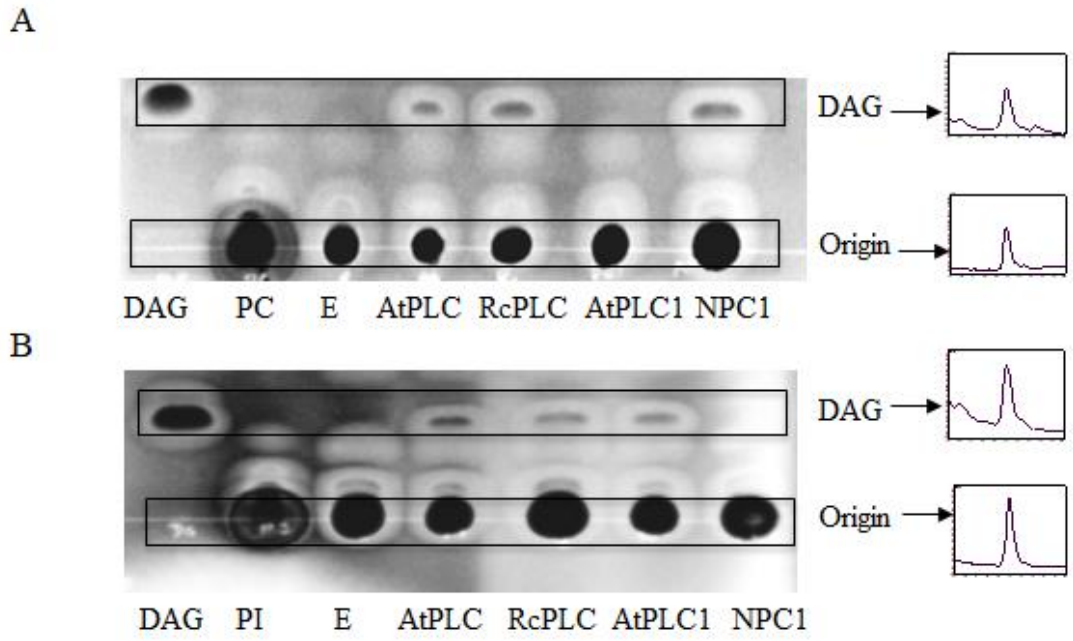


Fig 4. Phospolipase activity assay for AtPLC and RcPLC. Lipids were separated on TLC plates after being extracted from reactions with substrates Enzyme assay for AtPLC and RcPLC. A, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (16:0 PC); B, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoinositol (16:0 PI. E, PYES2 empty vector; AtPLC, PYES2-AtPLC; RcPLC, PYES2-RcPLCAtPLC1, PYES2-AtPLC1; NPC1, PYES2-NPC1. On the right, peaks are obtained by scrapping from TLC and running on GC of the origin and DAG bands. The 16:0 peaks in the origin and the DAG bands confirms that the product are from the origin.

FA Composition Altered in Oil from Mature Seeds of Mutants Compared to Wild Type

To investigate the roles of PLC-likes in TAG synthesis pathway in Arabidosps, I ordered T-DNA lines for five PLC-likes that were most closely related; AtPLCL1 to AtPLCL5 (alignment figure in appendices). Expression data from the TAIR showed that these these five genes were well expressed during the different stages of Arabidopsis seed development (figure in appendices).

FA composition from the mature seeds of homozygous line were then analyzed. FA composition for the mature seeds of mutant lines differed from the wild type (Table 3,

Fig 6). 16:0 and 18:0 FA were found to be similar among the wild type, *At1g49740* and *At4g36945* but was increased for *At1g13680, At5g67130* and *At3g19310*. 18:1 was increased in all the mutants. *At3g19310* had the highest increase; 19.07% against 16.07 % in the wild type. 18:2 was not changed much. 18:3 was decreased in the mutant lines. *At5g67130* had the lowest amount of 18:3; 15.18% against 18.09% in the wild type. 20:0 and 20:1 FA did not change much for the mutants when compared to the wild type. These changes in the FA composition suggest the role of PLC-like phosphodiesterases in the oil biosynthesis pathway of the developing Arabidopsis seeds. The decrease in polyunsaturated FAs such as 18:3 and increase in saturated FA like 16:0 and 18:0 may reveal the functions of these enzymes in accumulating the modified fatty acid in the oil. Non-specific phospholipase C (NPCs) have been predicted before to release the DAG from PC pool which contains the modified fatty acids. An increase in 18:1 and decrease in 18:3 projects towards the role of these PLC-likes in releasing the DAG from PC.

Table 3. Germplasm of the T-DNA lines for PLC-like phosphodiesterases and the primers used for confirmation of the homozygous mutant.

Gene	**Germplasm name**	**Primers**	**Size**
AT4G36945	SALK_026986	LP: ATCAGCTTTGCCACTGATCTC RP: CGGTTGATGATATGGTGAAGC	PRODUCT_SIZE 1011 BP+RP_PRODUCT_SIZE 486-786
AT1G13680	SALK_048688	LP: GCTATTCCTTCGGTTTCTTGC RP: AGGCTTGATCTGCAACTCTTG	PRODUCT_SIZE 1022 BP+RP_PRODUCT_SIZE 467-76
AT3G19310	SALK_128054	LP: TCTCCACATAAACGCAATTCC RP: CTTAGCCCTTCATATGCCCTC	PRODUCT_SIZE 1105 BP+RP_PRODUCT_SIZE 518-818
AT5G67130	SALK_209557	LP: GTCAGGAGGGAATAGAATGCC RP: CTCTTTTCGCAATTGCAGAAC	PRODUCT_SIZE 1209 BP+RP_PRODUCT_SIZE 557-857
At1g49740	SALK_086530	LP: ACTATCCACATTTGCCGTCAG RP: TTGCAGAATGGTGTAAGAGGG	PRODUCT_SIZE 1103 BP+RP_PRODUCT_SIZE 553-853

Table 4. FA composition of oil from mature seeds of wild type and the mutated lines. Data represent average±SD for 10 individual plants. *P<0.05.

	16:0	18:0	18:1	18:2	18:3	20:0	20:1
Col	7.97±0.3	2.66±0.1	16.70±1.1	29.49±0.7	18.09±0.7	2.35±0.2	22.74±0.5
At1g49740	7.95±0.3	2.84±0.2	18.50±1.8*	29.10±0.2	16.84±0.9*	2.37±0.1	22.41±0.4
At4g36945	7.40±1.5	2.97±0.3	18.88±1.6*	29.69±1.3	16.91±0.6*	2.05±0.9	22.11±1.1
At1g13680	10.24±3.6*	5.26±3.0*	18.13±0.5*	26.23±3.0	15.49±1.6*	3.23±1.4	21.43±2.6
At5g67130	11.24±2.6*	5.64±2.1*	18.86±0.5*	26.40±2.2	15.18±1.2*	2.65±0.2	20.03±2.0
At3g19310	10.82±2.1*	5.40±1.7*	19.07±0.7*	26.45±1.8	15.39±1.2*	2.63±0.3	20.24±1.5

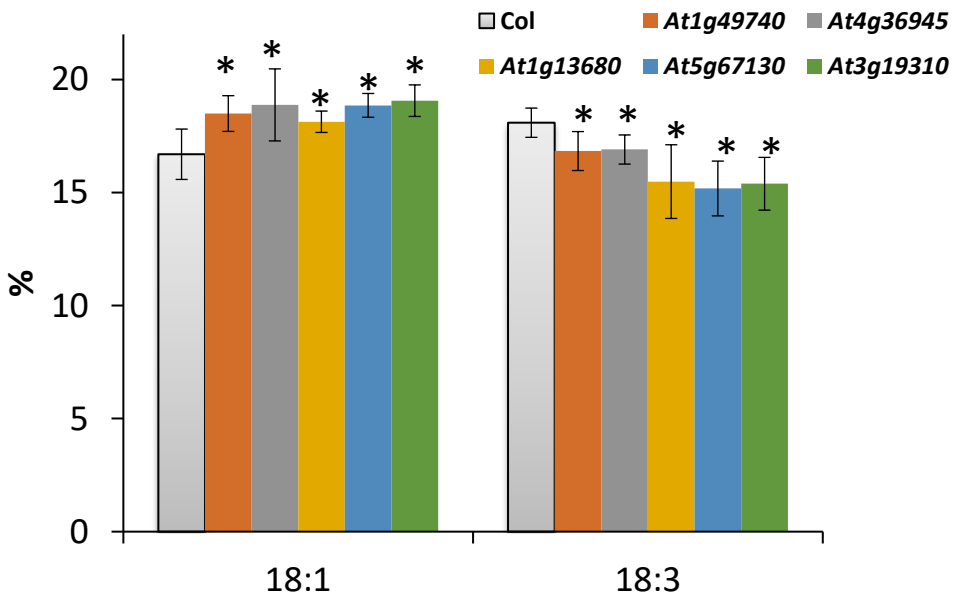


Fig 5. 18:1 FA and 18:3 FA for wild type and mutated lines. Data represent average±SD for 10 biological replicates. * P<0.05.

Conclusions

Plant phospholipases have important roles in routine metabolism of plants. These enzymes primarily hydrolyze the phospholipids which are the principle components of the membranes, such as primary cell and organelle membranes. Additionally, they serve as precursors of secondary messengers, associated with the fatty acid signaling pathway and for the formation of storage lipids (Xue, Chen, and Mei 2009). PLC particularly cleaves the head groups from phospholipids. In plants, two groups of phospholipids, PI-PLCs and NPCs are extensively studied. They have been found to play a diverse role in signaling pathway in response to different stresses (Chapter 1). A different set of phospholipases, PLC-likes are identified from the genomic sequences. These sequences have not been yet studied across the plant kingdom.

Previously, NPCs were characterized and shown that they have an activity on PC. I demonstrated that they might release the DAG from membrane PC in the developing seeds of Arabidopsis and thus contribute in the flux of polyunsaturated fatty acids out of PC (Chapter 2). However, these NPCs were predominantly expressed in the tissues other than endosperm of the HFA accumulating Castor plant. Indeed, PLC-likes were found to be expressed primarily in the developing endosperm. RcPLCL1, a strong candidate, and its closest homologue in Arabidopsis AtPLCL1 were further studied. PLC-likes were found to have been widely present in the plant kingdom and have an independent evolutionary existence. These proteins were found to be completely different from PI-PLCs and NPCs in Arabidopsis. Bioinformatic studies suggest that these proteins have phosphodiester activity. An in vitro characterization using yeast system showed that

PLC-likes possess both PC and PI activity. Characterization of PLC-likes were done using yeast expression system. The assay showed that PLC-likes have both PC and PI activity. It requires further research at the aminoacid level for these proteins to have a complete understanding of the mechanisms of PC and PI activity.

Furthermore, the mutation experiment of five PLC-likes in Arabidopsis showed that polyunsaturated FA (18:3) was decreased and monounsaturated FA (18:1) was increased in the seed oil. This suggests the role of PLC-likes in TAG synthesis in the developing seeds of Arabidopsis probably by releasing DAG from membrane PC.

This study, hence, identified and characterized the novel PLC-likes proteins in plant. It also demonstrated their roles in TAG-biosynthesis in the Arabidopsis seeds. The study also provided a suitable candidate enzyme to transform into the transgenic Camelina for increasing the amount of HFA in TAG.

CHAPTER FOUR

A PHOSPHOLIPASE C LIKE PROTEIN FROM *RICINUS COMMUNIS* INCREASES HYDROXY FATTY ACIDS ACCUMULATION IN TRANSGENIC SEEDS OF *CAMELINA SATIVA*

Abstract

There have been strong interests in producing unusual fatty acids in oilseed crops to provide renewable industrial feedstocks. Results are so far largely disappointing since much lower amounts of such fatty acids accumulate in genetically engineered seeds than in their original natural sources. Additional genes besides those encoding fatty acid modifying enzymes are needed to boost unusual fatty acid production in transgenic seeds. I show here that a phospholipase C like enzyme (RcPLCL1) from castor bean, which accumulates nearly 90% of the hydroxylated ricinoleic acid in its seed oil, increase the hydroxy fatty acids (HFAs) amount in transgenic camelina seed expressing the fatty acid hydroxylase (FAH12). RcPLCL1 has activities on both phosphatidylcholine (PC) and phosphatidylinositol substrates in our in vitro assay conditions (Chapter 3). HFAs are synthesized on PC. The PC-PLC activity of the RcPLCL1 increased the efficiency of HFA-PC to diacylglycerol conversion, thus increased HFA contents in triacyglycerols. The decreased HFA in the membrane lipid PC during seed development may also alleviate the potential detrimental effect of HFA on germination of the engineered camelina seeds. My results provide an additional tool to engineer elevated levels of HFAs in transgenic oilseeds.

Introduction

Triacylglycerols (TAGs) accumulated in seeds represent an important source for human consumption and industrial uses (Lu et al. 2011). Hydroxy fatty acids (HFAs) such as ricinoleic acid (12-hydroxyoctadec-cis-9-enoic acid; 18:1OH) and its derivatives are among the unusual fatty acids found in the seeds of castor (*Ricinus communis*) and a few other plant species such as *Physaria fendleri*, *Agonandra brasiliensis* and *Poliothyrsis sinensis* (https://phylofadb.bch.msu.edu/) (Atsmon 1989; Snapp and Lu 2013a; Horn et al. 2016b; Li et al. 2017). HFAs are a very valuable renewable resource for the chemical industry (Mutlu and Meier 2010), however, native plants like castor and Physaria have several drawbacks which limit their commercial production (Severino et al. 2012; Dierig et al. 2011). Production of such unusual fatty acids in transgenic oilseed crops may provide a vital alternative.

Despite decades of research, unusual fatty acids only accumulate to limited amounts in transgenic seeds that are far below the levels found in their native species (Snapp and Lu 2013a; Horn et al. 2016b). Transgenic Arabidopsis and camelina (*Camelina sativa*) expressing the castor fatty acid hydroxylase (RcFAH12) only produced 10-17% of HFAs in seeds, compared to nearly 90% in the castor oil (Lu et al. 2006; Lu and Kang 2008a; Snapp et al. 2014). However, engineering medium-chain fatty acids represents one of the few relatively successful efforts as lauric acid (12:0) can accumulate to nearly 60% of the TAGs in transgenic rapeseed by introducing an acyl-acyl carrier protein thioesterase, which terminates the fatty acyl chain elongation in the plastid (Voelker et al. 1996). The unusual acetyl-TAGs may also be accumulated to 70% of seed

oils in transgenic camelina and soybean expressing an *Euonymus alatus* diacylglycerol acetyltransferase (EaDAcT), which adds an acetate instead of a long-chain fatty acid at the *sn*-3 position on diacylglycerols (Liu et al. 2015). Unlike these unusual TAGs, HFAs are produced by modifying fatty acids (e.g., oleic acid; 18:1) esterified on phosphatidylcholine (PC) before being incorporated into TAG. In castor, this is done by an endoplasmic reticulum-located RcFAH12, a homolog of the fatty acid desaturase2 (FAD2) (van de Loo et al. 1995). Similarly, other divergent forms of FAD2 catalyze the introduction of triple or acetylenic bonds, epoxy groups, conjugated and *trans* double bonds on C18-fatty acids on PC (Jaworski and Cahoon 2003b). Therefore, inefficient flux of HFA and other unusual fatty acids from PC and subsequent incorporation onto TAG present a significant bottleneck for the accumulation of high amounts of such fatty acids in transgenic seed oils (Bates and Browse 2011). Consequently, this inefficiency may also inhibit fatty acid synthesis that resulted in low oil content in transgenic seeds (Bates and Browse 2011; Bates et al. 2014), and affect seed vigor with low germination ability (Dauk et al. 2007). By co-expressing with RcFAH12, a number of genes originated from the native HFA-accumulating species significantly increased HFA accumulation in transgenic seed, and at least partly restored the oil content and seed germination potential (Lu et al. 2006; Snapp et al. 2014; Hu, Ren, and Lu 2012; van Erp, Bates, Burgal, et al. 2011; Burgal et al. 2008b). These results suggested that many enzymes may have co-evolved with the RcFAH12 to specifically facilitate HFA metabolism in seed. Reducing the competition of endogenous isozymes that have low

specificity to unusual fatty acids may further increase their accumulation in transgenic seed (van Erp et al. 2015b).

Modified fatty acids on PC may enter the acyl-CoA pool for TAG assembly by the so-called acyl editing enzymes including lysophosphatidylcholine acyltransferases (LPCAT), acyl-CoA:glycerophosphocholine acyltransferases (GPCAT), and phospholipase A2 (PLA2) (Bates et al. 2012a; Lager et al. 2015; Bayon et al. 2015). PC may also be converted to diacylglycerol (DAG), the immediate precursor of TAG, by the head group exchange using the phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) (Lu et al. 2009). In addition, the diacylglycerol cholinephosphotransferase (CPT) and phospholipases C and D (PLC, PLD) may also play roles during the PC-DAG conversion. It was previously demonstrated that PDCT is responsible for nearly 40% of 18:1 entering PC for desaturation (Lu et al. 2009), and overexpression of a castor PDCT increased HFA accumulation in transgenic Arabidopsis seed (Hu, Ren, and Lu 2012). In this study, I investigated the function of a castor phospholipase C like (RcPLCL1) in PC-DAG conversion (Fig 1) and demonstrated that it increased HFA accumulation in transgenic camelina seed expressing a RcFAH12.

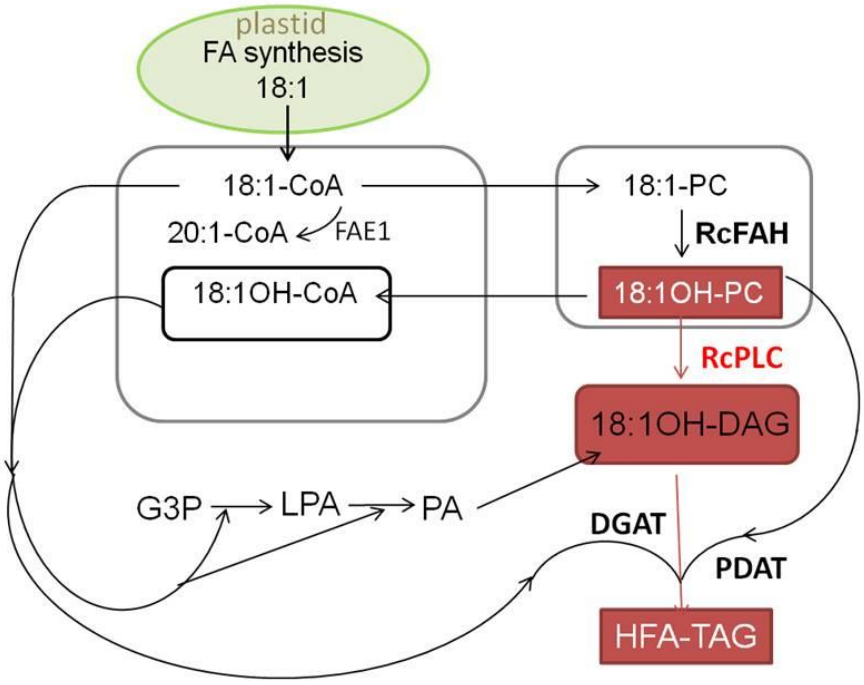


Fig 1. Proposed pathway for the accumulation of HFA in the camelina developing seeds coexpressing RcFAH and RcPLC.

Material and Methods

Plant Material and Green House Growth Condition

Camelina sativa RcFAH (#7-1) was produced previously by transforming a castor fatty acid hydroxylase (RcFAH12) under a seed-specific phaseolin promoter and the DsRed fluorescent marker (Lu and Kang 2008a). An untransformed camelina variety, Suneson, was used as the wild type plant line. Plants were grown in a 6" or 8" pot containing a 1:1 mix of MSU soil (equal parts by volume of loam soil : washed concrete sand : Canadian sphagnum peat moss with AquaGro 2000 G wetting agent blended in at 1 lb/cubic yard of soil. Aerated steam pasteurized at 70ºC for one hour) and Sunshine

Mix #1 (Bellevue, WA, USA). Greenhouse conditions were 22º/18ºC +/- 1 ºC for day/night temperatures, a relative humidity of 30%, and a 16-hour photoperiod of natural lighting supplemented when necessary by season.

Plasmid Construction and Plant Transformation

A fragment of ~1.2 Kb of the *RcPLC* gene was amplified from the castor cDNA library constructed previously (Lu et al. 2006) using primers ATGTTTGCGTGCTTCGCGGACTACTGTA and TAATAAAGATGCCATTAGTGAAA, and inserted into a binary vector, PinGlyBar1 at the restriction sites *EcoR*I and *Xho*I between the soybean Glycinin promoter and Glycinin terminator. The vector contained Kanamycin resistance for the *E. coli* selection and *bar1* for transgenic plant selection. The plasmid was transformed into the Agrobacterium strain GV3101, and transgenic plants were obtained by vacuum infiltration method as described (Lu and Kang 2008a). Seed from T0 plants was harvested, and then planted directly in flats to screen transgenics by spraying with glufosinate herbicide at a concentration of 23.4 ml/L (5.78% glufosinate ammonium, Power Force Grass and Weed Killer, Bayer, Birmingham, AL, USA). Surviving plants were also tested for the presence of the *RcPLC* gene by PCR using the above gene specific primers. The individual plants showing the highest hydroxy fatty acid amount were advanced to the T_4 generation to obtain homozygous lines.

Lipid Extraction and Fatty acid Analysis

Fatty acid methyl esters (FAMEs) were created from the total lipids of the mature seeds using: (1) TMSH method: Trimethylsulfonium hydroxide (TMSH) preparation was used for the 96-well plate screenings as described previously (Lu et al. 2006). (2) Acid derivation method: FAMEs were derived from seeds in 1 ml of 2.5% sulfuric acid in methanol at 80ºC for 90 min (Browse, McCourt, and Somerville 1986). FAMEs were injected into a Shimadzu 2010 GC fitted with a narrowbore column (HP-Innowax; 30 m × 0.25 mm i.d. × 0.25 μm; Agilent Technologies). The oven temperature was programmed at 190ºC initially followed by an increase of 20ºC/min to 250ºC and maintained for 9 min.

For stereochemical analysis, total lipids were extracted from a total of 15 seeds using a modified Blight and Dyer method (Hu, Ren, and Lu 2012). HFA-containing TAG (1OH TAG, 2OH TAG) and normal TAG were then separated by Thin Layer Chromatography (TLC) on silica gel plates (silica gel 60, 20x20cm, EMD Chemicals, Darmstadt, Germany) using the solvent system of 70:30:1 v/v Hexane : anhydrous ethyl ether : formic acid (Snapp et al. 2014). The separated bands were scrapped off the TLC plates and the above acid derivative method was used to analyze FAMEs by GC. Phospholipids (mainly PC) that remained at the sample loading spots on the TLC plates were also analyzed by GC.

Oil Content Measurement and Germination Test

Seed oil content was determined by a bench-top NMR seed analyzer (MQC23, Oxford Instruments, Concord, MA, USA) and by GC analysis using heptadecanoic acid

(17:0) as internal standard (10 mg/ml), which was added to test tubes prior to FAMEs derivatization.

For germination tests, 100 seeds from each line were placed on wet filter papers in covered petri dishes. Water was replenished as needed when filter paper dried and seeds were left to germinate for 7 days at room temperature. Germination was determined by radicle emergence.

Phylogenetic Analysis

A neighbor-joining tree was vreated using MEGA6 (Tamura et al. 2013). Sequences for different PLCs were obtained from NCBI (https://www.ncbi.nlm.nih.gov/) and the Arabidopsis TAIR (http://www.arabidopsis.org/) websites. The sequences were then aligned by ClustalW using the default parameters. Unaligned long flanking sequences were chopped off for both sides and a neighbor joining tree was built.

Results and Discussion

RcPLC Increases HFA Accumulation in Transgenic Camelina

To determine the role of RcPLC in HFA accumulation, its coding sequence was cloned into a plant transformation vector under a strong seed-specific glycinin promoter (Sengupta-Gopalan et al. 1985), and transformed into a camelina expressing fatty acid hydroxylase gene *RcFAH12* (7-1). A previously generated camelina line #7-1 (RcFAH) contains 15% HFA (Lu and Kang 2008a). The seed fatty acid composition was analyzed using gas chromatography (GC). Single seed analysis from different independent transgenic lines showed that HFA content in the oil of RcFAH-RcPLCL1 lines were

increased to as high as 26 % (Fig 1). I obtained 13 transgenic lines. HFA content was increased in all the transgenic lines ranging from 19-25% (Fig 2). AtPLCL1, the closest homologue of RcPLCL1 in Arabidopsis, was also transformed to the Camelina expressing RcFAH12 as a control. However, HFA was not increased for RcFAH-AtPLC1 (Fig 5). This could be because RcPLCL1 and AtPLCL1 were co-evolved in Castor and Arabidopsis respectively and hence they have different substrate preferences, but it requires in- depth experimentation at amino acid level for these enzymes to conclude on that.

I continued to characterize the RcPLCL1 plants. Comparision of FA composition among RcFAH and RcFAH-RcPLCL1 lines demonstrated that polyunsaturated fatty acids were decreased and HFA were increased for RcPLCL1 lines. FA profiling by GC indicated that the average HFAs, including ricinoleic acid (18:1OH), densipolic acid (18:2OH), lesquerolic acid (20:1OH), and auricolic acid (20:2OH), in RcFAH-RcPLCL1 transgenic lines significantly increased compared to those in the RcFAH line (Fig 3). Equivalently, linoleic (18:2) and linolenic acid (18:3) were decreased in RcPLCL1 lines. This shows that that the increase in HFAs was in expense of polyunsaturated FAs. Fatty Acid Desaturases (FADs) and RcFAH12 both utilize PC as a substrate. RcPLCL1 from Castor might have predominant preference to the HFA which might have caused primary release of HFA containing DAGs from PC. This type of substrate specificity has been demonstrated in caster genes, e.g., RcPDAT1A, RcDGAT2, RcPLA2a (Burgal et al. 2008b; van Erp, Bates, Burgal, et al. 2011; Bayon et al. 2015)

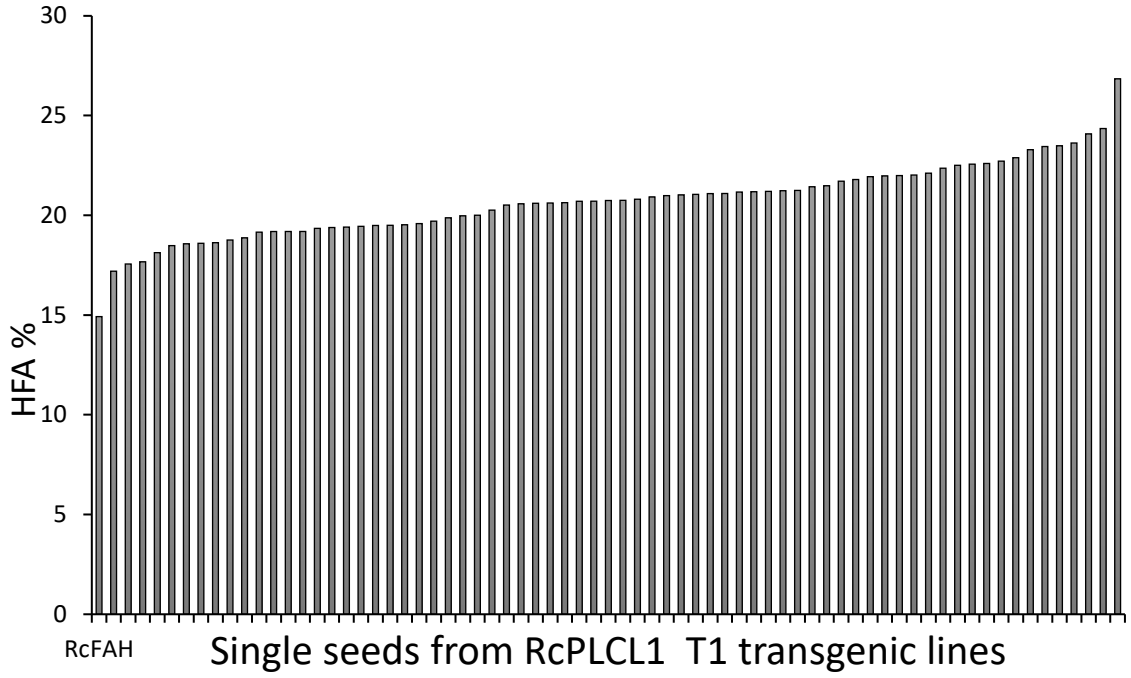


Fig 2. Accumulation of HFA in single seeds from different T1 transgenic lines.

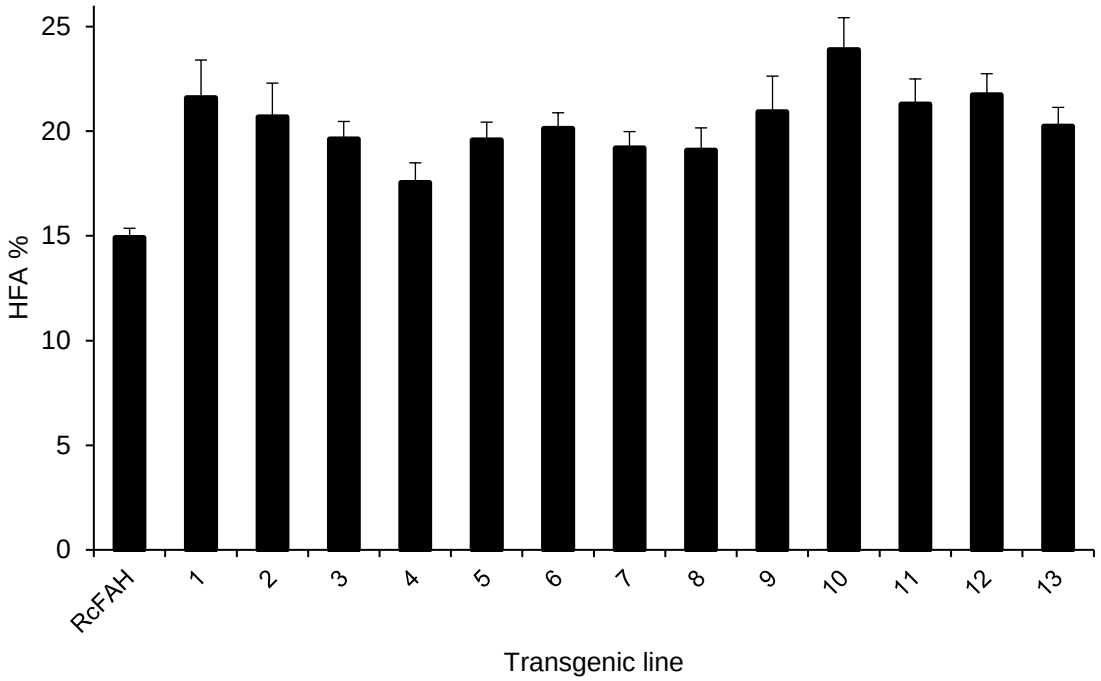


Fig 3. Accumulation of HFA in independent 7-1-RcPLCL1 transgenic lines. Data represents average±SD.

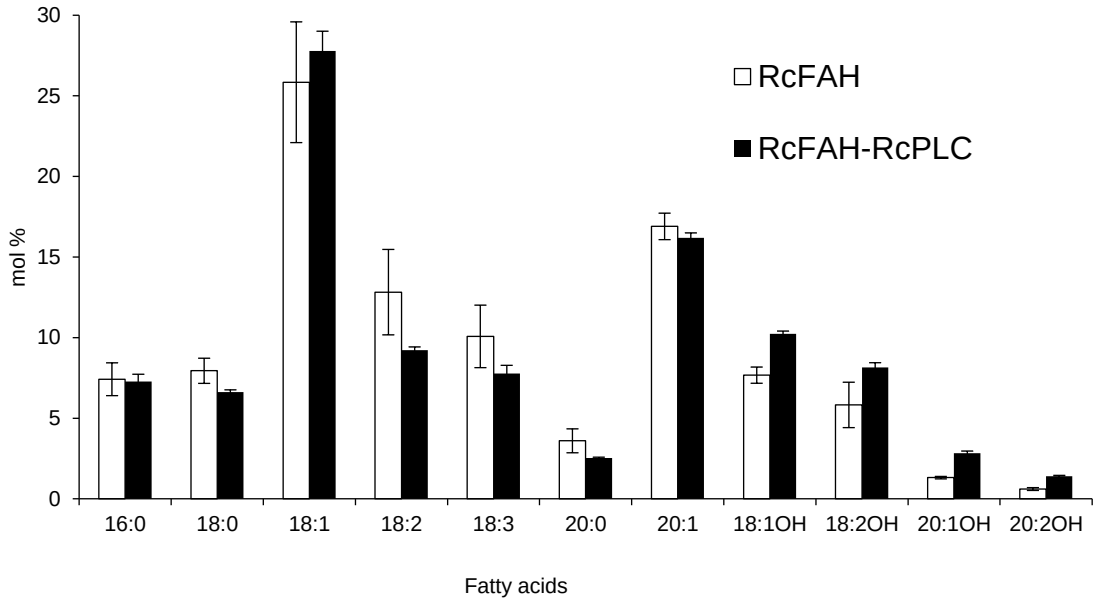


Fig 4. Fatty acid profiles of camelina transgenic seeds. Open bars: RcFAH, closed bars: RcFAH-RcPLCL1. Data are average ± SD.

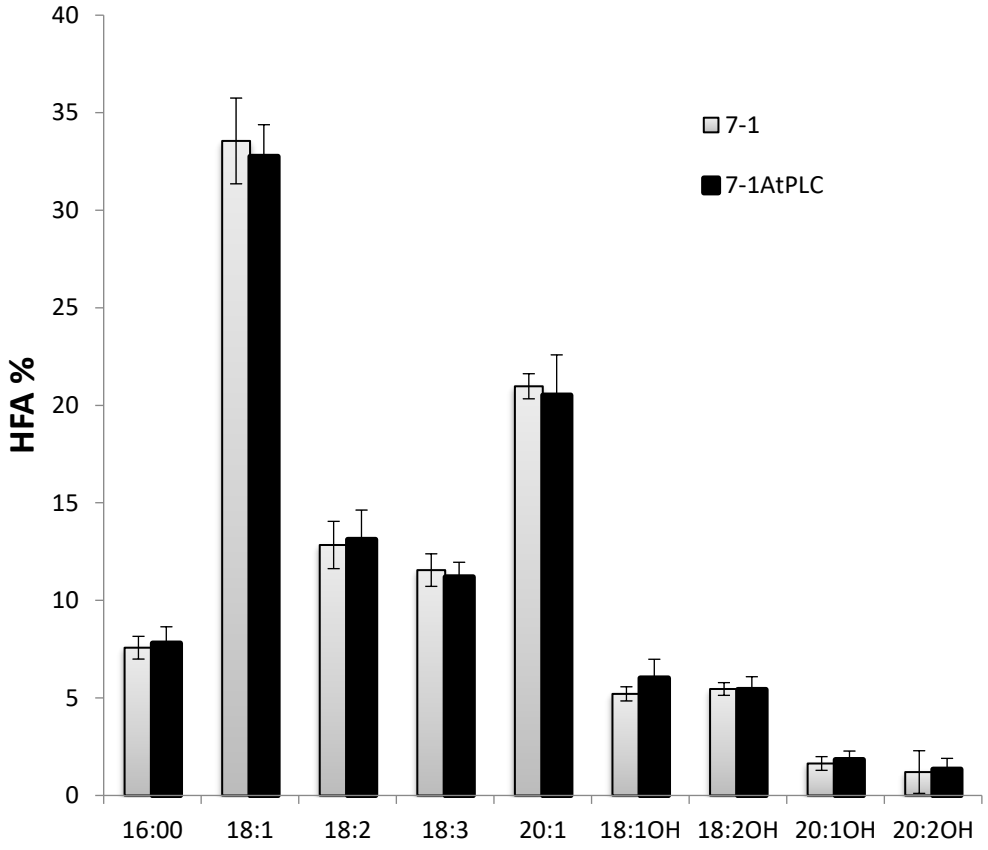


Fig 5. Fatty acid profiles of camelina transgenic seeds. Open bars: RcFAH, closed bars: RcFAH-AtPLCL1. Data are average ± SD.

HFA Accumulation During Camelina Seed Development

The above results demonstrated that RcPLC increased HFA accumulation when co-expressed with RcFAH in camelina seeds. RcPLC was placed under the family PLC-like phosphodiesterases, but our analysis indicated that it could also hydrolyze the PC substrate in our assay conditions. The PI-PLC activity may produce the signaling molecules phosphoinositols that potentially regulate biochemical processes. Though I could not rule out this possible effect on HFA metabolism, it is more likely that the PC-PLC metabolic activity of RcPLC in transgenic camelina could enhance the conversion of

HFA-containing PC into HFA-DAG, thus increased HFA accumulation in seed TAG. To test this hypothesis, I examined HFA accumulation during seed development. As shown previously, camelina seeds rapidly synthesize storage lipids during 8-30 days after flowering (DAF) (Rodriguez-Rodriguez et al. 2013). The RcFAH12-expressing camelina seeds accumulated less oil than the wildtype plants, and the most rapid TAG synthesis occurred during 10-20 DAF (Horn et al. 2016b). I excised seeds from 12, 16, and 20 DAF from the plants of RcPLC-RcFAH and RcFAH. Total oils were directly analyzed by GC using whole seeds, and PC fractions were analyzed after separating from neutral lipids on TLC plates as described previously (Snapp et al. 2014). As shown in Fig. 6A, HFA accounted for nearly 5% of total FAs in seed oils at 12 DAF and steadily increased as seeds matured in both groups of transgenic plants. At all developmental stages RcFAH-RcPLCL1 seeds contained significantly more amounts of HFAs compared to the RcFAH seeds collected at the same time points. HFAs in the PC fraction also increased during seed development, however, their amounts started to differ after 16 DAF with significantly lesser accumulation found in the RcFAH-RcPLCL1 seeds than the RcFAH controls. In mature seeds, RcFAH-RcPLCL1 seeds contained ~5% HFAs in the polar PC fraction, while the RcFAH seeds contained nearly 7.5% HFA in the PC (Fig. 6B). These observations clearly indicated that RcPLC increased the efficiency of HFAs removal from PC, where they were synthesized by the RcFAH, by converting to DAG and resulted in more HFA accumulation in the TAG of the RcPLC-expressing camelina seeds.

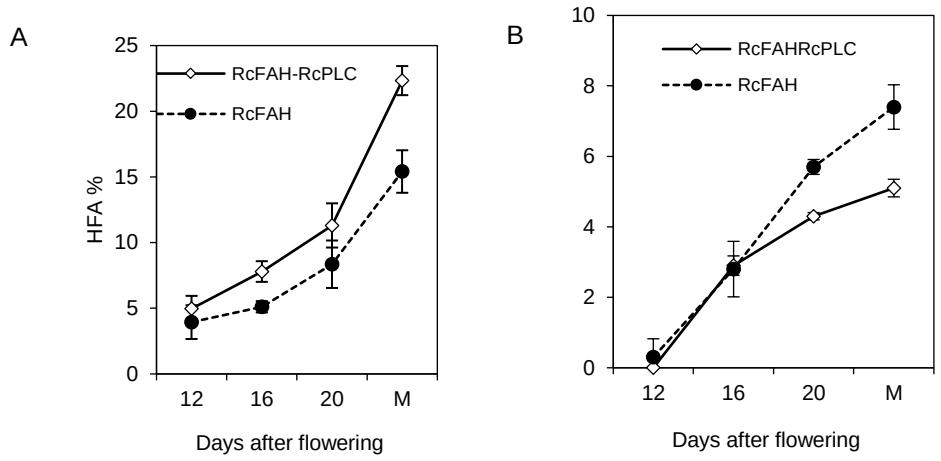


Fig 6. HFA accumulation in camelina developing seeds. A. Percentage of HFA in total seed oils. B. Percentage of HFA in polar lipid (PC) fraction. M: Mature seeds. Data represent average ±SD of 3 replicates.

Distribution of HFA in Different TAG Fractions of Oil

Different TAGs were separated with thin layer chromatography from the oil of mature seeds of MT5 (wildtype), RcFAH lines and RcPLCL1 lines. The HFA in the polar lipid or the PC fraction was decreased for RcPLCL1 lines compared to the RcFAH line; from 7.9 to 5.6%. The HFA was increased in 1OH and 2OH TAGs for RcPLCL1 lines; 51.30 to 54.47% in the 2OH TAG and 28.02 to 29.7% in 1OH TAGs. The results show that the HFA was increased in both 1OH and 2 OH TAG fractions. However, this does not tell us whether the increase was in sn1 or sn2 position.

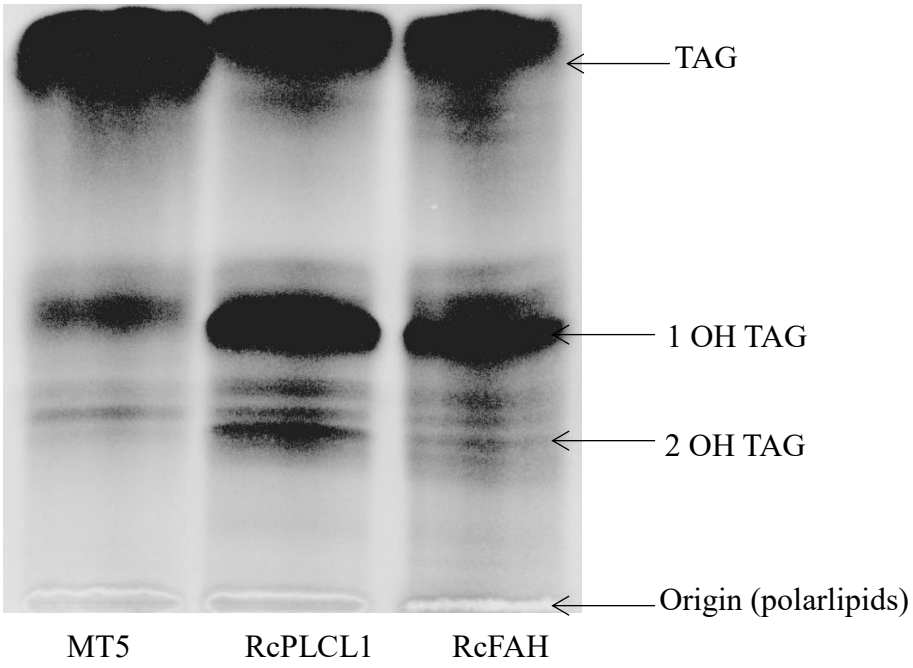


Fig 7. Separation of TAGs for the oil from the mature seeds of MT5 (wild type), RcPLCL1 and RcFAH lines.

Table 1. Distribution of HFA in different fractions of the oil for RcFAH and RcPLCL1 lines separated by thin layer chromatography.

	RcFAH	RcPLCl 1
Polar Lipid/PC	7.9	5.6
2 OH TAG	51.30	54.47
1 OH TAG	28.02	29.7

RcPLC Improved Germination of HFA-Camelina Seeds

To assess the effects of HFA accumulation in camelina seeds, I measured the oil content and performed germination tests of the transgenic seeds. Previous reports had shown that oil content decreased in HFA-accumulating transgenic seeds of camelina and Arabidopsis plants due primarily to the feedback inhibition during oil biosynthesis (Bates et al. 2014; Snapp et al. 2014; van Erp, Bates, Burgal, et al. 2011). The RcFAH camelina (7-1) seeds contained nearly 30% less oil than the non-transgenic control seeds (Fig. 5). The four lines of RcPLCL1 expressing seeds that I examined did not show significant differences from the RcFAH seeds. This result suggested that although it helped the flux of HFA from PC into DAG, RcPLCL1 was not able to alleviate the reduced TAG accumulation in the RcFAH transgenic seeds. Other mechanisms such as RcPDAT1 and RcDGAT2, which have been demonstrated in HFA-expressing Arabidopsis seed (Burgal et al. 2008b; van Erp, Bates, Burgal, et al. 2011), will be required to restore the oil content to the non-transgenic levels in camelina.

Homozygous T4 transgenic seeds were also tested for germination by plating on damp filter papers. Wild type seeds were also included as controls, which mostly germinated the next day showing radicle emergence and followed by successful seedling growth. Camelina RcFAH seeds had slower germination rate and nearly 40% failed to germinate at 7 days after imbibition. The germination rate for the RcFAH-RcPLC seeds were improved compared to the RcFAH plants. While 10-20% seeds still did not germinate during the test time-period, the RcFAH-RcPLCl1 seeds showed significantly increased germination rate and potential (Fig. 9). It was previously shown that HFA-accumulating transgenic Arabidopsis and camelina seeds suffered from decreased germination abilities. Co-expressing additional genes with RcFAH such as DGAT2, PDAT1, and PDCT from castor and KCS3 from *Physaria fendleri* at least partially restored the germination potential (Hu, Ren, and Lu 2012; Snapp et al. 2014; van Erp, Bates, Burgal, et al. 2011). In this study, I showed that RcPLCL1 decreased HFA accumulation in the PC fraction during seed development and in mature seeds, and consequently the RcFAH-RcPLCL1 seeds showed increased germination potential compared to the RcFAH seeds. This result suggested that the unusual HFA in the

membrane lipid could have destructive effect on seed viability.

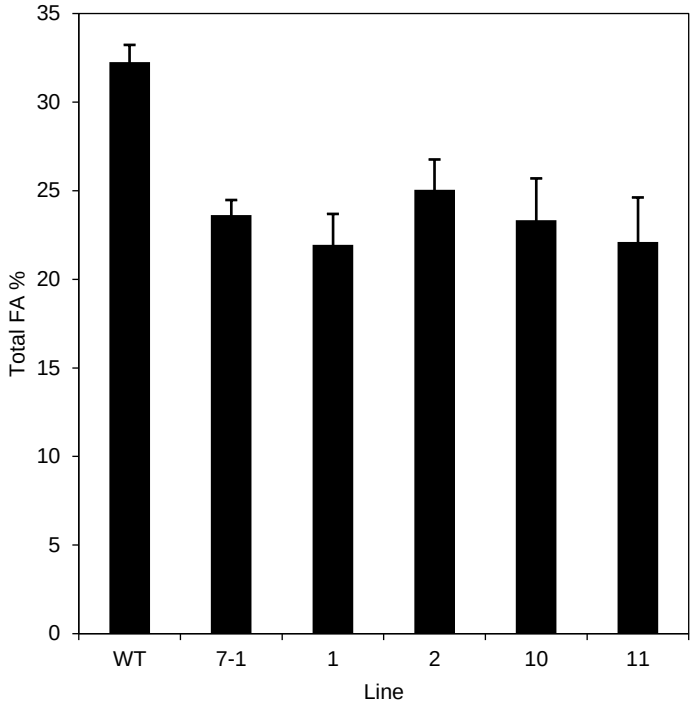


Fig 8. Total fatty acid content of camelina seeds. WT: non-transgenic control Suneson; 7-1: RcFAH12 transgenic seed; 1, 2, 10 and 11 are transgenic seeds of RcFAH-RcPLC. Data represent average ± SD for three replicates.

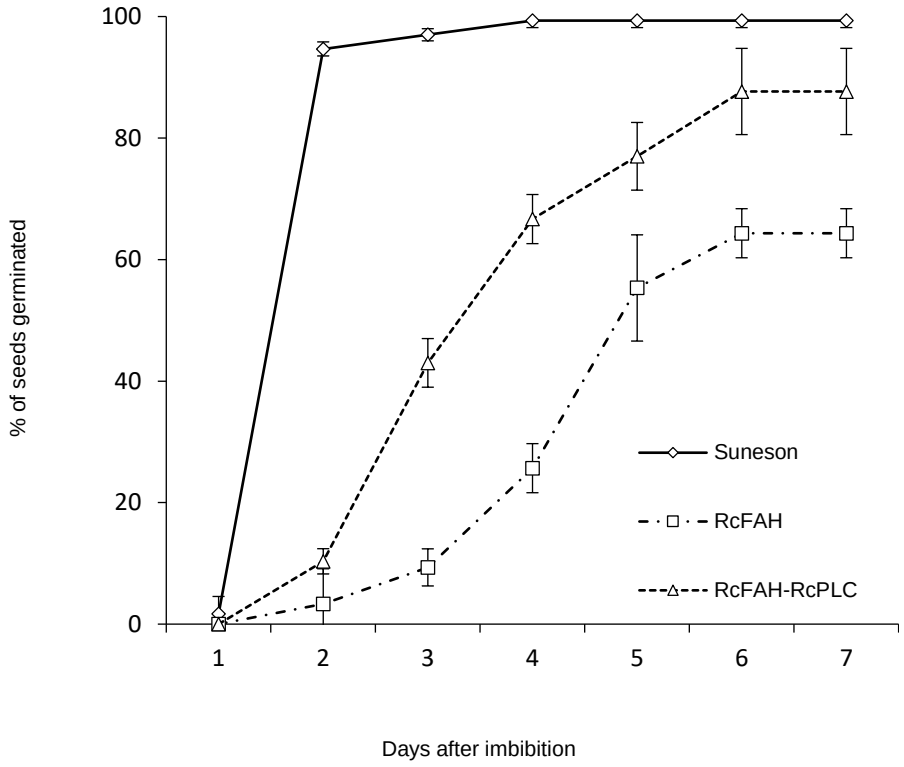


Fig 9. Germination rates of camelina wild type and transgenic seeds. Data are the average of three replicated experiments.

Conclusion

Hydroxy fatty acids are a valuable renewable source for many industrial products. Producing these unusual fatty acids in transgenic oilseed crops is an attractive way but has met great challenges to achieve large amounts of accumulation in engineered seeds. One of the bottlenecks in HFA metabolism is the efficient flux from PC, the site of synthesis, to storage TAG. I show here that co-expressing a phospholipase c like from castor, a native HFA accumulator, with the fatty acid hydroxylase may facilitate the transfer of HFAs from PC to DAG, thus increase their accumulation in TAG of

transgenic camelina. The reduced residual HFAs on the membrane lipids may also alleviate the potential detrimental effects of these unusual fatty acids on seed germination ability. Our results provide a useful tool to engineer high levels of HFAs in transgenic oilseeds such as the industrial crop *Camelina sativa*.

CHAPTER FIVE

FIELD EVALUATION OF HFA-PRODUCING CALMELINA

Abstract

An experiment was carried out for two years in the field at the Arthur H. Post Research farm of Montana State University to evaluate different traits of wild type (WT) and a transgenic line (7-1) that produce Hydroxy Fatty Acids (HFAs) in the seed of Camelina. The phenotypic trait measurements revealed significant differences compared to those taken in the greenhouse. The total oil content and plant height in field grown plants showed an increase for both wild type and 7-1 plants, though seed weight did not change much in both lines. The germination of field-harvested seeds showed a higher rate than those from greenhouse plants. HFA levels for the 7-1 line were decreased in the seeds of field grown plants. Overall, in the field plants demonstrated better performance, however, HFA levels decreased in the transgenic line.

Introduction

Overall performance of a plant completely depends upon the environment it grows in. The combination of several known and unknown biotic and abiotic factors affects the different traits of a plant, including yield. Plant scientists, including agronomists and biologists, grow plants in greenhouses for their experimental purposes. Abiotic conditions like light and temperature are manipulated to support plant growth in greenhouses. In addition, the plants are grown at a set distance so that the plant to plant

interactions are reduced. Fertilizers and insecticides are applied to increase plant growth and to reduce disease infection. However, in the field there is always a diverse climatic situation which cannot be controlled. Hence, there are always differences in measured plant traits between greenhouse and field grown plants.

Previous studies have shown differences in measurement of different traits when greenhouse and field grown plants are compared (Table 1). For example, Seed Specific Area (SSA) has been shown to increase in the field (Patterson et al. 1977, Poorter & De Jong 1999, Cornelissen et al. 2003). Non-photochemical quenching (NPQ) mutant Arabidopsis lines gave 3% lower yield than wild type in the greenhouse, while in the field, the yield decreased by 27% (Külheim, Agren, and Jansson 2002).

Transgenic plants are created in labs and often grown in greenhouses. When these plants are taken to the field for trials or for commercial production, there is possible bias compared to what was obtained from the greenhouse experiments. Several proteins or the enzymes in a normal or transgenic plant are affected by temperature, light and other environmental factors. These factors affect the enzymes at transcriptional or post translational levels. Omega-3 fatty acid desaturase (FAD3) works better and produces more product in cooler temperatures. However the FAD3 transcription was not altered much in cold and hot growth conditions. Indeed, the protein turnover was more for the cold temperature grown (O'Quin et al. 2010). Increased temperature altered the fatty acid composition in the seeds of safflower; an increase in oleate and a decrease in linoleate was observed (John Browse 1983). Storage lipid content and composition was changed in rapeseed when the night temperature was increased for the plant. Transcriptome analysis

revealed that the gibberellic acid signaling was upregulated, which in turn upregulated the fatty acid synthesis genes (Zhou et al. 2018).

In this experiment I have shown that performance of Camelina plants is different in the greenhouse and field. I have also measured the traits for transgenic Camelina that produce hydroxyl fatty acids (HFA) in its oil for greenhouse and field grown plants. The transgenic Camelina (7-1) contains Castor Fatty Acid Hydroxylase (RcFAH12) expressed under a seed specific promoter.

Table 1. Summary of previous studies on differences on different traits in greenhouse vs field grown plants.

Plant Species	**Trait**	**Values in greenhouse or growth chamber compared to the field**	**Reference**
Gossypium hirsutum	Photosynthetic activity/area	35% lower	(Patterson et al. 1977)
	SLA	55% higher	
	Chlorophyll/area	40% lower	
Nicotiana sylvestris	Alkaloid concentration in leaves	> 400% increase	Baldwin , 1998
Arabidopsis thaliana	Seed production per plant	*npq*-mutant had 3% lower value than WT in the GC, 29% lower in the field	(Külheim, Agren, and Jansson 2002)
22 herbaceous species	SLA	60% higher	Poorter & De Jong 1999
c. 100 woody species; young plants from the lab, adult plants in the field	SLA	125% higher	Cornelissen et al. 2003
	Leaf Nitrogen concentration	15% higher	

Material and Methods

Plant Growth in Greenhouse

MT5 (Wild type) and MT5RcFAH (7-1) lines were used for the transgenic lines production. 6 and 8 inches pots were used for growing the plants. 5 seeds were germinated in a pot. Sunshine Mix #1 (Bellevue, WA, USA) was used for growing the plants. 22º/18 ± 1 °C for day/night temperatures, relative humidity of 30 %, and a 16 h photoperiod of natural lighting were the greenhouse conditions. Plants were provided with fertilizer thrice through their lifetime; once in a week when the plants were ready for flowering.

Plant Growth in the Field

Wild type (MT5) and the MT5-RcFAH (7-1) were grown in Arthur H. Post Research Farm (Bozeman, MT) of Montana State University in the summer of 2016 and 2017 (June-September) (Fig 1). Plants were seeded in rows with a gap of 1.5 inches. No fertilizers or other external supplies were provided to the plants, except for irrigation that was given two times during very hot days.

Plant Harvest

Ten plants for both lines were harvested and further analyzed for both 2016 and 2017. The plants were uprooted and packed in separate bags which were then transported to the lab for analysis of different traits.

Measurement of Traits

Plant heights were measured after plants obtained the maximum height; when the plants change the color to yellowish from green. For seed weight, 100 seeds were counted and measured for every single plant then the single seed weight was calculated. Nuclear Magnetic Reader (OXFORD instruments, MQC) was used for measurement of total oil percentage of the seeds. Gas Chromatography was used for measuring the HFA content in the oil.

Statistical Analysis

Microsoft Excel was used for the statistical analyses. Two tail Student T Test was used to test the significance among the measurements. P value equal to or less than 0.01 was considered statically significant.

Lipid Extraction and Fatty Acids Analysis

Fatty acid methyl esters (FAMES) were created from the total lipids of the mature seeds with either the TMSH method (Lu et al. 2006) or acid derivation method (Browse, McCourt, and Somerville 1986). For the TMSH method, a 96 well-plate was used for crushing single seeds and TMSH was added to the crushed samples followed by addition of methanol after 15 minutes. For acid derivative method, 1 ml of 2.5% H2SO4 in methanol was added to the single seeds in glass tubes with Teflon-lined screw caps. Then it was heated for 90 minutes at 85-95^{0}C. 300ul hexane and 1.5 ml 0.9 M NaCl was then added to the samples and mixed properly and centrifuged to separate the upper layer FAMES. A program with parameters: 190^{0}C hold for 0.6 minute, 250^{0}C for 7 minutes

with 20^0C /min ramp was used for GC separation and measurement of fatty acid peaks. Helium was used as a carrier gas.

Germination Assay

100 seeds from three biological replicates each were imbibed on the wet filter paper. The germination was observed and accounted in every 24 hour. The final data was then prepared after 6 days when the graph was flat using the average of the biological samples.

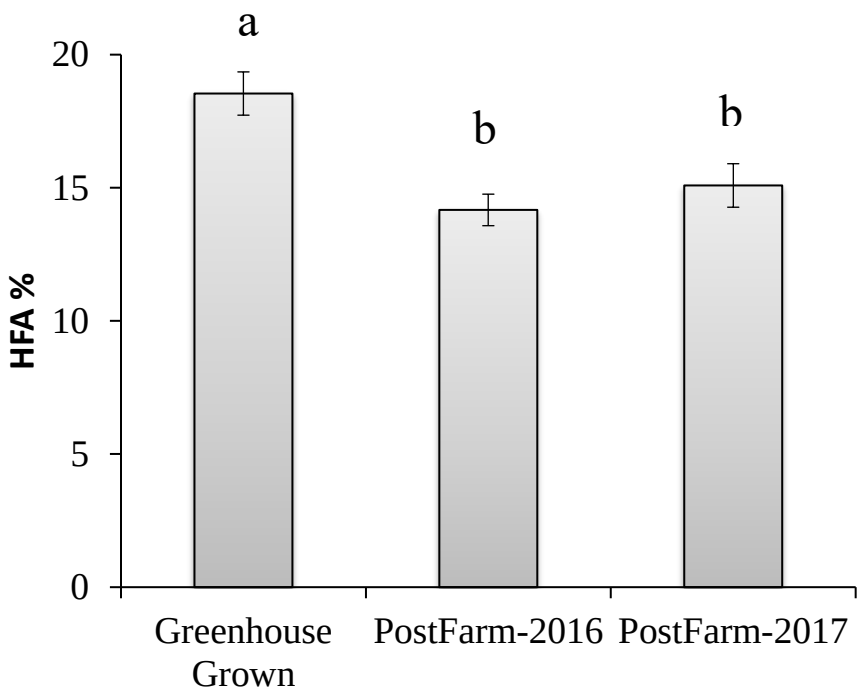
Fig 1. Camelina plants growing at the Arthur H. Post Research farm (8431 Huffine Ln, Bozeman, MT 59718).

Results and Discussion

HFA Level Decreased for the Plants Grown in Field

Level of HFA in the seeds from field were decreased compared to those grown in the greenhouse (fig2). The HFA level in greenhouse grown plants was 17% on average but decreased to an average of 14% in the field grown plants. The 2016 and 2017 field results were consistent, which verified the observed decreases in HFA content in the field. Single seed analysis gave as low as 12% HFA from the seeds grown in the field. The maximum decrease was observed in the 18:1OH, while 18:2OH did not change or had a slight increase in field grown plants (Fig 3). The decrease in HFA may have been caused by several known and unknown factors which cannot be concluded at this point.

A



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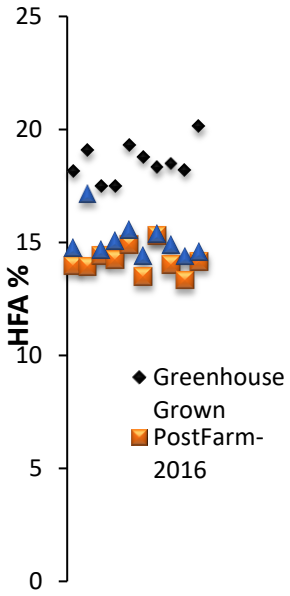

Fig 2. Accumulation of HFA in the mature seeds of 7-1 grown in greenhouse and post farm.Data is a mean±SD of 10 biological replicates. a and b are significantly different groups. P<0.05.

18:3 Increased for the Seeds from Field Grown Plants

Fatty acid hydroxylase uses phosphatidylcholine (PC) in the membrane of the endoplasmic reticulum as a substrate (Snapp and Lu 2013b). Fatty acid desaturases such as FAD2 and FAD3 also act on PC for FA modification (Kang, Snapp, and Lu 2011). Hence, when FAH12 is expressed in wild type Camelina, the metabolic activity of FAD enzymes may decrease as a result of competition with FAH12. The seeds from greenhouse grown plants have large decreases in 18:2 and 18:3, which are the products of FAD2 and FAD3 respectively. However, for the seeds from the field, 18:3 increased significantly compared to that from greenhouse seeds (Fig 3). Hence the decrease in HFA levels in the field could have resulted from an increase in 18:3.

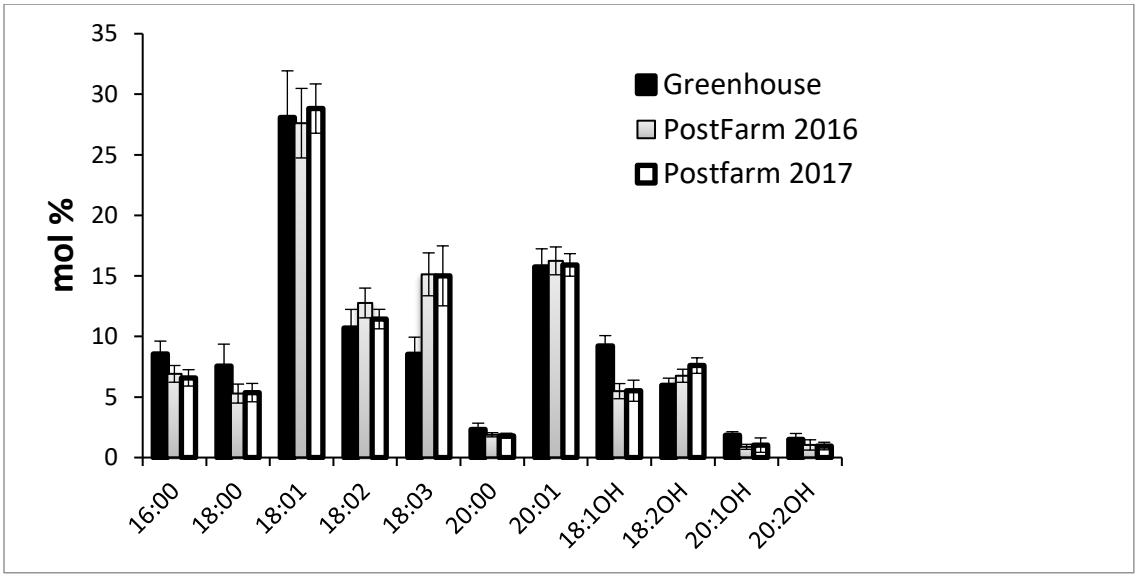

Fig 3. Composition of fatty acids in mature seeds of 7-1 grown in greenhouse and post farm. Datas are mean±SD of 10 biological replicates. P<0.05.

Oil Content Increased for Both MT5 and 7-1 Seeds from Field

Seeds from wild type (MT5) grown at the greenhouse contained about 30% oil. When RcFAH12 was expressed, the oil content decreased to 22-25% (Fig 4). When the plants were grown in the field, the oil content increased for the seeds from both wild type and 7-1. This increment could be because of healthier seeds produced as a result of favorable conditions in the field. Several known and unknown factors might have played role for this change in oil content. However, the oil content in 7-1 remained lower than wild type for greenhouse and field grown plants.

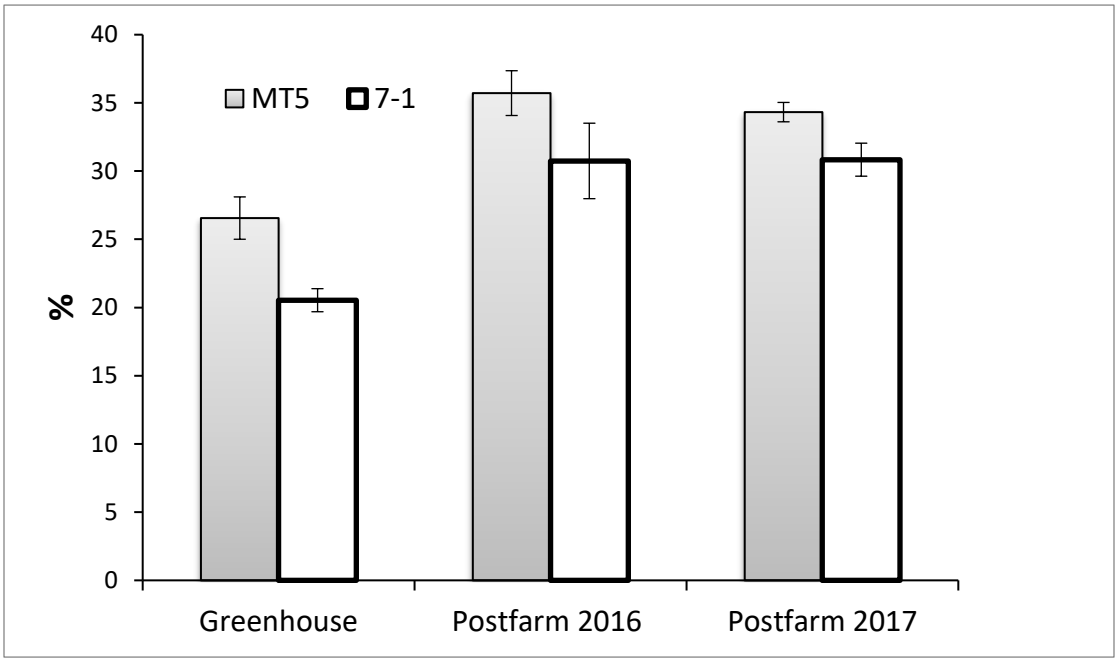

Fig 4. Oil content for mature seeds of MT5 and 7-1 grown in greenhouse and post farm. Datas are mean±SD of 10 biological replicates.

Single seed weight and number of seeds per pod did not change

Single seed weight did not change significantly for plants grown in the field compared to those grown in greenhouse. The individual seed weight varied from 0.7 mg to 0.9 mg for the greenhouse grown plants (Fig 5). The field harvested seeds weighed from 0.8 to 1.0 mg.

There was no significant difference in number of seeds per pod for the plants grown in greenhouse and the field (Fig 6). A single pod contained an average of 12 seeds for both greenhouse and field grown plants for either MT5 or 7-1.

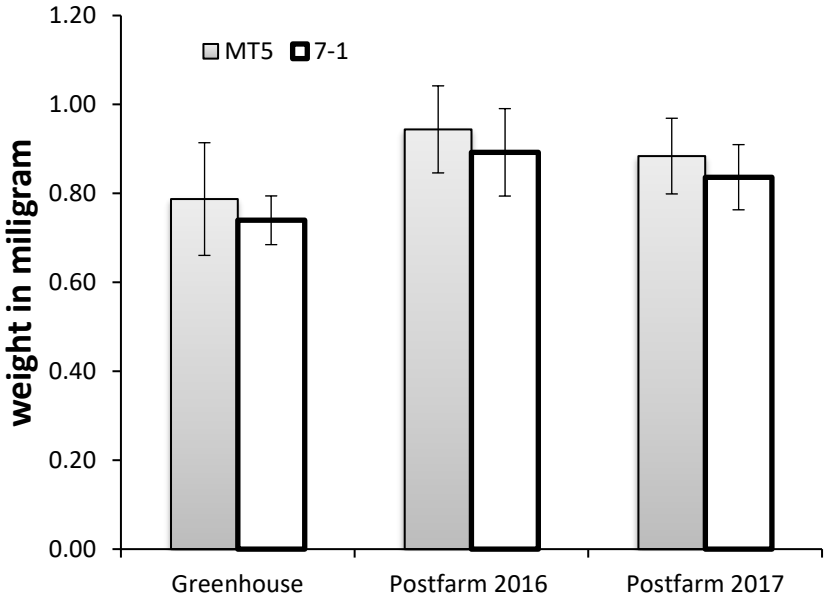

Fig 5. Individual seed weight for the mature seeds of MT5 and 7-1 grown in greenhouse and post farm. 100 seeds were counted and weighed and the weight for individual seeds were calculated. Datas are mean±SD of 10 biological replicates.

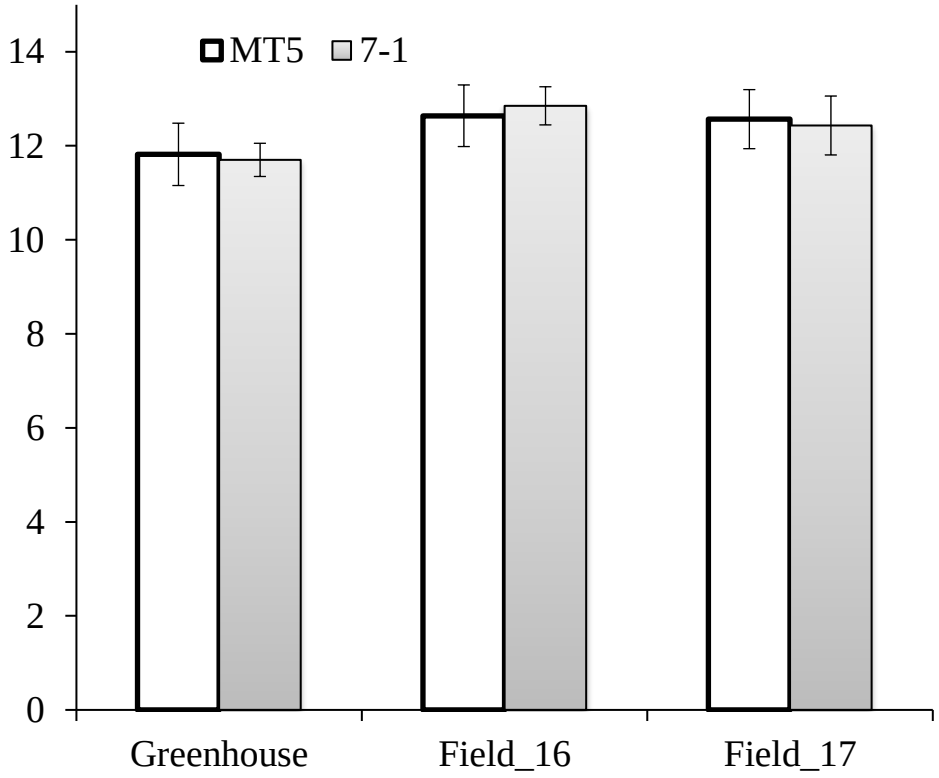

Fig 6. Number of seeds per pod. For every individual plant 10-15 pods were counted. The data represents average ± SD for 10 individual plants.

Plant Height Increased for the Field Grown Plants

For the plants grown in the field, the height was increased compared to those grown in the greenhouse. Plants grown in the greenhouse attained the height of 17-20 inches. The plants grown in the field attained the height of 23-27 inches (Fig 7). However there was no significant difference in plant height between MT5 and 7-1 in the greenhouse or in the field.

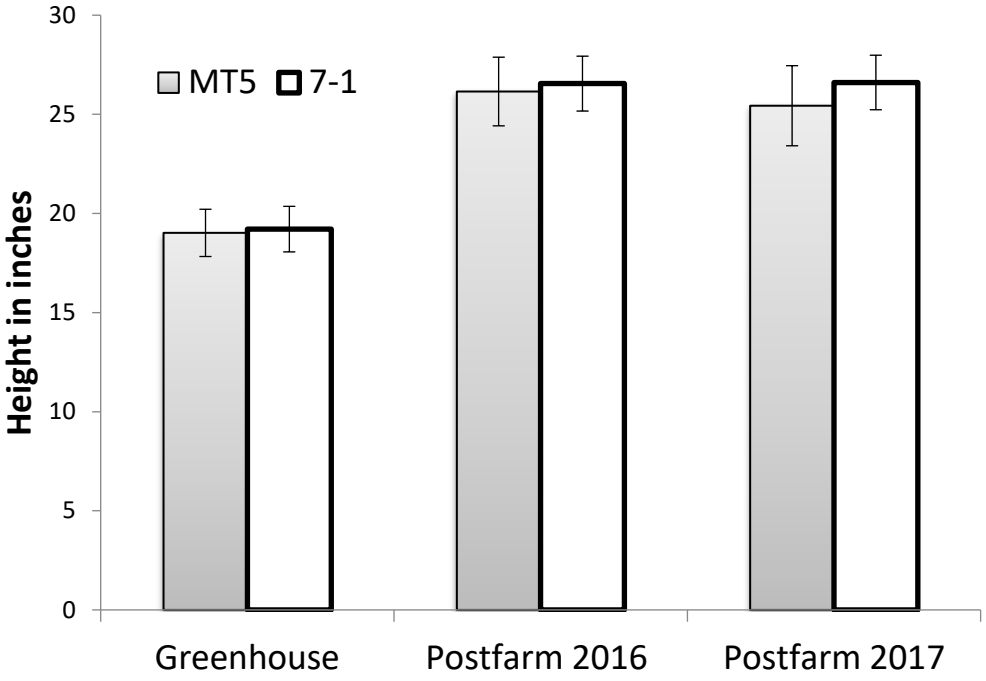

Fig 7. Plant height for MT5 and 7-1 grown in greenhouse and Postfarm. Datas are mean±SD of 10 biological replicates.

Yield Increased for Field Grown Plants

Yield per plant increased for the plants grown in field (Fig 8). The greenhouse grown plants had a very low yield compared to the field grown. Individual plants produced about 2.5 gram of seeds in the greenhouse for both MT5 and 7-1. There was a significant increase in yield compared to greenhouse for both 2016 and 2017 field grown plants. 2017 field plants produced much higher amount of seeds per plant than 2016. About 8 grams of seeds were produced by individual plant in 2016 while it increased to about 12 grams in average for the year 2017. However, there was no significant difference in yield per plant between MT5 and 7-1 in the greenhouse or field.

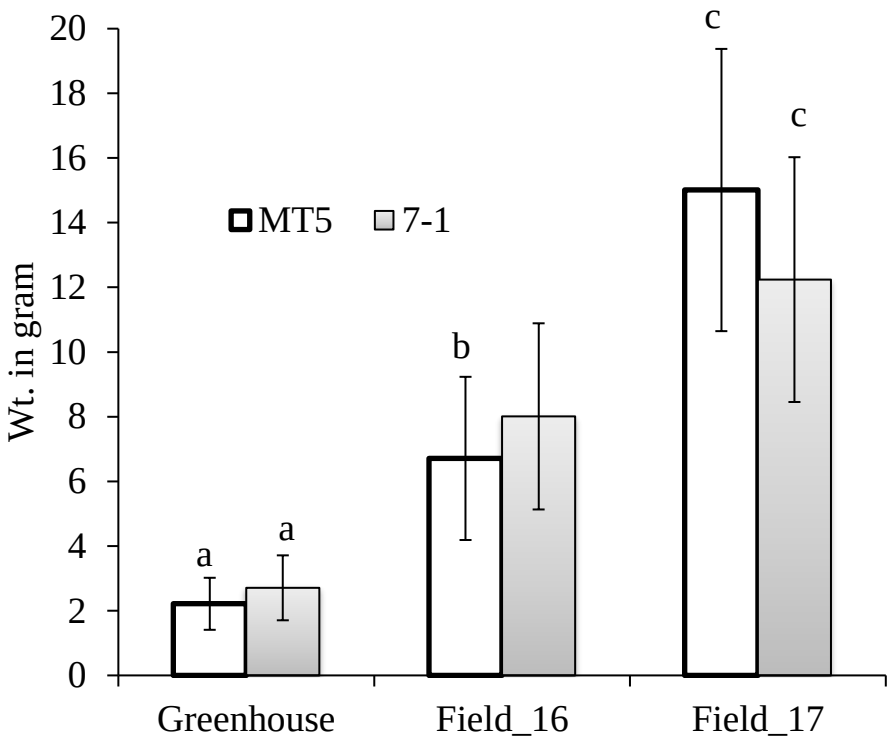

Fig 8. Yield per plant for the greenhouse and filed grown plants. Data represents average±SD for 10 biological replicates. a, b and c represent significantly similar groups. $p<0.05$.

Germination Rate Improved for the Field Harvested Seeds

A germination test was performed for the seeds harvested from greenhouse and the field. It was earlier shown that transgenic Camelina producing HFA in the seed oil has a low germination rate (Snapp et al. 2014). The germination rate for 7-1 seeds from the greenhouse harvest was about 60%. But when the field harvested seeds were tested for germination, the rate was about 85% (Fig 7). This increase in germination in field harvested 7-1 seeds could be attributed to the increase in oil content and decrease in

hydroxy fatty acid in the oil. However, for the wildtype (MT5) seeds, both greenhouse and field harvested had nearly 100% viability.

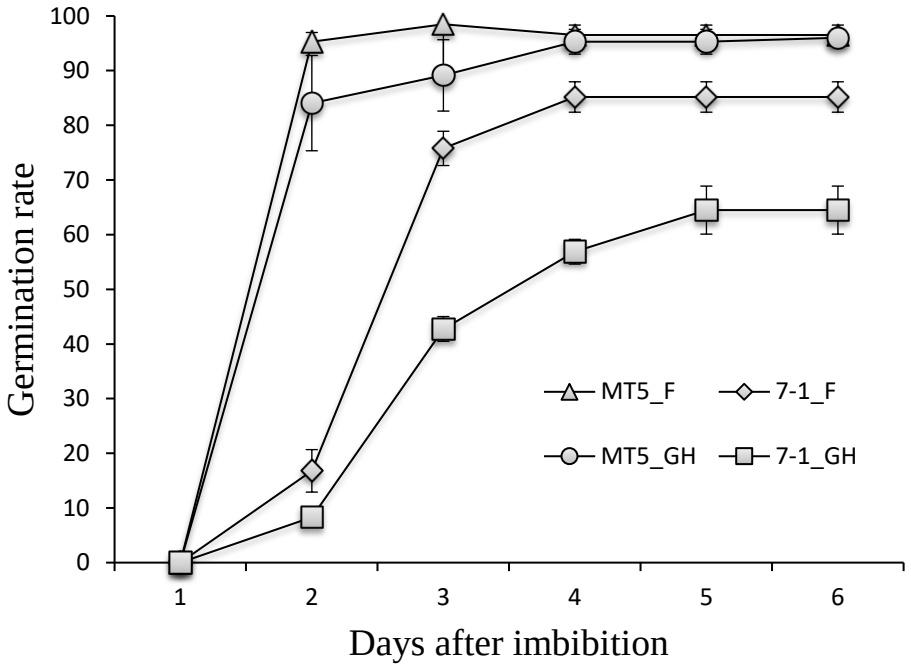

Fig 7. Germination test for the seeds harvested from post farm 2016 summer. Wet filter papers were used for the test. Datas are mean±SD of 3 biological replicates.

Conclusion

Plants are genetically modified in labs to produce novel and desirable products from them. However, those plants have to be taken to the field for trials before grown for commercial production of the new products. Though the HFA production of transgenic Camelina is still at a limited level, this experiment indicated that RcFAH12-expressing plants have different growth phenotypes in greenhouse and field conditions. Further and

in-depth experiments are required to explore the causative factors for the observed differences in transgenic plants when grown in the greenhouse and field.

CONCLUSION OF THE STUDY

The importance of plant oil has been booming in industrial applications and in nutrition. Fatty acids esterified to the glycerol backbone make oil. The characteristics, and hence the quality of the oil is decided by the FA attached to the glycerol backbone. To improve plant oils, it requires a complete understanding of the oil biosynthesis pathway in the developing seeds. Also, different unusual FAs are produced in some plants that have either not been domesticated yet or have several drawbacks which limit the possibility of large-scale production. HFA, one of the important unusual FA natively produced by *Ricinus* and *Physaria sps.*, have immense importance in industrial applications. These plants, however, have different shortcomings for the commercial production of HFA in them. This has led to a demand for the production of HFA in transgenic plants. Camelina, being a very convenient plant for the transgenic approach for several reasons, has great potential to produce the unusual, such as HFA, FAs in its seed.

Expressing the key enzyme, Fatty Acid Hydroxylase (FAH) from these plants, into Camelina or Arabidopsis produces 15-20 % HFA in the oil whereas the oil from native plants consists of 70-90% HFA. So far, despite immense efforts in research, there has not been much progress in increasing the amount of HFA or other modified FAs in the oil of transgenic plants. Several problems have been proposed to have limited this effort. One of the obstacles is releasing HFA from the PC. I have proposed and shown that PLCs have a role in this process.

I tested 6 NPCs, which were previously shown to act on PC, from Arabidopsis by using T-DNA lines for those genes. FA composition was analyzed from the mature seeds

of T-DNA lines. *npc1, npc4* and *npc6* showed an increase in monounsaturated (oleic;18:1) FA and a decrease in polyunsaturated (linoleic;18:2 and linolenic;18:3) FA. 18:1 FA was further increased for the *npc1/npc6* double cross. These changes in FA profile suggest the role of NPCs in TAG biosynthesis by releasing DAG from PC.

After verifying the roles of AtNPCs in TAG biosynthesis pathway in Arabidopsis, I examined the previously published expression pattern of phospholipases in Castor. RcNPC3 and RcNPC6 were expressed in the castor. RcNPC6 was predominantly expressed in leaves and male flower and had a very low expression in developing endosperm, which is the site for synthesis and storage of Castor seed oil. Interestingly, a different group of phospholipase C (PLC-likes were predominantly expressed in the endosperm. Out of 6 phospholipase C expressed in the endosperm, five of them were predicted as PLC-likes. I selected RcPLCL1 as my candidate and started investigation on it.

When I explored the characteristics of this phospholipase, the bioinformatics studies showed that it belongs to the PLC-like phosphodiesterase family which has not been characterized yet. Based on the sequence, the protein contains the X domain for the PI-activity but it lacks the Y and C2 domains. I could not predict any domain for PC-activity. Enzyme assay done using yeast showed that RcPLCL1 and AtPLCL1, the closest homologue of RcPLCL1 in Arabidopsis, have both PC and PI activity. From the genomic sequence analysis, I found 8 genes in Arabidopsis which are predicted as PLC-like phosphodiesterase. T-DNA lines were ordered for 5 closely related PLC-like genes. FA composition analysis from the mature seeds of these plants showed a decrease in 18:3

and an increase in 18:1. This change in FA composition suggests the role of these proteins in oil biosynthesis in Arabidopsis seeds. RcPLCL1 was transformed to the Camelina that had expressed RcFAH12. This increased the HFA in the seed oil form 15% in RcFAH line to as high as 24% in RcFAH-RcPLCL1 line. I observed a decrease in the amount of HFA in PC which supports my hypothesis, that RcPLCL1 releases DAG from PC. However, the coexpression of homologue PLC-like from Arabidopsis did not increase the HFA in seeds of Camelina. This could be because of the effect of co-evolution of RcPLCL1 and AtPLCL1 in two different plants. It requires further experimentation at the amino acid level for the concrete conclusion.

Most of the transgenic plants are produced in the labs. When they are grown in the field, expression of engineered targets and other traits may be different from what have been observed in the greenhouse. I wanted to test the performance of Camelina producing HFA in the field. I carried out a two-year experiment at the Arthur H. Post Research farm (8431 Huffine Ln, Bozeman, MT 59718) of Montana State University to measure the different traits of a wild type (WT) and a transgenic line (7-1). The total oil content and plant height in field grown plants showed an increase for both wild type and 7-1 plants. The germination of seeds harvested from the field showed higher rates than those from the greenhouse. HFA levels for the 7-1 line decreased in the seeds of field-grown plants. Overall, the plants in the field demonstrated better performance; however, HFA levels decreased in the transgenic line in the field.

HIGHLIGHTS

1. Of 6 NPCs in Arabidopsis (NPC1-6); NPC1, NPC4 and NPC6 have possible roles in TAG synthesis in Arabidopsis seeds.

2. PLC likes phosphodiesterases (RcPLCL) are dominant PLCs expressed in HFA-accumulating castor endosperm. From bioinformatics analysis, these PLC-likes are widely distributed in plant species and they are evolutionarily distinct from other PLCs in Arabidopsis.

3. Enzyme assay for RcPLCL1 and AtPLCL1 using yeast expression showed that the enzymes hydrolyze both PI and PC.

4. From the genomic sequence analysis, 8 PLC like genes were found in Arabidopsis. FA profiling from single mutants of 5 of those genes showed that they may have roles in TAG biosynthesis.

5. A phospholipase C from Castor, RcPLCL1 (30115.m001244), increases HFA in transgenic Camelina when co-expressed with RcFAH12. However, the homologue, AtPLCL1 (At5g67130), did not increase HFA.

6. Decrease in HFA in PC of the transgenic RcFAH-RcPLCL1 Camelina and its activity to PC observed from the enzyme assay indicated that RcPLCL1 may release HFA-DAG from HFA-PC in transgenic seeds.

7. HFA-producing Camelina showed decreased HFA content in seed from field grown plants compared to greenhouse plants.

DISCUSSION

Multiple forms of PLCs exist in plants and they differ in their substrate specificities. PI- and non-specific PC-hydrolyzing PLCs have been extensively studied previously, but mostly focused on lipid signaling (Hong et al. 2016b; Wang 2001). The roles of PLCs in seed storage lipid biosynthesis are unclear. In this study, I tested whether AtNPCs play roles in releasing polyunsaturated FA from PC. With an aim to increase HFA in transgenic Camelina, I wanted to express a suitable phospholipase from Castor. The castor NPC homologs would be the candidates to enhance HFA accumulation in transgenic seed since they hydrolyze PC, however the two putative *NPC* genes present in the Castor transcriptome are primarily expressed in leaf and male flower, suggesting their minor roles in storage lipid biosynthesis. I therefore tested the putative PLC-like proteins which were primarily expressed in the endosperm. I selected RcPLCL1 as my candidate gene and started investigation on it. The invitro enzyme assay showed that RcPLCL1 and AtPLCL1, the closest homologue of RcPLCL1, have both PC and PI hydrolyzing activities. In Arabidopsis, 8 PLC-likes were identified from the genomic sequence. Expression data from TIAR (www.Arabidopsis.org) showed that these PLC-likes are well expressed in the developing seeds of Arabidopsis. The single T-DNA mutant lines for these genes showed that they are involved in TAG biosynthesis pathway. RcPLCL1 was then expressed in the Camelina expressing RcFAH12. This increased the HFA in transgenic plant from 15% to as high as 24% in homozygous plants. From my study, it is more likely that the PC-hydrolyzing activity of RcPLCL1 was responsible in transgenic camelina that enhanced the HFA accumulation in seed TAG by converting

HFA-containing PC into HFA-DAG. This was supported by my observation that HFA contents in PC declined concomitantly with their increases in TAG during seed development of camelina co-expressing RcPLCL1 and RcFAH12.

In castor, the majority of ricinoleic acid may be released from PC after 18:1 hydroxylation and enter the acyl-CoA pool to participate in TAG biosynthesis through the Kennedy pathway. To some extent, ricinoleic-PC also may turn into DAG through phosphocholine headgroup exchange (Bafor et al. 1991). In transgenic Arabidopsis and camelina, a major pathway for HFA-TAG biosynthesis is reported through the PC-DAG interconversion (Bates and Browse 2011, 2012). Previously it was shown that PDCT is required for fatty acid hydroxylation in RcFAH-transgenic Arabidopsis and coexpressing a castor RcPDCT with RcFAH increased HFA accumulation (Hu, Ren, and Lu 2012). In this study, I showed that a PLC-like protein also increased total HFA content in RcFAH-expressing camelina seeds, accordingly the 1-OH-TAG and 2-OH-TAG fractions were also increased. These results suggest that, besides RcPDCT, RcPLCL1 is also involved in the conversion of PC to DAG. It is not clear whether the increase of HFA in TAG is caused by a general enhancement of PC to DAG conversion, but it is possible that the RcPLCL1 may specifically act on HFA-containing PC. This type of substrate specificity has been demonstrated in caster genes, e.g., RcPDAT1A, RcDGAT2, RcPLA2a (Burgal et al. 2008b; van Erp, Bates, Burgal, et al. 2011; Bayon et al. 2015). In our parallel experiment, an Arabidopsis homolog AtPLCL1 with 69% amino acid identity failed to increase HFA in the RcFAH-containing seed. This result suggests a similar substrate specificity of RcPLCL1. However, it cannot be drawn such a conclusion without supports

with more enzyme activity assays and transgenic experiments using additional homologous *AtPLCL* genes. The PLC-like phosphoesterases isolated in this study have not been characterized previously. Their homologous genes are widely present in other plants like Arabidopsis. More research is needed to understand the full spectrum of enzyme activities and biological roles of this class of phospholipases in plant development and metabolism.

The biotechnological challenges of producing HFAs in transgenic oilseeds include the detrimental effects on seed physiology such as decreased seed viability and oil accumulation (Snapp and Lu 2013a). RcPLCL1 effectively removed HFA from the membrane lipid PC during seed development and resulted in decreased residual HFA in seed PC. Consequently, seed germination potential improved in the RcPLCL1 lines compared to the RcFAH line. This result further supports previous notion that the unusual HFA in the membrane lipid could have destructive effect on seed viability (van Erp, Bates, Burgal, et al. 2011; Hu, Ren, and Lu 2012; Snapp et al. 2014). Although RcPLCL1 was able to increase HFA accumulation in TAG, the total FA contents in RcPLCL1-expressing seeds remained largely at the same levels as in RcFAH-only seeds. Other mechanisms such as RcPDAT1 and RcDGAT2, which have been demonstrated in HFA-expressing Arabidopsis seed (Burgal et al. 2008b; van Erp, Bates, Burgal, et al. 2011), will be required to restore the oil content to the non-transgenic levels in camelina. The decreased oil accumulation in HFA-producing seed is mainly due to feedback inhibition on fatty acid synthesis caused by inefficient incorporation of HFA onto TAG (Bates et al. 2014). Multiple mechanisms are at work to remove HFA from PC (e.g.,

PLA2, PDCT and PLC) and incorporate onto TAG (e.g., DGAT and PDAT) (Burgal et al. 2008b; van Erp, Bates, Burgal, et al. 2011; Hu, Ren, and Lu 2012; Bayon et al. 2015), systematic approaches will be needed to increase efficiencies of HFA synthesis and assembly.

My results advanced the knowledge of lipid metabolism in seed and will help design efficient strategies to engineer high levels of HFAs in transgenic oilseeds such as the industrial crop *Camelina sativa*. The study opens a window for the studies of PLC-like phosphodiesterases for the oil biosynthesis and as candidate for efficient production of modified fatty acids in transgenic oil-seed plants. As discussed in the different sections of this study, the overexpression of genes from HFA producing plants itself may not increase the unusual FA in the transgenic plants. Transcription factors and small RNAs could be studied for their coordination with several genes in an oil-biosynthesis pathway.

I also performed a two-year experiment in the field to see the performance of HFA containing Camelina. I observed a decrease in HFA in the field-grown plants when compared to the greenhouse-grown plants. However, oil contents and other physiological traits were improved. Though the Camelina I tested contained only 15% HFA in its oil, the study implies that it is essential to test the transgenic oil-seed crops in the field for their performance. The plants also could be tested in the different temperature and light conditions in the greenhouse.

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APPENDIX

Table. TRAVA - transcriptome variation analysis. Absolute read counts. Read counts normalized by Med. Format: average value. Details about samples can be found at http://travadb.org/samples/

	AtPLCL1 (At5g67130)	AtPLCL2 (At3g19310)	AtPLCL3 (At1G13680)	AtPLCL4 (At1g49740)	AtPLCL5 (At4g36945)
Young seeds 1	1701	7	65	1068	200
Young seeds 2	1741	29	61	1029	181
Young seeds 3	1632	49	68	1124	147
Young seeds 4	1557	68	35	1192	133
Young seeds 5	1481	35	64	1111	160
Silique 2	992	49	3	1921	615
Silique 4	1074	61	13	1597	701
Silique 6	1250	128	68	1424	674
Silique 8	1723	113	90	1210	599

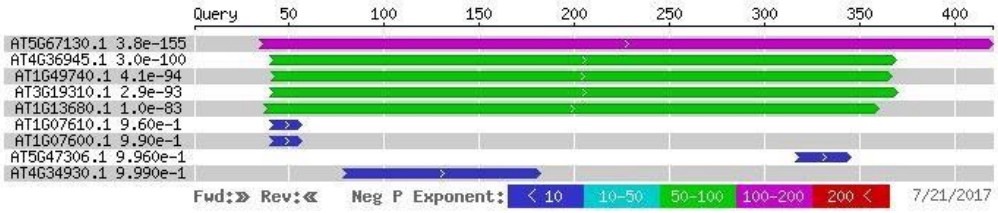
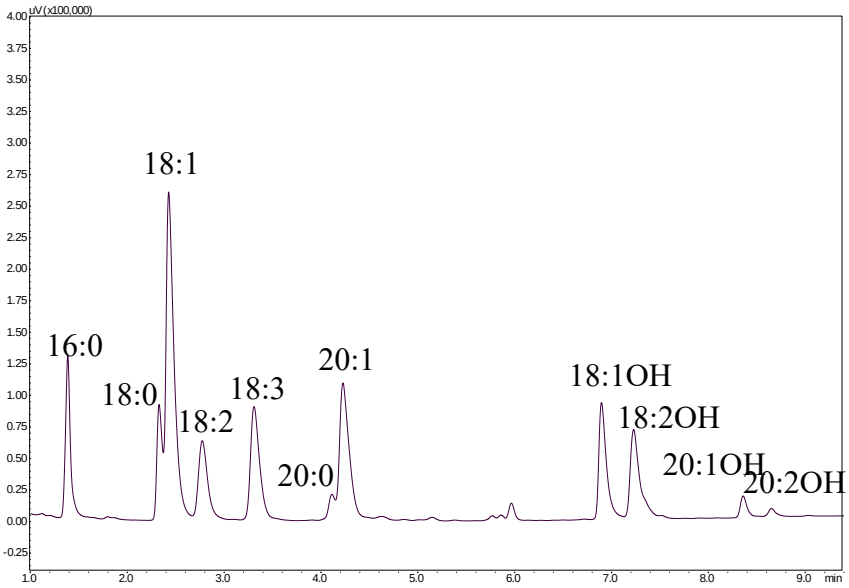


Fig. Alignment of AtPLC-likes.

A



B

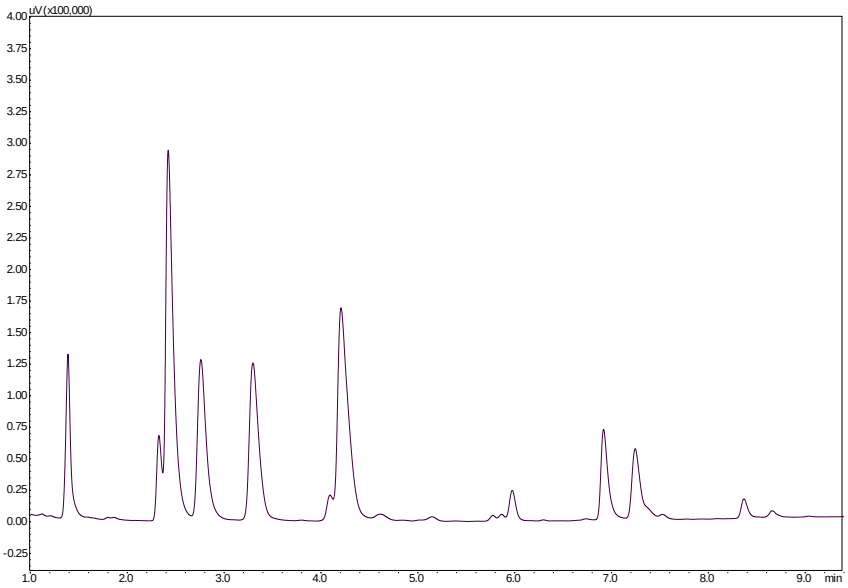


Fig 1. Peaks for different fatty acids of oil from A) 7-1 RcPLC B)7-1 mature seeds

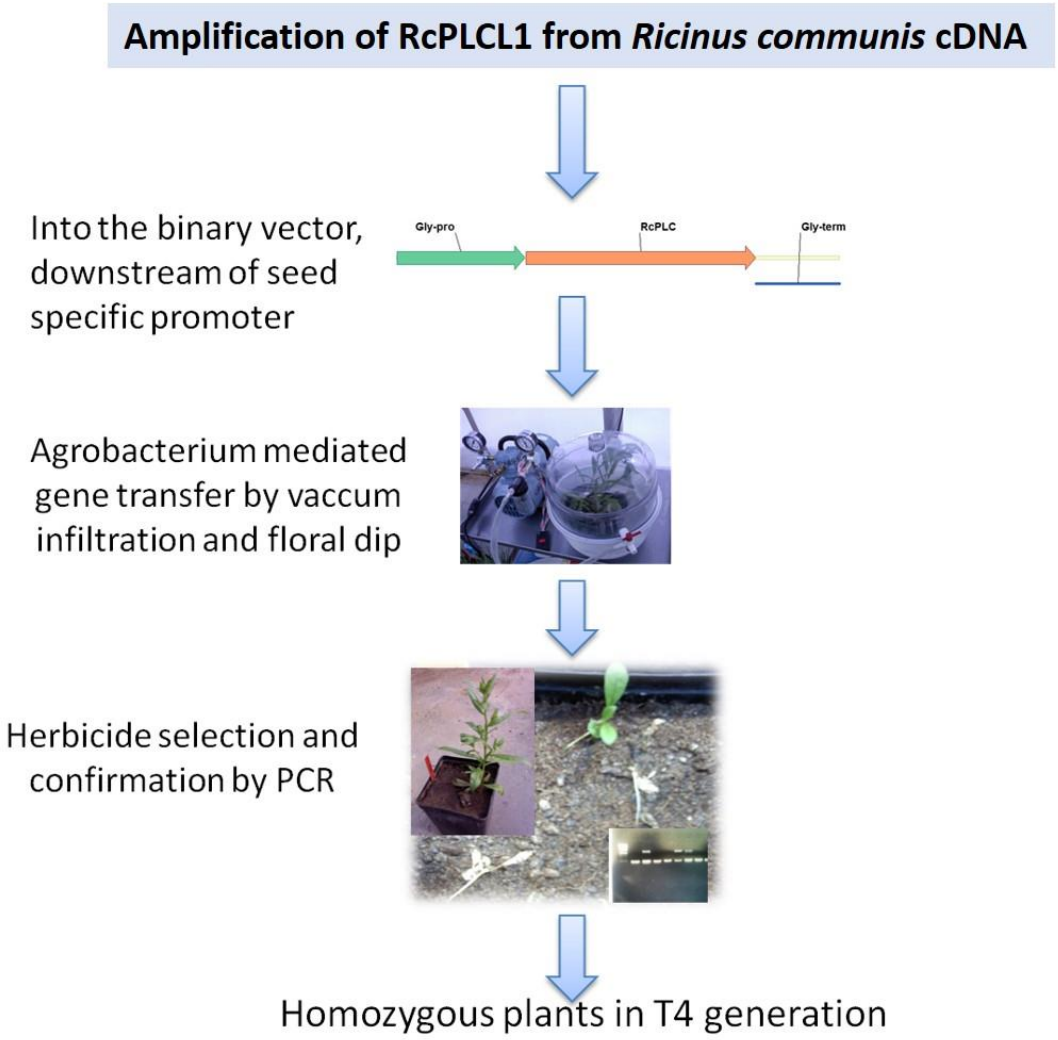


Fig: Methodology for generation of RcFAH-RcPLCL1 transgenic line.

cds sequences of PLC like Phospholipases in Arabidopsis

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>30147.m014488 Phospholipase C 4 precursor, putative

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>29847.m000249 phospholipase C, putative

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>29847.m000250 phospholipase C, putative

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