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Expression and Functional Characterization of Avocado DGAT1 and PDAT1 in Arabidopsis and Camelina

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Expression and Functional Characterization of Avocado DGAT1 and PDAT1 in Arabidopsis and Camelina

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by

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ABSTRACT

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The study is aimed to determine the role of avocado DGAT1 and PDAT1 in seed oil synthesis. Triacylglycerol (TAG) has a nutritional and industrial value and is essential for plant growth. DGAT1 and PDAT1 catalyze the final step of TAG Assembly. We hypothesized that both Pa*PDAT1* and Pa*DGAT1,* although predominantly expressed in non-seed tissues, could contribute to oil accumulation in seeds. *Agrobacterium* transformants with PaPDAT1 and PaDGAT1 cloned in pCAMBIA were generated to test this. Subsequently, the *Agrobacterium*-mediated transformation of Arabidopsis mutant lines and camelina was carried out by floral dipping. The T_1 camelina seeds expressing the genes of interest were selected using fluorescence screening. Homozygous T_3 lines were generated. The transgenic camelina seeds were evaluated for TAG content and fatty acid composition relative to wild-type seeds. Line D1 3-3-2 expressing Pa*DGAT1* and line P1 7-8 expressing Pa*PDAT1* showed a significant increase in C18:1 compared to the wild type.

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ABBREVIATIONS

ABRC	Arabidopsis Biological Resource Center
AtDGAT1	*Arabidopsis thaliana* diacylglycerol acyltransferase1
AtPDAT1	*Arabidopsis thaliana* phospholipid: diacylglycerol acyltransferase1
CoA	Coenzyme A
DsRed	*Discosoma* sp. Red
FAME	Fatty Acid Methyl Esters.
GC	Gas Chromatography
GC-FID	Gas Chromatography-Flame Ionization Detector
HCL	Hydrochloric Acid
KOH	Potassium Hydroxide
LB	Luria Bertani
NEB	New England Biolabs
OD	Optical Density
PaDGAT1	*Persea americana* diacylglycerol acyltransferase1
PaPDAT1	*Persea americana* phospholipid: diacylglycerol acyltransferase1
PCR	Polymerase Chain Reaction
LDAPs	Lipid Droplets-Associated Proteins

CHAPTER 1. INTRODUCTION

Triacylglycerols (TAGs) and their Significance

Triacylglycerols (TAGs) also known as vegetable oil belongs to a class of neutral lipids that consist of three fatty acids esterified into one glycerol molecule. In most terrestrial plants and microalgae, TAG exists as a high energy storage compound in form of lipid droplets in developing seeds and/or non-seed tissues (Sánchez and Harwood 2002). The role and value of TAGs are directly derived from the fatty acid composition which in turn determines the market value of these plants' oils. Nutritionally, TAGs provide dietary sources of calories to humans, which is 2.25x more compared to the number of calories derived from consuming a similar amount of carbohydrates. TAG also has industrial and pharmaceutical applications.

There is also increasing interest in the use of TAGs as a source of biofuel, a form of renewable energy. So far, several studies have been conducted in an attempt to identify and isolate the rate-limiting steps in TAG biosynthesis. Thus, many genes contributing to TAG turnover have been characterized. Among these genes, acyl-CoA: diacylglycerol1 (DGAT1) and phospholipid: diacylglycerol1 (PDAT1) were shown to catalyze the terminal and committed step of fatty acid biosynthesis in plants by converting diacylglycerol (DAG) to triacylglycerol (TAG) (Banaś et al. 2013). Both DGAT1 and PDAT1 play a pivotal role in the assembly and the determination of the composition of fatty acids of TAGs. DGAT1 was first isolated and characterized in mice in 1998 (Cases et al. 1998) whereas PDAT1 was first isolated and characterized in yeast in 2000 (Dahlqvist et al. 2000). Since then, various isoforms of these genes have been identified and characterized in different plant species.

In angiosperms and algae, TAG biosynthesis occurs through acyl-CoA-dependent or acyl-CoA-independent pathways. The acyl-CoA-dependent pathway is catalyzed by DGAT whereas the Acyl-CoA-independent pathway is catalyzed by PDAT (Yuan et al. 2017). *PDAT1*

and *DGAT1* genes are highly conserved in higher plants and microalgae (Dahlqvist et al. 2000; Banaś et al. 2013). DGAT and PDAT play crucial roles in determining the flux of carbon into seed TAG. Thus, they have been considered the key targets for engineering oil production in higher plants (Weselake et al. 2009;Vanhercke et al. 2019).

Fatty Acid Biosynthesis in Plants

The rate of TAG synthesis primarily depends on the supply of fatty acids from the plastid (Bao and Ohlrogge 1999). Fatty acid biosynthesis is a basic yet dynamic process that is developmentally and physiologically regulated (Baud and Lepiniec, 2009, 2010). Fatty acid biosynthesis in plants is quite different from other eukaryotes in that it occurs inside the plastid of plant cells such as chloroplasts in green tissues or leucoplasts in non-green tissues. In addition, the plant fatty acid synthase (FAS) is a dissociable complex with separate proteins for the acyl carrier proteins (ACP) and each enzyme (Chapman and Ohlrogge 2012). Glucose which is produced from photosynthesis supplies carbon that is channeled into the fatty acid pathway.

The conversion of glucose to pyruvate occurs inside the plastid or cytoplasmic sub-compartments. However, only plastidial pyruvate is converted into acetyl-CoA. Acetyl CoA is the initial substrate for the synthesis of the backbone of all fatty acids. Inside the plastids, pyruvate dehydrogenase catalyzes the formation of acetyl-CoA from pyruvate. Acetyl-CoA carboxylase (ACCase) then catalyzes the formation of malonyl-CoA from acetyl-CoA and bicarbonate (Sasaki and Nagano 2004). ACCase is a key rate-determining step that controls FA synthesis (Donaldson 1979; Wright et al. 2006). The activity of ACCase is regulated by light, phosphorylation, thioredoxin, PII protein, and product feedback control. A homodimeric form of ACCase is found in plastids of all plants except Poaceae (grass family) whereas a heterodimeric form is found in the cytosol of all plants and the plastids of members of the grass family (Sasaki and Nagano 2004). Before getting incorporated into the FA synthesis pathway, the malonyl

group of malonyl-CoA produced by ACCase is transferred from CoA to an acyl carrier protein (ACP) by a malonyl-CoA: ACP malonyl transferase (Bao and Ohlrogge 1999). Acyl carrier protein (ACP) possesses a phosphopantetheine prosthetic group that is covalently bound to a serine. This prosthetic group provides an elongated and flexible arm that can reach into active sites and a terminal sulfhydryl group for the attachment of acyl groups through a thioester linkage (Zhang et al. 2001). Fatty acids are grown by sequential condensation of two-carbon units by enzymes of the fatty acid synthase (FAS) complex followed by a series of dehydration and reduction reactions (Chapman and Ohlrogge 2012).

Three isoforms of ketoacyl-ACP synthase (KAS) all found in plants function cooperatively to elongate acyl chains on an ACP platform to C16 or C18. KASIII initiates FA synthesis by condensation of acetyl-CoA and malonyl-ACP (Andre et al. 2012). KASI continues elongation to C16:0 whereas KASII provides the final elongation step from C16:0 to C18:0. Subsequently, 18:0 ACP has desaturated the enzyme stearoyl-ACP desaturase (SAD) to produce 18:1 ACP, and FATA and FATB thioesterases release newly synthesized 16:0, 18:0, and 18:1 fatty acids in the plastid stroma. The NADPH and ATP for FAS can be generated by photosystems depending upon the plant species and the presence of thylakoid membranes (Baud and Lepiniec 2010; Chapman and Ohlrogge 2012). Thus, the main products of plastid FA synthesis are 16:0 and 18:1 free acid. Their relative abundance is mainly determined by the activities of FATA, FATB, 18:0-ACP desaturase (SAD), and KASII (Bates et al. 2013). Transgenic studies have indicated that seed FA chain length and saturation can be altered by the manipulation of any of these four enzymes. For example, expression of FATB specific for lauric acid led to the production of 60% lauric acid (12:0) in transgenic *Brassica napus* (Voelker et al.1996).

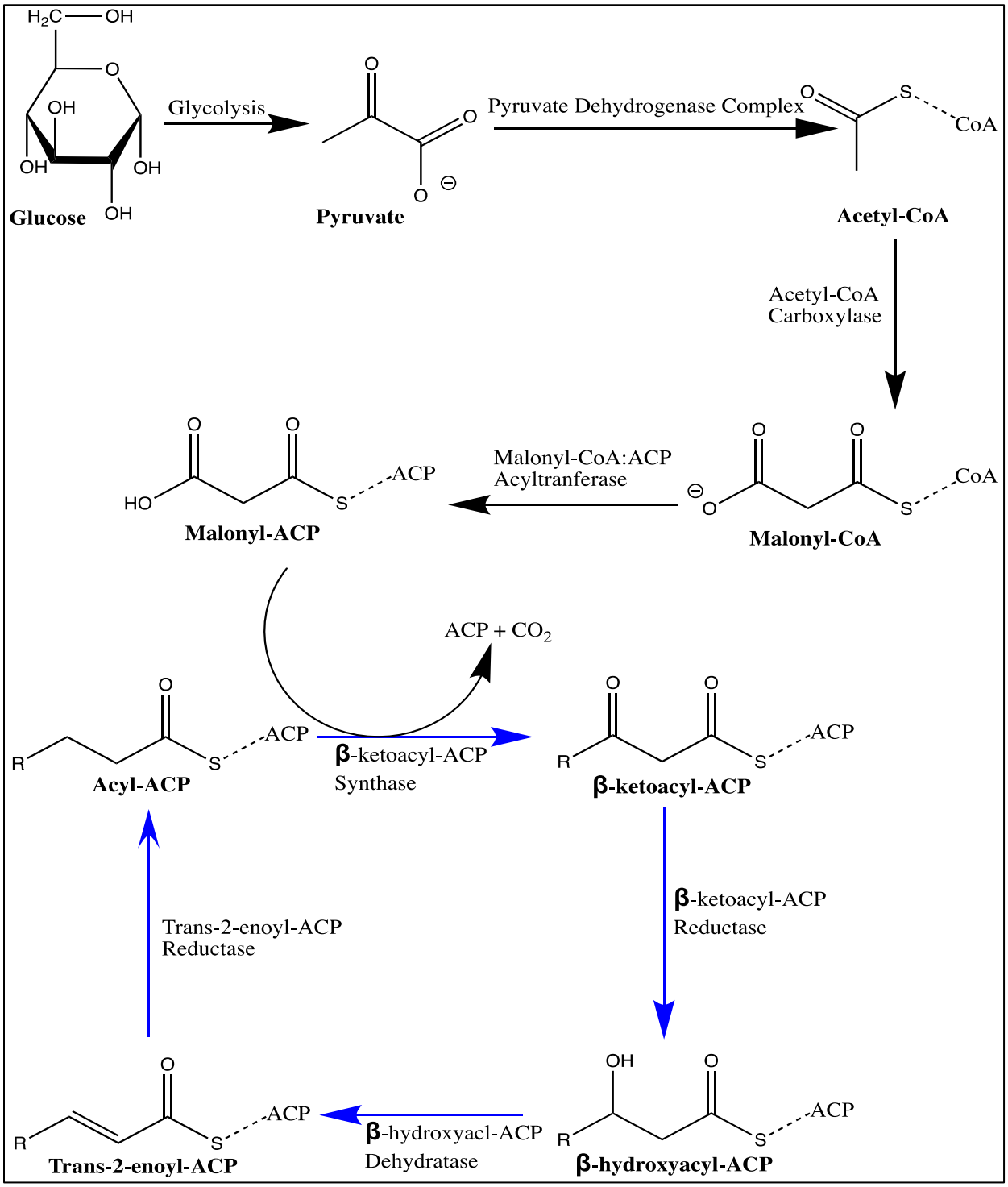


Figure 1. A schematic illustration of the fatty acid synthesis pathway in higher plants

Regulation of Fatty acid Biosynthesis in Plants

The process of fatty acid biosynthesis is under the transcriptional control of the *WRINKLED1* transcription factor (Cernac and Benning 2004; Ma et al. 2015; Vogel et al. 2019). The WRI1 transcription factor regulates the expression of at least 15 enzymes that are involved in the late glycolytic and fatty acid biosynthetic pathways (Baud and Lepiniec 2010). WRI1 expression in plant tissues is, therefore, key to directing the carbon flux into FA synthesis. *Wri1* knockouts demonstrated a decrease in oil contents. However, overexpression of WRI1 in maize embryos increased seed oil in the embryo by over 30% while total oil yield increased by 20% (Shen et al. 2010). In recent years, the identification of WRI1 co-regulators as well as elucidation of structural features and molecular functions of WRI1 have contributed to the understanding of the mechanism of the transcriptional regulation of plant oil biosynthesis (Kilaru et al. 2015; Behera et al. 2021).

Transcriptome studies of oil-rich non-seed tissues such as avocado, oil palm, and olive revealed several similarities in the expression pattern of genes involved in the plastidial fatty acid synthesis. However, unique variations were observed in the expression of genes involved in TAG assembly (Kilaru et al. 2015; Rahman et al. 2016). In seed tissues, many of the master regulators of embryogenesis and seed maturation, such as LEAFY COTYLEDON (LEC) genes LEC1, LEC1-like (L1L), LEC2 and FUSCA3 (FUS3), and abscisic acid (ABA) - insensitive3 (ABI3) regulate TAG synthesis directly or indirectly through the downstream transcription factor WRINKLED (Kagaya et al. 2005; Baud and Lepiniec 2010).

Once Fatty acids are synthesized, they are elongated by the fatty acid synthase (FAS) complex, with two carbon increments. Fatty acids are then exported from plastids to ER, in the form of acyl-CoAs. Each acyl chain is hydrolyzed by an acyl-ACP thioesterase (FAT) into free fatty acids and then reactivated by long-chain acyl-CoA synthetase (LACS). Although free fatty acids may transit the chloroplast inner envelope membrane via simple diffusion, fatty acid export 1 (FAX1), an integral protein embedded in the inner envelope membrane through alpha-helical membrane-spanning domains emerged as a likely transporter (Li et al. 2015; He et al. 2020). Mutation of FAX1 decreased levels of ER-derived lipids but its overexpression increased lipid content (Li et al. 2015). The FAX family consists of 7 members. Whereas the role of FAX1 is more pronounced in leaves and flowers (Li et al. 2015) and the mesocarp of plants such as avocado and oil palm trees (Kilaru et al .2015), FAX2 and FAX4 are important for seed oil accumulation (Tian et al. 2019; Dastmalchi 2020; Li et al. 2020).

Triacylglycerol Assembly

Plants synthesize oil mainly TAG which is required by germinating seeds as a source of energy as well as playing other vital roles in non-seed tissues. The oil biosynthesis pathway is conserved in the plant kingdom but the molecular and biochemical factors responsible for TAG synthesis appear to have been altered during plant evolution leading to diversity in oil content and composition as seen in different plant species and/or different plant tissues. TAG is made up of three fatty acids esterified to a glycerol molecule (Fig. 2). Triacylglycerols are assembled in the endoplasmic reticulum (ER). Recent studies have shown that the enzymes involved in TAG assembly-DGAT1 and PDAT1- are under transcriptional control of the MYB96 transcription factor (Lee et al. 2018). Once fatty acids are synthesized in the plastid, acyl-CoA synthetase catalyzes the formation of acyl-CoA thioester from fatty acids that are exported from the plastid to the endoplasmic reticulum (ER) for TAG assembly (Kennedy 1961; Huang 1996). TAG is

formed via sequential acylation of the *sn-1, sn-2*, and *sn-3* positions of glycerol-3 phosphate with acyl-CoA in the *de novo* Kennedy pathway. The enzyme glycerol-3-phosphate acyltransferase catalyzes the first acylation and the lysophosphatidic acid generated in the initial step is converted to phosphatidic acid by lysophosphatidic acid acyltransferase, which is further hydrolyzed by phosphatidic acid phosphatase to generate diacylglycerol (DAG) (Bates et al. 2013)

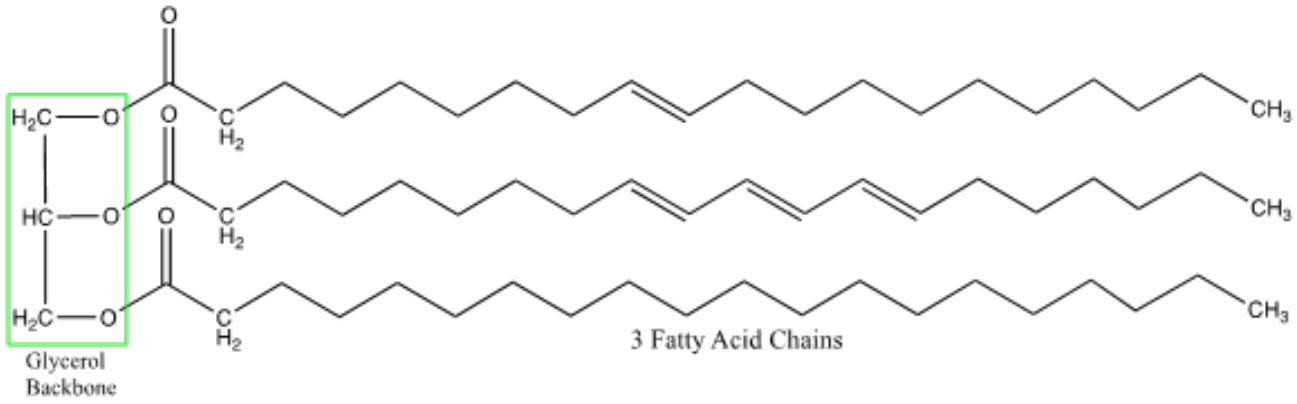


Figure 2. Structure of Triacylglycerol

The fatty acids which make up TAG can either be saturated or unsaturated and their lengths vary depending on the number of carbon chains.

Acyl-CoA Dependent TAG Synthesis

DGAT catalyzes the last step of TAG accumulation by transferring acyl moiety from an acyl-COA to the *sn-3* position of DAG (Fig.3). Membrane-bound **(**DGAT1 and DGAT2) and a soluble type (DGAT3) are the three unrelated families of DGAT that have been identified in various plant species. DGAT1 and DGAT2 were the only ones shown to participate in TAG synthesis (Ichihara et al. 1988; Turchetto-Zolet et al. 2016). Though functional, DGAT1 and DGAT2 were not found to share similar amino acid sequences (Kalscheuer and Steinbüchel

2003). In *Arabidopsis*, DGAT1 is about 59KDa whereas DGAT2 is approximately 36KDa. DGATs are membrane-localized and associated with oil bodies (Gurr et al.1974; Siaut et al. 2011) and/or ER (Lung and Weselake 2006). The mechanism by which the DGATs are localized in the oil bodies is not well understood but is thought that the oil bodies are generated from the ER and imported to the cytoplasm, with DGAT in the membrane of the oil bodies (Huang 1996). On the other hand, some studies have suggested that oil bodies are not associated with the ER and when nascent oil bodies are translocated from the ER, they are surrounded by oil body components by other factors, in which case DGATs would not appear in their membranes (Ichihara and Noda 1982). These observations varied with plant species and more studies are required to understand the activity of DGAT, especially in those plant species where oil bodies protected by oleosins are not well documented. Both DGAT1 and DGAT2 are involved in TAG biosynthesis in several seeds and non-seed tissues. While in most tissues one of the enzymes may play a predominant role, in several others, they may function together in an overlapping manner. In seed tissues, such as embryos of *Arabidopsis* and *Brassica napus* (rapeseed),

DGAT1 was highly expressed but DGAT2 was hardly expressed (Li et al. 2010; Troncoso-Ponce et al. 2011). The role of DGAT1 in TAG accumulation in *Arabidopsis* seeds has been further confirmed by the overexpression of DGAT1 in seed tissues which led to enhanced TAG content, but its perturbation resulted in only a 20 - 40% reduction in oil content (Andrianov et al. 2010). Apart from reduced seed oil content, *Arabidopsis dgat1* exhibits increased levels of 18:3 fatty acids and reduced levels of 18:1 and 20:1 fatty acids, reduced germination rate, and limited activity during seed development.

Acyl-CoA Independent Triacylglycerol Synthesis

Acyl-CoA-independent TAG formation is mediated by PDAT that is found in yeast and plants (Fig.3). PDAT uses phospholipids as acyl donors for the conversion of DAG to TAG (Dahlqvist et al. 2000). During this process where PDAT is involved, an acyl group from the *sn-2* position of phosphatidylcholine (PC) is transferred to the *sn-3* position of DAG forming TAG and *sn*-1 also-PC (Banaś et al. 2013). PC is the site for ER fatty acid desaturation and acyl editing and therefore accumulation of unusual fatty acids is more likely to occur (Bates and Browse 2011). At least six *PDAT* like genes have been identified in *Arabidopsis.* However, only *PDAT1* accounted for most of its PDAT activity (Ståhl et al. 2004).

Disruption or overexpression of *PDAT1* does not affect TAG accumulation much in *Arabidopsis* seeds (Ståhl et al. 2004; Mhaske et al. 2005). However, silencing of *PDAT1* expression by RNAi mechanism in a *dgat*1-1/- mutant background reduces TAG content in seeds by 70–80% (Zhang et al. 2009). This suggests that *PDAT1* most likely compensates for the loss or absence of *DGAT1* and vice versa and that the acyl-CoA-dependent and independent pathways are cooperatively involved in TAG synthesis in many oil-rich parts of plants. *Arabidopsis* double mutant *pdat1*/*dgat1* was characterized by abnormal, sterile pollen and poorly developed embryo hence it is generally considered lethal (Zhang et al. 2009).

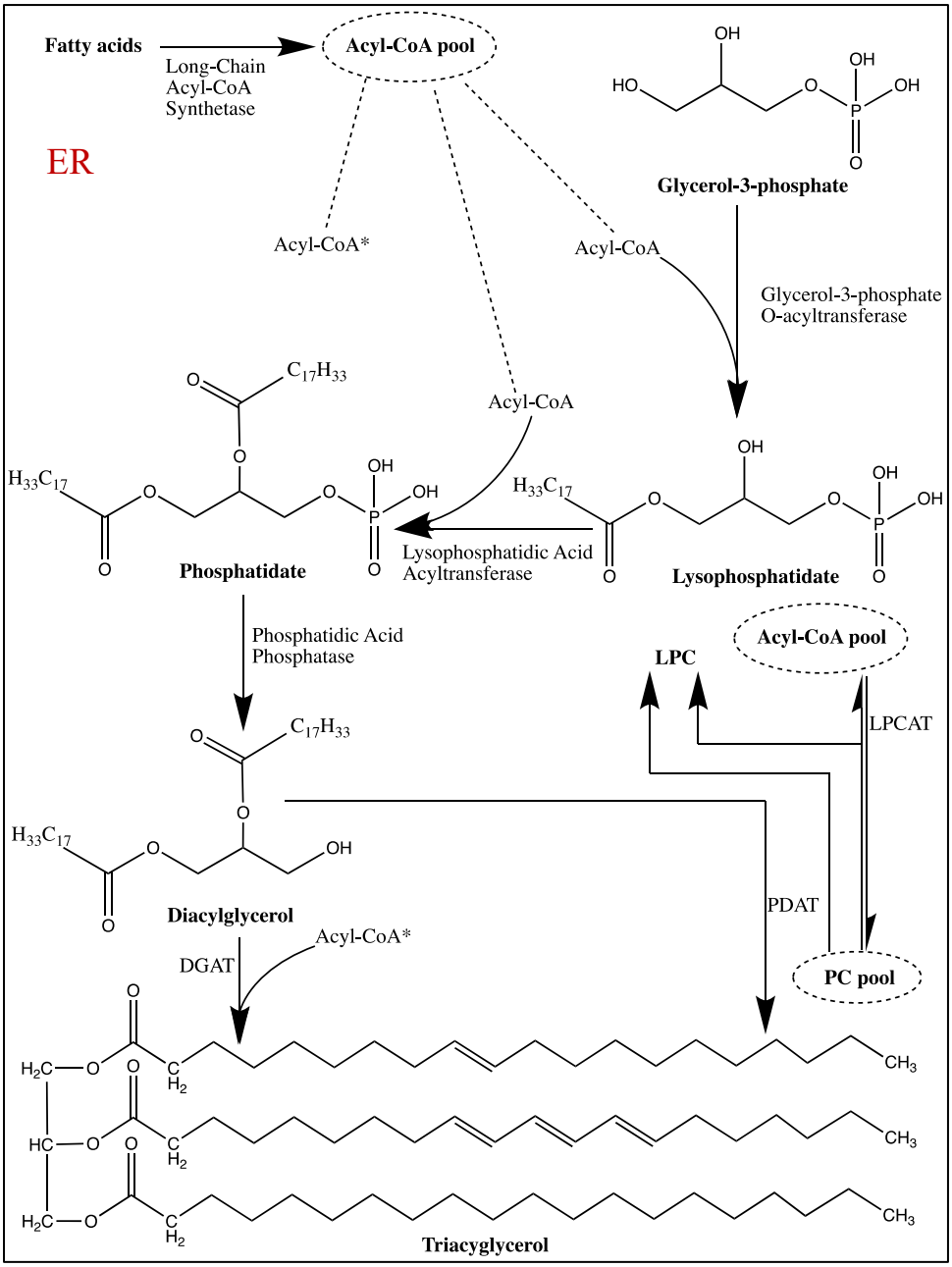


Figure 3. Triacylglycerol assembly in plants

DGAT1 uses acyl-CoA to convert diacylglycerol (DAG) to Triacylglycerol (TAG). PDAT1 uses phospholipids such as phosphatidylcholine (PC) as a source of acyl chains to covert DAG into TAG.

Camelina as an Oilseed Crop

Camelina sativa, a member of the Brassicaceae family, is an emerging oilseed crop designed for biofuel production as well as a source of edible oil and for making bioproducts. It has elicited increasing interest in recent years due to its positive agronomic attributes (Vollmann et al. 2007; Zanetti et al. 2017), geographic adaptability (Kang et al. 2011), availability of genetic information, ability to be engineered easily (Abdullah et al. 2016) and the wide range of applications for its oil as well as other biological products (Shen et al. 2010). The use of camelina as a biofuel crop dates back to the 1950s in part due to its high seed oil content (30-40% of dry weight) , short growth length between 85-100 days with low agricultural input (Putnam et al. 1993), ability to be grown as an annual crop,high levels of omega 3 fatty acids and its excellent adaptability to drought conditions (Séguin-Swartz et al. 2009). Although camelina has potential benefits for use as biodiesel, its seeds need to have higher oil yield to be a successful biofuel crop hence a need to engineer it for increased oil yield as well as fatty acid composition.

Avocado PDAT1 and DGAT1

Avocado (*Persea americana)* which belongs to the Lauraceae family*,* is a commercially important plant. It is a basal angiosperm (Soltis and Soltis 2004). It accumulates about 70 % of oil (table 1) comprising mainly oleic acid (C18:1) in the mesocarp (Kilaru et al. 2015). Recent avocado genome sequencing has revealed that avocado lineage underwent two lineage-specific polyploidy events during its evolutionary history and that duplicate genes descending from polyploidy expanded the transcription factor diversity of avocado (Rendón-Anaya et al. 2019). The avocado fruit takes approximately 9 months to develop and grow. Studies have shown that fruit development, growth, and accumulation of its storage metabolites (TAG) are highly

coordinated processes that are regulated by crosstalk between various hormones and genes involved in TAG synthesis. For instance, treatment with exogenous ABA enhanced TAG accumulation by inducing the expression of various lipid biosynthesis genes in the developing seeds of castor bean (Chandrasekaran et al. 2014).

The rate of TAG accumulation in the avocado mesocarp and changes in its composition are directly correlated with fruit development and an increase in its biomass (Kilaru et al. 2015; Battistelli et al. 2019). The fatty acid composition in avocado was found to be tissue-specific and varied with the development of mesocarp. Among the major fatty acids, oleic acid (18:1) was most abundant in mesocarp which coincided with high expression of stearoyl-ACP desaturase genes, while in seeds linoleic acid was predominant throughout the development (Kilaru et al. 2015; Battistelli et al. 2019; Ge et al. 2021). There was a small variation in mesocarp composition for 16:0, 16:1, and 18:0, during the mid to late stage of development, a steady increase in 18:1 and a concurrent decline in 18:2 proportion. Seeds showed very little variation in fatty acid composition during the development but contained a higher proportion of linolenic acid and a lower 16:1(Kilaru et al. 2015). The higher levels of sugars in the early stages of fruit growth might play a role in regulating the initiation of oil biosynthesis, further suggesting that TAG synthesis is coordinated with complex metabolic networks (Kilaru et al. 2015; Zhai et al. 2021). Although avocado contains about 70% oleic acid in the mesocarp (Kilaru et al. 2015), camelina contains just 21 % of oleic acid in the seeds (Abdullah et al. 2017) while Arabidopsis contains 15% of oleic acid in the seeds as shown in Table 1. An increase in oleic acid content in Arabidopsis seeds through functional complementation of mutants would allow us to translate the concept into camelina as well as canola.

Table 1. Comparison Between Avocado, Arabidopsis, and Camelina Oil Composition

Fatty Acid	Avocado Mesocarp Oil (%)	Arabidopsis Mature seed oil (%)	Camelina Mature Seed oil (%)
Palmitic acid (16:0)	20	10.2 ±1.2	0.16 ± 0.02
Palmitoleic acid (16:1)	4	-	3.38 ± 0.08
Stearic acid (18:0)	1	2.5 ± 0.3	11.84 ± 0.15
Oleic acid (18:1)	70	15.4 ±1.1	21.04 ± 0.14
Linoleic acid (18:2)	18.5.	32.7 ±1.9	30.28 ± 0.27
Linolenic acid (18:3)	1.5	20.3 ±1.2	2.44 ± 0.04

Most of the information known so far about PaPDAT1 and PaDGAT1 acyltransferases involved in oil biosynthesis in avocado have been derived from available transcriptome data (Kilaru et al. 2015). Multiple sequence alignment has shown that PaPDAT1 and PaDGAT1 share significant homology with other known eukaryotic PDATs and DGATs (Rahman, 2018, unpublished). To date, the role of avocado *PaPDGAT1* and *PaDGAT1* in seed oil biosynthesis has not been elucidated. Their role in seed oil production must be investigated because the world demand for vegetable oil is surging daily and research into alternative sources of oil is vital to meet this demand.

Rationale, Hypothesis, and Specific Aims

The main aim of this project was to elucidate the role of PaPDAT1 and PaDGAT1 in seed oil biosynthesis using Arabidopsis and camelina as model organisms. Avocado provides a rich source of monounsaturated fatty acids and stores about 80% of oil in the mesocarp. Since PDAT1 and DGAT1 are conserved in higher plants, it is expected that PaPDAT1 and PaDGAT1 will play a role in seed oil synthesis in Arabidopsis and camelina even though oil accumulates predominantly in the mesocarp of avocado fruit but not in the seed. Previously, the two genes were transiently expressed in the leaves of *Nicotiana benthamiana* and shown to be capable of increasing total lipid content acid content and oleic acid. However, their role in the accumulation

of TAG in seed tissues remains elusive. Wild-type Arabidopsis seeds typically show 20-30% TAG by dry weight. Both *Atdgat1* and *Atpdat1* mutant lines show a 50-80% reduction in seed oil. Mutant seeds also showed changes in seed morphology and germination rate (Zhang et al. 2009). An increase in camelina oil yield would boost its candidacy for use as a biofuel as well as act as a reservoir for oleic-rich oil. It is pertinent that functional validation of PaPDAT1 and PaDGAT1 in Arabidopsis and camelina be carried out to increase the prospect of generating transgenic plants with large amounts of TAG in the future for biofuel production as well as for sustainable sources of heart-healthy oils.

Hypothesis and Specific Goals

I hypothesize that PaPDAT1 and PaDGAT1 will affect monounsaturated oil content and composition in Arabidopsis and camelina. To test this hypothesis, the following specific aims are proposed:

Aim 1: To carry out functional complementation of Arabidopsis mutants by avocado DGAT1 and PaPDAT1.

Aim 2: To overexpress avocado DGAT1 and PDAT1 in wild type camelina plants.

CHAPTER 2. MATERIALS AND METHODS

Arabidopsis Growth Conditions

Arabidopsis thaliana ecotype Col was used as control of wild-type plants for the different ecotype mutants. SALK mutant lines, Col ecotype, were obtained from the Salk Institute via the ABRC (Ohio State University, Columbus, OH). Seeds in pots were stratified for 3 d at 4℃ in the dark before being transferred to a controlled growth chamber (Lindsey et al. 2017). The plants were grown in a soil mixture (3:1:1 mixture of peat moss–enriched soil: vermiculite: perlite) in a growth chamber with 16 h light (200 mmol m22 s21 radiation) and 8 h dark at 22℃. For growth on plates, seeds were surface sterilized for 20 min in 20% (v/v) bleach and rinsed three times with sterile water. Seeds were then plated on an agar medium containing half-strength Murashige and Skoog medium salts and 0.75% Phytoblend with/without 1.5% (w/v) sucrose, adjusted to pH 5.7 using KOH before autoclaving. Kanamycin (50 mg/L) was added or omitted after the media was autoclaved. Non-sterilized seeds were also germinated on wet filter paper in a Petri dish and washed every day to reduce possible microbial growth. Germination was determined after 7 days. All seedlings developing on plates were cultured under the same conditions as are planted in pots. Approximately six-week-old Arabidopsis plants, with inflorescences trimmed once, were used for transformation by the floral dip method (Clough and Bent 1998).

Camelina Growth Conditions

Camelina plants were grown in the greenhouse in pots at 22°C under natural light conditions supplemented with high-pressure sodium lights (566 mol/m^2/s) with 16 h of light and 8 h of darkness at a minimum humidity (50 %). The plants were fertilized with 200 ppm N (Peters Professional) and 20-10-20 Peat-lite water-soluble fertilizer and then watered regularly.

Generation of Agrobacterium Clones

Pa*DGAT1* and Pa*PDAT1* were previously cloned in the entry vector pK34 which has a double CaMV35S promoter and a terminator sequence (Shockey et al. 2015). The pK34 vector was digested with the *AscI* restriction enzyme to get the gene insertion, promoter sequence, and terminator sequence for entry into the pCAMBIA DsRed vector. The gene cassette and the digested vector were ligated overnight by T4 DNA ligase from NEB. The resulting plasmid construct was chemically transformed into Top 10 *E. coli* cells for screening. Restriction digestion followed by gel electrophoresis was done to confirm the positive colonies. The plasmids were ex from the positive colonies for further transformation into *Agrobacterium* GV3101.

Preparation and Transformation of Agrobacterium Competent Cells

Agrobacterium GV3101 strain was inoculated in 5 mL of LB media containing 10 µg /mL of rifampicin antibiotic overnight at 28 °C with shaking at 250 rpm. Two mL of overnight grown culture was diluted to 50 mL with LB media and was incubated for another 2-4 hours at 28 °C with shaking at 250 rpm until the OD reached 0.5 to 1.0. Cultures were collected in an ice-chilled 50 mL falcon tube and centrifuged for 20 minutes at 4 °C in a swing bucket centrifuge at 4500 x *g*. The supernatant was discarded, and the pellet was resuspended in one ml ice-cold 20 mM $CaCl_2$. After treating with ice-cold 20 mM $CaCl_2$, the cells became competent and ready to use for transformation. These prepared competent cells were used immediately, and the remaining competent cells were collected into prechilled screw cap tubes. Tubes were then frozen in liquid nitrogen and kept at -80 °C for further use. Competent cells were thawed on ice and 100 µL of competent cells were used for each Pa*DGAT1* and Pa*PDAT1* transformation.
5 µL of 100-1000 ng pCAMBIA vector containing the gene insert

either Pa*DGAT1* or Pa*PDAT1* were added in the competent cells and mixed by flicking the tube. The mixture was kept on ice for 5 minutes and then immediately transferred to liquid nitrogen for 5 minutes. The mixture was collected from liquid nitrogen and incubated for 5 minutes at 37 °C in a water bath. Afterward, 100 µL of the mixture was transferred to a corning tube containing one mL LB media and incubated at 28 °C for 2 hours, shaking at 250 rpm. After that, the mixture was centrifuged at 4500 x *g* for 2 minutes at room temperature and the pellet was collected. The pellet was then resuspended in 50-100 µL of LB media and spread onto LB agar plates containing antibiotic (kanamycin). Plates were incubated for 3 days at 28 °C for colony formation. Positive colonies were confirmed by restriction digestion followed by gel electrophoresis.

Genotyping of Arabidopsis Mutants by PCR

Primers that were used for identifying SALK T-DNA insertion mutants, were designed using the Signal T-DNA Express Arabidopsis Gene Mapping Tool (http://signal.salk.edu/tdna primers.2.html, provided by the Salk Institute Genomic Analysis Laboratory (Alonso et al. 2003). The *pdat1* mutant, designated in the SALK collection as line S065334, was genotyped using left genomic primer S065334LP, right genomic primer S065334RP, and T-DNA left border primer LBb1.3 while the dgat1 mutant designated in the SALK collection as line S039456 was genotyped using left genomic primer S039456LP, right genomic primer S039456RP and LBb1.3. PCR reactions were carried out following the manufacturer's instructions using Promega GoTaq® Green Master Mix (Catalog no: M7123). Table 2 shows the primers used in genotyping the Arabidopsis mutant lines.

Table 2. Gene-specific Primers used in Genotyping Arabidopsis Mutant Lines

Primers	Sequence (5'-3')
*AtP*1-FP	CATGTGGTGTTGCATTTTCAG
*AtP*1-RP	TTTTGTTTTCGGTCTTGTTGG
*AtD*1-FP	GGGTAAAAGTGTTTGCTGCAC
*AtD*1-RP	CTTGTTTTTCAGACACTCGCC
LBb 1.3	ATTTTGCCGATTTCGGAAC

Transformation by Floral Dipping

The binary vector (pCAMBIA) was used for Arabidopsis and Camelina transformation according to the method described by (Clough and Bent 1998; Zhang et al. 2006). About 5 ml culture of *Agrobacterium* containing the construct for transformation was grown, shaking overnight at 28 ℃ in LB with 10 µl 50 mg/ml rifampicin and 10 µl 50 mg/ml kanamycin. This was added to 500 ml LB/Rif/Kan, prewarmed, and shaken overnight at 28 ℃. The mixture was centrifuged at 5K for 20 minutes. While spinning down *Agrobacterium*, a dipping solution was prepared by mixing 25 g sucrose, 250 µl Tween 20, and topped up to 500 ml with MilliQ water. The supernatant was discarded. The dipping solution was added to the *Agrobacterium* pellet and mixed gently with the pipette tip until the pellet is completely dispersed. The wetting agent Silwet L-77 (0.4%v/v) was added before dipping. Half of the mix was poured into a dipping tray and the plants (3-5 per pot) were dipped for 3-5 seconds. Only the inflorescence and all above-ground tissues were dipped. The remaining dipping solution was used for the second dipping of plants after one week.

Selection of Transformants

The T_0 transgenic seeds were screened with a red filter under green light. DsRed has an excitation peak of 546 to 558 nm and an emission of 575 to 640 nm (Zhang et al. 2009). Camelina T_1 generation expressed DsRed, and the positive seeds were isolated. However, DsRed was not detected in Arabidopsis seeds after floral dipping despite multiple attempts. The Arabidopsis T_0 seeds were further screened in hygromycin to select the positive transformants after the fluorescence detection failed but even that did not yield results. All the seedlings died after one week. The selected camelina T_1 seeds were grown for two generations to yield homozygous T_3 seeds. The T_3 lines were used for all subsequent experiments.

Lipid Extraction from Camelina

20 mg of camelina seeds were weighed and put in a glass tube. The seeds were washed with deionized water and then rinsed. The rinsed seeds were put into preheated isopropanol (85 °C) for 15 minutes. The seeds were ground thoroughly with a homogenizer. Three ml of hexane and 2.5 ml of 6.6 % Na_2SO_4 were added and vortexed to mix thoroughly to induce phase separation then kept at room temperature for 30 minutes. The top lipid phase was collected and transferred into a new Teflon-screwed glass tube. The mixture was washed with hexane: isopropanol (7:2) three times. The top phases (hexane layer) were combined. The samples were evaporated under a stream of nitrogen gas at 40 °C to dry the lipid samples. The samples were stored at -20 °C until further use.

Transmethylation Reaction and Fatty Acid Analysis by GC-FID

For fatty acid analyses, the procedure described by Li et al. 2006 was used with some modifications. To 20 mg of lipid extract, 0.2 mL of 2 M methanolic KOH and 2 mL of hexane

were added and vortexed for 2 minutes. The reaction was allowed to proceed for 5 minutes at room temperature. The reaction mixture was vortexed with 400 µL of 2 M HCl (~pH 3–4) to neutralize the alkaline solution to stop the reaction and then centrifuged for 5 min at 4415 x g. The upper hexane phase was carefully transferred to a new Teflon-lined screw-cap glass tube. The mixture was extracted three times with 2 mL of hexane. The organic phases were combined and dried using nitrogen gas and a dry bath then resuspended in 400 µL hexane to achieve a final concentration of ~1 mg/ µL lipid. 1 µL/sample was auto-injected to a GC with an RTX-5MS column (30 m × 0.25 mm × 0.25 µm).

The injector was washed with hexane before loading standard (37-FAME mix) and samples. Helium was used as a carrier gas at a total flow rate of 50 mL/min. The inlet temperature was set to 250 °C, pressure to 27 psi, and split ratio to 0.1:0. The FID detector temperature was set to 300 °C with a hydrogen flow rate of 30 mL/ min, airflow rate of 400 mL/ min, and helium flow rate of 25 mL/ min. The initial oven temperature was set e to 150 °C for 3 min and then ramped to 220 °C at a rate of 6 °C/ min. The injector was washed with hexane before and after each injection. The retention time for each fatty acid was determined using the FAME mix standard. The data obtained from GC-FID was analyzed, and fatty acids were quantified relative to the 17:0 spiked internal standard**.** The peak areas of fatty acids identified were used in the calculation of the fatty acid composition relative to the internal standard (C17:0) using the following formula:

Fatty acid composition (mg/gFW) = amount of internal standard (mg) x (peak area of individual fame/peak area of internal standard)/tissue weight (g). There were 4 replicates for each line. The percent composition of each fatty acid in each line was also calculated from the total fatty acid content for each line.

Statistical Analyses

Student t-test was used to determine significant differences in fatty acid composition in different camelina lines relative to the wild-type. P values <0.05 were considered significant.

CHAPTER 3. RESULTS

Confirmation of PaDGAT1 and PaPDAT1 cloned into pCAMBIA Vector

Pa*DGAT1* and Pa*PDAT1* were subcloned into pCAMBIA for the transformation of camelina and Arabidopsis. The pCAMBIA has a napin promoter that is seed-specific and expresses a DsRed, a fluorescence label that allows for selection. Both genes were put under beta-conglycinin promoter which is also a seed-specific promoter. The pCAMBIA vector has two restriction sites for the *Asc1* restriction enzyme that was used to introduce the gene cassette from the pK34 entry vector and to confirm gene insertion. Figure 4 indicates the cloning sites of Pa*DGAT1* and Pa*PDAT1*.

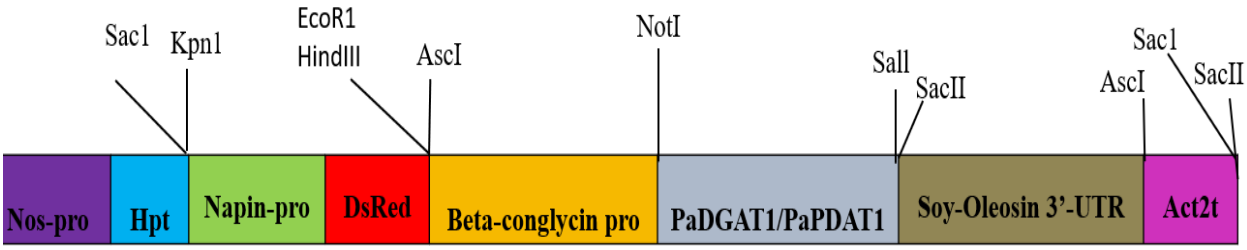


Figure 4: Vector construct showing the cloning site of PaDGAT1 and PaPDAT1 in pCAMBIA The vector has a napin promoter which allows the expression of genes in seeds and DsRed which is a fluorescence label to enable detection under fluorescence light.

After subcloning Pa*DGAT1* and Pa*PDAT1* into the pCAMBIA DsRed vector, restriction digestion using the *Asc1* restriction enzyme followed by gel electrophoresis gave the expected band which includes the whole gene cassette. This confirmation of the insert was done by isolating plasmids from colonies of Pa*DGAT1*/Pa*PDAT1* transformed *Agrobacterium* following through *Asc1* digestion. The results revealed the expected product size of approximately 3kb as shown below.

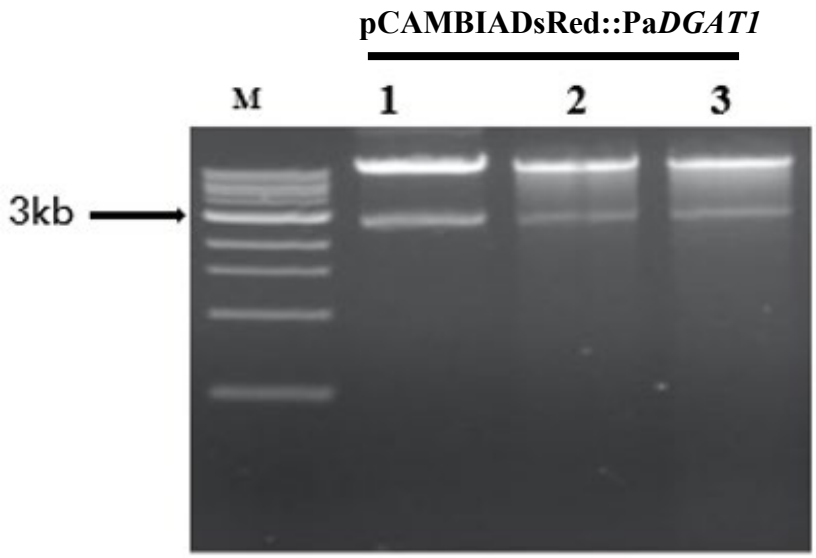


Figure 5. PCAMBIA DsRed::*PaDGAT1* cut with AscI restriction enzyme

The agarose gel electrophoresis confirmed the presence of the Pa*DGAT1* gene cassette in the pCAMBIA vector. The expected product size is about 3000 bp. All three colonies confirmed the insertion of Pa*DGAT1* in *Agrobacterium.*

Similarly, plasmids extracted from 3 colonies of Pa*PDAT1* transformed *Agrobacterium* were digested with the *AscI* restriction enzyme. The agarose gel results confirmed the gene insert of the expected size of 3kb as indicated below.

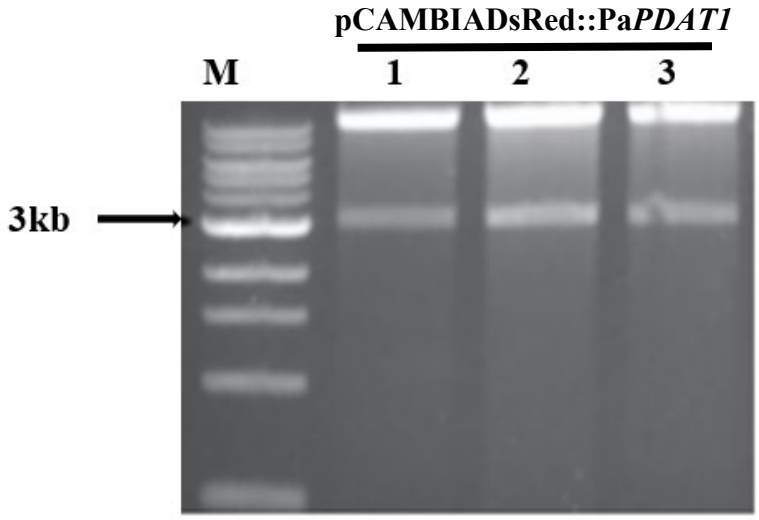


Figure 6. pCAMBIA DsRed::PaPDAT1cut with *AscI* restriction enzyme

The expected product size of PaPDAT1::pCAMBIA DsRed in *Agro* GV3101 confirmed the presence of the PaPDAT1 gene cassette. The expected size was 3200 bp. All three colonies confirmed the presence of gene insert.

Genotyping of Arabidopsis Mutants

The mutants were confirmed by using a pair of forward and reverse primers to amplify the genomic DNA and left border primer and reverse primer to amplify the T-DNA insertion. Using the left border primer and reverse primer, four homozygous mutant lines of *Atpdat1* were identified (Fig.6) but no mutant lines were detected when the forward and reverse primers were used as only the wild type was genomic DNA was amplified (Fig.7). Similarly, using the left border primer and reverse primer, the expected product size was obtained and 4 homozygous mutant lines of *Atdgat1* were identified but no mutant lines were detected when the forward and reverse primers were used as the primers only amplified the genomic DNA (Fig.8). The confirmed mutants were transformed through the *Agrobacterium*-mediated method.

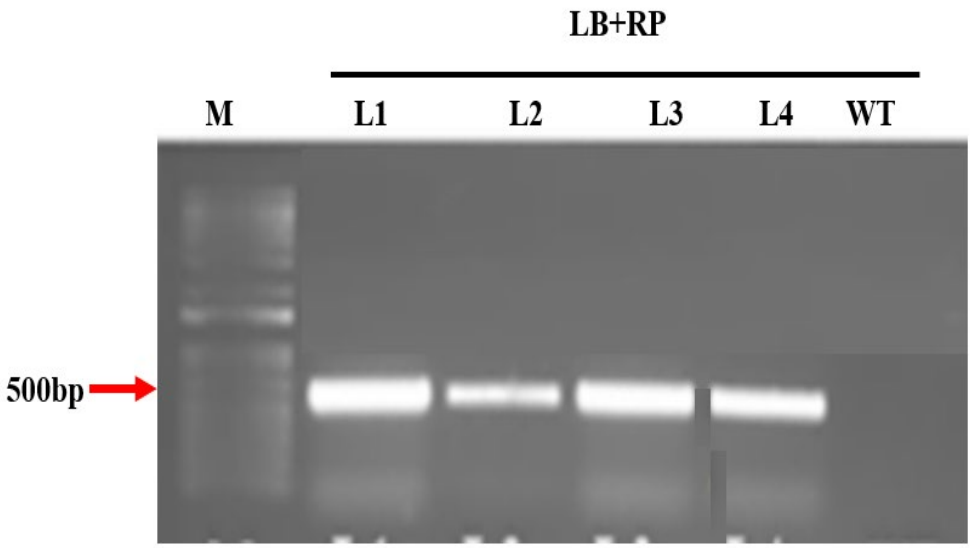


Figure 7**.** PCR analysis of *Atpdat1* mutant line using Left Border primer (LB) and Reverse Primer (RP).

The expected product size of 500bp confirmed the presence of T-DNA insertion.

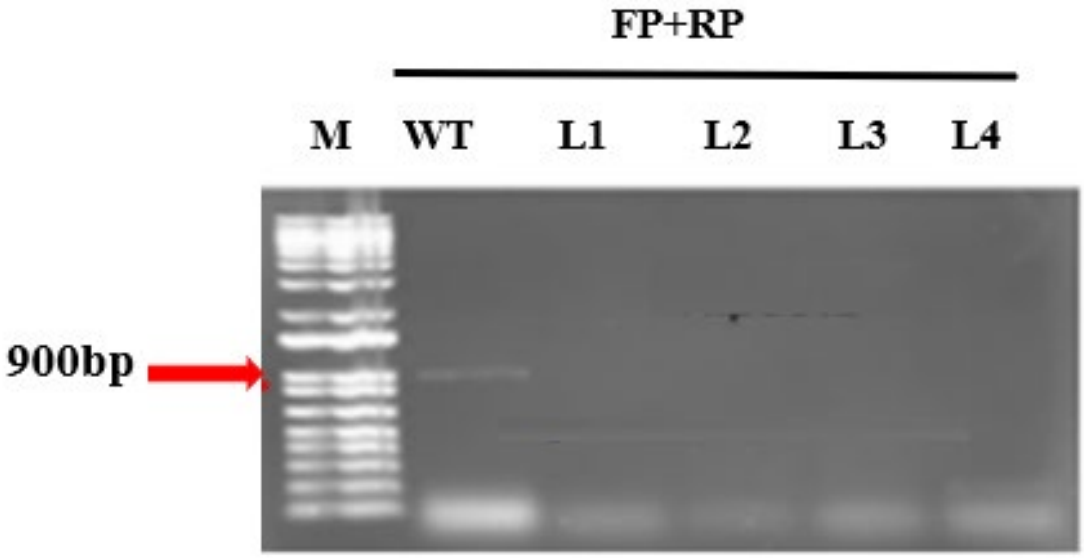


Figure 8. PCR analysis of *Atpdat1* mutant line using Forward Primer (FP) and Reverse Primer (RP)

Both pairs confirmed that *Atpdat1* is a homozygous mutant because only one band was observed when TDNA insertion was amplified using LB primer and reverse primer. No band was observed when PCR was repeated using forward and reverse primers.

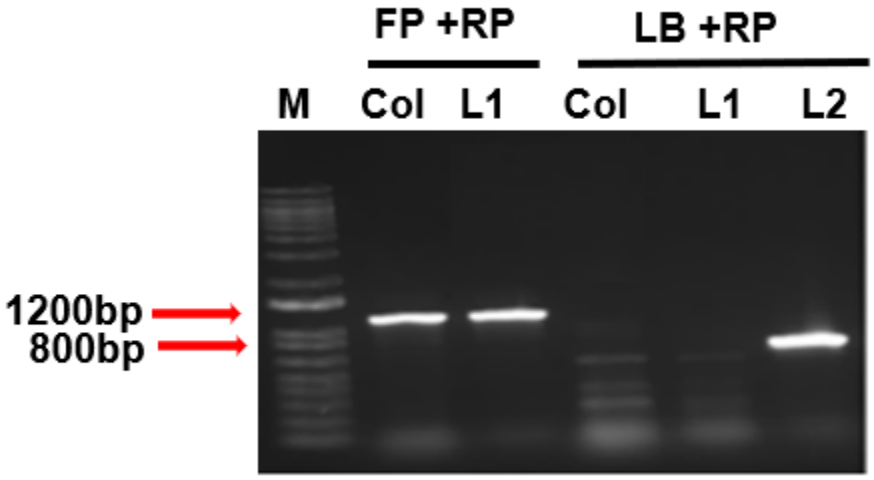


Figure 9. PCR analysis of Atdgat1 mutant line using a pair of forwarding Primer (FP) +Reverse Primer (RP) and a pair of Left Border (LB) and Reverse Primer (RP).

The analysis identified L2 that was homozygous.

Selection of Transformed Seeds

Floral dipping was used in Arabidopsis and camelina transformation. The DsRed in pCAMBIA was used to determine which seeds had *PaDGAT1* and *PaPDAT1*. Screening of Arabidopsis T_1 seeds for DsRed expression did not yield any results. In Fig.10, the various Arabidopsis seed phenotype are shown.

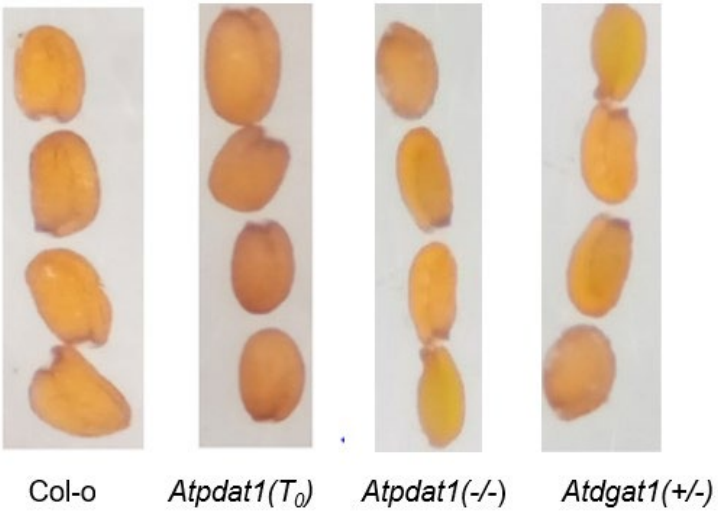
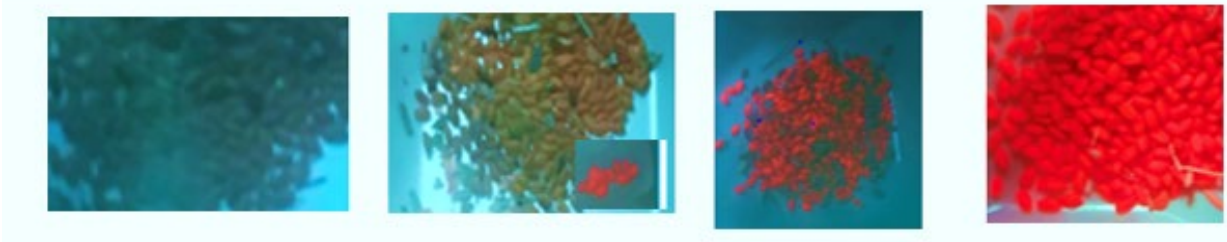


Figure 10. Arabidopsis seed phenotype.

Col-o stands for Colombia-0 (wild type), and *Atpdat1*(T_0) are the seeds harvested after transforming Arabidopsis knockout mutant with avocado *PDAT1*. *Atpdat1-/-* is the homozygous Arabidopsis *PDAT1*-knockout mutant and *Atdgat+/-* is the heterozygous Arabidopsis *DGAT1*-knockout mutant.

Although DsRed was not detected in Arabidopsis T_0 seeds, it was readily detected in camelina seeds. Wild-type seeds do not express DsRed. After the few seeds were selected in T_1 (Fig. 10b), the positive lines were grown to get T_2 lines of which only 75% were positive following the mendelian genetics. The T_2 seeds were further grown to get T_3 lines. The T_3 camelina seeds were used in fatty acid analyses of camelina oil.



a) WT seeds **b) T_1** **c) T_2** **d) $T_{3)}$**

Figure 11. Selection of positive seeds expressing DsRed

Fig. 11a shows wild-type seeds with no DsRed expression; **Fig. 11b** shows T_1 seeds with very few positive seeds; **Fig. 11c** shows T_2 seeds with 75% of them expressing DsRed and **Fig. 11d** shows T_3 homozygous seeds, all of them expressing DsRed.

FAME Analysis by GC-FID

To determine the substrate specificity of PaDGAT1 and PaDGAT1 expressed in seeds tissue, the fatty acid profile in camelina seeds was examined using GC-FID. A total of 12 fatty acids were identified after GC-FID analyses by comparing the peaks with the FAME (37) reference standard. The fatty acids identified were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), a-linolenic acid C18:3), arachidonic acid (C20:0), gondoic acid (C20:1), eicosadienoic acid (C20:2), arachidic acid (22:0), erucic acid (C22:1), tetracosanoic acid C24:0) and nervonic acid (C24:1). The identity of each fatty acid was further confirmed by doing a quick analysis of a few samples using GC-MS and doing a similarity search in the compound database for each peak on the chromatogram.

Among the different lines expressing PaDGAT1, lines D1 3-3-2, D1 7-8-4, and D1 10-4-4 showed a significant increase in C18:1, C18:3, and C20:1 ($P<0.05$) compared to the wild type (Fig. 14). Among the lines expressing PaPDAT1, only line P1 7-8 showed a significant increase in C18:1 and C18:3 (Fig.16). The total fatty acid content was significantly higher in line D1 3-3-2 compared to the wildtype (Fig.12). However, the lines expressing PaPDAT1 did not show a significant increase in total fatty acids when compared to the wild type (Fig.13).

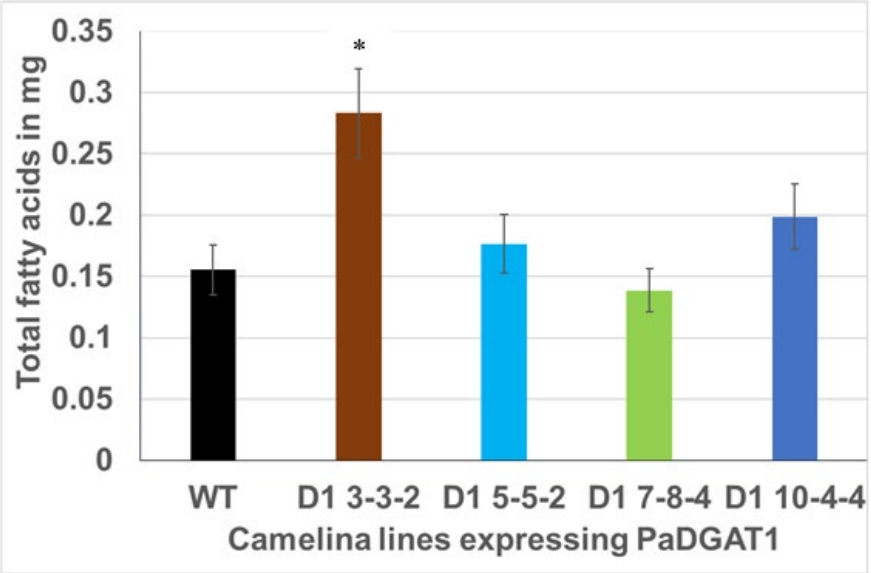


Figure 12. Total fatty acids in camelina lines expressing Pa*DGAT1 c*ompared to the wild type

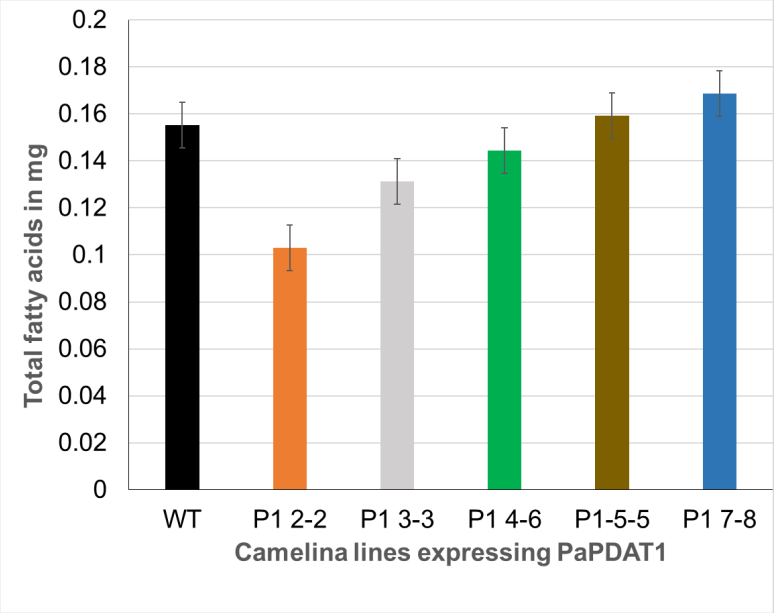


Figure 13. Total fatty acids in camelina lines expressing Pa*PDAT1* compared to the wild type

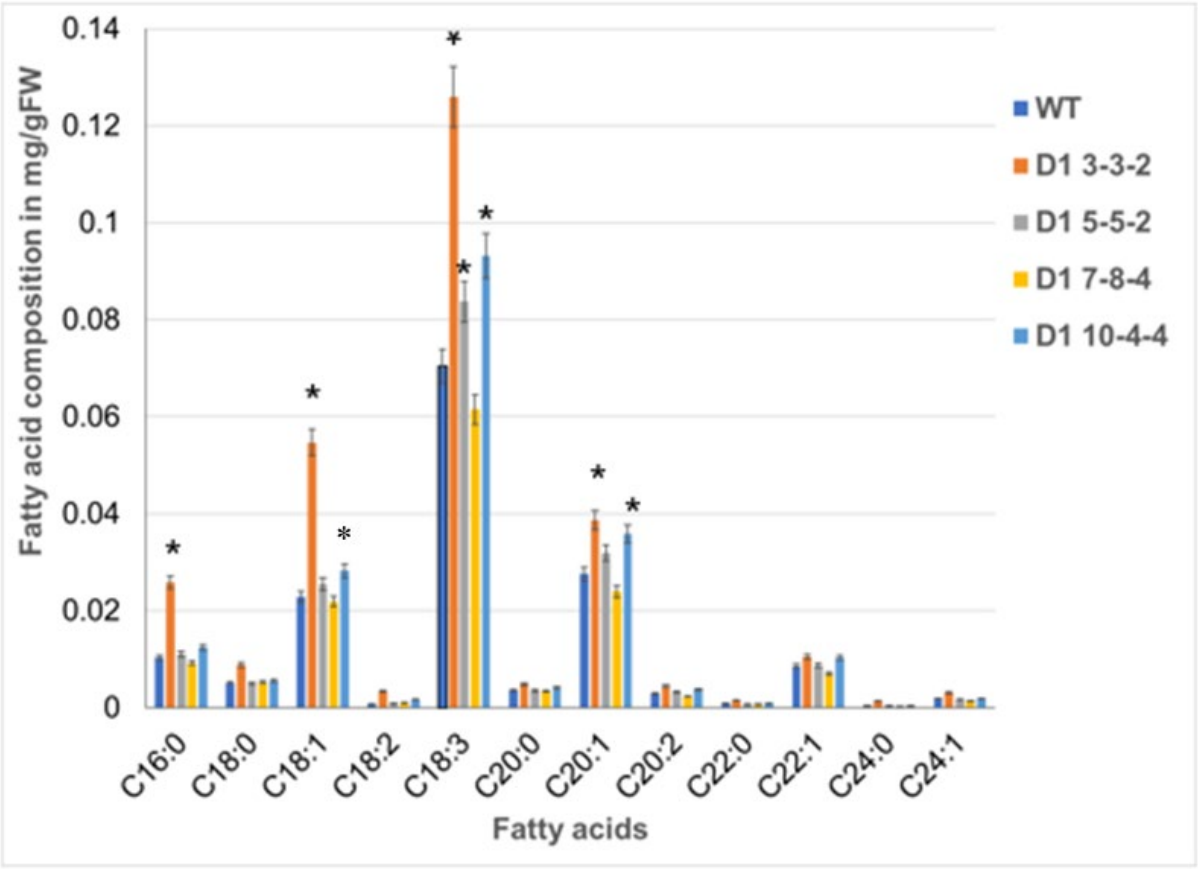


Figure 14. Average fatty acid composition in camelina lines expressing PaDGAT1 compared to the wild type.

The asterisk (*) represents significant values. Lines D1 3-3-2 and line D1 10-4-4 had a significant increase in C18:1 compared to the wild type.

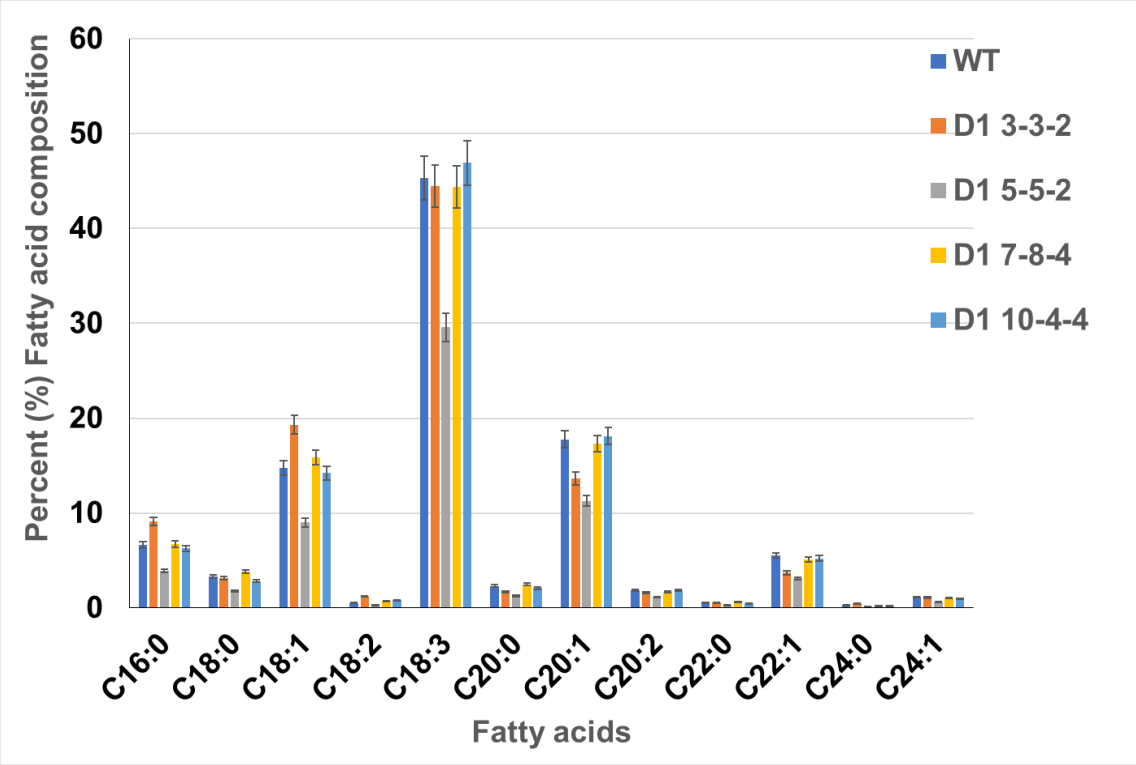


Figure 15. Percent change in oil composition in transgenic camelina lines expressing PaDGAT1 Line D1 3-3-2 showed a 4% increase in C18:1 compared to the wild type.

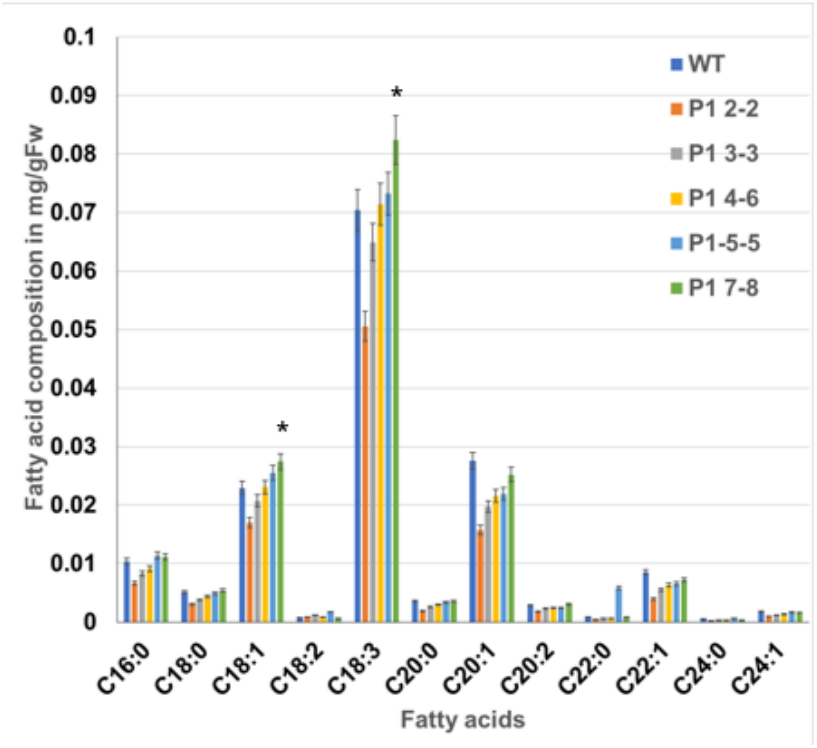


Figure 16. Average fatty acid composition in camelina lines expressing PaPDAT1 compared to the wild type

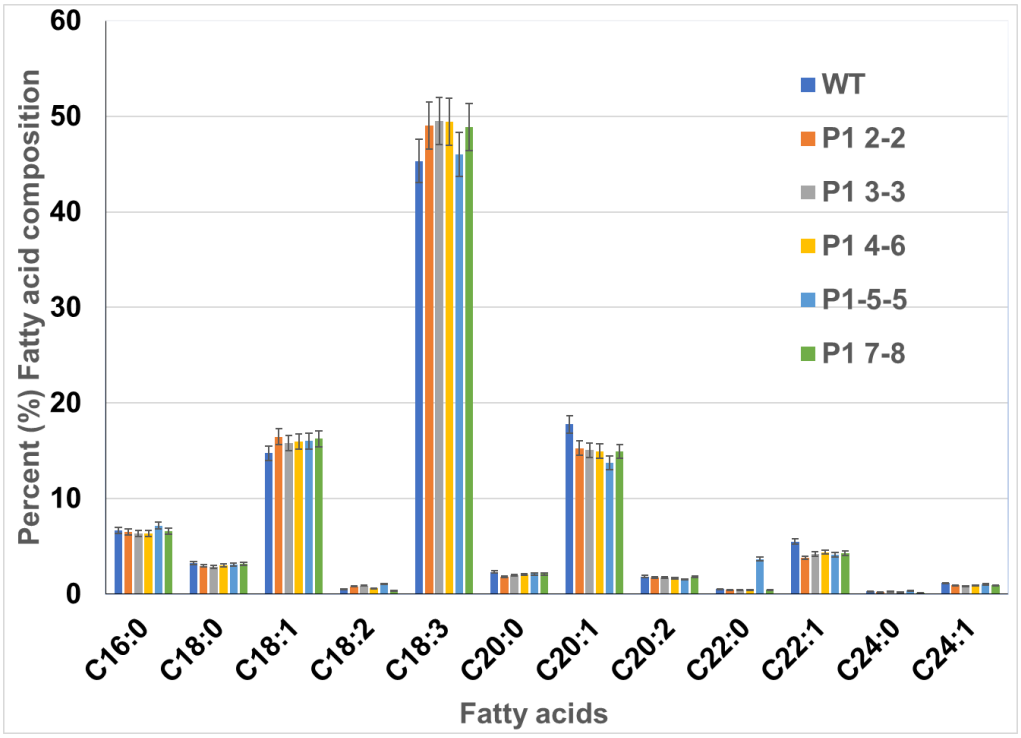


Figure 17. Percent change in oil composition in transgenic camelina lines expressing *PaPDAT1* There is not much difference in percentage oil composition in all lines expressing PaPDAT1

PaDGAT1 Increased C18:1 more than the PaPDAT1 in Camelina Seeds

A comparison was made between transgenic camelina line D1 3-3-2 expressing avocado *DGAT1* and P1 7-8 expressing avocado *PDAT1*. Both lines showed a significant increase in C18:1 However, the results showed that the camelina line expressing avocado DGAT1 (Fig. 17) had a 2-fold increase in C18:1 while the camelina line expressing avocado PDAT1 (Fig. 18) had a 1.2-fold increase in C18:1 which was less than that of camelina PaDGAT1.

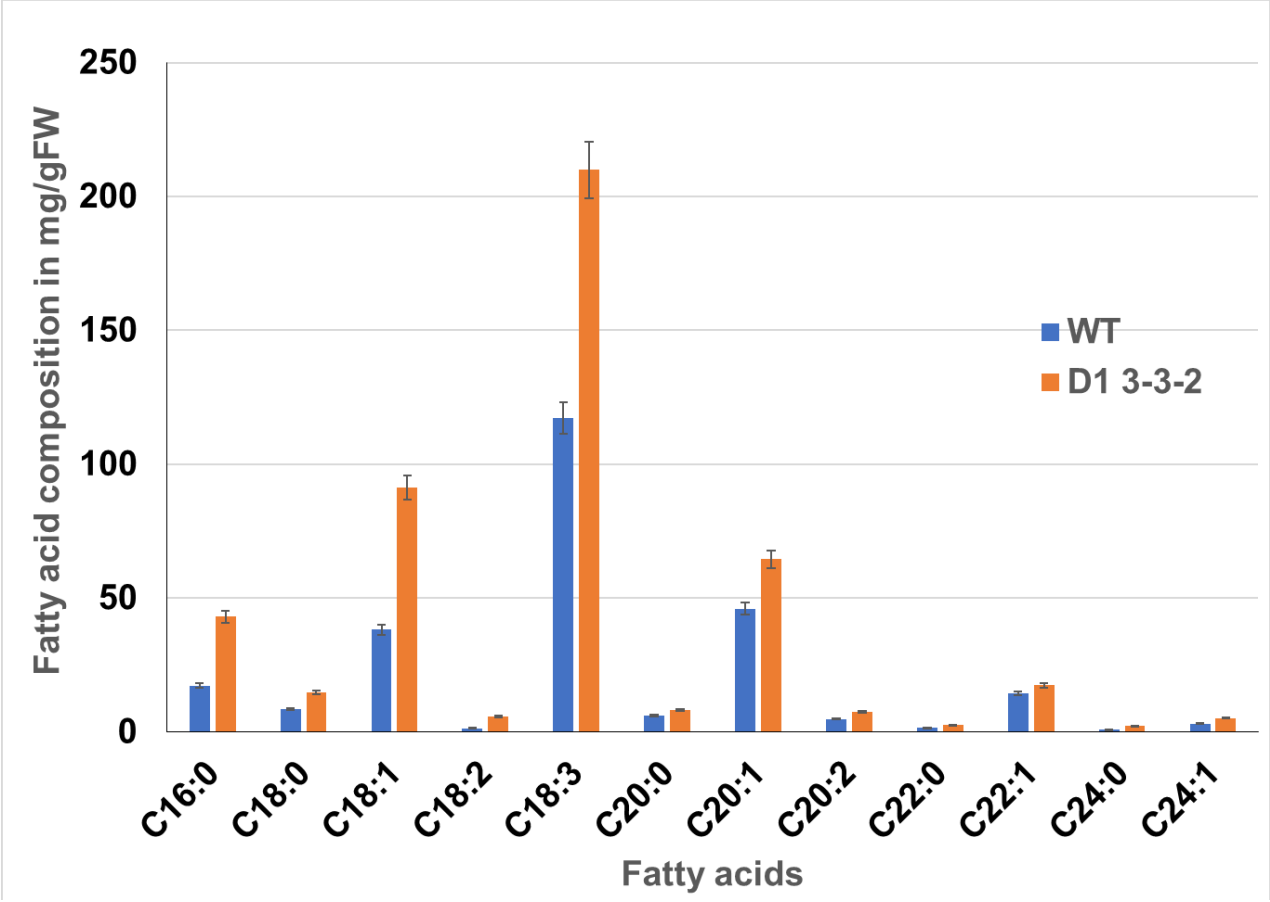


Figure 18. Camelina D1 3-3-2 expressing avocado DGAT1 compared to the wild type.

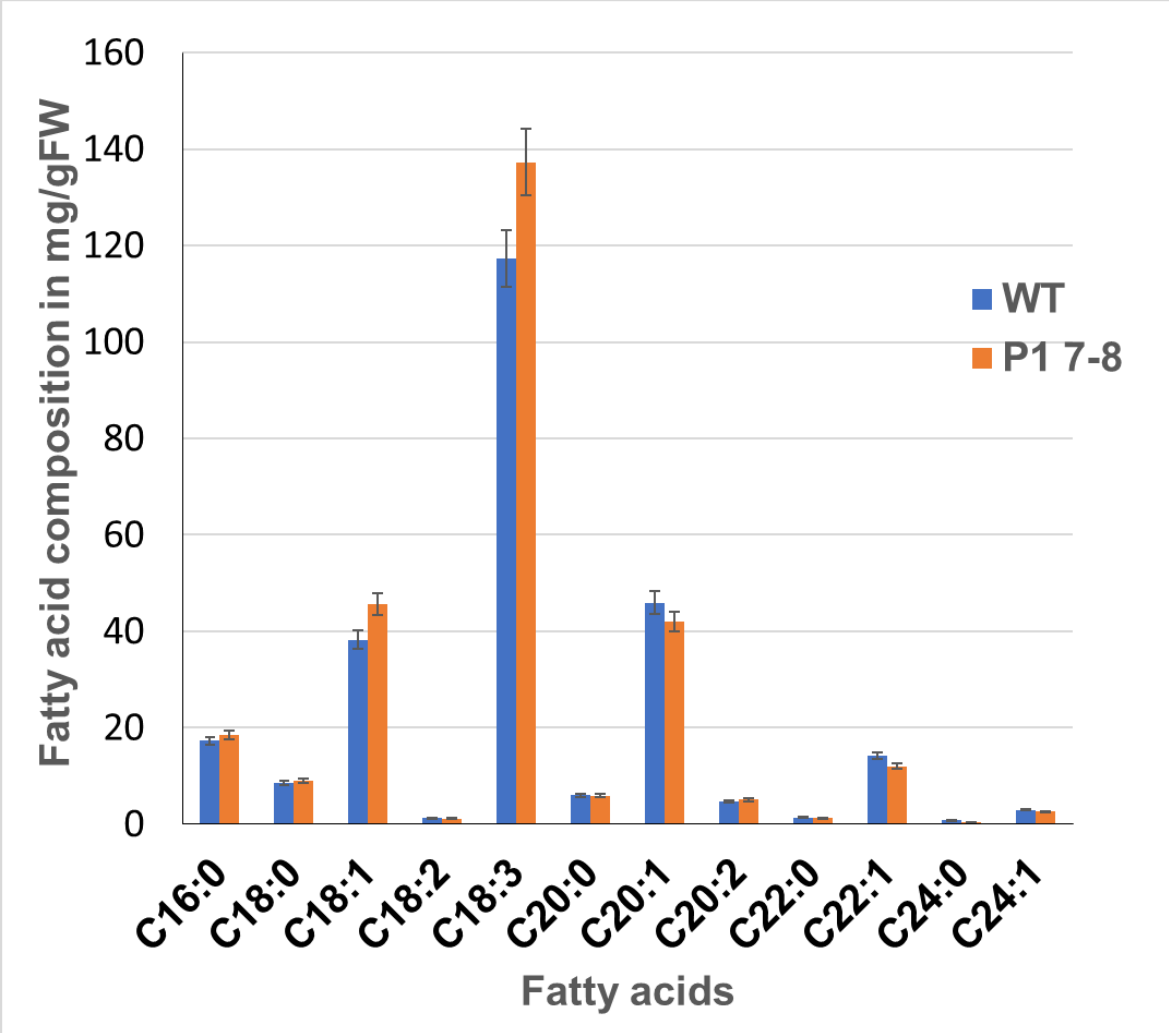


Figure 19: Camelina P1 7-8 expressing avocado PDAT1 compared to the wild type

CHAPTER 4. DISCUSSION

Due to the increasing global demand for vegetable oil, understanding the factors that regulate oil content and composition, particularly in non-seed tissues, is crucial. TAG derived from oil-rich non-seed tissues has attracted increasing attention in recent years. Biotechnology is a promising approach to generating enhanced levels of oil in high biomass plant non-seed tissues. Most of the genes used currently in metabolic engineering to increase oil content and composition in non-seed tissues originate mainly from oilseed tissues. Since the discovery of LDAPs from oil-rich non-seed tissues (Gidda et al. 2016), it is thought that such genes with a non-seed origin are more likely to enhance the oil content in non-seed tissues with little pleiotropic effects on plant growth and agronomic traits (Wan et al. 2019).

Comparative transcriptome studies of oil-rich non-seed tissues such as avocado, oil palm, and olive uncovered several similarities in the expression pattern of genes involved in the plastidial fatty acid synthesis. However, unique variations were observed in the expression of genes involved in TAG assembly (Kilaru et al. 2015; Rahman *et al.* 2016). In mature avocado fruits, more than 85% by dry weight of TAG in mesocarp is predominantly stored as monounsaturated oleic acid (18:1), which increases during fruit development while linoleic acid (18:2) decreases. Besides 18:1 and 18:2, mesocarp also contains 16:0, 16:1, and 18:0, and their distribution was almost constant during the fruit development (Platt-aloia and Thomson 1981; Kilaru et al. 2015; Kerlavage et al. 2018).

A previous study showed that transient expression of PaDGAT1 and PaPDAT1 in the leaves of *Nicotiana benthamiana* led to an increase in total oil content and composition (Mahbubur et al. 2018, unpublished). Expression of PaDGAT1 and PaPDAT1 in Arabidopsis

mutants did not yield any results as DsRed was not detected even with repeated transformation. Screening of the Arabidopsis T_0 seeds in hygromycin did not identify any transformants because all the seeds died after one week of germination. Some of the reasons that could have impacted this outcome could be associated with the vector construct itself, or even post-transcriptional silencing of the genes. We, therefore, chose to overexpress the PaPDAT1 and PaDGAT1 in the camelina. The results of overexpression of the genes suggest that PaDGAT1 and PaPDAT1 are capable of affecting oil composition and content in camelina seeds. While the fatty acid profiles of transgenic camelina lines expressing PaDGAT1 or PaPDAT1 differed, the increase in the level of C18:1 compared to C16:0, particularly in line D1 3-3-2 was remarkable. C18:1 is a monounsaturated fatty acid that is good for heart health. The present results suggest that Pa*DGAT1* expression in D1 3-3-2 increased both 16:0 and 18:1 but it showed a marked preference for 18:1 (Fig. 14). The transgenic line D1 3-3-2 also had a significant increase in total fatty acid content compared with the wildtype (Figs. 12,18). In camelina lines expressing PaPDAT1, lines P1 7-8 expressing PaPDAT1 showed a significant increase in C18:1 and C18:3 (Figs.16,19) compared to the wildtype. However, none of the lines expressing PaPDAT1 had a significant increase in total oil content when compared with the wild type (Fig. 13). A recent study showed that overexpression of Arabidopsis PDAT1 in *Brassica napus* resulted in a small but consistent decrease in seed oil content and altered TAG's fatty acid composition (Fenyk et al. 2022). PDAT1 is known to accumulate unusual fatty acids (Bates and Browse 2011). PDAT1 also uses a different pathway which does not appear to be more efficient compared to the Kennedy pathway. Previous studies have shown that silencing or knocking down PDAT1 in a DGAT background in Arabidopsis did not show any significant increase in fatty acid content suggesting that DGAT1 contributes more to TAG assembly in absence of PDAT1 (Zhang et al.

2009). The substrate preference for DGAT1 in plants differs depending on the species and tissue types and on the availability of the substrates in that species (Shockey et al. 2006). Plant DGAT1 has a wide range of substrate specificity from medium to long-chain fatty acids with mono or polyunsaturation. A high preference for 18:1 compared to 16:0 in camelina line D1 3-3-2 expressing PaDGAT1 is not unusual for plant DGAT1s. A previous study showed that the ancestral maize DGAT1-2 has a high preference for oleic acid 18:1 but it lost its specificity for oleic acid significantly and the enzyme efficiency when one specific phenylalanine (469 position) was deleted during the breeding (Zheng et al. 2008). This suggests DGAT1 orthologs may show different substrate specificities when they differ by only one amino acid residue. Another study on *Brassica napus* showed that DGAT1 had a higher preference for 18:1 compared to 16:0. The high preference of *Brassica* DGAT1 for 18:1 correlated with the seed fatty acid composition which is characterized by a high percentage of 18:1 (Aznar-Moreno et al. 2015). Similarly, in avocado, the preference of DGAT1 towards 18:1 correlates with the fatty acid composition of avocado mesocarp (Kilaru et al. 2015).

Currently, the underlying mechanism that dictates the fatty acid composition of TAG accumulation is not completely understood. TAG synthesis which commences from acetyl-CoAs involves about 30 reactions. Thus, regulation can occur in several steps (Ohlrogge & Jaworsk 1997). Nonetheless, current literature suggests that DGAT1 and PDAT1 are crucial enzymes and play a role in TAG biosynthesis by determining the TAG composition. Although not all transgenic lines exhibited an increase in oil content and composition, overall, the current study suggests that PaDGAT1 and PaPDAT1 are capable of affecting oil content and composition in seeds. Camelina seed consists of about 43% oil in dry matter with the content of unsaturated fatty acids in the oil being about 90% and 50% of the total fatty acids being

polyunsaturated-linoleic acid (18:2*n*− 6) and α-linolenic acid (18:3*n*− 3) (Zubr 1997). Engineering camelina seeds with avocado acyltransferases could provide a platform for generating enhanced oil content with a high amount of heart healthy C18:1.

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

This study was undertaken due to the paucity of information on the role of avocado acyltransferases in seed oil synthesis and their potential implication in the regulation of TAG assembly. The work has been used to study the effect of overexpression of Pa*DGAT1* and Pa*PDAT1* in camelina which has not been previously associated with TAG accumulation in seed tissues but plays a role in regulating TAG levels in non-seed tissues. The fatty acid profile suggests that Pa*DGAT1* and Pa*PDAT1* are functional and could be expressed in other plants to increase oil content and alter composition since DGAT1 and PDAT1 are evolutionary conserved. However, with the presence of native genes in camelina, pinpointing the individual contribution of each gene presents a challenge. Future studies will use this information to examine how the expression of Pa*DGAT1* and Pa*PDAT1* in knockout mutant lines can rescue TAG-deficient phenotype and affect seed oil content and composition. The creation of mutant knockouts could be achieved through the use of genome editing tools such as CRISPR/Cas9. Additionally, the mechanism of regulation of DGAT1 and PDAT1 is poorly understood. Thus, a thorough investigation of the factors that regulate DGAT1 and PDAT1 genes is needed. While the GC-FID was used to determine oil content and composition, GC-MS could be used to determine the molecular species affected by the expression of PaDGAT1 and PaPDAT1 in camelina. Site-directed mutagenesis could also help understand the functional motifs responsible for DGAT and PDAT activity.

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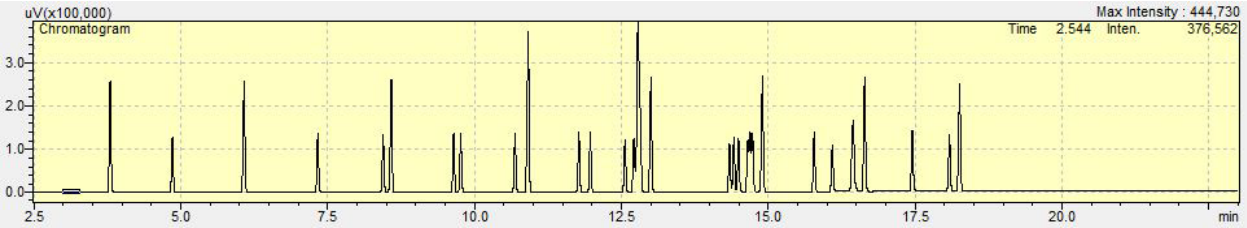
APPENDICES. GC-FID Chromatograms

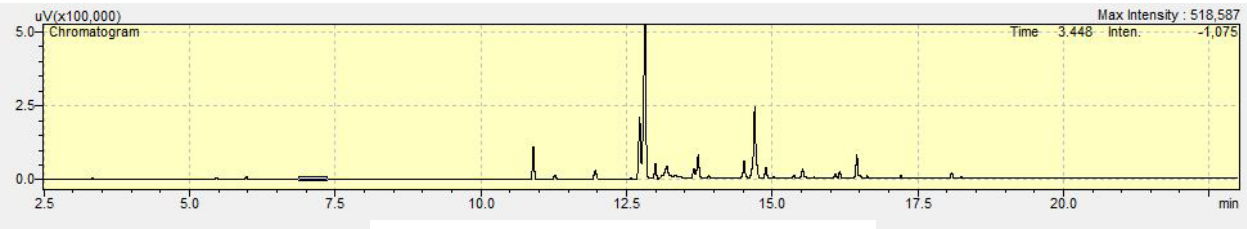
Appendix I: GC-FID Chromatogram of 37 FAME Reference Standard



Retention time

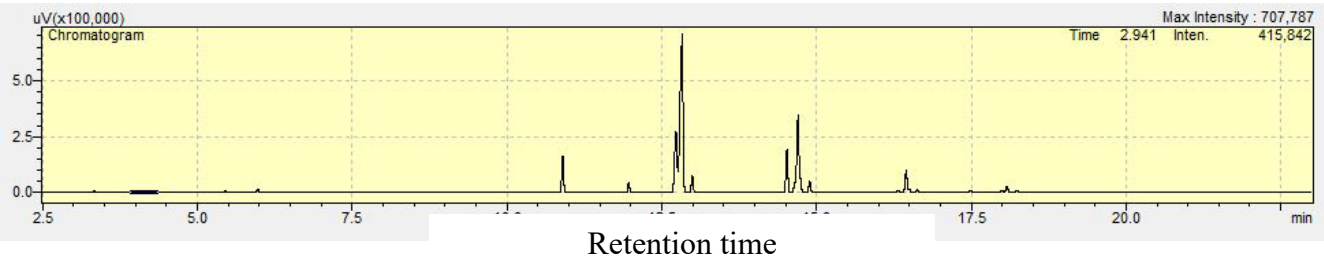
Appendix II: GC-FID Chromatogram Showing Fatty Acid Profile of Wild Type Camelina

:



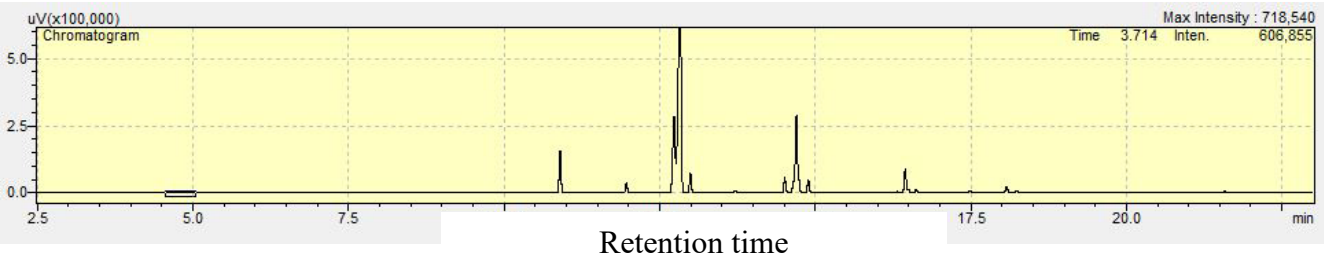
Retention time

Appendix III: GC-FID Chromatogram Showing Fatty Acid Profile of Transgenic Camelina DGAT1 Line



Retention time

Appendix IV: GC-FID Chromatogram showing Fatty Acid Profile of Transgenic Camelina PDAT1 Line



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