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Characterization of Acyltransferases and WRINKLED Orthologs Involved in TAG Biosynthesis in Avocado

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A dissertation

presented to

the faculty of the Department of Biomedical and Biological Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy in Biomedical Sciences

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by

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December 2018

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ABSTRACT

Characterization of Acyltransferases and WRINKLED Orthologs Involved in TAG Biosynthesis in Avocado

by

Md Mahbubur Rahman

Triacylglycerols (TAG) or storage oils in plants are utilized by humans for nutrition, production of biomaterials and fuels. Since nonseed tissues comprise the bulk biomass, it is pertinent to understand how to improve their TAG content. Typically, the final step in TAG biosynthesis is catalyzed by diacylglycerol (DAG) acyltransferases (DGAT) and/or phospholipid: diacylglycerol acyltransferases (PDAT), which also determine the content and composition of TAG. Besides enzymatic regulation of TAG synthesis, transcription factors such as WRINKLED1 (WRI1) play a critical role during fatty acid synthesis. In this study, mesocarp of *Persea americana*, with > 60% TAG by dry weight and oleic acid as the major constituent was used as a model system to explore TAG synthesis in nonseed tissues. Based on the transcriptome data of avocado, it was hypothesized that both DGAT and PDAT are likely to catalyze the conversion of DAG to TAG, and orthologs of WRI1 transcription factors regulate fatty acid biosynthesis. Here, with comprehensive *in silico* analyses, putative PamDGAT1 and 2 (Pam; *Persea americana*), PamPDAT1, and PamWRI1 and 2 were identified. When acyltransferases were expressed into TAG-deficient mutant yeast strain (H1246), only DGAT1 restored TAG synthesis capacity, with a preference for oleic acid. Furthermore, even *in planta*, when transiently expressed in *Nicotiana benthamiana* leaves, while PamDGAT1, PamPDAT1, PamWRI1 and PamWRI2 increased lipid content, PamDGAT2 remained inactive. The data reveals that putative PamDGAT1, PamPDAT1 are functional and preferred acyltransferases in avocado and both PamWRI1 and 2 regulate fatty

acid synthesis. In conclusion, while nonseed tissue of a basal angiosperm has certain distinct regulatory features, DAG to TAG conversion remains highly conserved.

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CHAPTER 1

INTRODUCTION

Background

Triacylglycerol (TAG) is the main form of storage lipid in plants found in both seed and nonseed tissues. TAG is composed of a glycerol backbone esterified with three fatty acids, which can be either saturated, monounsaturated or polyunsaturated. In seed tissues, TAG is mostly stored in embryo or endosperm. For example, TAG is accumulated in the embryo in sunflower and rapeseed, endosperm in castor bean and carrot and both embryo and endosperm in tobacco (Murphy 1990). Among nonseed tissues TAG is abundant in mesocarp of oil palm (Bourgis et al. 2011; Tranbarger et al. 2011; Dussert et al. 2013), avocado (Ibarra-Laclette et al. 2015; Kilaru et al. 2015) and olive (Alagna et al. 2009), tuber of yellow nutsedge (Thieffry 2014), fruit surface of bayberry (Simpson and Ohlrogge 2016), tallow of Chinese tallow (Divi et al. 2016; Divi 2016) and leaves of Arabidopsis (Tjellström et al. 2015). Several of these diverse plant species contribute to nearly 100 billion kilograms of oil stored in their various tissues, which has a monetary value equivalent to US $140 billion. Nevertheless, according to USDA, vegetable oil consumption is expected to be doubled by 2030 (USDA, 2011), pushing the need to meet the demand.

Plants use TAGs as a carbon and energy source during seed germination. Humans consume TAG to fulfill their nutritional supply and for non-food applications such as biofuel production and industrial feedstock. Currently, around 15% of vegetable oil is used in the industry for the production of soaps, lubricants, paints, surfactants, solvents, detergent, and chemical feedstocks (Carlsson 2009). The use of plants oils for the production of biofuel has attracted attention to replace fossil fuels (Durrett et al. 2008). The unusual fatty acids

produced by plants are used as chemical and industrial feedstock as replacements for petroleum products (Carlsson 2009).

Seed oil contains a wide range of saturated and unsaturated fatty acids and composition is specific to the species and tissue type (Aitzetmüller et al. 2003; Shockey et al. 2006; Ohlrogge et al. 2018). Rapeseed (*B. napus*) contains 21-46% of oil by dry weight (DW), which is characterized by oleic acid (61%) and an unusual fatty acid, erucic acid (23%). The erucic acid has an industrial value and its TAG is categorized based on high or low erucic acid content (Sharafi et al. 2015). Flaxseed (*L. usitatissimum*) contains approximately 40% of oil by DW and contains mostly 60% α-linolenic acid (C18:3), which is primarily used for industrial purposes (Zhang et al. 2018). Castor (*R. communis*) seed contains 39.6 – 59.5% oil by DW, which contains up to 90% ricinoleic acid that mainly used for industrial application (Chen et al. 2007). On the other hand, coconut oils mostly contain medium-chain fatty acids (Ramos et al. 2008). Beside seed tissues, nonseed tissues such as mesocarp also contain a wide range of saturated and unsaturated fatty acids. The mesocarp of oil palm that contains 80-90% TAG on a DW is characterized by 44% palmitic acid (16:0), 39% oleic acid (18:1), and 11% linoleic acid (18:2) (Rosli et al. 2018), whereas lauric acid (12:0) is the predominant fatty acid found in the palm kernel oil extracted from the endosperm of palm oil (Dussert et al. 2013). In olive, 28-30% of the total fresh weight of mesocarp is TAG with oleic acid contributing to 75-80% of the total TAG. Other fatty acids like linoleic acid (C18:2), palmitic acid (C16:0), stearic acid (C18:0) and linolenic acid (C18:3) are also present in olive mesocarp but in small quantities (Alagna *et al.*, 2009). Avocado mesocarp contains 65-80% TAG on a DW which is dominated by oleic acid (18:1 ; 60-80%) (Kikuta and Erickson 1968; Luza et al. 1987) with the varying distribution of fatty acids during fruit development (Kilaru et al. 2015).

There are several pathways in eukaryotes by which TAG can be synthesized. The Kennedy pathway is acyl-CoA dependent and comprises three sequential acylations to the glycerol backbone; it is considered as one of the major pathways for TAG biosynthesis in plants (Weiss et al. 1960; Kennedy 1961). The acylation of *sn*-3 position of the glycerol backbone is catalyzed by diacylglycerol acyltransferase (DGAT); it is considered to be a rate-limiting step and well-studied in yeasts, mammals, and plants (Bouvier-Nave et al. 2000; Shockey et al. 2006; Beopoulos et al. 2012). Alternatively, TAG synthesis is catalyzed by an acyl-CoA independent pathway where last step conversion of diacylglycerol (DAG) to TAG catalyzed by phospholipid:diacylglycerol acyltransferase (PDAT), which is found mainly in yeasts and vascular plants (Dahlqvist et al. 2000). In this pathway, PDAT catalyzes the transfer of a fatty acyl group from phosphatidylcholine (PC) to DAG. Lastly, a diacylglycerol transacylase (DGTA), identified in microsomal preparation of the intestine of *Rattus norvegicus* and developing seeds of safflower, was shown to utilize two DAG molecules as both acyl donor and acceptor to form TAG and monoacylglycerol (MAG) (Yoon et al. 2012). In plants, these pathways were extensively studied in seed tissues (Shockey et al. 2006; Zhang et al. 2009) but largely uncharacterized in nonseed tissues (Rahman et al. 2016). The long-term focus of this study is to understand TAG biosynthesis in nonseed tissues such as avocado mesocarp.

Triacylglycerol Biosynthesis

Triacylglycerol biosynthesis in plants involves fatty acid biosynthesis in the plastid and TAG assembly in the endoplasmic reticulum (ER). In organisms other than plants, *de novo* biosynthesis of fatty acids for TAG or membrane lipids takes place in the cytosol (Athenstaedt and Daum 2006). While in plants, the reactions in green tissues occur in the chloroplasts, for non-green tissues, they take place in the leucoplasts. These newly synthesized fatty acids in the

plastid can be utilized for plastidial lipid biosynthesis or transported to the ER for TAG assembly or further modifications by acyl editing (Stymne and Stobart 1984; Bates et al. 2007; Ma et al. 2013).

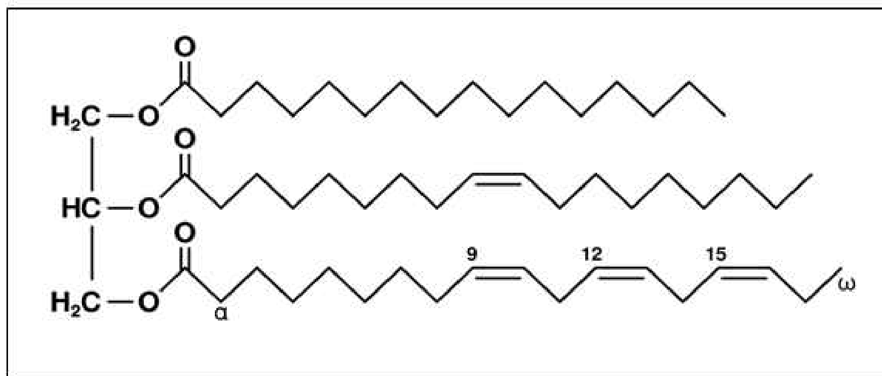

Figure 1.1 Schematic representation of a triacylglycerol molecule. A generalized diagram of the triacylglycerol molecule showing the 3-sn positions on the glycerol backbone where the fatty acids are attached in a stepwise fashion.

In plants, TAGs are stored in small compartments known as lipid droplets (LDs). These LDs are different from most other cellular compartments in that they have a single layer of phospholipid membrane, with the polar head groups in contact with the cytosol and the non-polar tails in contact with the internal lipids (Murphy and Vance 1999; Krahmer et al. 2009; Chapman et al. 2012; Gidda et al. 2016). Oleosins are small proteins unique to plants that coat the LDs, helping to maintain size and shape and preventing coalescence of the LDs (Tzen and Huang 1992; Leprince et al. 1997; Hsieh 2004). At least, 17 oleosin genes have been detected in Arabidopsis, which are differentially expressed in various tissues suggesting that these genes are highly regulated even within a single plant species (Siloto et al. 2006; Shimada et al. 2008). These typical oleosins are not highly expressed in nonseed oil accumulating tissues such as the mesocarp of olive, oil palm and avocado (Troncoso-Ponce et al. 2011; Murphy 2012; Huang et

al. 2013; Kilaru et al. 2015). However, lipid droplet-associated proteins, identified in nonseed tissues might play a similar role as oleosins (Horn et al. 2013).

Synthesis of TAG Precursor (DAG)

TAG assembly starts with glycerol-3-phosphate (G3P) undergoing sequential reactions to incorporate saturated or unsaturated fatty acids at the three *sn*-positions. This sequential incorporation is catalyzed by three specific acyltransferases namely, glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT), and DGAT (Martin and Wilson 1984). GPAT catalyzes the first incorporation of fatty acid from acyl-CoA or acyl-ACP (where CoA is coenzyme A and ACP is acyl carrier protein) to the *sn-1* position of G3P to form lysophosphatidic acid (LPA). In plants, this reaction may occur in three distinct subcellular compartments: plastid, ER, and mitochondria (Chen and Smith 2012). Plants have two biosynthetic pathways and each pathway has distinct GPAT enzymes for the production of lipid; the plastidial prokaryotic pathway uses a soluble acyl-ACP-dependent GPAT (Frentzen et al. 1983, 1987) whereas the eukaryotic pathway uses a membrane-bound GPAT (Weiss et al. 1960; Kennedy 1961). In *Arabidopsis*, nine ER-localized GPATs (Slabas et al. 2002; Coleman and Lee 2004; Xu et al. 2006) have been detected; however, most of these GPATs are mainly responsible for the biosynthesis of cutin or suberin rather than storage or membrane lipids (Yang et al. 2010). Among these, GPAT9 is considered as the best candidate for incorporation of acyl groups at *sn-1* position (Cao et al. 2006; Gidda et al. 2009; Shockey et al. 2016). Constraints in isolating and purifying membrane-bound enzymes have limited the understanding of GPAT9 (Chapman and Ohlrogge 2012; Shockey et al. 2016; Singer et al. 2016; Waschburger et al. 2018).

The incorporation of the second fatty acid at *sn-2* position to form phosphatidic acid (PA) from LPA is catalyzed by LPAAT. Like GPAT, LPAAT may also be located in mitochondria,

ER, and plastids (Coleman and Lee 2004; Yu et al. 2004). The gene that encodes for LPAAT was identified in eggplant (*Limnanthes douglasii*) (Brown et al. 2002), corn (Brown et al. 1994), turnip rape (Bafor et al. 1990), coconut (Lassner et al. 1995), *Arabidopsis thaliana* (Kim and Huang 2004), rapeseed (Maisonneuve et al. 2000), and soybean (Rao and Hildebrand 2009). Five LPAAT genes were isolated from *A. thaliana* genome; three of them (LPAAT1, LPAAT2, and LPAAT3) were found to be essential for normal plant development. Nonetheless, the specific role of LPAAT in lipid biosynthesis, particularly in TAG accumulation is yet to be identified (Maisonneuve et al. 2010). Several transcriptome and enzymatic studies suggest that LPAAT2 might be the crucial isoform for catalyzing the conversion of PA from LPA (Körbes et al. 2016).

Phosphatidic acid phosphatase (PAP) is responsible for the catalysis of phosphatidic acid dephosphorylation to form DAG. PAP has been detected within the inner envelope of the chloroplast, cytosol, and ER (Joyard and Douce 1979). In *R. communis* four putative PAP genes were identified *in silico*. These PAPs from castor and numerous other species have six transmembrane domains and three conserved phosphatase sequence motifs (Cagliari et al. 2010). In plants such as in Arabidopsis, PAP1 and PAP2 are active and involved in lipid biosynthesis (Pierrugues et al. 2001).

TAG synthesis from DAG by Acyl-CoA Dependent Pathway

The last acyltransferase (DGAT) is one of the regulatory enzymes for TAG accumulation

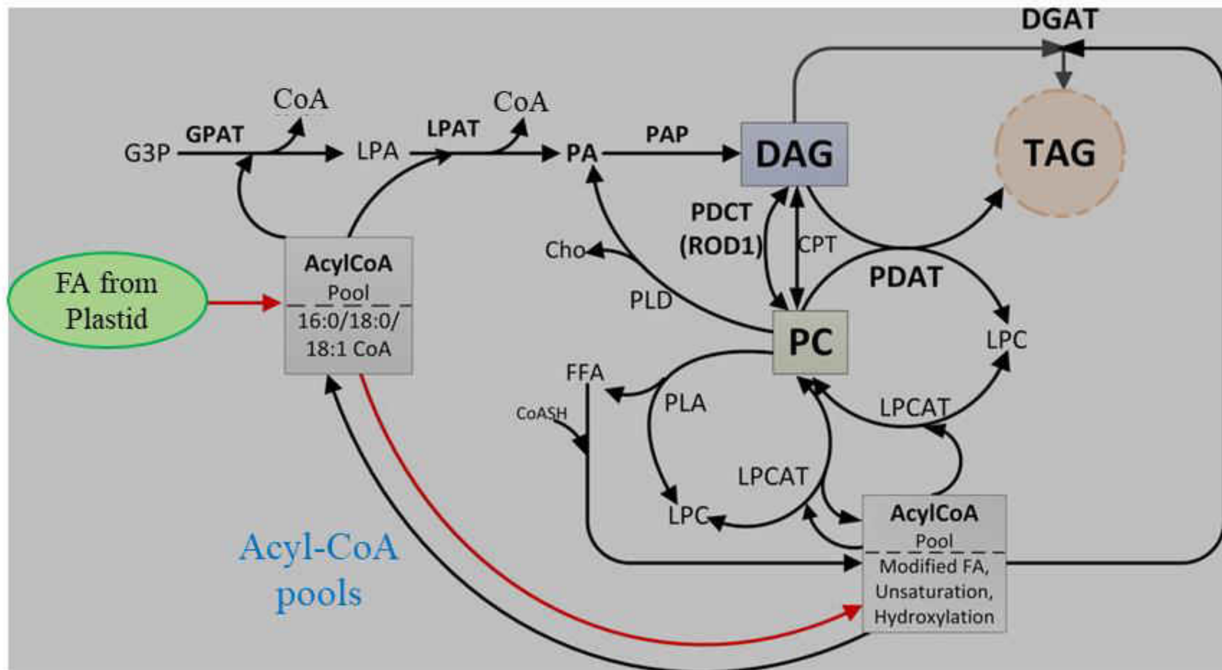

Figure 1.2 A schematic diagram of triacylglycerol biosynthesis pathway.

A simplified representation of acyl-CoA dependent and -independent pathway for TAG biosynthesis. GPDH – glycerol-3-phosphate dehydrogenase; GPAT – glycerol-3-phosphate acyltransferase.

in the acyl-CoA dependent pathway or Kennedy pathway. At least two types of *DGAT* genes namely *DGAT1* and *DGAT2* show DGAT activity in various eukaryotic cells. The first DGAT1 enzyme was identified in the mouse and initially, mouse DGAT1, which is 20% identical to mouse acyl CoA:cholesterol acyltransferase (ACAT), was thought to be responsible for cholesterol esterification but was later shown to participate in TAG biosynthesis (Cases et al. 1998). Subsequently, mouse DGAT1 homolog was identified in *Arabidopsis* (*AthDGAT1*) and in other plants (Hobbs et al. 1999; Routaboul et al. 1999; Zou et al. 1999; Bouvier-Nave et al. 2000; He et al. 2004; Shockey et al. 2006; Aymé et al. 2015). The *AthDGAT1* is located on the upper arm of chromosome II and has a long 5´ UTR, 15 introns, and it encodes a 530 amino acid long peptide/protein (Hobbs et al. 1999). The protein is predicted to contain 9 or 10 transmembrane

domains with the N-terminal region interacting with the CoA of the acyl-CoA for transport (Wang et al. 2006; Weselake et al. 2000).

DGAT1 is expressed in various plant tissues that accumulate oil including roots, leaves, stems, petals, flowers, anthers, developing siliques, young seedlings, and germinating seeds (Hobbs et al. 1999; Routaboul et al. 1999; Li et al. 2010) (Hobbs et al. 1999; Routaboul et al. 1999; Zou et al. 1999; Giannoulia et al. 2000; He et al. 2004; Yu et al. 2004; Lardizabal et al. 2008; Li et al. 2010). The expression of *DGAT1* is associated with the ER in seeds and chloroplast envelopes in leaves (Kaup et al. 2002). In *B. napus*, *DGAT1* is expressed in the leaves, stems, embryos, and flowers (Hobbs et al. 1999). In Arabidopsis, the expression of *DGAT1* is tissue-specific and dependent on the developmental stages. *DGAT1* was highly expressed in the pollen and developing seeds along with germination and seedling growth in Arabidopsis (Lu et al. 2003). In oil palm, *DGAT1* was expressed both in mesocarp and endosperm tissues (Rosli et al. 2018).

The role of DGAT1 in TAG biosynthesis in plants has been shown by both overexpression and mutational down-regulation. The total seed oil content in Arabidopsis is increased by 10% to 70% with the overexpression of *AthDGAT1* (Jako et al. 2001). When *DGAT1* of *A. thaliana* or *B. napus* was transformed into canola, total oil increased by nearly 7% (Taylor et al. 2009). TAG accumulation is reduced by 45% in the seeds of Arabidopsis when the *DGAT1* gene function was disrupted (Katavic et al. 1995; Routaboul et al. 1999) and seed oil content of rapeseed reduced by 30% with the depletion of DGAT1 function (Lock et al. 2009). The TAG content changed in castor bean in respect to the DGAT1 activity which implies the role of DGAT1 in seed TAG accumulation (He et al. 2004). In addition, differences in DGAT activity were correlated with differences in the total oil concentration in soybean genotypes which also

suggested the possible role of DGAT1 in TAG accumulation in soybean seed (Settlage et al. 1998). Overexpression of *DGAT1* in non-seed tissues also enhanced TAG accumulation (Routaboul et al. 1999). For example, overexpression of Arabidopsis *DGAT1* in the tobacco leaves resulted in an increase of TAG by 7 to 20-fold (Bouvier-Nave et al. 2000; Andrianov et al. 2010) and elevated total lipid was also observed in the leaf of *J. curcas* and *L. perenne* when *AthDGAT1* was overexpressed (Winichayakul et al. 2013; Maravi et al. 2016). Tobacco with knock out DGAT1 showed up to 49% reduction in total seed oil content (Zhang et al. 2005).

Oil accumulation coincided with the increase of *DGAT1* expression which is one of the major enzymes in seed oil accumulation across several species (Li et al. 2010). DGAT1 has been shown to a broad substrates preference for fatty acids (Cao and Huang 1986; Lung and Weselake 2006). For example, DGAT1 from the seeds of *B. napus* has a preference for oleic acid (18:1) (Aznar-Moreno et al. 2015) whereas oil palm EguDGAT1 prefers medium chain fatty acids (Aymé et al. 2015). In Arabidopsis, mutational downregulation of AthDGAT1 by methylsulfonate increased polyunsaturated fatty acids (18:2 and 18:3) and reduced monounsaturated fatty acids (18:1 and 22:1) (Lung and Weselake, 2006). Various *in vitro* assays suggested that substrate specificity changes with temperature; the DGAT1 of canola has a preference for oleic acid at 24 ºC, but at 40 ºC it prefers erucic acid (Cao and Huang 1987). Other factors including ATP, Co-enzyme A, and magnesium, along with other unidentified small organic compounds can influence the level of DGAT1 activity in cell culture (Byers et al. 1999). When bovine serum albumin (BSA) was added to the microsomal mixtures containing DGAT from maturing sunflower seeds, oleic acid was incorporated at a higher rate along with overall enzymatic activity (Triki et al. 2000). One amino acid can dictate the specificity of DGAT1. For instance, a maize-QTL-associated with high oil and oleic acid content is present in ancestral

genotypes and possesses phenylalanine inserted at 469, but this amino acid is missing from modern corn genotypes. When the ancestral gene was transformed into modern maize line, there was a marked increase in both oil and oleic acid content (Zheng et al. 2008).

Like DGAT1, DGAT2 also catalyzes the terminal incorporation of fatty acid to DAG for the biosynthesis of TAG. DGAT2 is localized in the ER, but to a different subdomain than that of DGAT1 (Shockey et al. 2006). DGAT2 has a length between 315 and 373 amino acids (Cao 2011). Unlike DGAT1 protein, DGAT2 is predicted to have only two transmembrane domains (Weselake et al. 2000; Shockey et al. 2006). Typically, DGAT2 incorporates unusual fatty acid into the TAG (Li et al. 2010). In Tung tree, DGAT2 is expressed during oil biosynthesis and preferentially catalyzes the incorporation of eleostearic acid – the main fatty acid in tung oil (Shockey et al. 2006). During seed development of castor bean, DGAT2 was highly expressed than DGAT1 and contributes to the accumulation of ricinoleic acid (Kroon et al. 2006; Cagliari et al. 2010). More recent studies have shown that DGAT2 may not always be associated with unusual fatty acid incorporation. For instance, a DGAT2 from *P. tricornutum* increased palmitic acid (16:0) and oleic acid (18:1) when expressed in the yeast system (Gong et al. 2013). Soybean expressing fungal DGAT2A showed a 1.5% increase in total oil in seeds, but the protein content and yield remained unchanged (Lardizabal et al. 2008).

TAG Biosynthesis from DAG by Acyl-CoA Independent Pathway

Acyl-CoA independent pathway for TAG accumulation was first discovered in yeast and plants (Dahlqvist et al. 2000). Overexpression of *PDAT* increased TAG content, which led to the isolation and characterization of this acyl-CoA independent enzyme, PDAT that is responsible for the synthesis of a TAG from DAG and PC (Dahlqvist et al. 2000) (Figure 1.2). In some plant species, PDAT incorporates unusual fatty acids, like the epoxy and hydroxy fatty acids

(Dahlqvist et al. 2000; Li et al. 2010). For example, in castor bean, PDAT predominately incorporates ricinoleic (a hydroxy fatty acid) and vernolic (an epoxy fatty acid) acids in the glycerol backbone (Dahlqvist et al. 2000). Several genes encode for PDAT1 in castor bean, and all of these copies are expressed at different times during seed development (Cagliari et al. 2010). One isoform, PDAT1A increased hydroxy fatty acids by 27% in transgenic Arabidopsis (van Erp et al. 2011; Kim et al. 2011). In Arabidopsis, PDAT was shown to be most active with fatty acids that contained multiple double bonds, epoxy or hydroxy groups (Ståhl et al. 2004). Moreover, in Arabidopsis, PDAT1 function overlapped with DGAT1 and was shown to be essential for normal pollen and seed development (Zhang et al. 2009). The *sn-2* fatty acyl moiety of PC for PDAT is regulated by phosphatidylcholine: diacylglycerol phosphocholine transferase (PDCT) by channeling unsaturated fatty acids into the TAG (Chapman and Ohlrogge 2012; Lu et al. 2009).

Regulation of Fatty Acids Synthesis Necessary for TAG Assembly

The current understanding of transcriptional regulation of fatty acid biosynthesis is mostly limited to seed tissues. Master regulators of seed maturation and embryogenesis such as ABSCISIC ACID INSENSITIVE3 (ABI3), FUSCA3 (FUS3), and LEAFY COTYLEDON1 and 2 (LEC1 and LEC2) play an essential role in the regulation of oil content. These master regulators, however, act *via* downstream transcription factors that directly interact with the genes encoding for late glycolysis and fatty acid synthesis (Kagaya et al. 2005; Mu et al. 2008; Baud et al. 2009). Among such downstream transcription regulators that control lipid biosynthesis, WRINKLED 1 (WRI1) protein was the most studied. Orthologs of WRI1 in various other plant species were also shown to regulate fatty acid biosynthesis and thus TAG content in seed tissues including corn and rapeseed , and nonseed tissue of oil palm (Cernac and

Benning 2004; Liu et al. 2010; Ma et al. 2013; An et al. 2017), leaf blades of *Brachypodium distachyon*, stem of poplar (*Populus trichocarpa*) and tuber parenchyma of nutsedge (Grimberg et al. 2015; Yang et al. 2015). Orthologs of four *WRI* genes that likely encode for WRI1, WRI2, WRI3, and WRI4 have been identified in avocado mesocarp, but their functional role has not been characterized (Kilaru et al. 2015; Dabbs 2015). The expression of Arabidopsis *WRI1* in corn, camelina and *B. napus* increased TAG content by 14-30% compared to wild-type seeds (Cernac and Benning 2004; Liu et al. 2010; Shen et al. 2010; Wu et al. 2014). Similarly, transient expression of *WRI1* orthologs from potato, oat, nutsedge, Arabidopsis in *Nicotiana bethamiana* leaves also increased TAG content by 0.05% to 2.20% (Vanhercke et al. 2013; Grimberg et al. 2015).

Orthologs of WRINKLED Transcription Factor

Four *WRI* genes that belong to the AP2/EREBP family and encode for WRI1, WRI2, WRI3, and WRI4 proteins have been identified in Arabidopsis (To et al. 2012). Until recently, only WRI1 was shown to involved to regulate fatty acid biosynthesis in TAG accumulating seed and nonseed tissues (To et al. 2012; Chen et al. 2018; Ma et al. 2013). The exact functions of other homologs such as WRI2, WRI3, and WRI4, however, are not fully characterized. The *WRI1* gene was originally discovered as a low seed oil mutant in *Arabidopsis thaliana* that showed impairment in seed development (Focks and Benning 1998). The homozygous *wri1* mutant showed 80% lower seed oil content, wrinkled seed coats, and impairment in seed germination and seedling establishment (Focks and Benning 1998). The gene was later shown to code for a transcription factor and acts to regulate oil accumulation in seed tissues (Cernac and Benning 2004). Expression studies suggest that WRI1 primarily regulates genes involved in late glycolysis and fatty acid synthesis such as plastidial pyruvate kinase, sucrose synthase, pyruvate

dehydrogenase and BCCP (a subunit of ACCase), enoyl-ACP reductase, β-ketoacyl-ACP reductase, fatty acid desaturase 2 and acyl-carrier protein, respectively (Baud et al. 2007; To et al. 2012). Orthologs of WRI1 in *Brassica napus* and *Zea mays* have also been shown to regulate many of the same proteins involved in glycolysis and fatty acid synthesis (Liu et al. 2010; Pouvreau et al. 2011).

In Arabidopsis, other WRI paralogs (WRI3 and WRI4) are capable of complementing the *arabidopsis wri1* mutant but do not affect seed oil accumulation. Both are expressed highly in nonseed tissues of the plant, such as flowers, stems, and roots and are thought to play a role in the tissue-specific synthesis of fatty acids for cutin biosynthesis. Arabidopsis WRI2 is not capable to complement *wri1* mutant (To et al. 2012). In maize, where a duplication event has created two *WRI1* genes, *ZmaWRI1a* and *ZmaWRI1b* played an important role to regulate the fatty acid synthesis in the species (Pouvreau et al. 2011). Both *ZmaWRI1a* and *ZmWRI1b* genes complement fatty acid content in Arabidopsis *wri1-4* mutant (Pouvreau et al. 2011). The protein sequence of ZmaWRI1a and ZmaWRI2 are more closely related to Arabidopsis WRI1 compared to any of the other WRI paralogs in Arabidopsis. Additionally, maize does have orthologs to WRI2, WRI3, and WRI4 but their functions were not characterized yet (Pouvreau et al. 2011). The functional role of WRI2 as a transcription factor for fatty acid biosynthesis, however, has not been discovered yet in any plant species (To et al. 2012).

Studies have also shown that WRI1 homologs also play a role in directing TAG accumulation outside of the developing seed. A WRI1 homolog from oil palm was highly expressed in the mesocarp, compared to the closely related date palm (which does not accumulate oil), and coordinately with an increase in TAG levels (Bourgis et al. 2011). The WRI1 homolog from oil palm has also been shown to rescue the *wri1-4* mutant phenotype of

Arabidopsis (Ma et al. 2013). Interestingly the WRI homologs found in nonseed oil accumulating plants are most closely related to Arabidopsis WRI1 according to amino acid sequence and not to other WRI homologs (WRI2, WRI3 and WRI4) of Arabidopsis (Bourgis et al. 2011). In Arabidopsis, other WRI homologs such as WRI3 and WRI4 were shown to stimulate the production of acyl chains for cutin biosynthesis in the floral tissues. Although, WRI2 was detected in Arabidopsis but did not show any transcriptional activity (To et al. 2012). The higher transcript level of *WRI2* was not reported in other oil-rich tissues except in avocado mesocarp, which suggests that the function of WRI2 might be retained in avocado mesocarp but not in other plant tissues (Kilaru et al. 2015).

Avocado as a Model System to Study TAG Accumulation in Nonseed Tissues

Avocado History

Avocado (*Persea americana*) belongs to the family *Lauraceae* which contains 2500 to 3000 species in over 50 genera (Rohwer 1993). Avocado is one of the basal angiosperms, which is considered the first and the oldest family of flowering plants diverged from ancestral angiosperms has been used as a model system to understand lipid biosynthesis in nonseed tissue with respect to evolution (Soltis et al. 1990; Chanderbali et al. 2008). Because of the pivotal position *Persea americana* occupies in plant phylogeny, it serves as an excellent model system to understand the evolution of regulatory and biochemical mechanisms in lipid synthesis. Based on their origin and fruit characteristics, there are three types of avocado: Guatemalan (*P. nubigena* var. guatemalensis L. Ws.), Mexican (*P. americana* var. drymifolia Blake), and West Indian (*P. americana* Mill. Var. americana) (Requejo-Tapia et al. 2000). Additionally, cultivar crosses of Guatemalan and Mexican avocado resulted in ‘Fuerte’, which was introduced in California from Mexico in 1911. But because of low yield and disease (Slater et al. 1975),

‘Fuerte’ avocado has been replaced by the ‘Hass’ cultivar, which was also a hybrid of Guatemalan and Mexican avocado. Avocados of ‘Hass’ variety were able to produce better quality fruit, 50% more oil yield, and displayed longer shelf life, and endured transportation (Requejo-Tapia et al. 2000).

Economic and Nutritional Value

Despite being native to Central America, Avocado is cultivable in tropical and Mediterranean climates and as a result, it has become popular throughout the world including the United States of America, Dominican Republic, Mexico, Peru, Chile, Argentina, Spain, Israel, South Africa, Australia, New Zealand and Japan (World Avocado Production Statistics-USDA; Takenaga et al. 2008). This is economically significant, as the avocado from 2004 to 2006 yielded ~3.5 million tons and was valued as more than two billion dollars (USDA 2011).

The fleshy mesocarp, edible part of avocado contributes to 50 to 80% of total weight, while seed comprises to 10 to 25%. In addition, mesocarp had a significantly higher amount of oil that is mostly composed of oleic acid (18:1), a precursor for essential fatty acids and considered a healthy oil (Bates 1970). Avocado contains abundant amounts of vitamin A, B, and E, and has been a resource for developing nutraceuticals (Slater et al. 1975; Nelson et al. 2008). Avocado, therefore, in addition to its evolutionary significance, has been of great interest to researchers because of its nutritional, medicinal, and economic value.

Avocado Fruit Development

Developmental process and stages of fruits have been well studies in various plants, which usually starts with the fertilization and the stage continues until maturation of the fruit. Generally, there are three phases of fruit development which begin with the phase of fruit set when an ovary is developed and determine the process whether this stage is further developed or

aborted. This stage is followed by the second phase of cell division and finally cell expansion to mature the fruits when the fruits attain in its final extent (Gillaspy et al. 1993). Although the development of fruits is following a similar pattern, however, the period of the second stage of development that includes cell division is variable among different fruits (Gillaspy et al. 1993). For example, in apples, the cell division stage lasts for three weeks after full blooming and pollination followed by cell expansion to increase the final size of the pome (Schroeder 1953). In contrast, in peaches, this cell division phase holds for thirty days after fruit set stage.

Unlike most of the other fruits, the developmental pattern of fruit in avocado is unique. As mentioned previously, in most cases, cell division stage entirely terminate to initiate the cell expansion phase, whereas in avocado fruits, both of these stages continue in parallel that leads to an increase in cell size and cell number throughout the development slowly until fruit remains on the tree (Taylor and Cowan 2001). In general, fruit growth process is the combined effect of both cell division and expansion phases, however, to determine the final fruit size of an avocado, cell division stage plays the dominant role which is predominant in early fruit growth and continues further throughout the fruit development. Moreover, fruit ripening process of avocado fruits are unique; although avocado is called climacteric fruits and at maturity of the fruit, it produces ethylene, however, they are not fully ripening unless harvested from the tree (Schroeder 1953).

Additionally, some varieties of avocado have a propensity towards alternate bearing and production of small size fruit. Especially when the tree ages, there is a high propensity to produce small-sized avocadoes in some varieties including ‘Hass.' Usually, ‘Hass' produces normal fruit (NF) and phenotypically small fruit (SF) with an unidentified pattern of distribution on the tree (Dahan et al. 2010). Depending on several factors including climate, tree health or even under favorable conditions, ‘Hass’ SF portion may constitute ~20 to 60% of harvested fruit

(Cowan et al. 1997). Moreover, the number of SF increases during a heavy bearing year that may lead to an enormous economic loss. Although fruit size primarily varies due to the cell number (Sabag et al. 2013), the precise underlying mechanisms that lead to variation in avocado fruit phenotypes are still unknown.

Oil Biosynthesis in Avocado Mesocarp

TAG is the predominant lipid (more than 85% of the total lipid content) in mature avocado fruit (Platt-Aloia and Thomson 1981) and contains primarily six different fatty acids including saturated and unsaturated. The saturated fatty acids are palmitic (C16:0) and stearic (C18:0) acids, the monounsaturated fatty acids are oleic (C18:1) and palmitoleic (C16:1) acids, and polyunsaturated fatty acids are linoleic (C18:2) and linolenic (C18:3) acids. Oleic acid comprising ~ 60% of the total TAG is the main fatty acid among all these fatty acids in the mesocarp of mature avocado (Kikuta and Erickson, 1968; Luza et al. 1987) even though the distribution of fatty acids vary during developmental stages of avocado fruit. While linoleic acid is the predominant fatty acid in the earlier stage of fruit development, the content of linoleic acid gradually decreases in the later stages with the increase of oleic acid content. Palmitic acid followed by linoleic acid are the second and third most ample fatty acids in the mature avocado fruit, respectively (Sung 2013; Ibarra-Laclette et al. 2015; Kilaru et al. 2015). Recent transcriptome analysis of avocado mesocarp reveals the relative transcript abundance of the fatty acid synthesis genes and TAG biosynthesis genes coincide with the lipid accumulation in avocado mesocarp (Ibarra-Laclette et al. 2015; Kilaru et al. 2015). Nevertheless, the underlying molecular mechanisms that regulate the composition of TAG especially the abundance of oleic acid in mature avocado mesocarp tissue are largely unknown.

Until recently, extensive studies have been carried in seed tissues (Troncoso-Ponce et al. 2011), e.g., in Arabidopsis (Baud and Lepiniec 2009; Baud et al. 2008; Lu et al. 2003; Li et al. 2006), Rapeseed (*Brassica napus)* (Weselake et al. 2008), Castor bean (*Ricinus communis*) (Kroon et al. 2006; He et al. 2004), soybean (*Glycine max*) (Bates et al. 2009; Lardizabal et al. 2008; Wang et al. 2006), Tung tree (*Vernicia fordii*) (Shockey et al. 2006), flax (*Linum usitatissimum*) (Pan et al. 2013) and safflower (*Carthamus tinctorius*) (Stobart et al. 1997; Stobart and Stymne 1985) to understand TAG biosynthesis and the regulation of TAG biosynthesis both in transcriptional and translational level. These studies revealed that TAG can be regulated both in transcriptional level by the transcription factors such as WRINKLED and translational level by the enzymes such as DGAT and PDAT. On the other hand, a moderate number of studies have been conducted in nonseed tissues including mesocarp of oil palm, tallow of Chine*se* tallow to understand the regulation of TAG biosynthesis in these nonseed tissues (Aymé et al. 2015; Divi 2016). Current transcriptome studies of oil palm, olive, and avocado mesocarp have suggested that the regulation of TAG biosynthesis in nonseed tissues is likely to be different from that of seed tissues (Banilas et al. 2011; Bourgis et al. 2011; Horn et al. 2013; Kilaru et al. 2015). In contrast, the transcript level of the majority of TAG biosynthesizing enzymes in tuber of *Cyperus esculentus* showed a similar pattern to those in seed tissues (Yang et al. 2016). Transcriptome analyses of nonseed tissues also revealed the absence of oleosin proteins (Banilas et al. 2011; Bourgis et al. 2011; Horn et al. 2013; Ibarra-Laclette et al. 2015; Kilaru et al. 2015), which typically stabilize and regulate the size of oil bodies in seeds and pollen (Huang 1992; Ross et al. 1993). Absence of oleosins seems unique among lipid-rich nonseed tissues; it is presumed that oleosins may not be essential in tissues where desiccation is non-detrimental to propagation and/or where lipids are not required to be stored by the plant on a

long-term basis, unlike in seeds that are desiccated and required to store resources until germination (Ross et al. 1993). A recent proteomic analysis combined with transcriptomics, however, revealed a new class of lipid droplet-associated proteins (LDAP) in avocado and oil palm mesocarp, which may play a role in stabilization of lipids (Horn et al. 2013). Avocado transcriptome data also provided insights into genes likely involved with TAG assembly in mesocarp tissue, including the acyltransferases, which encode for enzymes that catalyze the conversion of DAG to TAG (Ibarra-Laclette et al. 2015; Kilaru et al. 2015). The transcript level for *DGAT1*, an acyl-CoA dependent enzyme and *PDAT1*, an acyl-CoA independent enzyme is higher than that of *DGAT2* in the fruit developmental stages of avocado mesocarp. Which suggests there might be both acyl-CoA dependent and independent pathways are active for TAG accumulation in avocado mesocarp. Transcript level of *DGAT1* was gradually increased along with the fruit development and reached to its peak in the middle stage and then there is a decline. The expression pattern of *DGAT1* is correlated with the elevation of oleic acid during fruit development (Sung 2013; Ibarra-Laclette et al. 2015; Kilaru et al. 2015). Transcriptome analysis of oil palm revealed that *WRI1* is highly expressed in the seed as well as in the mesocarp tissue with the coordination of oil accumulation in these tissues (Bourgis et al. 2011; Tranbarger et al. 2011; Dussert et al. 2013; Ma et al. 2013). Interestingly, the transcript level of *WRI2* and *WRI3* are higher in avocado mesocarp along with *WRI1*, which suggests *WRI2* or *WRI3* or both might be playing an additional role as a transcriptional regulator for fatty acid biosynthesis in nonseed tissue (Sung 2013; Kilaru et al. 2015).

Rationale, Hypothesis, and Specific Aims

The underlying mechanisms of TAG accumulation in seed tissues are well known in many plants such as in Arabidopsis and Tung tree, but limited information is available for

nonseed tissues such as avocado mesocarp. TAG biosynthesis can be regulated both transcriptionally and translationally. Transcriptional regulation of fatty acid biosynthesis is well documented in both seed and nonseed tissues. The WRI transcription factor was shown to be an important regulator of oil biosynthesis in plants. So far, only WRI1 was shown to be involved in lipid regulation although genes for other isoforms (WRI2, WRI3, WRI4) were also identified in Arabidopsis. While orthologs of both *WRI1* and *WRI2* genes were highly expressed in avocado mesocarp during fruit development but their function as transcriptional regulators in TAG biosynthesis in nonseed tissue has not been evaluated yet. The enzymatic regulation of TAG accumulation in ER, specifically the acylation of *sn-3* position of the glycerol backbone in nonseed tissues also remains unresolved. DAG represents a critical branch point between the storage and membrane lipid synthesis and the enzymes DGAT and/or PDAT catalyze the conversion of DAG to TAG. In many plants, two different unrelated types of DGAT namely DGAT1 and DGAT2 have been identified. In Arabidopsis, though DGAT1 and DGAT2 are expressed, DGAT1 has a predominant role in TAG biosynthesis in the seed. On the other hand, DGAT2 mainly incorporates the unusual fatty acid in the glycerol backbone of TAG in *R. communis* and Tung seed oil. However, in oil palm and olive mesocarp, which accumulate normal TAG, expression of *DGAT2* was much higher than DGAT1 (Manuel et al., 2011). The overall goal of this research is to characterize the terminal enzymes in TAG biosynthesis and WRI orthologs as regulators of fatty acid synthesis in avocado mesocarp.

Based on transcriptome data of avocado mesocarp, orthologs of DGAT, PDAT1, and WRI are considered to play an important role in TAG accumulation. Therefore, it is hypothesized that:

1. *Putative PamDGAT and PamPDAT are likely to catalyze the conversion of DAG to TAG. and*
2. *Orthologs of WRI1 transcription factors regulate fatty acid biosynthesis in avocado mesocarp tissue*

To test these hypotheses, the following specific aims are proposed.

1. Identification and *in silico* analysis of putative avocado DGAT1 and 2 and PDAT1.
2. Cloning and heterologous expression of the acyltransferases into TAG-deficient mutant yeast.
3. *In planta* expression of avocado DGAT1 and 2 and PDAT1.
4. Transient expression of PamWRI1 and 2 in *Nicotiana benthamiana* leaves.

General Experimental Method

Using the avocado transcriptome database, full-length coding sequences of avocado DGAT1, DGAT2, and PDAT1 were identified. These full-length cDNA sequences were translated into the putative protein sequence using the ExPASy protein translation tool (http://web.expasy.org/translate/). *In silico* analyses including multiple sequence alignment, the probability of start codon, prediction of transmembrane domains, and phylogenetic analysis were conducted to get the putative acyltransferase for further functional characterization.

RNA was extracted, and cDNA was prepared from the extracted RNA. Coding sequences of avocado DGAT1, DGAT2, and PDAT1 were amplified using gene-specific forward and reverse primers. Amplified coding sequences of these genes were cloned into a galactose-inducible vector (pESC-URA) for heterologous expression in the mutant H1246 yeast strain.

Cloning of these genes was confirmed by colony PCR using one gene-specific and one plasmid-specific primers followed by sequencing. Western blot confirmed the successful transformation of these genes into H1246 yeast strains. Functional complementation was carried out by lipotoxicity rescue assays followed by lipid analysis by Nile Red staining, TAG separation on TLC, quantifying and analyzing the lipid content. In vitro enzyme activity was conducted with radiolabeled substrates to determine the specificity of the enzyme.

For transient expression of three acyltransferases (PamDGAT1, PamDGAT2 and PadmPDAT1) and two transcriptions factors (PamWRI1 and PamWRI2) were cloned into plant expression vector, pB110 containing CaMV 35S promoter for the constitution expression in *N. benthamiana* leaves. Functional characterization was carried out by analyzing the lipid content and composition in the transient tobacco plants expression PamDGAT1, PamPDAT1, PamWRI1, and PamWRI2.

CHAPTER 2

IDENTIFICATION AND FUNCTIONAL VALIDATION OF DIACYLGLYCEROL ACYLTRANSFERASES OF AVOCADO

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Abstract

Avocado mesocarp, a nonseed tissue, accumulates significant amounts of triacylglycerol (TAG; ~ 70% by dry weight) that is rich in oleic acid. Two unrelated enzymes, diacylglycerol acyltransferases 1 and 2 (DGAT1 and 2), catalyze the terminal step in TAG biosynthesis both in seed and nonseed tissues. In this study, we identified, isolated and cloned putative avocado DGAT1 and DGAT2. Avocado DGAT1, when expressed in a quadruple yeast mutant (H1246) that is defective in TAG biosynthesis restored accumulation of neutral lipids. Putative DGAT2, however, both in native and codon-optimized forms, failed to show DGAT activity when expressed in the yeast system. Heterologous expression of avocado DGAT1 increased the lipid content significantly when compared with the mutant H1246 strain. Furthermore*, in vitro* enzyme assays with radiolabeled [1- 14C] acyl-CoAs (palmitoyl-CoA (16:0) or oleoyl-CoA (18:1) showed that avocado DGAT1 has acyltransferase activity with a preference for oleic acid (18:1) to incorporate into diacylglycerol to generate TAG. Understanding substrate specificity and regulation of DGAT1 in nonseed tissues in synthesizing TAG will allow for generation of molecular tools necessary to improve and increase oil content and composition in agronomically relevant species.

Keywords: Triacylglycerol, nonseed tissue, DGAT1, oleic Acid, avocado, yeast quadruple mutant H1246

Introduction

Avocado (*Persea americana*), a basal angiosperm species, is an evolutionarily important cash crop that accumulates nutritionally rich triacylglycerols (TAGs) in nonseed tissues (Takenaga *et al.*, 2008). Typically, neutral lipids such as TAGs are the main storage lipids in plants and accumulate in fruits, developing seeds, anthers, pollen grains and flower petals (Murphy and Vance, 1999; Stymne and Stobart, 1987; Oo and Chew, 1992; Xue *et al.*, 1997). These storage lipids serve as a main reservoir for carbon and the metabolic energy source for seed germination, sexual reproduction and pollen development in diverse plants (Slocombe *et al.*, 1994; Zheng *et al.*, 2003; Wolters-Arts *et al.*, 1998). During fruit development, the fatty acid composition of TAG varied but the content in avocado mesocarp was highly correlated with the total fruit weight (Kilaru *et al.*, 2015). In mature avocado fruits, TAG in mesocarp (more than 85% by dry weight) is predominantly stored as oleic acid (18:1), which increases during fruit development while linoleic acid (18:2) decreases. Besides 18:1, 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; Sung, 2013).

Monounsaturated fatty acids are precursors for essential fatty acids and are considered as healthy oils to humans (Burr and Burr, 1930). Understanding the factors that regulate oil content and composition, particularly in nonseed tissues has been of great interest to many researchers. Transcriptome studies of oil-rich nonseed tissues such as avocado, oil palm, and olive revealed several similarities in the expression pattern of genes involved in plastidial fatty acid synthesis, while unique variations were observed in the expression of genes involved in TAG assembly (Rahman *et al.*, 2016). In plants, subsequent to two sequential acylations and formation of diacylglycerol (DAG) in the endoplasmic reticulum (ER), *sn*-3 position is typically acylated by a

diacylglycerol acyltransferase (DGAT) and/or a phospholipid: diacylglycerol acyltransferase (PDAT) (Dahlqvist *et al.*, 2000; Banas *et al.*, 2000). Specifically, the gene expression pattern for these enzymes involved in catalyzing the terminal and rate-limiting step in TAG accumulation is species-specific and tissue-dependent (Shockey *et al.*, 2006).

Eukaryotic DGATs are members of a major class of membrane-bound protein family (Shockey *et al.*, 2006; Turchetto-Zolet *et al.*, 2011; McFie *et al.*, 2010). At least two structurally unrelated polypeptides, namely DGAT1 and DGAT2, catalyze the transfer of a fatty acid from acyl-coenzyme A (CoA) to DAG to generate TAG (Zheng, *et al.*, 1998; Hobbs *et al.*, 1999; Cases *et al.*, 2001; Routaboul *et al.*, 1999). While DGAT1 belongs to membrane-bound O-acyltransferase (MBOAT) superfamily (Hofmann, 2000), DGAT2 belongs to a family that includes acyl-CoA: monoacylglycerol acyltransferases (MGAT) and acyl-CoA wax alcohol acyltransferases (AWAT) (Yen *et al.*, 2008). Furthermore, the peptide length of DGAT1 enzymes are longer than DGAT2 and contain six to nine transmembrane domains while DGAT2 has only one or two predicted transmembrane domains (Yen *et al.*, 2008; Turchetto-Zolet *et al.*, 2011). In mammals, both DGATs contribute to TAG biosynthesis (Cases *et al.*, 1998) and exhibit non-redundant physiological functions (Chen *et al.*, 2002; Smith *et al.*, 2000; Stone *et al.*, 2004). In plants, particularly in developing seeds, DGATs were shown to play an independent and/or synergistic role in channeling carbon towards TAG synthesis (Ohlrogge and Browse, 1995).

In transgenic studies, TAG content was enhanced when Arabidopsis DGAT1 was overexpressed in yeast cells and tobacco plants (Jako et al., 2001). Conversely, inactivation of DGAT1 in Arabidopsis reduced the seed TAG content by 45% (Routaboul *et al.*, 1999). In addition, DGAT1s from different species have a unique preference for fatty acid incorporation in

the TAG (Jako *et al.*, 2001; Katavic *et al.*, 1995; Wang *et al.*, 2015; Aznar-Moreno *et al.*, 2015). Arabidopsis DGAT1 prefers long chain fatty acids to generate TAGs while oil palm (*Elaeis guineensis*) EgDGAT1-1 prefers medium-chain fatty acids to produce TAGs when expressed in yeast (Aymé et al., 2015). The other DGAT enzymes (DGAT2) incorporate unusual fatty acids into TAGs (Cahoon et al., 2007), as for instance, Tung (*Virnicia fordii*) DGAT2 incorporates eleostearic acid, a polyunsaturated fatty acid (Shockey et al., 2006) and Castor (*Ricinus communis)* DGAT2 incorporates ricinoleic acid, a hydroxy fatty acid (Kroon et al., 2006) during TAG synthesis. In addition, the expression levels of DGAT2 in developing seeds of Tung tree and castor are higher than those of DGAT1 suggesting that DGAT2 might play a more prominent role than DGAT1 in the selective accumulation of unusual fatty acids (Liu et al., 2012).

Typically, the enzyme activity of DGAT proteins is analyzed by heterologous expression of the protein in yeast strain followed by the isolation of microsomes and conducting *in vitro* assays with radiolabeled substrates (Oelkers *et al.*, 2002; Cao and Huang, 1986; Settlage *et al.*, 1995; Lacey and Hills, 1996). Substrate preferences of DGAT including specificity and selectivity were tested in different plant species by such *in vitro* assays where microsomal fractions are incubated with a single or an equimolar mixture of different acyl-CoAs (Lung and Weselake, 2006). The fatty acid incorporation by DGAT enzyme might also be affected by availability of the acyl-CoA substrate and DAG molecules (Shockey et al., 2006).

In this study, we identified the coding sequences for putative avocado DGAT1 and DGAT2 and comprehensive *in silico* analyses were conducted to determine their respective start codons, full-length coding sequences, predicted transmembrane domains, predicted protein structures, and phylogenetic relationship with other known DGAT 1 and DGAT2 proteins,

respectively. These data reveal that the putative DGAT1 and 2 of a basal angiosperm species retain features that are conserved not only in angiosperms but also in other eukaryotes. Both genes were isolated, cloned and expressed in a TAG-deficient yeast strain (H1246) for further biochemical characterization. Lipotoxicity rescue assays and *in vitro* enzyme assays revealed acyltransferase activity for the enzyme suggesting that it is the likely DGAT1 that regulates TAG content and composition in avocado mesocarp.

Materials and Methods

Identification and In silico Analyses of PamDGAT1 and 2

Transcripts for the *PamDGAT1* and *2* (*Persea americana*) sequences were retrieved from the avocado transcriptome database (Kilaru *et al.*, 2015); the sequence was also verified against avocado genome data (Ibarra-Laclette *et al.*, 2015). The predicted full-length *PamDGAT1* and *2* sequences were then translated using the ExPASy protein translation tool. The coding sequence for the longest open reading frame was selected from the six translated frames as a candidate of *DGAT1* and *2* in avocado. Nucleotide sequences were removed before start codon and after the stop codon that resulted in 1608 bp long putative full-length sequence for DGAT1 and 996 bp for DGAT2. For further confirmation, the nucleotide sequence of *PamDGAT1* and *2* was analyzed to the check the probability of start codon using NetStart1.0 prediction software (http://www.cbs.dtu.dk/services/NetStart/). In this tool, avocado *PamDGAT1* and *2* nucleotide sequences were checked with Arabidopsis genome to determine the probability of start codon of these putative sequences. For each methionine (ATG), its probability as a start codon is scored and if the score is 0.5 or more it can be considered as a probable start codon. In addition to this, putative PamDGAT1 protein sequence was aligned by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) with other known DGAT1 protein sequences *of*

Lindera communis (*Lco*)*, Brassica napus (Bna), Olea europaea (Oeu). (*Accession number: AthDGAT1 (NP_179535.1*), LcoDGAT1* (AHN93288)*,* BnaDGAT1 (AAD40881.1), OeuDGAT1 (AAS01606.1) to analyze the sequences similarity of PamDGAT1 with biochemically characterized or putative DGAT1 proteins. Similarly, putative PamDGAT2 protein sequence was aligned by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) with *Arabidopsis thaliana (Ath), Olea europaea (Oeu), Virnicia fordii (Vfo)*. Accession number: AthDGAT2 (OAP06431.1), OeuDGAT2 (ADG22608.1), VfoDGAT2 (DQ356682) to analyze the sequences similarity of PamDGAT1 with biochemically characterized or putative DGAT2 proteins. Probable transmembrane domains were also predicted for PamDGAT1 and 2 by TMHMM transmembrane domain prediction software (http://www.cbs.dtu.dk/services/TMHMM/) and were compared with transmembrane domains of characterized DGAT1 and 2 proteins. A total of 53 DGAT1 and 26 DGAT2 protein sequences were analyzed by molecular evolutionary phylogenetic analysis (MEGA) software. The phylogenetic tree was constructed by maximum likelihood method based on the Poisson correction model (Zuckerkandl and Pauling, 1965) and the tree with the highest log likelihood was shown. The phylogenetic figure was drawn to scale and evolutionary analyses were performed in MEGA X (Kumar *et al.*, 2018). The name of each protein consists of first letter of the species followed by first two letters of the genus. The organisms and GenBank accession numbers for DGAT1 proteins used are as follows: AthDGAT1 (*Arabidopsis thaliana;* AEC06882.1), BnaDGAT1a (*Brassica napus*; AAY40785.1), BnaDGAT1b (*Brassica napus*; AAD40881.1), OeuDGAT1 (*Olea europaea*; AAS01606.1), EalDGAT1 (*Euonymus alatus*; AAV31083.1), EpiDGAT1 (*Echium pitardii*; ACO55635.1), GmaDGAT1a (*Glycine max*; AAS78662.1), GmaDGAT1b (*Glycine max*; BAE93461.1), JcuDGAT1 (*Jatropha curcas*;

ABB84383.1/ AFV61669.1), LjaDGAT1 (*Lotus japonicas*; AAW51456.1), MtrDGAT1 (*Medicago truncatula*; ABN09107.1), NtaDGAT1 (*Nicotiana tabacum*; AAF19345.1), OsaDGAT1 (*Oryza sativa*; NP_001054869.2), PfrDGAT1 (*Perilla frutescens*; AAG23696.1), RcoDGAT1 (*Ricinus communis*; XP_002514132.1), TmaDGAT1 (*Tropaeolum majus*; AAM03340.2), VfoDGAT1 (*Virnicia fordii*; ABC94471.1), VgaDGAT1 (*Vernonia galamensis*; ABV21945.1), VviDGAT1 (*Vitis vinifera*; XP_002279345.1), ZmaDGAT1 (*Zea mays*; ABV91586.1), CclDGAT1 (*Caenorhabditis elegans*; CAB07399.2), LusDGAT1 (*Linum usitatissimum*; AHA57450.1), BjuDGAT1a (*Brassica juncea*; AAY40784.1), BjuDGAT1a (*Brassica juncea*; AAY40785.1), PtrDGAT1a (*Populus trichocarpa*, XP_002308278.2), PtrDGAT1b (*Populus trichocarpa*; XP_002308278.2), SbiDGAT1 (Sorghum bicolor; XP_002437165.1), AaeDGAT1 (*Aedes aegypti*; XP_001658299), BtaDGAT1 (*Bos taurus*; NP_777118.2), BbuDGAT1 (*Bubalus bubalis*; AAZ22403.1), ChiDGAT1 (Capra hircus; ABD59375.1), DmeDGAT1 (*Drosophila melanogaster*; NP_609813.1), DreDGAT1 (*Danio rerio*; NP_956024.1), HsaDGAT1 (*Homo sapiens*; NP_036211.2), MmuDGAT1 (Mus musculus; NP_034176.1), MmulDGAT1 (*Macaca mulatta*; XP_001090134.1), PtrDGAT1 (*Pan troglodytes*; XP_520014.2), RnoDGAT1 (*Rattus norvegicus*; NP_445889.1), SscDGAT1 (*Sus scrofa*; NP_999216.1), SkoDGAT1 (*Saccoglossus kowalevskii*; XP_002736160.1), TcaDGAT1 (*Tribolium castaneum*; XP_975142.1), TgoDGAT1 (*Toxoplasma gondii*; AAP94209.1). The organisms and GenBank accession numbers for DGAT2 proteins used are as follows: OsaDGAT2 (*Oryza sativa*; ADG22608.1), ZmaDGAT2 (*Zea mays*; NP_001150174.1), EolDGAT2 (*Elaeis oleifera*; ACO35365.1), PamDGAT2 (*Persea americana*), HanDGAT2 (*Helianthus annuus;* ABU50328.1), OeuDGAT2 (*Olea europaea;* ADG22608.1), VviDGAT2 (*Vitis vinifera*; XP_002263626), AthDGAT2 (*Arabidopsis thaliana*; NP_566952), BnaDGAT2

(*Brassica napus*; ACO90188), PpaDGAT2 (*Physcomitrella patens;* XP_001758758.1), EalDGAT2 (*Euonymus alatus*; ADF57328.1), GmaDGAT2 (*Glycine max*; ACU20344.1), VfoDGAT2 (*Virnicia fordii*; DQ356682.1), RcoDGAT2 (*Ricinus communis*; XP_002528531.1), VgaDGAT2 (*Vernonia galamensis*; ACV40232.1), BtaDGAT2 (*Bos taurus*; XP_875499.3), MmuDGAT2 (*Mus musculus*; NP_080660.1), RnoDGAT2 (*Rattus norvegicus*; NP_001012345.1), HsaDGAT2 (*Homo sapiens;*), AcaDGAT2 (*Anolis carolinensis*; XP_003225477.1), XtrDGAT2 (*Xenopus tropicalis*; NP_989372.1), DreDGAT2 (*Danio rerio*; NP_001025367.1), IpuDGAT2 (*Ictalurus punctatus*; NP_001188005.1), PpalDGAT2 (*Polysphondylium pallidum*; EFA83646.1), TguDGAT2 (*Taeniopygia guttate*; XP_002187643.1), TcaDGAT2 (*Tribolium castaneum*; XP_975146.1). The 3-D structure of PamDGAT1 and PamDGAT2 was predicted by I-TASSER (Zhang, 2009).

Cloning of Putative PamDGAT1 and 2

For cloning into yeast, pESC-URA, a bi-directional expression vector was used to direct galactose-inducible expression of different proteins in the yeast. Total RNA from avocado mesocarp was used to generate full-length DGAT1 and 2 cDNA. First strand cDNA was synthesized using oligo (dT) primer and AMV Reverse Transcriptase (Promega). For PCR amplification, forward and reverse primers were designed to include *Not*I and *Pac*I restriction sites, respectively. Hemagglutinin (HA) tag was added with the forward primer before the start codon for detection by western blot. Additionally, “ACC” nucleotides were added in front of HA tag in the forward primer to create a better match to the Kozak consensus that will aid in increasing translation rates of the transcript *in vivo* (supplementary table 2.2). The full-length cDNA of PamDGAT1 and PamDGAT2 were amplified (figure 2.3) by their respective primers using Advantage 2 PCR (clontech) kit. The resulted PCR product and the vector were cut with

Not1 and Sac1 (New England Biolabs (NEB)) according to manufacturer's protocol. Briefly, 1 µg gene product or vector, 1 uL (10 units) of each restriction enzymes, 2 µL of cut smart buffer and an adequate amount of nuclease-free water was added to make the reaction volume to 20 µL. The reaction mixture was kept for one hour at 37 °C for digestion. Digested vector treated with 0.2 µL calf alkaline phosphatase (CIP; NEB) for 20 minutes to avoid self-ligation followed by heat inactivation at 65 °C for 20 minutes. Both the digested product and CIP treated vector were gel purified. For ligation, 50 ng gel purified gene product, 25 ng gel purified vector, 2 µL 10X reaction buffer, 0.5 µL T4 DNA ligase (NEB) and nuclease-free water were added to make 20 µL reaction mixture. The reaction mixture was kept at 16 °C for overnight ligation. The resulted vector containing *PamDGAT1* or *2* was chemically transformed into *E. coli* competent cells (Top10) by incubating at 42 °C for 50 seconds followed by in ice for 10 minutes**.** These transformed *E. coli* cells were plated and grown overnight at 37 °C without shaking. Colony PCR was done using a vector-specific and gene-specific primers to select positive colonies. Plasmid was isolated from the cell culture of positive colonies and the insert was confirmed by sequencing and was prepared for yeast transformation.

Construction and Maintenance of Different Saccharomyces Cerevisiae Strains

The *S. cerevisiae* quadruple mutant strain H1246 (*Δdga1*, *Δlro1*, *Δare1*, and *Δare2*) was transformed either with empty pESC-URA cassette for negative control or pESC-VfoDGAT1-URA (*V. fordii* DGAT1) for positive control or the pESC-PamDGAT1-URA or pESC-PamDGAT2-URA cassette by S. c. Easy Comp Transformation Kit from Invitrogen. The mutant H1246 yeast strain can survive well but cannot synthesize the neutral lipids or TAG. The transformed yeast strain was grown initially in SD-URA (synthetic dextrose -URA) media, 2% glucose, 0.67% yeast nitrogen base, and synthetic complete mixture of amino acids minus uracil.

Then these strains were induced to grow in SRGG-URA (synthetic raffinose, glycerol, galactose -URA) media containing 1% raffinose, 0.25% glycerol, 2% β-galactose (Difco), 0.67% yeast nitrogen base (Difco), and 1X synthetic complete mixture amino acids minus uracil. Several colonies were selected and grown in SRGG-URA liquid media for 24 hours. All cultures were grown at 28 °C on a shaker at 250 rpm. Both the liquid yeast culture and the plates were kept at 4 °C for further experiments.

Indirect DGAT Activity Analysis via Lipotoxicity Rescue Assay

Lipotoxicity rescue assays were performed as described previously (Garbarino et al., 2009; Sandager et al., 2002; Siloto et al., 2009). Competent cells were prepared from H1246, a quadruple yeast mutant. The coding sequence VfoDGAT1 (*Virnicia fordii* DGAT1 or Tung DGAT1) was transformed into H1246 under the control of GAL promoter. H1246 yeast strain lacks four acyltransferases and thus devoid of neutral lipids biosynthesis (Sandager *et al.*, 2002). Complementation of H1246 by Tung DGAT1 was previously validated and was used as a positive control in this study (Shockey *et al.*, 2006). The H1246, transformed with empty pESC-URA was used as a negative control. Aliquots of competent cells were transformed for negative and positive controls, and with PamDGAT1 and 2 in pESC- URA for the experimental group. All transformed strains grew on uracil selected SD-URA solid media. Plates were incubated at 28 °C for 2-3 days until colonies emerged. Several colonies were inoculated into a small volume (~5 mL) of SD-URA liquid media and incubated with shaking at 28 °C for overnight. Some of this culture was used to prepare for glycerol stock by adding 25% glycerol and kept in -80 °C and the remaining was used to start the induction culture with SRGG-URA at 28 °C with shaking for overnight. Cell density (usually using 1/10 dilutions of the overnight cultures, measured as OD_{600nm} on spectrophotometer) was measured from the overnight cultures. New SRGG-URA

liquid media was prepared and one portion of the SRGG-URA liquid media contained base components with 0.2% Tergitol detergent alone. The other portion of the liquid media contained base components, 0.2% Tergitol and 1.0 mM free fatty acids (16:0, 18:1 or 18:2, prepared as stock 0.5 M in ethanol). Both types of media were warmed at 28 °C at least 15-30 minutes prior adding to the yeast cells to ensure even dispersal of fatty acid in the media. Based on the OD readings, these cultures were inoculated at an initial concentration of 0.1 OD/ml into SRGG +/- free acids. Cultures were incubated for 26 hours at 28 °C with shaking at 250 rpm and at various time points e.g. 0, 12, 22 and 26 hours OD readings were taken, and incubation was stopped when cultures reached late log or stationary phase (O.D. 600 nm of approximate 8-12/ml). OD readings were plotted against various times points to generate yeast growth curve. Three technical replicates and two biological replicates were done to ensure the reproducibility of the experiments.

Microsome Isolation and Western Blot Analysis

Microsomes were prepared from H126 yeast strains expressing PamDGAT1 according to the method as previously described (Stymne and Stobart, 1984; Shockey *et al.*, 2006). Briefly, yeast cells were grown in liquid SRGG-URA media for overnight and cells were harvested by centrifugation at 1500 x *g* for 10 minutes. Harvested yeast cells were washed twice with sterile dH_20 and with 1X phosphate saline buffer (PBS) buffer. After washing, the pellets were resuspended in cell lysis buffer I (1x PBS containing Mini EDTA-free protease cocktail tablets from Roche Diagnostics; 1 tablet for 50 mL buffer) and transferred to a 15-mL glass tube. An equal volume of 0.50 mm acid-washed glass beads was added to the cells and were lysed by vortexing. Debris and unbroken cells were removed by centrifugation at 1500 x *g* for 15 minutes at 4 °C. The supernatant was recovered and centrifuged again at 100,000 x *g* for 1 hour at 4 °C in

a Thermo WX80 Ultracentrifuge. Microsomal pellets from the high-speed spin were collected and resuspended in 50 mM Tris-HCl and 25% glycerol, pH 8.0. A portion of microsomes was used for SDS-PAGE, western blot analysis and *in vitro* enzyme activity assays and the remaining were stored in aliquots at −80 °C for future use.

For SDS-PAGE and western blot analysis, two polyacrylamides (10%) gels were prepared in a 1.0 mm gel cassette. Samples were prepared by adding 5 µL 4X SDS-PAGE sample buffer with 15 µL microsomal preparation. Then the sample was denatured by boiling 5 minutes in a water bath before running in a mini-gel tank (Invitrogen). Protein Ladder (PageRuler™, Invitrogen) was used as a marker in this experiment. Two SDS gels were prepared, samples and marker were loaded in each gel. The gels were electrophoresed for 90 minutes at 100 V. One gel was stained with Coomassie blue for 20 minutes and destained overnight and the remaining gel was used for western blot analysis. For western blot analysis, the dry transfer technique was used; briefly, the gel was placed on iBlot® 2 Transfer Stacks, PVDF, mini (Invitrogen). It takes 7 minutes at 20 V to transfer the gel to a PVDF membrane using iBlot dry blotting system (Invitrogen). PVDF membrane was blocked overnight at 4 °C with 5% not-fat milk (Topco Associate LLC) solution in 50 mM 1X Tris-buffered saline (TBS). After overnight incubation, the PVDF membrane was washed twice with washing solution (sodium azide in TBS). Then, the membrane was incubated with rabbit anti-hemagglutinin (HA) antibody (Bethyl Laboratories A190-108A) diluted (1: 2500) in TBS with freshly prepared 0.02% sodium azide for additional 3 hours with shaking at room temperature. After incubating with primary antibody, the membrane was washed three times again with TBS solution. Subsequently, F(ab')2 Goat anti-Rabbit IgG-F (ab')2 fragment of secondary antibody AP-conjugated (Bethyl Laboratories A120-112AP), was added and kept at room temperature for two hours with shaking.

Thereafter, the membrane was washed two times with TBS and soaked in alkaline phosphatase buffer for 5 minutes. After that, expression of PamDGAT1 and 2 and VfoDGAT1 was detected using nitro-blue tetrazolium chloride and BCIP (5-bromo-4- chloro-3'-indolyphosphate p-toluidine salt) substrate (Invitrogen) which imparted insoluble black-purple precipitate with alkaline phosphatase for a positive reaction. The developed membrane was transferred into a solution containing 50 mL of 1X TBS buffer and 200 µL of 0.5 M EDTA for 5-10 minutes to stop the AP reaction. Finally, the membrane was air dried and scanned for the record of western blot analysis.

Lipid Extraction from Yeast Cell Cultures

Yeast cultures were induced at the stationary phase at 28 °C with shaking at 250 rpm. Cells were harvested from 50 mL induced yeast culture at 1300 x *g* for 10 minutes. Harvested cells were washed twice with sterile distilled water and with 1X PBS. Cells were treated with isopropanol at 80 °C for 10–15 minutes to stop the lipolytic activity. After that, isopropanol was evaporated under nitrogen gas before lipid extraction according to a modified Bligh and Dyer Method (Bligh and Dyer, 1959). Glyceryl triheptadecanoate (17:0) was added as an internal standard during lipid extraction because this biological system usually does not produce any odd number carbon containing fatty acids. An equal volume of acid-washed glass beads (0.5 mm) was added and the suspension was vigorously vortexed. For a monophasic system, 5 mL of water: isopropanol: hexane (1:4:6) were added to the sample. The sample was vortexed well and allowed to stand for a few minutes. For phase separation, 2.5 mL aqueous sodium sulfate solution (6.6 g anhydrous sodium sulfate in 100 mL) was added and vortexed vigorously sample. Then the sample was spin down for 5 minutes at 5000 x *g* at room temperature and the upper hexane phase was carefully transferred to another pre-weighed glass tube and dried under

nitrogen gas. The total lipid was quantified before resuspended in 1 mL hexane and kept in -20 °C for further analysis.

Triacylglycerol Separation and Quantification from Total Lipid Extracts

TAGs were separated from the total lipid extracts by thin layer chromatography (TLC) on Silica Gel 60 plates (Whatman). Lipid extracts were spotted on the TLC plate and developed in a solvent tank containing petroleum ether: diethyl ether: glacial acetic acid (70: 30: 1, v/v). TAGs were visualized by a brief exposure to iodine vapor. For charring, plates were sprayed with a solution of 10% (v/v) H_2SO_4 in methanol, and heated until spots appeared. TAGs were scratched from the TLC plate with a razor and were extracted from the silica plates using the lipid extraction procedure described previously. Lipids were then quantified gravimetrically and were processed for composition analyses by gas chromatography (GC).

Determination of Fatty Acid Composition of TAG

The fatty acid content and composition of transformed yeast strains were determined using a previously described protocol (Browse *et al.*, 1986). Briefly, 100 µL of TAG extract was dried under liquid nitrogen and 2 mL of hexane was added and mixed well by vortexing. For transmethylation, 200 µL of KOH/MeOH (2M) was added and vortexed for 2 minutes. To acidify (~ pH 3-4), 400 µL of 2M HCl acid was added to the mixture. After adding 2 mL of hexane, the sample was centrifuged for 5 minutes at 3000 x g and the upper hexane phase was recovered into a new test tube and dried under nitrogen gas. The dried fatty acid methyl ester was resuspended in 1 mL hexane for analysis by GC coupled with a flame ionization detector (GC-FID, Varian), to analyze the fatty acid profile (Christie and Han, 1982). A capillary column (DB-23; 30 m x 0.32 mm I.D., 0.25 µm) was used in the chromatography with the helium carrier gas

(flow rate of 1.5 mL/minute). The GC-FID Column was equilibrated with 2 µL hexane (blank) injection before sample injection (Hara and Radin, 1978). The sample was injected for three minutes at a temperature of 150 °C. Then samples were separated at 150 °C for three minutes followed by 150-240 °C at 6 °C/minute, where the detection temperature was 300 °C. Each sample was analyzed in triplicates (Hara and Radin, 1978).

Nile Red Fluorescence Staining of Neutral Lipid

The Nile Red staining of LDs was carried out as described previously (Greenspan *et al.*, 1985) and was visualized under fluorescence confocal microscopy as an indirect measurement of TAG production. In this assay, yeast cells were induced by providing galactose into the growth media for 24 h and harvested at 1300 x *g* for 10 minutes at room temperature. Cells were then washed twice with 1X PBS and sterile distilled water. For sample preparation, 2 µL of Nile Red solution (0.8 mg/mL stock in methanol) was added to 200 µL of yeast culture (1:100 ratio of Nile Read and yeast cells) and was incubated at room temperature for 20 minutes. After incubation, 10-15 µL prepared sample was put on a microscopic slide and covered with a coverslip. Slides were immediately observed under the Leica TCS SP8 Confocal Fluorescence Microscope. Nile Red fluorescence was observed with an excitation of 488 nm and an emission of 600-650 nm wavelength (n = 3).

In Vitro Enzyme Activity and Substrate Specificity of PamDGAT1

Enzyme activity and substrate specificity of DGAT1 were measured by the incubation of microsomal fractions with different combinations of radiolabeled fatty acyl-CoA substrate and DAG as described previously with some modifications (Shockey et al., 2006). Briefly, 25 µg of microsomal membrane proteins were incubated with 10 µM [1- 14C] acyl-CoAs and 400 µM DAG (stock of 40 mM in methanol). The acyl-CoAs are palmitoyl-CoA (16:0) and oleoyl-CoA

(18:1) from PerkinElmer; Specific activity, 20,000 CPM/µmol. The DAGs are di-palmitin (di16:0) and diolein (di18:1). The reaction mixture volume was made 100 µL by 100 µM Tris-HCl, pH 8.0 buffer. The reaction mixture was incubated at 37 °C for one hour in a water bath. Lipids were extracted as described earlier and dissolved in 20 µL chloroform. Lipids were spotted on the TLC plate and separated as described previously (Janero and Barrnett, 1981). After running, the TLC plate was dried for 10 minutes and placed on AR-2000 radio-TLC imaging scanner to detect and quantify the radioactive compounds. Radioactive substrates including other components except microsomal proteins were used as a negative control. In a functional enzymatic reaction, the fatty acid from radiolabeled acyl-CoA will be incorporated into the *sn*-3 position of the glycerol backbone of DAG. Radioactive peaks were detected and quantified as counts per minute (CPM). DGAT1 activity was tested in triplicate with palmitoyl-CoA (16:0) or oleoyl-CoA (18:1) substrates. The average value of CPM with standard deviation for each substrate was used to determine the activity.

Statistical Analysis

Statistical analysis was conducted using Minitab software. Data were expressed as their mean value and standard deviation (SD). Student's paired t-test was used to determine significant differences between the data set.

Results and Discussion

Putative PamDGAT1 and 2 reveal conserved motifs and show sequence similarity with their corresponding plant DGATs

Coding sequences for putative *PamDGAT1* and *PamDGAT*2 were obtained from avocado transcriptome data (Kilaru *et al.*, 2015). Protein sequences were inferred by conceptual translation of the coding sequences. For *PamDGAT1* and *PamDGAT2,* these were 535 and 331

amino acid residues long, respectively excluding the stop codon. The probability of start codon of these putative sequences was analyzed by the NetStart1.0 prediction software by comparing with Arabidopsis genome database and found that start codon of PamDGAT1 and PamDGAT2 sequences have the probability of 76.6% and 86.0%, respectively (Table 2.1).

Table 2.1 Features of putative PamDGAT1 and PamDGAT2

***In silico* analyses**	***PamDGAT1***	***PamDGAT2***
Full length cDNA	2079 bp	1386 bp
Coding sequence length	1608 bp	996 bp
Probability of start codon	78.60%	86.00%
Protein length	535 aa	331 aa
Estimated MW	61.2 kD	37.2 kD
# of transmembrane domains	9	5
Conserved motif	6	3
Catalytic residues	6	Not known

Further *in silico* analysis of putative PamDGAT1 revealed high sequence similarity with other putative, predicted or biochemically characterized DGAT1 protein sequences (Figure 2.1). A 95.5% similarity with putative LcoDGAT1 *(L. communis)*, which contains 56.4% oleic acid in sarcocarp; 65% and 64.9% identity with biochemically characterized OeuDGAT1 (*O. europaea*) and BnaDGAT1 (*B. napus*), respectively. Multiple sequence alignment of these DGAT1 protein sequences with putative PamDGAT1 also predicted a set of conserved domains including acyl-CoA binding site, fatty acid binding site, DAG binding site and active site, which were initially identified in DGAT1 of Arabidopsis (Figure 2.1; Jako *et al.*, 2001; Hobbs *et al.*, 1999; Zou *et al.*, 1999) and *T. majus* (Xu *et al.*, 2008). An ER retrieval motif that is necessary for ER targeting as shown in Tung tree (Shockey *et al.*, 2006) was also was found in PamDGAT1. Previously, 55

DGAT1 sequences from plants, animals, and fungi were shown to contain 41 conserved amino acids (Cao, 2011), which are also retained in putative PamDGAT1. A membrane protein topology prediction method revealed nine transmembrane domains for PamDGAT1 (Figure S1), similar to DGAT1 in Arabidopsis, *V. fordii* and *B. napus*. Monocots *O. sativa* and *Z. mays*,

Figure 2.1 Sequence alignment, phylogenetic analysis of DGAT1 and DGAT2 proteins from different plant species. Multiple sequence alignment, prediction of conserved motifs and amino acids (showed by giving asterisk mark) in plant DGAT1(2.1 A) and DGAT2 (2.1B) protein sequences. Phylogenetic analysis of DGAT1 (2.1C) and DGAT2 (2.1D) proteins. DGAT1 and DGAT2 protein sequences were collected from NCBI and then phylogenetic analysis was conducted using Molecular Evolutionary Genetic Analysis (MEGA) software.

on the other hand, were predicted to contain eight transmembrane domains. Multiple sequence alignment of putative PamDGAT2 also showed high similarity with biochemically characterized OeuDGAT2 (61.0%), AthDGAT2 (58.4%) and VfoDGAT2 (56.9%). Similar to other plant DGAT2 sequences predicted (Cao, 2011), PamDGAT2 also retained 16 conserved amino acids. However, unlike DGAT2 from other monocots and dicots that contain two or three transmembrane domains, PamDGAT2 was predicted to have five transmembrane domains (Figure 2.2B). PamDGAT1 and PamDGAT2 were phylogenetically closer to orthologs from monocots *O. sativa* (Figure 2.1C) and *Z. mays* (Figure 2.1D), respectively. The tertiary structure of PamDGAT1 (Figure 2.2C) and PamDGAT2 (Figure 2.2f) predicted by I-TASSER and revealed the predicted α-helices of these proteins.

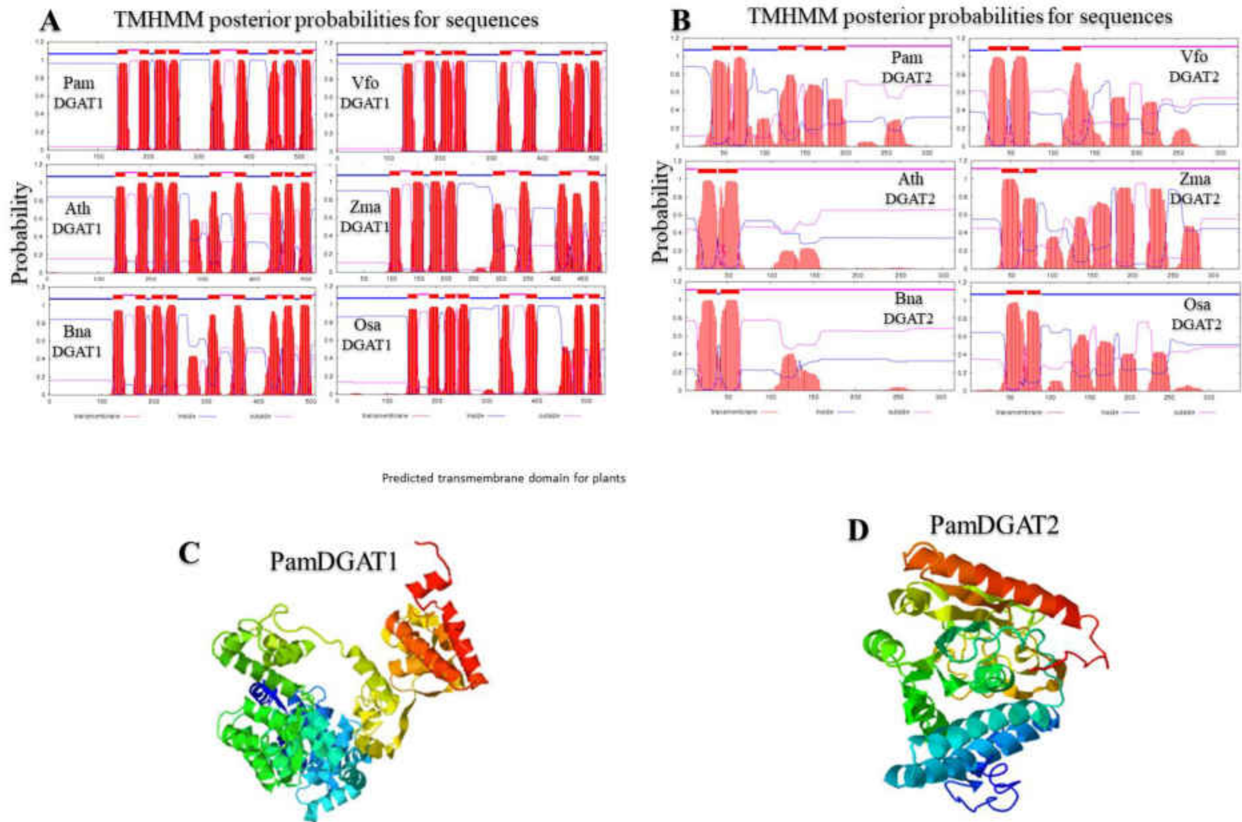

Figure 2.2 Prediction of transmembrane domains and 3-D structure of avocado DGAT1 and DGAT2. Prediction of transmembrane domains of putative PamDGAT1 (2.2A) and PamDGAT2 (2.2B) using TMHMM software version 2.0. Pink and blue color in the sequences are representing whether the sequences are outside or inside the membrane respectively. The N-terminal and C-terminal regions of a sequence are predicted to face towards the cytosol if they have even number transmembrane helices and one terminal towards cytosol and other faces toward ER. Prediction of 3-D structure of PamDGAT1 (2.2C) and PamDGAT2 (2.2D) by I-TASSER.

Together, *in silico* analyses further established the validity of putative DGAT sequences from avocado as potential candidate genes for further biochemical characterization.

Isolation and expression of PamDGAT1 and 2 in TAG-deficient yeast

The predicted full-length PamDGAT1 and 2 sequences were amplified by PCR from cDNA and the expected bands were 1608 bp and 996 bp detected by gel electrophoresis,

respectively (Figure 2.3A and B). The amplified genes of *DGAT1* and *2* were cloned into the pESC-URA vector. After the sequences were confirmed, vector containing the genes were transformed into the H1246 for expression.

The yeast strains were induced by galactose containing growth media for 24 hours and the

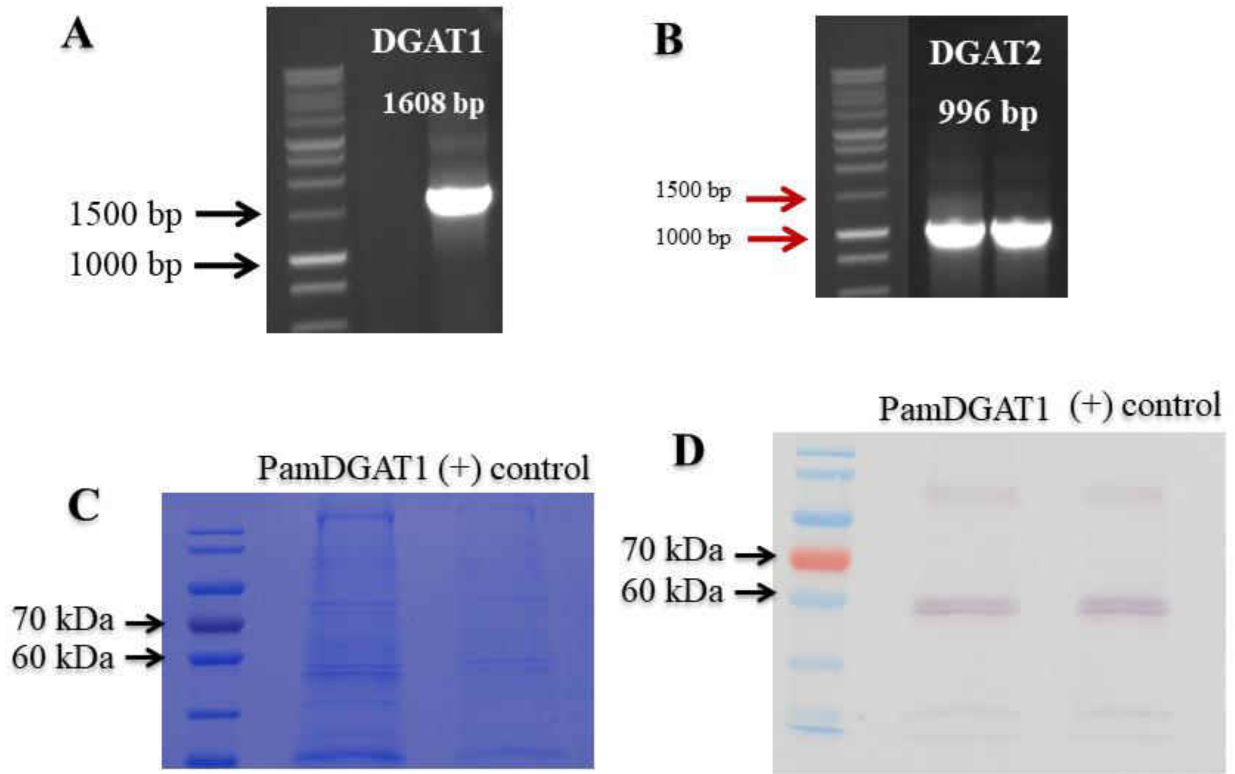

Figure 2.3 PCR amplification and verification of heterologously expressed PamDGAT1 and 2. Putative full-length avocado DGAT1 and DGAT2 genes were amplified using primers with restriction sites and predicted length of DGAT1 (2.3A) and DGAT2 (2.3B) was observed on gel electrophoresis. Yeast microsomes were extracted as a protein source of DGAT1 and were separated in an SDS-PAGE (2.3C) and PamDGAT1 was detected in western blot analysis along with the positive control (2.3D). Protein Ladder (PageRuler™, Invitrogen) was used as a marker in this western blot analysis.

microsomal fractions were isolated. The proteins of microsomal fractions were separated on

SDS-PAGE (Figure 2.3C) and to confirm the expression of PamDGAT1 and PamDGAT2, western blot analysis was conducted (Figure 2.3D. Results showed that a PamDGAT1 band corresponding to the size of ~58 kDa was detected in complemented H1246 yeast cell by PamDGAT1 and the positive control that is Tung DGAT1 expressed in H1246 also had a band close to PamDGAT1 (Figure 2.3D). A similar result was found in HEK293 cell lysates where DGAT1 was detected but no protein band was found for DGAT2 (Qi et al., 2010). Multiple bands were detected in case of DGAT1 proteins, which might be due oligomerization of DGAT1 proteins.

Functional complementation of H1246 with avocado DGAT1 and 2

The H1246 mutant yeast strain lacks four acyltransferases (Are1p, Are2p, Dga1p, and Lro1p) necessary for TAG and steryl ester synthesis and is devoid of neutral lipids (Sandager *et al.*, 2002). This TAG-deficient mutant is thus suitable for complementation assays and functional characterization of putative DGAT genes. It is well established that TAG-deficient yeast will experience lipotoxicity if the growth media is supplemented with free fatty acids; functional complementation would, however, successfully rescue the mutant yeast strain as the active DGAT would utilize the free fatty acids and accumulate TAG (Pan *et al.*, 2013; Siloto *et al.*, 2009). Here, a functional complementation analysis of TAG-deficient yeast was carried out with putative avocado DGAT1 and 2 using a lipotoxicity rescue assay. Yeast mutant strains transformed with PamDGAT1 and 2, along with a positive and a negative control were grown in the presence of saturated (16:0) and unsaturated (18:1 and 18:2) free fatty acids and their growth pattern were observed for 26 hours (Figure 2.4). While 16:0 did not affect the growth of any yeast culture (data not shown), both oleic (18:1) and linoleic (18:2) acid halted growth of the negative control (yeast mutant without any complementing gene). On the other hand, both the

positive control and H1246 complemented with DGAT1 maintained their normal growth pattern in the presence of 18:1 or 18:2 fatty acids suggesting their ability to utilize free fatty acids from the media and thus they were free from the lipotoxic effects. The growth curve showed that initially, it takes few hours for complemented yeast strains to adjust to the new growth media containing galactose instead of glucose, however, as time progressed the propagation rate increased rapidly (Figure 2.4A and B). Putative PamDGAT2 however, was unable to complement H1246 in its native or codon-optimized form (Figure 2.4C and D).

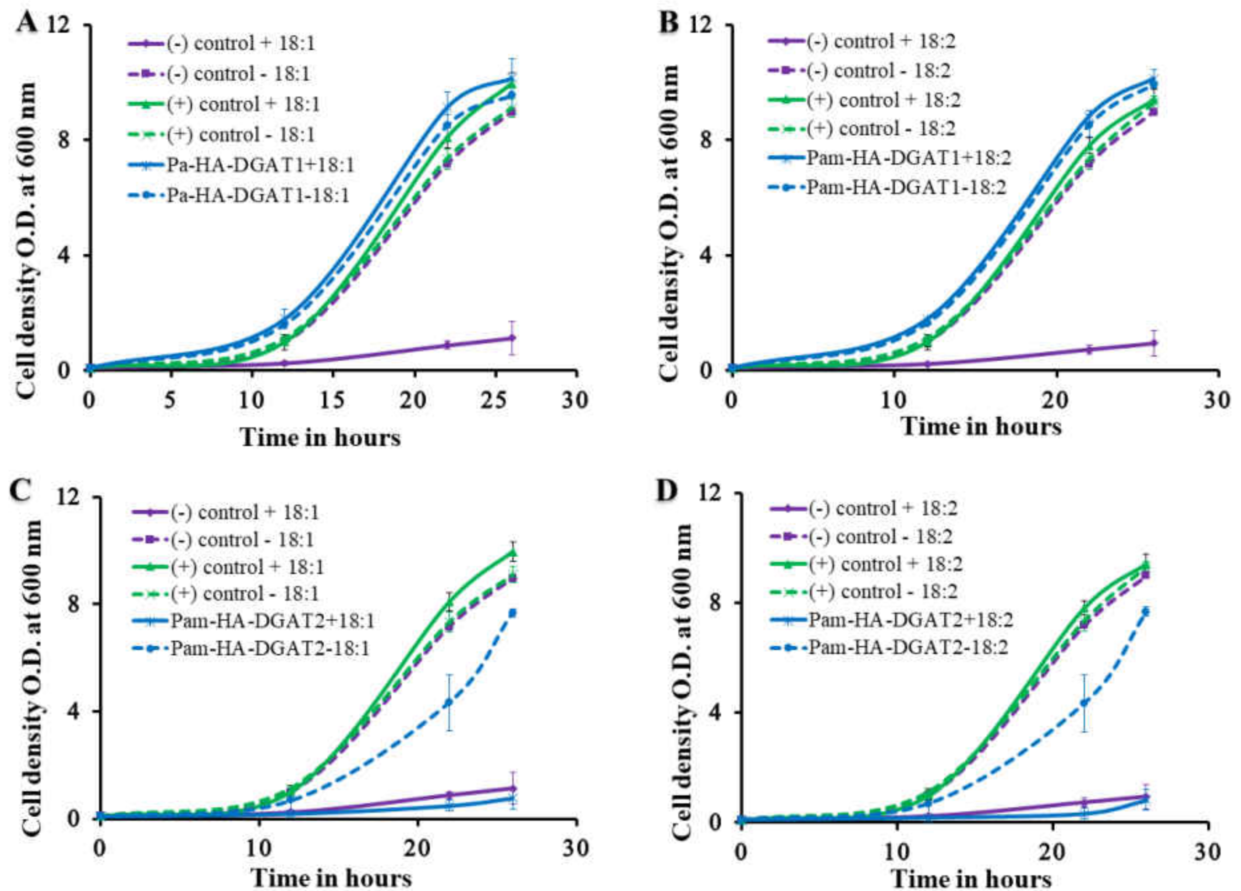

Figure 2.4 Lipotoxicity rescue assays to confirm initial complementation of H1246 yeast strain by PamDGAT1 and 2. The transformed yeast strains with PamDGAT1 and PamDGAT2 along with negative (H1246 with transformed empty pESC-URA vector) and positive (H1246 transformed with Tung DGAT1) controls were challenged with 1 mM free oleic acid (18:1) (Figure 2.4A and 2.4C, respectively) and 1 mM free linoleic acid (18:2) (Figure 2.4B and 2.4D, respectively) by supplementing into the growth media. Lipotoxicity assays were conducted as an indirect means of determining the function of TAG synthesizing genes.

Generally, yeast strains with lipid synthesizing genes can uptake free fatty acids from the media and utilize them for lipid biosynthesis without experiencing toxicity. To overcome the lipotoxic effect, these free fatty acids are utilized for esterification of DAG to TAG or sterols to steryl esters. Neutral lipids thus generated are cytoprotective as they buffer excess free fatty acids inside the cellular system (Garbarino and Sturley, 2009; Listenberger *et al.*, 2003). Previous

studies showed that reverse complementation of H1246 with TAG synthesizing genes (Dga1p and Lro1p), but not steryl ester synthesizing genes (Are1p and Are2p), prevented lipotoxicity effect of free fatty acids (Petschnigg et al., 2009). Because putative avocado DGAT1 complemented and rescued quadruple mutant yeast strain H1246 in the presence of free fatty acids, it can be postulated that PamDGAT1 was able to utilize free fatty acids to overcome toxicity by accumulating TAG rather than steryl ester synthesis (Petschnigg *et al.*, 2009).

When unsaturated fatty acids are not utilized, this results in accumulation of reactive oxygen species (ROS), impaired cell survival, misfolded proteins leading to apoptosis (Garbarino and Sturley, 2009) and necrotic cell death (Rockenfeller *et al.*, 2010). Curiously, both native and codon-optimized putative PamDGAT2 failed to rescue H1246 in a lipotoxicity assay with 18:1 or 18:2 in the growth media. Several studies have reported that heterologous expression of native DGAT2 from several plants including *Arabidopsi*s (Wang *et al.*, 2015), and the fungus *Umbelopsis ramanniana* (Lardizabal *et al.*, 2008) showed almost no DGAT activity although their codon optimized DGAT2s were active. Typically, DGAT2 incorporates unusual fatty acids to TAG (Napier, 2007) and since avocado does not accumulate unusual fatty acids, it might be possible that the putative PamDGAT2 is not functional in avocado. In addition, the transcript level of DGAT2 was very low in all developing stages of avocado mesocarp, relative to DGAT1 and PDAT1 (Kilaru *et al.*, 2015). Further *in planta* expression studies might confirm the functional nature of PamDGAT2.

The lack of any effect of 16:0 fatty acid on yeast growth was also observed in previous studies. For example, mutant H1246 yeast strain expressing Arabidopsis DGAT1 was successfully rescued from lipotoxic effect when the growth media was supplemented with unsaturated (18:1) fatty acid but not with medium-chain saturated fatty acids (Iskandarov *et al.*,

2017). Similarly human DGAT2 reverted the lipotoxicity in H1246 in the presence of unsaturated but not saturated fatty acid (Garbarino and Sturley, 2009). Conversely, *Cuphea* DGAT1 was able to rescue the lipotoxic effect when media contained medium chain saturated fatty acid but not with unsaturated (18:1) fatty acid (Iskandarov *et al.*, 2017). Therefore, it may be that PamDGAT1 is functional in yeast and may contribute to TAG biosynthesis in avocado.

Avocado DGAT1 Restores Neutral Lipid in H1246 Mutant Yeast Strain

The survival and growth of yeast H1246 complemented with PamDGAT1 in the presence of free fatty acids in the media were to be associated with accumulation of neutral lipids such as TAG stored in lipid droplets (LDs). Such yeast H1246 cells expressing PamDGAT1 and VfoDGAT1 (positive control), when stained with the Nile red fluorescence dye that binds to neutral lipids accumulated in small cytoplasmic inclusions, are revealed by confocal microscopy. LDs found in the transgenic yeast strain with PamDGAT1 (Figure 2.5). On the contrary, TAG-deficient mutant cells (negative control) do not accumulate any neutral lipid dye. These results suggest that PamDGAT1 not only rescues H1246 cells in the presence of free fatty acid supplement but also encodes for a protein that is capable of synthesizing neutral lipids. Based on the substrate that is provided in the media, it could be that PamDGAT1 likely served as an acyltransferase by incorporating acyl-CoA to generate neutral lipids.

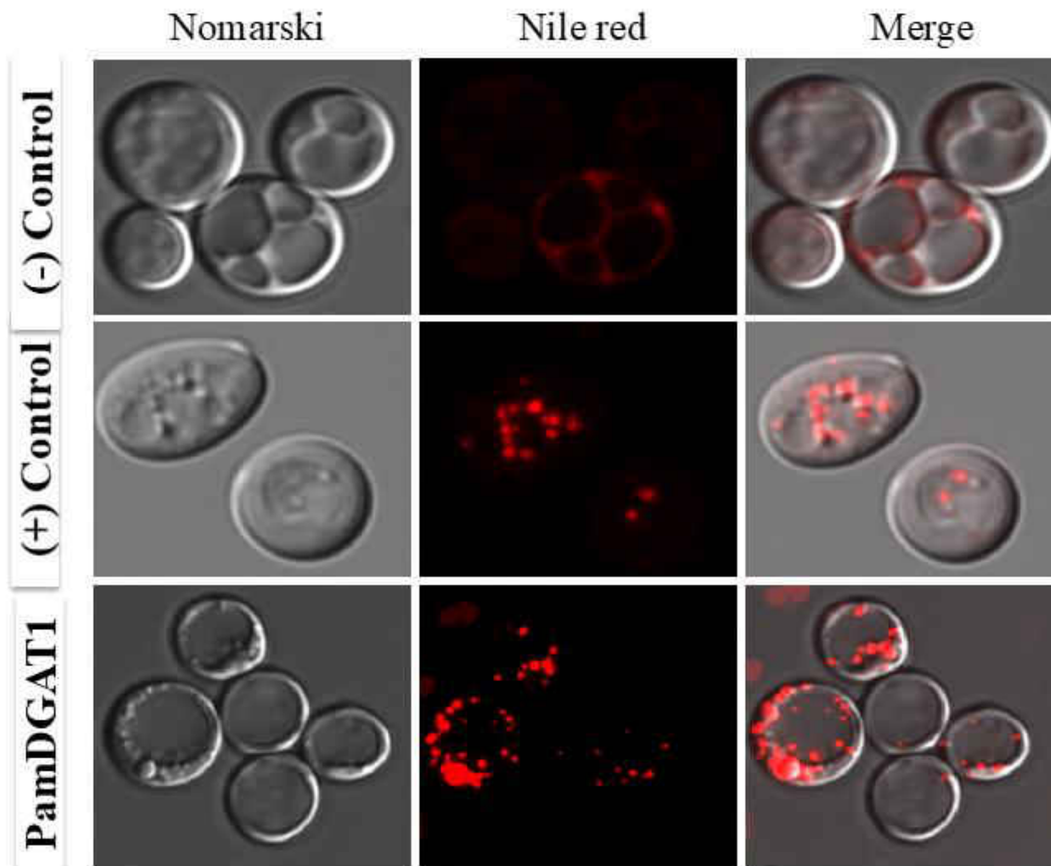

Figure 2.5 Observation of neosynthesized lipid droplets in complemented yeast strain. Yeast cells expressing PamDGAT1 restored lipid body formation. Avocado DGAT1 restore TAG synthesis in mutant yeast strain visualized in the lipid bodies in the yeast cells with Nile red fluorescence dye. The neutral lipid deficit quadruple mutant H1246 strain harboring VfoDGAT1 was used as a positive control. The quadruple mutant H1246 strain containing empty plasmid was used as a negative control. Nile Red fluorescence was observed with an excitation of 488 nm and an emission of 600-650 nm wavelength.

Further, total lipids were extracted from the complemented yeast strains and analyzed by TLC. As expected, TAG was detected for both the positive control and yeast transformed with PamDGAT1, while no TAG was found in the case of the negative control (Figure 2.6A). These results confirm that PamDGAT1 encodes for a protein with TAG biosynthetic activity.

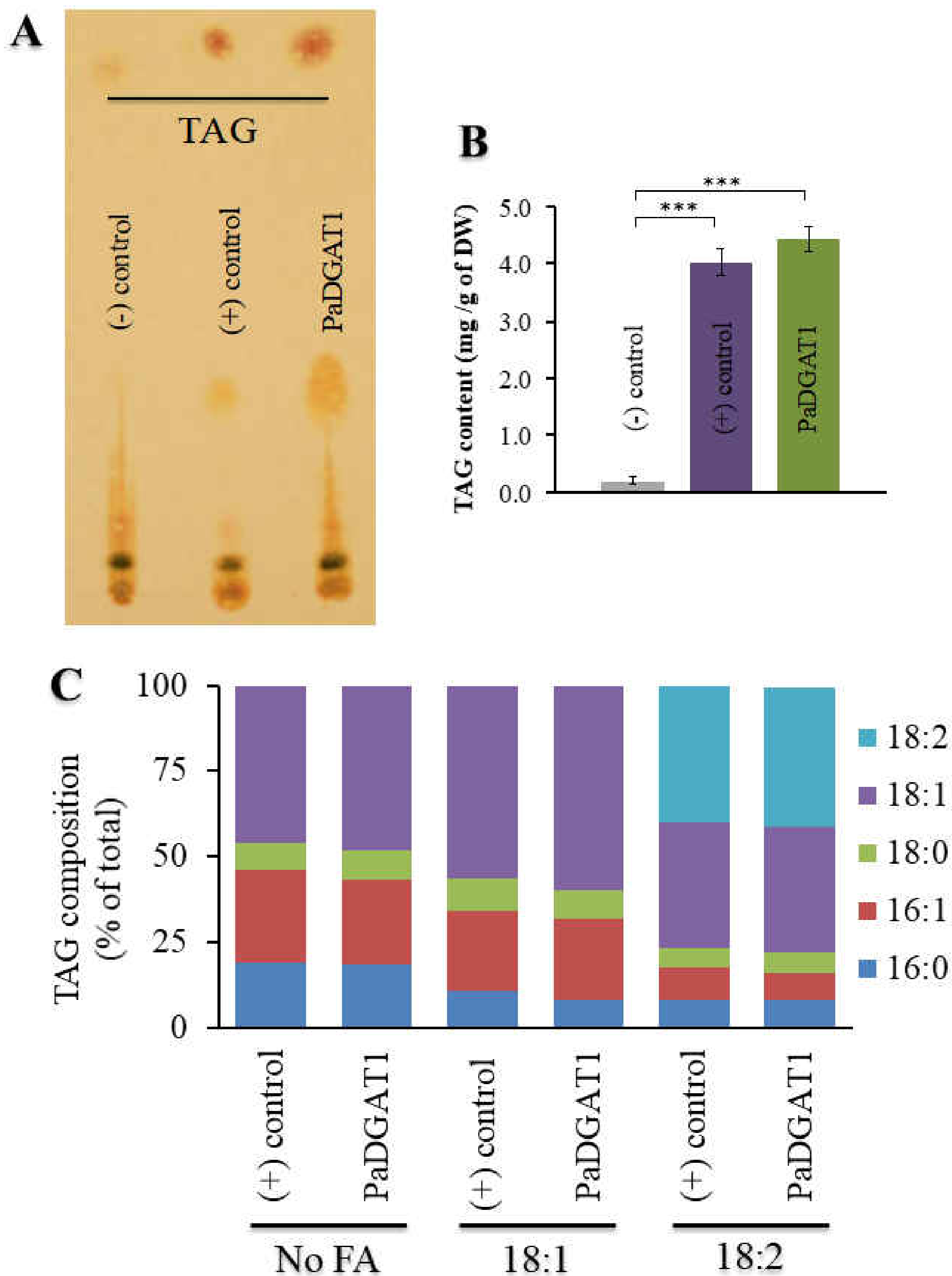

Figure 2.6 Separation and quantification of triacylglycerol from yeast strains with or without complementation. Lipids were extracted from 24 hours induced yeast culture and then was separated by running into the TLC plate (2.6A). Triacylglycerol spot from the TLC was collected and extracted (2.6B). The

amount of TAGs was measured in each case; negative control (H126 transformed with empty plasmid), positive control (H1246 transformed with Tung DGAT1) and H1246 transformed with avocado DGAT1. Amounts of TAG (mg / g dry weight) formed shown for each condition. Experiments were done in triplicate and the results are means ± SD, n=3. H1246 yeast strains expressing PaDGAT1 and Tung DGAT1 were induced for 24 hours and cells were harvested by centrifugation. TAG was extracted and transmethylated. The resulting FAMEs were analyzed by GC-FID. Fatty acids were quantified based on the internal standard Glyceryl triheptadecanoate (17:0). Fatty acid content was analyzed when media contain oleic acid (18:1) and linoleic acid (18:2) (2.6C).

The enzyme properties of PamDGAT1 were further examined in transgenic yeast expressing the enzyme *in vivo*. The fatty acid composition was analyzed in transgenic yeast without any fatty acid supplementation and then subsequently with 18:1 and 18:2 in the media. Usually, yeast can uptake fatty acids from the medium by esterification to fatty acyl-CoAs (Færgeman *et al.*, 2001) and the supplemented fatty acids can provide an excess amount of fatty acid as a substrate for the expressed PamDGAT1 enzyme *in vivo*. After 48 hours, yeast cells were harvested, and fatty acid composition was analyzed from total TAG content. In a yeast cell, the amount of 16-carbon containing fatty acid is produced by yeast itself and 16:1, is produced by the action of yeast OLE1p Δ9 desaturase enzyme. While 18:0 was produced by the yeast itself, 18:1 is the sum of supplements in the media and what was produced by the enzyme OLE1p from 18:0. Yeast does not have any intrinsic 18:2, therefore, the amount of 18:2 reflects only the amount of fatty acid provided in the media as a supplement. In the absence of a fatty acid supplement, the fatty acid composition which is incorporated by PamDGAT1 is dominant by 18:1, ~ 46% of the total fatty acid. In yeast media supplemented with 18:1 helps to increase up to ~70% 18:1 and supplemented with 18:2 increase its content by ~ 40% (Figure 2.6C).

Avocado DGAT1 Preferentially Incorporates Oleic Acid (18:1) During TAG Accumulation

The substrate specificity of DGAT1 was first examined with 16:0 and then with 18:1 by supplying radiolabeled CoAs separately to the reaction mixture. Our results suggest that

PamDGAT1 incorporates both 16:0 and

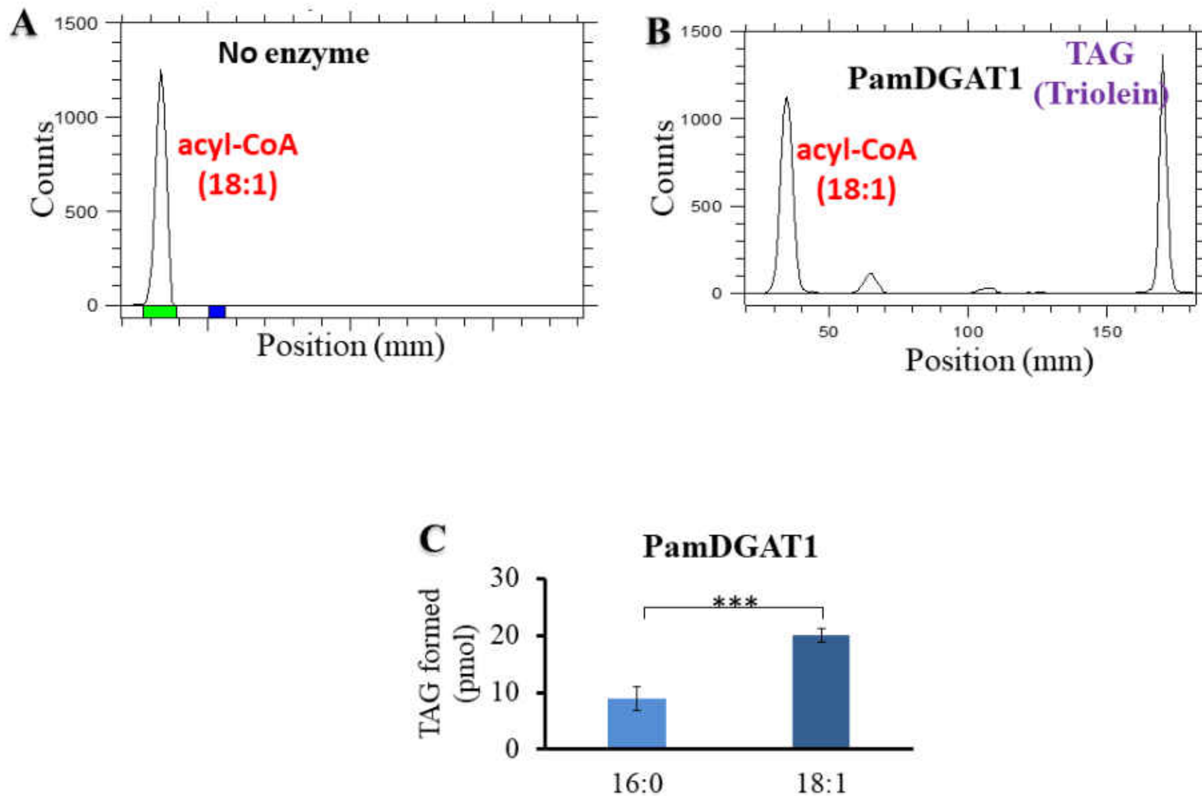

Figure 2.7 In vitro enzyme activity and specificity assays of PamDGAT1. Avocado DGAT1 with a N-terminal HA tag was expressed in H1246 yeast strain and was induced for 24 hours. Microsomal fractions were prepared and then incubated with radiolabeled fatty acyl-CoAs and diacylglycerols. Saturated, palmitoyl-CoA (16:0-CoA) and unsaturated oleoyl-CoA (18:1-CoA) were used in these assays. Diacylglycerols were diolein (di18:1) and dilinolein (di18:2). In each reaction, 25 mg microsomal fractions along with diacylglycerol and radiolabeled acyl-CoAs were provided. After a one-hour incubation, lipid was extracted and separated in TLC. Radiolabeled substances were measured by scanning the TLC plates under bioscanner. Amounts of radiolabeled TAG formed (in picomoles per hour) shown for each condition. Experiments done in triplicate and results are means ± SD, (n=3).

18:1 but it showed a marked preference (almost two-fold higher than 16:0: Figure 2.7C) towards 18:1. 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 for18:1 (almost two-fold higher than 16:0) of avocado DGAT1 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). Therefore, it can be deduced that 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 has a correlation 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 starting from acetyl-CoAs involves 30 reactions, therefore, regulation can occur in several steps (Ohlrogge and Jaworski, 1997). Nonetheless, current literature suggested that DGAT1 is one of the crucial enzymes in TAG biosynthesis and playing an important role to determine the TAG composition.

Conclusions

We have identified and characterized avocado DGAT1 by complementation of yeast quadruple mutant H1246 while native avocado DGAT2 did not show any functional activity in the yeast. Even codon optimized PamDGAT2 for yeast expression system has failed to show DGAT activity when expressed in the mutant yeast. Heterologous expression and *in vitro* enzyme activity confirmed diacylglycerol acyltransferase activity of putative avocado DGAT1 and specificity towards oleic acid (18:1). Overall, our findings suggest that the transgenic

expression of PamDGAT1 in the TAG deficient mutant yeast not only increases the TAG content but also alters the fatty acid composition. This study adds to our current understanding of TAG biosynthesis as well as the role of DGAT1 in nonseed tissues of a basal angiosperm.

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Supplemental tables and figures

Table 2.2 Primer sequences used in this study

Primer name	Sequence
PamD1-5HANotI	ACTTTGCGGCCGCCACCATGGCTTATCCTTATGATGTTCCTGATTATGCTGCGATCTCGACTCCGGAAGAAACCCT
PamD1-3PacI	TCACGTTAATTAATCACTCTGTTCTCACTCTCCGGTTCA
PamD2-5HANotI	ACTTTGCGGCCGCCACCATGGCTTATCCTTATGATGTTCCTGATTATGCTGATAGAGTTGGTGAGGAAAAGGAGAC
PamD2-3PacI	TCACGTTAATTAATCAAAGTATTCTCAACTGAACATCAC
Gal10P-SeqFor2	AATGGGGCTCTTTACATTTCCACAAC
ADH1Term-SeqRev	CAACCTTGATTGGAGACTTGACCAA
DGAT1-5N	CACTAATGATCTTGACTTGCGTTG
DGAT1-3N	ATTGAAAGGCGCGTCACCCTTATC

Figure 2.8 Probability of start codon for PamDGAT1 and 2. The probability of start codon PamDGAT1 (Figure 2.8A) and PamDGAT2 (Figure 2.8B) predicted by NetStart software by comparing gene sequences with Arabidopsis genome.

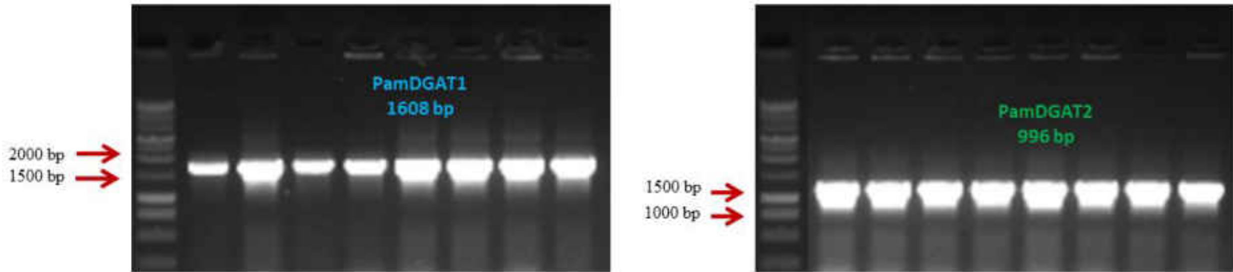

Figure 2.9 Colony PCR for PamDGAT1 and 2 to select positive transformation. Selection of PamDGAT1 (Figure 2.9A) and PamDGAT2 (Figure 2.9B) transformed positive colonies by colony PCR using one vector specific and one gene-specific primers.

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CHAPTER 3

TRANSIENT EXPRESSION OF AVOCADO DGAT1 AND PDAT1 INCREASE LIPID CONTENT IN NONSEED TISSUES OF *NICOTIANA BENTHAMIANA*

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Abstract

In plants, the final and committed step in triacylglycerol (TAG) biosynthesis is catalyzed by diacylglycerol acyltransferases (DGAT) and/or a phospholipid: diacylglycerol acyltransferases (PDAT). However, the preferred pathway for TAG biosynthesis is not well-studied in nonseed tissues such as mesocarp of avocado. In this study, putative PDAT1 was identified and comprehensive *in silico* analyses were conducted to predict the general features of avocado PDAT1. To compare the activity and substrate preference of PamPDAT1 with PamDGAT1 in nonseed tissues, both genes were transiently expressed in *N. benthamiana* leaves by *Agrobacterium*-mediated transformation. TAG accumulated in the lipid droplets was visualized by Nile Red staining, and lipid content and composition were analyzed by thin layer and gas chromatography. In leaf tissue, avocado DGAT1 and PDAT1 increased lipid content significantly and fatty acid analysis revealed their preference for oleic acid (18:1) as a substrate. These results suggest that avocado DGAT1 and PDAT1 are functional and capable of synthesizing TAG enriched in oleic acid (18:1) when expressed in nonseed tissues.

Introduction

Avocado *(Persea americana*) is one of the oldest flowering plants with oleaginous fruits belonging to the *Lauraceae* family. Unlike many other fruits, avocado contains mostly oil in its mesocarp instead of sugars. Avocado is one of the most nutritious fruits as its fleshy mesocarp contains mostly lipids that are beneficial for human health (Schaffer et al., 2013). Additionally, mesocarp also is comprised of carbohydrates, vitamin precursors, protein and some antioxidants such as vitamin E and carotenoids (Knight, 2002). The lipids in the mesocarp are mostly in the form of triacylglycerol (TAG; 60-70% dry weight (DW)) that is abundant with oleic acid. A detectable amount of linoleic acid (18:2) and palmitic acid (16:1) are also found in the TAG (Ozdemir and Topuz, 2004; Kilaru et al., 2015). The fruit ripening or softening process is considered as a unique feature of avocado because the ripening process does not start during fruit maturation period in the tree, rather it starts several days after picking the fruit from the tree. The oil content of the avocado increases after a few weeks of fruit development and continues to further increase biomass and oil content for a couple of months after fruit maturation (Martinez and Moreno, 1995). However, the underlying mechanisms of oil accumulation in this nonseed tissue are largely uncharacterized.

The major components of plant oils such as TAGs serve as storage energy for the plant cell. Plants use these storage lipids for germination and seedling establishment. As a source of highly reduced carbon molecules, TAG is also used for feed, food, and fuel (Murphy, 2005). In plants, TAG can be produced by several different pathways and all pathways require a series of enzymatic reactions that involve incorporation of an acyl group on to the glycerol backbone (Wu *et al.*, 2012; Chapman and Ohlrogge, 2012). In the acyl-CoA dependent Kennedy pathway, the biosynthesis of TAG starts with the sequential acylation of glycerol-3-phosphate (G3P) that is

catalyzed by the glycerol-3-phosphate acyltransferase to produce lysophosphatidic acid (LPA). The second acylation involves the conversion of LPA to phosphatic acid (PA) by the action of lysophosphatidic acid acyltransferase. Before the final and third acylation, PA undergoes a dephosphorylation reaction to generate *sn-1,2*-diacylglycerol (DAG) by phosphatidic acid phosphatase. The third acylation of *sn-3* position of DAG is catalyzed by diacylglycerol acyltransferase (DGAT) to produce TAG by using acyl-CoA pool as a donor for an acyl group (Bates *et al.*, 2013; Chapman and Ohlrogge, 2012; Ohlrogge and Browse, 1995). Differing from Kennedy pathway, DAG can accept an acyl group transferred from the *sn-2* position of phosphatidylcholine (PC) to yield TAG and lyso-PC, a reaction catalyzed by phospholipid:diacylglycerol acyltransferase (PDAT) (Dahlqvist *et al.*, 2000; Banaś *et al.*, 2000). In addition to these two pathways, one molecule of TAG and monoacylglycerol (MAG) can be formed from two molecules of DAG catalyzed by a DAG/DAG transacylase (Stobart *et al.*, 1997). The final acylation of TAG biosynthesis catalyzed by either DGAT or PDAT is considered as one of the rate-limiting steps in TAG biosynthesis (Li *et al.*, 2010; Li *et al.*, 2013).

Although numerous studies have revealed the importance of PDATs in TAG biosynthesis, the information is very limited (Zhang *et al.*, 2009; Wu *et al.*, 2012; Yuan *et al.*, 2017; Banaś *et al.*, 2013). In the exponential growth phase of yeast (*Saccharomyces cerevisiae*), *LRO1*, an ortholog of *PDAT1* (Banaś et al., 2000) serves as the major enzyme for TAG synthesis (Oelkers *et al.*, 2002). In Arabidopsis, two close homologs of yeast *PDAT* gene were identified (Ståhl *et al.*, 2004). Consequently, PDATs from castor bean *(Ricinus communis)* (RcoPDAT1 and RcoPDAT2; Kim et al., 2011), flax (*Linum usitatissimum*) (LusPDAT1 and LusPDAT2; Pan et al., 2013), *Myrmecia incise* (*MinPDAT*; Liu et al., 2016) and two green microalga *Chlamydomonas reinhardtii* (CrePDAT; Yoon et al., 2012) were also identified and

characterized. Nonetheless, it is not well known to what extent these PDATs play a role in TAG biosynthesis in plants. Increased PDAT activity was found in the microsomes isolated from root and leaf of Arabidopsis when the *PDAT* was overexpressed; however, the fatty acid content and composition was not altered (Ståhl *et al.*, 2004). Additionally, fatty acid content and composition did not change significantly in *pdat1* knockouts of Arabidopsis (Mhaske et al., 2005). Although PDAT1 has not been reported as a major contributor of TAG biosynthesis in seed, it may have a compensatory or overlapping role. To this extent, AthPDAT1 was shown to have an overlapping function with AtDGAT1 in TAG biosynthesis in the development of pollen grain in a single knockout dgat1-/-,; arabidopsis double knockout (dgat1-/- and pdat1-/-) was however lethal suggesting the significance of the redundancy provided by PDAT in the absence of DGATs (Zhang *et al.*, 2009).

In yeast *Saccharomyces cerevisiae*, both DGAT2 encoded by *DGA1* and PDAT encoded by LRO1 are functional and contribute TAG biosynthesis at different time points of growth phases (Kalscheuer *et al.*, 2004; Oelkers *et al.*, 2002). While the ortholog of PDAT is mainly responsible for TAG biosynthesis during cell division (Oelkers *et al.*, 2002), DGA1 is more active in synthesizing storage lipids during stationary growth (Sandager *et al.*, 2002; Oelkers *et al.*, 2002). Although DGA1 is predominant in TAG synthesis, only a 50% reduction in TAG was observed in a dga1-/- knock out. When both *DGA1* and *LRO1* were mutated, only 1% TAG was synthesized (Sandager *et al.*, 2002) suggesting a compensatory role for the PDAT ortholog.

Previously, among the two DGAT enzymes in avocado, DGAT1 was heterologously expressed and was shown to be functional *in vitro*. In this present study, we identified and cloned PDAT1 and transiently expressed in tobacco (*Nicotiana benthamiana)* leaves. To determine which acyltransferase is predominant in nonseed tissues, avocado DGAT1 was also transiently

expressed. Both enzymes were evaluated for their function and activity in *N. benthamiana* leaves.

Methods and Materials

In silico Analyses of PamPDAT1

Using avocado transcriptome database, the complete gene sequence of putative Pam*PDAT1 (Persea americana* PDAT1) was obtained. The *PamPDAT1* DNA sequence was then translated into six possible putative protein sequences using the Expasy protein translation tool (http://web.expasy.org/translate/) and the longest open reading frame was considered for full -length coding DNA sequence (cDNA) of *PamPDAT1*. Putative acyltransferase sequences from avocado were aligned with other eukaryotic acyltransferases, using CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2/), to gain an evolutionary perspective of avocado genes. Transmembrane domains were predicted by TMHMM transmembrane domain prediction software (http://www.cbs.dtu.dk/services/TMHMM/).

Plasmid Construction for PamDGAT1 and PamPDAT1

Previously extracted total RNA was used to synthesize cDNA using Promega superscript reverse transcriptase and oligo dT primer. The forward and reverse primers for PamDGAT1 and PamPDAT1 were designed with *Nco*I and *Sac*II restriction enzymes, respectively to amplify the coding sequence of these genes to clone into pK34 entry vector. The amplified PCR products of PamDGAT1 and PamPDAT1 were digested with restriction enzymes *Nco*I and *Sac*II. Similarly, the entry vector pK34 was digested with the same restriction enzymes and treated with calf alkaline phosphatase (CIP; from NEB) to prevent self-ligation. Each digested product and vector were ligated together using T4 DNA ligase (NEB) and incubated overnight at 12 °C. The recombinant pK34 vector was then chemically transformed to Top10 *E. coli* competent cells for

screening purpose. The positive transformed colonies were screened by colony PCR using one gene-specific and one plasmid-specific primers to check the correct orientation of the gene insertion in the entry vector. Further, the correct gene sequence was confirmed by sequencing. The sequenced confirmed gene inserted into the entry vector is now ready for clone into plant expression vector, pB110. The entry vector pK34 has double CaMV 35S promoter and a terminator sequence (Shockey *et al., 2015*). The entry vector was digested with AscI restriction enzyme to get the gene insertion, promoter sequence and terminator sequence for entry into pB110. The gene cassette and the digested vector with AscI ligated for overnight by T4 DNA ligase from NEB. The resulted plasmid construct was chemically transformed into Top 10 *E. coli* cells for screening. Colony PCR was done to confirm the positive colonies. The plasmid was extracted from the positive colonies for further transformation into *Agrobacterium*.

Agrobacterium Competent Cell Preparation and Transformation

Agrobacterium LBA4404 strain was inoculated in 5 mL of LB media containing 10 µg /mL of rifampicin antibiotic for 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 reaches 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*. 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 PamDGAT1 and PamPDAT1 transformation. Ten µL of 100-1000 ng of plasmid DNA (pB110) containing the gene insert either *PamDGAT1* or *PamPDAT1* was 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. From here, 100 µL of the mixture was transferred in a corning tube containing one mL LB media and incubated for at 28 °C for 2-4 hours with 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 100-200 µL of LB media and spread onto LB agar plates containing appropriate antibiotic (kanamycin). Plates were incubated for 3-6 days at 28 °C for colony formation.

Culture Condition of N. benthamiana and Agrobacterium Infiltration

Agrobacterium tumefaciens LBA4404 strains containing the plant expression vector (pB110) cloned with PamDGAT1, PamPDAT1, or P19 were grown overnight in LB media with kanamycin at 28 °C with shaking (250 rpm). The following morning, the turbid culture was supplemented with 100 mM acetosyringone and preincubated for another 2 hours. Pellets were collected by centrifugation at 400 x g for 5 minutes and resuspended in infiltration buffer containing 5 mM $MgSO_4$, pH 5.7, 100 mM acetosyringone and 5 mM MES and to an O.D. of 2.0. Then it was diluted to an O.D. of 0.3 for *N. benthamiana* infiltration by adding infiltration buffer.

N. benthamiana plants were grown in a 24 °C plant growth room with overhead lighting using 9:15 light: dark cycle. Five/six-day old *N. benthamiana* plants were chosen for infiltration. Generally, three leaves counting from the top (not too young or too old) were selected. Two

leaves were infiltrated entirely with one of the combinations of LBA4404 strain containing PamDGAT1 or PamPDAT1. The last leaf was used for two controls- mock and negative control. To facilitate infiltration, a small nick was created with a needle in the epidermis of the back side the leaf. After infiltration, plants were then transferred into the growth chamber and allowed to grow for 6/7 days to express the protein.

Nile Red Staining and Confocal Microscopy

Leaf disc was collected from the tobacco plants after 6/7 days of infiltration and placed in the 50-mL Falcon tube containing 4% formaldehyde (paraformaldehyde) in 1X PBS (phosphate-buffered saline). The samples were then shaken on a rotational shaker at 75 rpm for one hour. Leaf discs were washed three times with 1X PBS. After that, leaf discs were stained with 4 µg/mL Nile Red (4mg/mL stock in DMSO) in 1X PBS for 15 minutes at room temperature in a rotational shaker at 100 rpm in the dark. Leaves were washed three times: 15 minutes each time with 1X PBS on a rotational shaker at 100 rpm and lastly washed with ddH_2O. Each leaf disc was then mounted in H_2O with coverslips and slides were immediately observed under the Leica TCS SP8 Confocal Fluorescence Microscope**.** The excitation wavelength for Nile Red is 488 nm and the emission wavelength is 560 to 620 nm.

The total number of lipid droplets (LDs) were counted for each experiment by ImageJ software. At least, three panels from each biological replicate in a total of nine panels were taken to quantify the LDs. Statistical analysis was conducted by performing one-way analysis of variance (ANOVA) with Tukey's post-test using the Minitab18 statistical software.

Analysis of plant lipids

N. benthamiana leaves were collected at 6/7 days after infiltration and 200 mg fresh weight (FW) of tobacco leaf was taken for thin-layer chromatography (TLC). Hexane:

isopropanol method (Hara and Radin, 1978) was used to extract total lipids from plant leaves. Briefly, 200 mg of plant tissue (FW) was ground in liquid nitrogen with a motor and pestle. Two milliliters of hot isopropanol were added into the sample and collected the homogenate into a clean glass tube. To inactivate the internal lipase, the sample was incubated at 70 °C for 30 minutes in a water bath. After the sample was cooled to room temperature, one mL of chloroform and 250 µL water were added to make the ratio, 2 mL isopropanol: 1 mL chloroform: 0.45 ml water/tissue, vortexed thoroughly and stored at 4 °C overnight. On the following day, samples were equilibrated to room temperature and phase separation was achieved after vigorous vortex with one mL of chloroform and two mL of 1M KCl followed by centrifugation at 5000 x g for 5 minutes. The upper aqueous phase and the interphase were aspirated by Pasteur pipette and the phase separation steps were repeated twice with two mL of 1M KCl. Finally, the organic phase was transferred to a clean glass tube and dried under nitrogen gas. Dried lipid was dissolved in approximately one mL of chloroform and transferred into a pre-weighed glass vial, dried again and weighed to obtain the total lipids. For further TLC analysis, total lipid was dissolved in 1 mL of chloroform and stored at -20 °C.

Lipid Extraction and Analysis by TLC

For TLC analysis, 100 mL solvent of hexane: diethyl ether: acetic acid (80: 20: 1) was added to the TLC tank, along with a piece of filter paper to saturate the chamber. Samples and standard were spotted on the silica plate, dried under nitrogen and the plate was placed in the TLC tank to allow for separation of lipids. After 40 min, the plate dried and stained with primulin spray (0.005% acetone: water = 4:1); color was developed after drying the plate at 65 °C. The stained TLC plate was imaged, and the separated lipids were identified by associating the bands with standard.

Fatty Acid Analysis by GC-FID

For TAG composition analysis, plant leaves were collected (200 mg fresh weight) from *N. benthamiana* after 5/6 days of *Agrobacterium* infiltration. To the sample, 100 µg of heptadecanoic acid (17:0, Sigma-Aldrich) was added and homogenized. Total lipids were extracted with the same protocol as described earlier. The extracted total lipids were esterified to fatty acid methyl esterification (FAME) by heating the samples with two mL of 1 N HCl methanolic for 2 hours at 85 °C in a water bath. Samples were cooled after the transesterification reaction and FAMEs were extracted in the organic phase after vortexing with one mL KCl and one mL hexane followed by centrifugation for 5 minutes at 5000 x g. The upper organic phase hexane containing FAME was collected into a new test tube and dried under nitrogen gas at 40 °C. The sample was then resuspended in one mL of hexane and evaporated under nitrogen gas at 40 °C and again resuspended in 100 µL of hexane. Following this, one µL of FAME sample was injected into the column for separation and detection by GC-FID (SHIMADZU). Retention time for each fatty acid was determined using Supelco FAME mix standard. The data obtained from GC-FID were analyzed and fatty acids were quantified relative to 17:0 spiked internal standard.

Statistical analysis

Statistical analysis was conducted using Minitab software. Data were expressed as their mean with standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's post-test was used to determine the significant differences.

Results and Discussion

Identification and In silico Analysis of Avocado PDAT1

Full-length putative *PamPDAT1* (*Persea americana* PDAT1) was obtained from avocado transcriptome database (Kilaru *et al.*, 2015) and Ibarra-Laclette et al., (2015). The *in silico*

analyses (Table 3.1) revealed that the putative PamPDAT1 DNA sequence is 2052 bp long and the corresponding protein sequence of the longest open reading frame contains 682 amino acids without a stop codon. This putative protein sequence has a stop codon preceding its start codon suggesting that it is a full-length protein sequence with a complete N-terminal sequence. Furthermore, the start codon, ATG of *PamDGAT1* coding sequence has a probability of 98.8% to be a true start codon when compared with *A. thaliana* genome (Figure S3.3). Multiple sequence alignment of PamPDAT1 with other known PDAT1 protein sequences such as *Arabidopsis thaliana, Ricinus communis,* and *Glycine max* showed similarities among the PDAT1 proteins and also revealed that PDAT1 proteins are ~670 amino acids in length (Figure 3.1A). Avocado PDAT1 retained conserved N-terminal region with one probable transmembrane domain, similar to the PDAT1s from monocotyledons and eudicots including Arabidopsis (Supplemental figure S3.2), and yeast (Yoon *et al.*, 2012; Ghosal *et al.*, 2007).

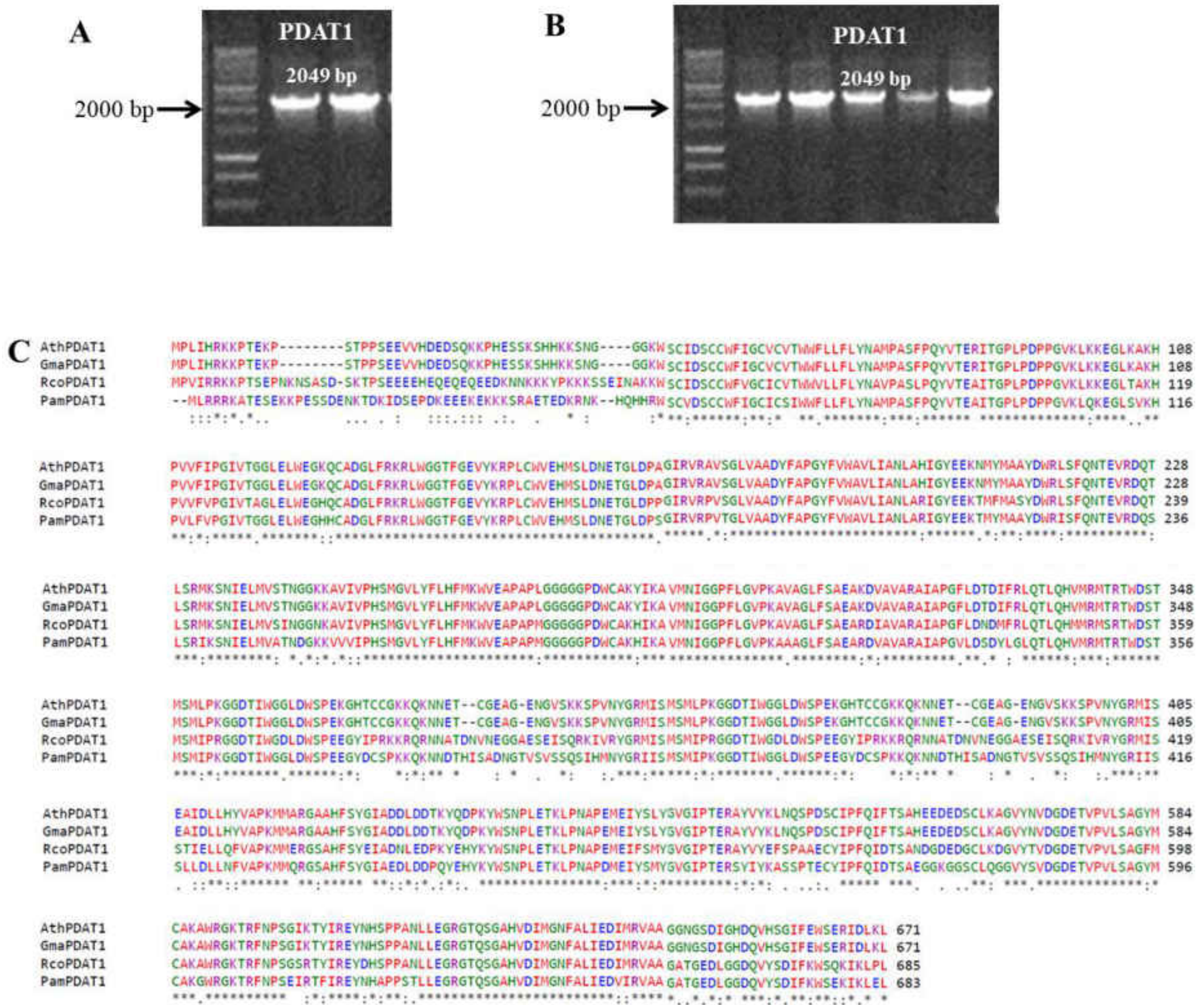

Figure 3.1 Identification and in silico analysis of PamDGAT1. Amplification of PamPDAT1 by PCR (3.1 A). Colony PCR for screening positive clones containing PamPDAT1 (3.1 B). Multiple sequence alignment of different PDAT1 proteins (3.1 C).

Collectively, these data obtained by comparing putative PamPDAT1 with other characterized PDAT1 proteins indicates that PamPDAT1 contains features common to that of PDAT family and suggests it might be functionally conserved and synthesize TAG by transferring the acyl group of *sn-2* position of phosphatidylcholine (PC) to DAG.

Table 3.1 Features of putative PamPDAT1

***In silico* analyses**	***PamPDAT1***
Full length cDNA	2079 bp
Coding sequence length	2052 bp
Probability of start codon	89.80%
Protein length	682 aa
# of transmembrane domains	1

For further characterization, 2052 bp putative *PamPDAT1* was isolated from avocado mesocarp RNA by reverse transcription followed by PCR amplification (Figure 3.1A) and cloned into a plant expression vector, pB110 to express into *N. benthamiana* leaves.

Expression of Avocado DGAT1 and PDAT1 in N. benthamiana Leaves

Avocado DGAT1 and PDAT1 were transiently expressed in *N. benthamiana* leaves individually or with the viral silencing suppressor protein P19 under the control of CaMV 35S promoter (Figure 3.2A) to examine the possible roles of these proteins *in planta*. Particularly, the role of PamDGAT1 and PamPDAT1 in TAG biosynthesis was examined in *Nicotiana benthamiana* leaves by observing LDs under confocal fluorescence microscopy. In this experiment, both mock control and co-infiltration of DGAT1 and PDAT1 along with P19 were examined. The phenotypic observation revealed that PamDGAT1 and PamPDAT1 produce higher LDs compared to wild-type which ultimately provides the evidence for TAG biosynthesis by these enzymes (Figure 3.2B). The number of LDs produced by PamDGAT1 in *Nicotiana*

benthamiana leaves is almost 10-fold and two-fold higher than those of in wild control (P-value < 0.01) and P19 control (P-value < 0.05), respectively. The combinatory effect of PamDGAT1 with P19 protein has a more robust expression where the number of LDs increased by 37-fold and 7-fold compared to wild-type (P-value < 0.001) and P19 control (P-value < 0.001), respectively (Figure 3.2C). A similar trend was observed when PamPDAT1 was transiently expressed in *N. benthamiana* leaves where the number of LDs increased by 10-fold and two-fold compared to wild-type control and P19 control, respectively. Co-infiltration of PamPDAT1 with P19 has an elevation of LDs by 22-fold and four-fold compared to wild-type (P-value <0.001) and P19 control (P-value <0.001), respectively, which were less than those of in PamDGAT1 (Figure 3.2D).

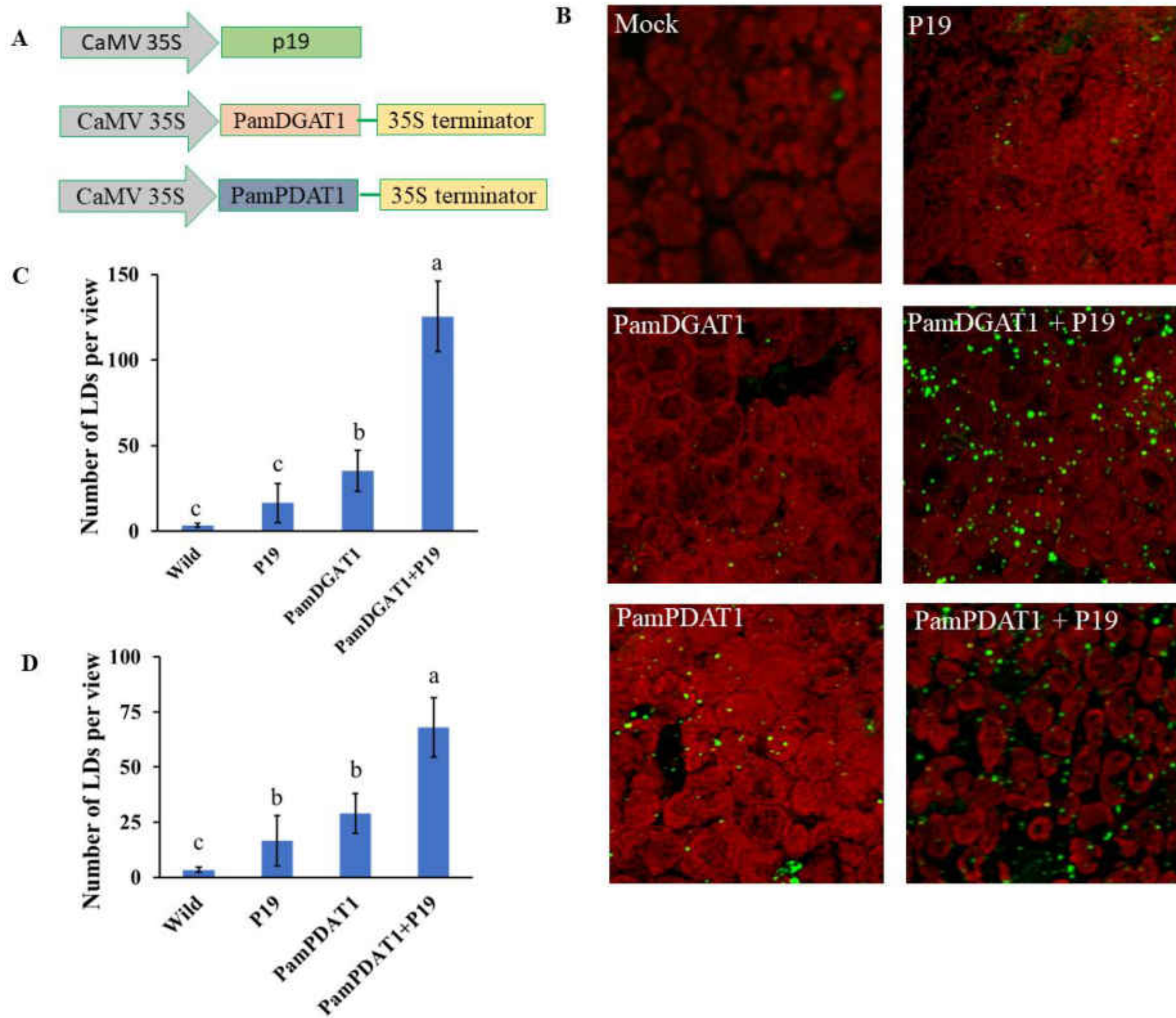

Figure 3.2 Vector constructs, LD visualization, and quantification LDs in N. benthamiana leaves expressing PamDGAT1 and PamPDAT1. Schematic diagram of vector constructs for Agrobacterium transformation of P19, PamDGAT1, and PamPDAT1 in N. benthamiana leaves (3.2A). Phenotypic visualization of LDs in different N. benthamiana leaves expressing P19, PamDGAT1 and PamPDAT1 alone or co-expression of PamDGAT1 and PamPDAT1 with P19. LDs were stained with Nile Red to visualize under confocal microscopy and shown in green color (3.2B). Quantification of LDs in different N. benthamiana leaves expressing PamDGAT1 (3.2C) and PamPDAT1 (3.2D). Data represent mean ±SD of three independent experiments and different letters indicate significant differences ($P < 0.05$), as determined by one-way analysis of variance (ANOVA) with Tukey's post-test.

Fluorescent dyes such as Nile Red, Nile Blue, BODIPY have been used routinely to measure

neutral lipid in both semiquantitative (Elsey *et al.*, 2007; Huang *et al.*, 2009) and quantitative (Sitepu *et al.*, 2012; Govender *et al.*, 2012) fashion. Staining of neutral lipid with Nile Red for quantitative analysis is widely used for the measurement of LDs in algae (Huang *et al.*, 2009), yeast (Sitepu *et al.*, 2012), seed and nonseed tissues of plants (Cai *et al.*, 2015). An elevated number of LDs were also observed when Arabidopsis DGAT1 was expressed in tobacco leaves (Vanhercke *et al.*, 2017; Vanhercke *et al.*, 2014). Taken together, these results suggest overexpression of avocado DGAT1 and PDAT1 increased the total LDs in *N. benthamiana* leaves as an indication of their ability to synthesize TAGs in the vegetative plant tissue. Therefore, it can be concluded that both avocado DGAT1 and PDAT1 are functional and capable of making TAG in nonseed tissues.

Total Lipid Content in N. benthamiana Leaves Expressing Avocado DGAT1 and PDAT1

The ability of DGAT1 and PDAT1 to increase lipid content was tested by transient expression of these proteins in *N. benthamiana* leaves. Total lipid extracted from infiltrated leaves expressing PamDGAT1 (Figure 3.3A) or PamPDAT1 and controls was separated on TLC plate (Figure 3.3C). Expression of PamDGAT1 significantly increased (more than two-fold) total lipid content compared to wild control or P19 control (P-value < 0.01) (Figure 3.3B). On the other hand, total lipid content was increased by nearly two-fold with PamPDAT1 expressed in *N. benthamiana* leaves (Figure 3.3D).

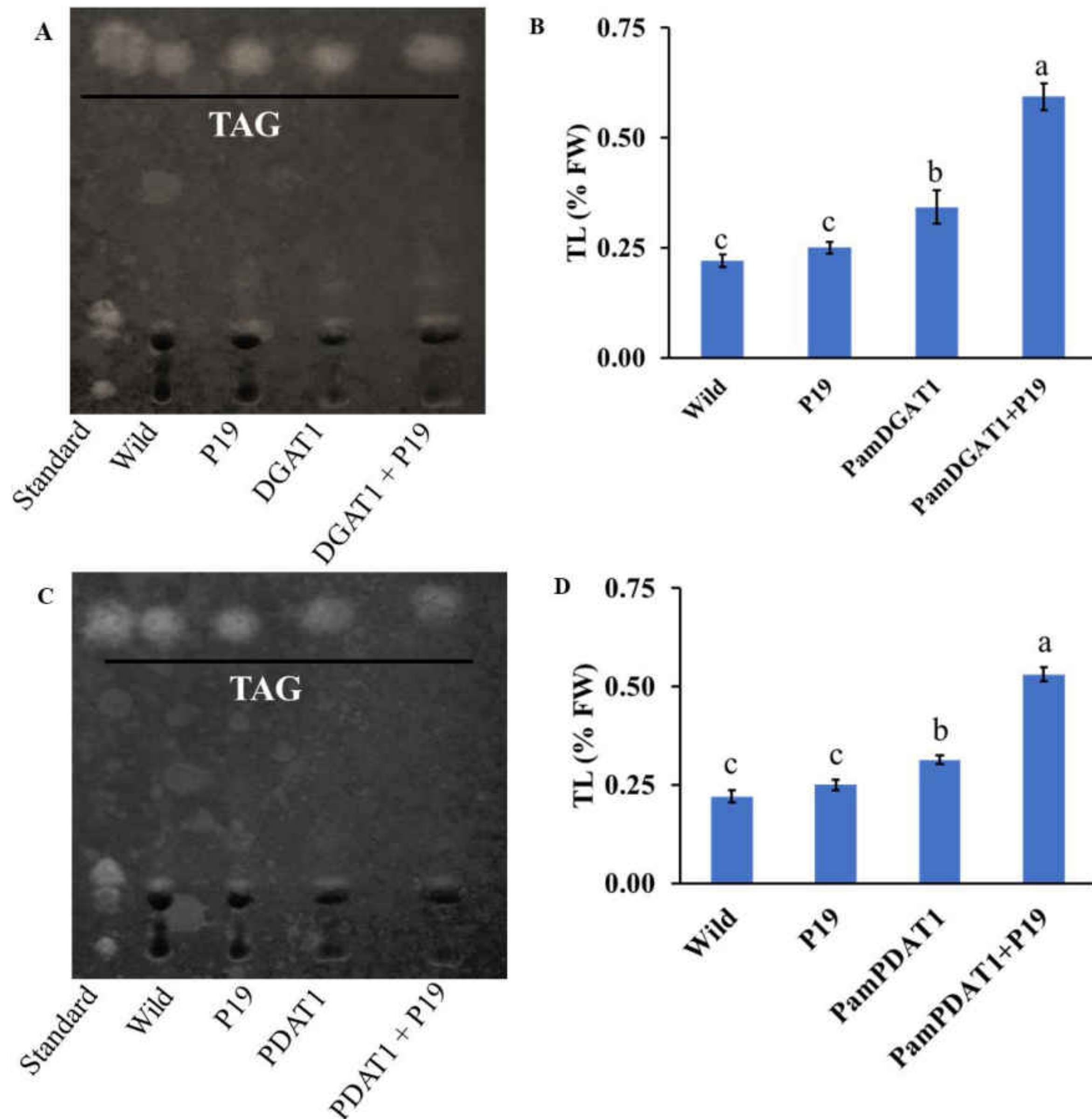

Figure 3.3 Quantification of total lipid in different N. benthamiana leaves expressing PamDGAT1 and PamPDAT1. Separation of total lipid by thin layer chromatography (TLC) from different N. benthamiana leaves expressing PamDGAT1 (3.3A) and PamPDAT1 (3.3C). Quantification of total lipid from N. benthamiana leaves expressing PamDGAT1 (3.3B) and PamPDAT1 (3.3D) respectively. Values represent average and SD of three independent experiments and different letters indicate significant differences ($P < 0.05$), as determined by one-way analysis of variance (ANOVA) with Tukey's post-test.

u35S promoter elevated the lipid content in leaves by five to sevenfold (Bouvier-Nave *et al.*, 2000). Overexpression of Arabidopsis DGAT1 with WRINKLED 1 transcription factor increased TAG content in tobacco leaves up to 15% by dry weight (Reynolds *et al.*, 2015; Vanhercke *et al.*, 2013). These results indicate that transient overexpression of avocado DGAT1 and PDAT1 increased total lipid content in *N. benthamiana* leaves by making more TAGs. Further complementation of Arabidopsis *dgat1* and *pdat1* mutants by avocado DGAT1 and PDAT1, respectively are required to test their role in seed oil biosynthesis.

Fatty Acid Profile of Leaves Expressing Avocado DGAT1 and PDAT1

The fatty acid profile was examined by GC-FID in *N. benthamiana* leaves expressing PamDGAT1 and PamPDAT1 in order to determine their role in regulating TAG composition in plant vegetative tissue. It was found that the content of major fatty acids including palmitic acid (16:1), palmitoleic acid (16:1) and linolenic acid (18:3) did not change significantly by PamDGAT1 (Figure 3.4 A) or PamPDAT1 expression (Figure 3.4B) compared to the wild-type or P19 control. However, PamDGAT1 significantly increase the amount of oleic acid (18:1) than wild-type (P-value < 0.05) when expressed in *N. benthamiana* leaves. Even the influence on the content of oleic acid (18:1) was greater when the PamDGAT1 was co-infiltrated with the viral silencing suppressor protein P19 compared to wild-type (P-value < 0.001) or *N. benthamiana* leaves expressing P19 (P-value < 0.05) (Figure 3.4A). Similarly, in *N. benthamiana*, PamPDAT1 significantly increased the amount of oleic acid (18:1) compared to the wild-type (P-value < 0.05) or P19 (P-value < 0.05) and a similar effect on oleic acid (18:1) was observed when PamPDAT1 was co-infiltrated with P19 (Figure 3.4B). Another major fatty acid- linoleic acid (18:2) did not change significantly either with PamDGAT1 (Figure 3.4A) or PamPDAT1 (Figure

3.4B). But there is a tendency to increase the content of linoleic acid (18:2) compared to wild-type.

Table 3.2 Analysis of fatty acid of total lipid in *N. benthamiana* leaves expressing PamDGAT1 and PamPDAT1

	Wild	P19	PamDGAT1	PamDGAT1+P19	PamPDAT1	PamPDAT1+P19
C16:0	18.24 ± 1.10	15.61 ± 0.59	17.80 ± 1.27	16.77 ± 1.01	18.39 ± 1.81	17.04 ± 2.17
C16:1	3.74 ± 0.32	3.05 ± 0.05	2.74 ± 0.43	2.69 ± 0.39	2.37 ± 0.37	2.11 ± 1.58
C18:1	0.72 ± 1.10	1.40 ± 0.15	3.24 ± 0.89	3.71 ± 0.77	3.68 ± 0.39	3.16 ± 0.74
C18:2	3.38 ± 5.23	7.82 ± 0.68	9.45 ± 0.29	7.29 ± 0.49	8.15 ± 0.99	7.82 ± 1.13
C18:3	68.78± 1.14	69.32 ± 0.27	65.47 ± 0.65	68.44 ± 1.07	67.14 ± 1.65	67.91 ± 1.63

Previously, we characterized avocado DGAT1 by complementing mutant H1246 strain and *in vitro* enzyme activity assays showed that avocado DGAT1 has a substrate specificity towards oleic acid (18:1) (unpublished work). Results from this experiment also showed the consistency with our previous results. Generally, the fatty acid composition in different plant species (Hitchcock and Nichols, 1971) including tobacco (Koiwai *et al.*, 1983) is fairly constant. Nevertheless, the fatty acid profile changing in this experiment is not surprising and is supported by some other studies (Andrianov *et al.*, 2010; Winichayakul *et al.*, 2013; Vanhercke *et al.*, 2014), where they found that fatty acid profile has been changed with upregulation of lipid biosynthesis. Arabidopsis DGAT1 alters the fatty acid profile, particularly oleic acid (18:1) in the vegetative tissue when expressing in tobacco leaves (Vanhercke *et al.*, 2014).

Transient expression of PamPDAT1 also significantly increased oleic acid (18:1) content, suggesting a substrate preference for 18:1; further validation *by in vitro* enzyme activity assays is needed. Although Arabidopsis PDAT retains a broader substrate specificity for acyl chains varying in length, it has a strong preference for fatty acid containing hydroxy or epoxy group or multiple double bonds (Ståhl *et al.*, 2004). Substrate preference of PDAT also reported in camelina when a specific member of camelina PDATs namely PDAT1-A was transiently

expressed in tobacco leaves. They found a significant increase in α-linoleic acid (18:3) compared to wild control (Yuan *et al.*, 2017). Another study in castor bean showed that RcoPDAT1A showed a specificity toward ricinoleic acid but the other member of PDATs did not show any specificity (van Erp *et al.*, 2011). In addition to this, flax PDAT1 has a strong preference for α-linoleic acid (18:3) but PDAT2 has a preference polyunsaturated fatty acids other than α-linoleic acid (18:3) (Pan *et al.*, 2013). Different substrate preference for different PDATs once again described the divergence functions of PDAT paralogs in plants.

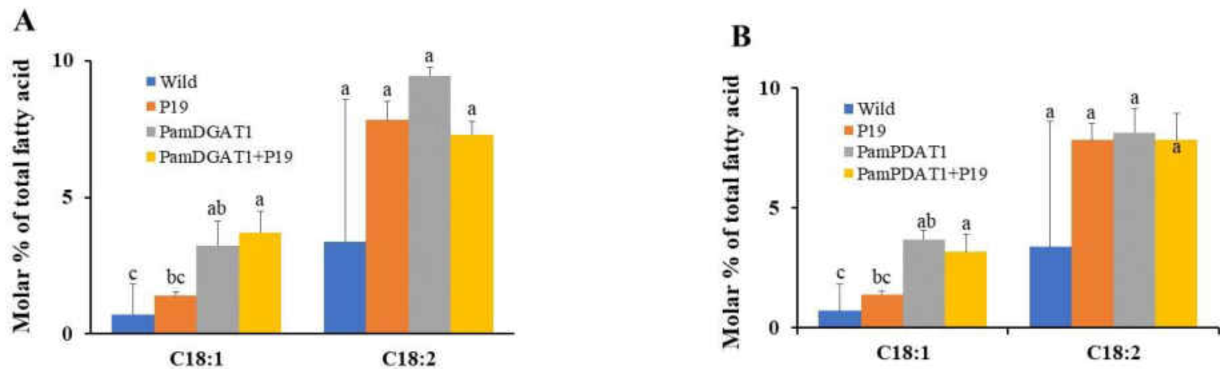

Figure 3.4 Analysis of fatty acid of total lipid in N. benthamiana leaves expressing PamDGAT1 and PamPDAT1. Fatty acids were analyzed and quantified by GC-FID in N. benthamiana leaves expressing PamDGAT1 (3.4A) and PamPDAT1 (3.4B), respectively. Values represent average and SD of three independent experiments and different letters indicate significant differences (P < 0.05), as determined by one-way analysis of variance (ANOVA) with Tukey's post-test.

Together, the data presented in Figure 3.4 suggested that avocado DGAT1 and PDAT1 have elevated oleic acid (18:1), the predominant fatty acid found in avocado mesocarp when expressed in *Nicotiana benthamiana* leaves.

Conclusions

Our study provides the general properties of avocado PDAT1 by *in silico* analyses covering the predicted full DNA sequence, prediction of transmembrane domains and

conserveness of the protein sequence with other PDAT proteins. Transient expression of avocado DGAT1 and PDAT1 in *Nicotiana benthamiana* leaves revealed the role of these enzymes in TAG biosynthesis. Moreover, our data suggested that both avocado DGAT1 and PDAT1 has a substrate preference for oleic acid (18:1) when transiently expressed in *Nicotiana benthamiana* leaves. This finding provides new insight into the underlying mechanism of lipid biosynthesis in avocado mesocarp. The results obtained from this study will enrich our knowledge towards an understanding of lipid biosynthesis in nonseed tissue and can be translated into other plant species or less complex organisms to produce oil enriched in oleic acid (18:1). Future studies will be conducted to complement the Arabidopsis mutant to characterize their function in seed tissues.

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Appendix

Supplemental tables and figures

Putative PDAT1 protein sequence

TYRSLADYQKDSSRRRSSTQTPR Stop MTTRRVSCHPQSFSSCNRKNLIPLSFWKTEKLGLGFP Stop NPNHHFLLLLPSDA
AVQALVITAAAEHLQISDFHFPH Stop SSVRVSKASSSSSSSTPFRCSLEGSEYCSSRAFKDLCFPFFLIDHRKSVRKMLRR
RKATESEKKPESSDENKTDKIDSEPDKEEEKEKKKSRAETEDKRNKHQHHRWSCVDSCCWFIGCICSIWWFLLFLYN
AMPASFPQYVTEAITGPLPDPPGVKLQKEGLSVKHPVLFVPGIVTGGLELWEGHHCADGLFRKRLWGGTFGEVYKRPL
CWVEHMSLDNETGLDPSGIRVRPVTGLVAADYFAPGYFVWAVLIANLARIGYEEKTMYMAAYDWRISFQNTEVRDQS
LSRIKSNIELMVATNDGKKVVVIPHSMGVLYFLHFMKWVEAPAPMGGGGGPDWCAKHIKAVMNIGGPFLGVPKAAA
GLFSAEARDVAVARAIAPGVLDSDYLGLQTLQHVMRMTRTWDSTMSMIPKGGDTIWGGLDWSPEEGYDCSPKKQKN
NDTHISADNGTVSVSSQSIHMNYGRIISFGKDVAEAHSSQIEQIEFRGAVKGNKLAKSNVSCDIWTEYHEMGLGGIKAV
ADYKVYTAGSLLDLLNFVAPKMMQRGSAHFSYGIAEDLDDPQYEHYKYWSNPLETKLPNAPDMEIYSMYGVGIPTERS
YIYKASSPTECYIPFQIDTSAEGGKGGSCLQGGVYSVDGDETVPVLSAGYMCAKGWRGKTRFNPSEIRTFIREYNHAPP
STLLEGRGTQSGAHVDIMGNFALIEDVIRVAAGATGEDLGGDQVYSDIFKWSEKIKLEL Stop FQPVLFETYLFNGLLGG
WMYMM Stop CVTFSPSPYFIFIFIF Stop MVSFNLLSFCTFTEEMSLKIL

Putative CDS length=2052

ATGTTGAGACGGAGAAAAGCGACTGAATCGGAGAAGAAGCCCGAGTCCTCAGATGAGAATAAAACGGATAAGATCGATAGCGAACCAGACAAGGAGGAG
GAGAAAGAGAAGAAGAAGAGCAGAGCAGAAACGGAGGACAAGCGTAATAAGCACCAACATCACAGATGGTCGTGCGTGGACAGCTGTTGCTGGTTCATT
GGCTGCATATGTTCGATTTGGTGGTTTCTGCTGTTTCTCTATAACGCCATGCCGGCGTCCTTCCCGCAGTATGTGACGGAGGCGATCACGGGCCCGTTA
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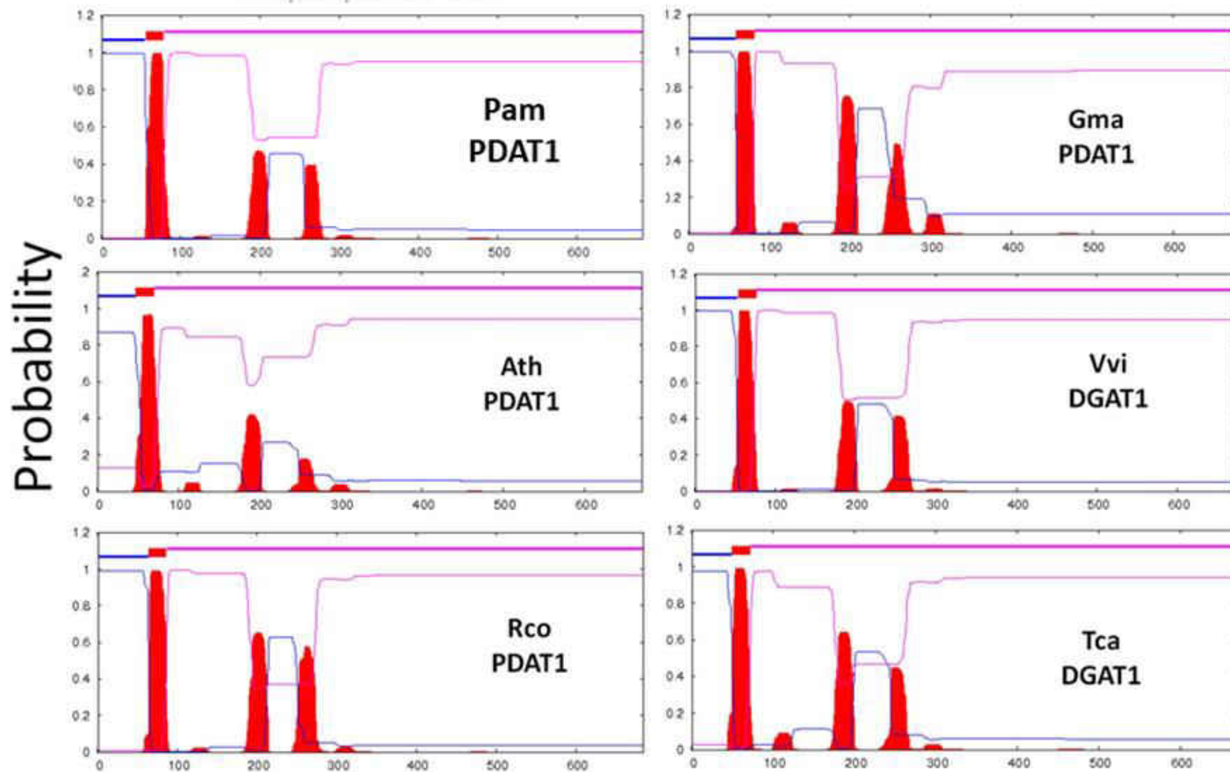
GAGCAAATCGAATTTAGGGGTGCTGTTAAGGGCAATAAGCTTGCTAAATCAAATGTGTCTTGCGATATATGGACGGAGTACCATGAGATGGGTTTGGGA
GGCATCAAAGCTGTTGCTGACTACAAAGTCTACACCGCCGGATCCCTGTTGGATCTGCTCAATTTTGTTGCTCCAAAAATGATGCAGCGTGGAAGTGCT
CACTTTTCATATGGTATTGCAGAGGATTTGGATGACCCACAGTATGAGCACTACAAATATTGGTCAAACCCCTTAGAAACGAAATTACCAAATGCTCCC
GATATGGAGATCTATTCTATGTATGGGGTTGGAATACCTACTGAAAGATCATATATTTACAAGGCATCATCCCCGACTGAATGCTACATTCCCTTTCAG
ATTGATACCTCAGCTGAGGGCGGAAAGGGTGGCAGCTGTTTACAAGGTGGAGTCTATTCAGTTGATGGGGATGAGACCGTGCCTGTTTTAAGTGCAGGC
TACATGTGTGCCAAAGGTTGGCGCGGGAAAACCAGATTTAATCCTTCAGAAATTCGGACTTTCATCCGGGAGTATAATCATGCCCCGCCATCAACTCTT
TTGGAGGGACGAGGCACCCAGAGTGGCGCTCATGTTGATATAATGGGGAACTTTGCATTGATTGAAGATGTTATAAGAGTGGCAGCAGGAGCTACAGGT
GAAGACTTGGGCGGCGATCAAGTTTATTCAGATATCTTTAAATGGTCCGAGAAAATCAAACTAGAACTCTAG

Figure 3.5 Full-length coding DNA sequences of putative PamPDAT1

TMHMM Posterior Probabilities for sequences



Predicted transmembrane domain for plants *P. americana* (Pam), *A. thaliana* (Ath), *R. communis* (Rco), *G. max* (Gma), *V. vinifera* (Vvi) and *T. castaneum* (Tca) PDAT1 protein sequences

Figure 3.6 Prediction of transmembrane domains by TMHMM software

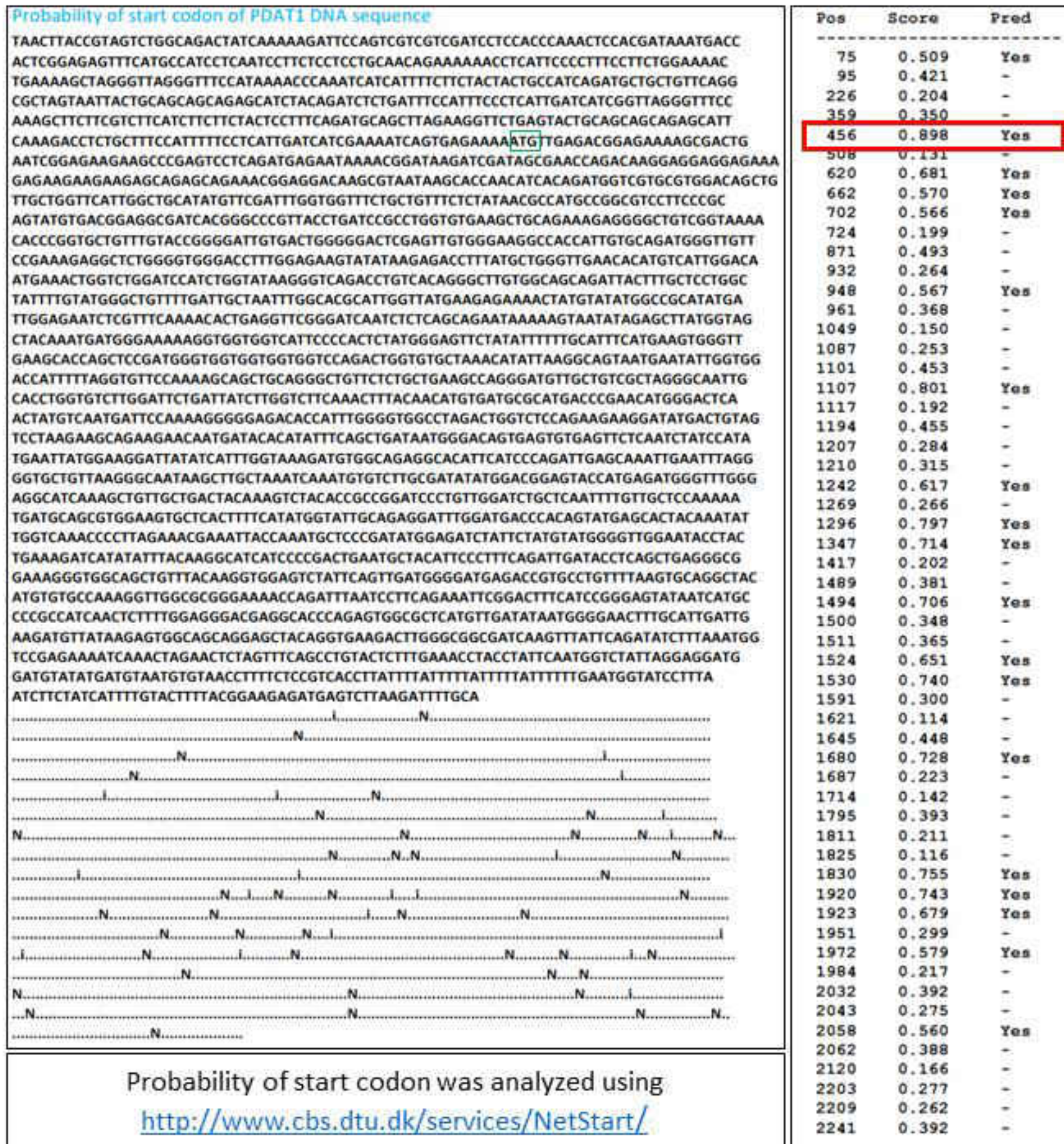


Figure 3.7 Probability of start codon PamPDAT1 predicted by NetStart software by comparing gene sequences with Arabidopsis genome.

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CHAPTER 4

TRANSIENT EXPRESSION OF AVOCADO WRINKLED ORTHOLOGS IN *NICOTIANA BENTHAMIANA* LEAVES

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Abstract

Fatty acid synthesis provides acyl chains necessary for triacylglycerol (TAG) assembly and thus contributes to content and composition of storage lipids in plants. A tight regulation of fatty acid biosynthesis is maintained to meet the demand for TAG accumulation in diverse oil-rich tissues. Transcription factor WRINKLED1 (WRI1) was established a necessary regulator of fatty acid biosynthesis in oilseeds. In this study, we describe the characterization of putative avocado WRI1 and its paralogs. Avocado homologs of AtWRI1 and AtWRI2, which were highly expressed in mesocarp tissue during oil accumulation, were identified, cloned and expressed in a binary vector. Using *Agrobacterium*-mediated transformation, both PamWRI1 and 2 were transiently expressed in *Nicotiana benthamiana* leaves. Both putative candidates from avocado increased the total lipid content and number of lipid droplets in leaves. Data obtained from this study indicate that both PamWRI1 and PamWRI2 are functional and capable of modulation of TAG content in plants, most likely through transcriptional regulation of fatty acid synthesis.

Introduction

Fatty acyl lipids are essential components of all cells and perform diverse functions. First, they are an indispensable component of eukaryotic cell membrane and are utilized to repair the cells. Second, acyl chains are components of the major storage neutral lipids such as triacylglycerols (TAGs) found mainly in seed tissues and in some nonseed tissues of plants; TAGs are the main carbon and energy source used for seed germination (Baud and Lepiniec, 2010). Third, a minor portion of plant lipids and metabolites are used to synthesize plant hormones such as jasmonate for plant signaling pathway (Wasternack, 2007). Lastly, cutin and wax are examples of cuticular lipids that cover the outer surface of plant epidermis and provide a hydrophobic surface which in turn protects the plant from pathogens and prevent water loss (Kunst and Samuels, 2009). Although the properties and structures of acyl lipids are distinct, they are synthesized in the same glycerolipid pathway from the same kind of fatty acid (Harwood, 1996). Generally, in the plants, plastid is the main site for fatty acid biosynthesis. Briefly, acetyl-CoA, which is generated from pyruvate by the action of pyruvate dehydrogenase serves as a carbon source for fatty acid biosynthesis in plastids. Synthesis of fatty acids begins with conversion of acetyl-CoA to malonyl-CoA by the enzyme acetyl-CoA carboxylase. The acyl carrier protein (ACP) is then attached with malonyl group of malonyl-CoA catalyzed by a malonyl-CoA: acyl carrier protein malonyltransferase. Finally, acyl chain is produced by the fatty acid synthase enzyme complex.

In Arabidopsis seeds, several master regulators including ABSCISIC ACID INSENSITIVE3 (ABI3), FUSCA3 (FUS3), LEAFY COTYLEDON1 (LEC1), and LEC2 form the AFL (ABI3/FUS3/LEC2) network that is necessary for oil biosynthesis (North *et al.*, 2010; Roscoe *et al.*, 2015; Devic and Roscoe, 2016). These transcription factors directly or indirectly

regulate the expression of genes that encode for storage lipids and proteins. An unrelated transcription factor from AP2 family, WRINKLED1 (WRI1), was shown to be crucial for lipid accumulation and function downstream to AFL network (Roscoe *et al.*, 2015). However, ABI3 is not known to have a direct regulatory effect on the expression of WRI1 (Devic and Roscoe, 2016). Ectopic expression of FUS3 has a regulatory role on WRI1 expression (Yamamoto *et al.*, 2010); in seeds, LEC2 also has regulation over fatty acid biosynthesis genes through direct regulation of WRI1(Baud *et al.*, 2007). In addition to regulatory role of AFL network in seed oil biosynthesis, they synergistically regulate seed maturation (Devic and Roscoe, 2016).

Transcription factor WRI1 is an AP2 (APETALA2) /EREBP (ethylene-responsive element binding protein) member and was first identified in an Arabidopsis mutant that displayed wrinkled seed phenotype with low seed oil content (Focks and Benning, 1998). Subsequently, WRI1 was shown to act as a regulator for several glycolytic enzymes including phosphoglycerate mutase, plastidial pyruvate kinase β subunit 1 (PI-PKβ1), and pyruvate dehydrogenase (PDHE1α) enzymes. Expression of WRI1 can increase the amount of pyruvate during acetyl-CoA synthesis (Baud *et al.*, 2007; Maeo *et al.*, 2009; Fukuda *et al.*, 2013). Furthermore, WRI1 directly regulates TAG accumulation through direct binding to the promoter region of key enzymes in fatty acid biosynthesis, such as biotin carboxyl carrier protein isoform2 (BCCP2), acyl carrier protein1(ACP1), and keto-ACP synthase1(KAS1) during seed maturation (Baud *et al.*, 2009; Maeo *et al.*, 2009). It is proposed that WRI1 recognized the AW-box containing a conserved nucleotide sequence [CnTn(n)7CG] of the proximal upstream regions of the several target genes (Maeo *et al.*, 2009). Several recent studies performed in plant species including in maize suggest that both these cis- and trans-regulatory elements might be conserved in nature in monocot and dicots (Shen *et al.*, 2010).

Mutation in Arabidopsis WRI1 reduced TAG content by ~ 80% due to the absence of transcriptional activator of fatty acid biosynthesis and the shape of the seed became wrinkled and increased sucrose levels compared to the wildtype seeds implying that WRI1 regulates carbon allocation between sucrose and fatty acids in developing seeds (Focks and Benning, 1998; Li *et al.*, 2015). Nevertheless, *wri1* mutant line possesses a basal level of transcriptional activity of fatty acid and late glycolytic biosynthetic gene to maintain the normal vegetative growth of the plant (Baud *et al.*, 2009). The findings along with others indicate that the transcriptional control of lipid biosynthesis is probably mediated by multiple regulatory proteins. In this context, the functional homolog of WRI1 or even transcriptional factors of redundant regulatory pathways might participate to control the production of fatty acyl chains. Discovery of AthWRI1 allowed for identification of a number of orthologs in seed tissues such as soybean, rapeseed , camelina, corn , and oil palm (Liu *et al.*, 2010; Pouvreau *et al.*, 2011; Ma *et al.*, 2013; Wu *et al.*, 2014; An *et al.*, 2017). On the other hand, seed-specific expression of Arabidopsis WRI1 increased TAG content by ~14–30% (DW) compared to the wild-type seeds of *Arabidopsis*, rapeseed, corn, and Camelina (Cernac and Benning, 2004; Liu *et al.*, 2010; Pouvreau *et al.*, 2011; Wu *et al.*, 2014; Shen *et al.*, 2010; An and Suh, 2015).

Additionally, like in seed tissues, WRI1 homologs have been found in nonseed oil containing tissues such as the stem of poplar (*Populus trichocarpa*), tuber parenchyma of nutsedge (*Cyperus esculentus*), leaf blades of *Brachypodium distachyon,* and mesocarp of Oil palm (*Elaeis guineensis*) and Avocado (*Persea americana*) (Bourgis *et al.*, 2011; Dussert *et al.*, 2013; Kilaru *et al.*, 2015; Grimberg *et al.*, 2015; Y., Yang *et al.*, 2015). Ectopic expression of WRI1 led to increase oil production in the nonseed tissues (Vanhercke *et al.*, 2013; Nookaraju *et al.*, 2014; Zale *et al.*, 2016), for example, expression of AthWRI1 in *Nicotiana benthamiana*

leaves increased TAG levels from 0.05% to 2.20% compared to wild-type (Grimberg *et al.*, 2015; J., Yang *et al.*, 2015). In another study, TAG content is increased by 1.1% on a DW in the tuber of transgenic potato overexpressing AthWRI1 (Hofvander *et al.*, 2016).

WRI paralogs WRI2, WRI3 and WRI4 other than WRI1 were identified in Arabidopsis and functional characterization reveals that AthWRI3 and AthWRI4 are belong to the AP2-EREBP transcription factor family. Although AthWRI3 and AthWRI4 complemented *wri1-4* mutant, did not show any transcriptional activity for fatty acid biosynthesis in the seeds of Arabidopsis rather they stimulate acyl chain production in the developing flower for the cutin biosynthesis to prevent organ adherence. Arabidopsis WRI2 neither complemented *wri1-4* mutant nor showed any transcriptional activity (To *et al.*, 2012). So far, only WRI1 showed transcriptional activity for seed fatty acid biosynthesis in TAG-storing seed and nonseed tissues (Ma *et al.*, 2013; To *et al.*, 2012; Chen *et al.*, 2018).

Previous transcriptome study revealed orthologs of Arabidopsis WRI1, WRI2, WRI3 and WRI4 in avocado mesocarp. Of the four orthologs, putative PamWRI1 and 2 were highly expressed, during the period of TAG accumulation in the mesocarp tissue (Kilaru *et al.,* 2015). In this study, complete coding sequences of these candidate WRI1 and 2 genes have been isolated, amplified and cloned into a plant expression vector. Here, the function of putative avocado WRI1 and WRI2 was characterized by transient expression in *N. benthamiana* leaves.

Methods and Materials

Plasmid Construction

From avocado mesocarp, total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instruction. Following that, cDNA was synthesized using superscript reverse transcriptase (Promega) from extracted RNA using oligo dT primer. For

amplification of the coding sequence of putative *PamWRI1* and *PamWRI2* and to clone into the entry vector, forward and reverse primers were designed with restriction enzymes *Nco*I and *Sac*II, respectively. Both the amplified PCR product and entry vector pK34 were digested with restriction enzymes *Nco*I and *Sac*II and to prevent self-ligation, the entry vector was treated with calf alkaline phosphatase (CIP; from NEB). Using T4 DNA ligase (NEB), ligation of the PCR product and vector was performed and was incubated overnight at 12 °C. The recombinant pK34 vector containing either *PamWRI1* or *PamWRI2* was chemically transformed to Top10 *E. coli* competent cells and positive transformed colonies were screened by colony PCR. Primers for colony PCR included both gene-specific and plasmid specific primers to verify the correct orientation of the gene insertion into the vector. The gene insertion was fully confirmed by sequencing.

Agrobacterium Transformation

For plant transformation, competent cells of *Agrobacterium* LBA4404 strain were used. Here, 100-1000 ng DNA was added to the 100 µL of competent cells and mixed gently. Following that, the mixture was kept in ice for 5 minutes and immediately transferred into liquid nitrogen. From liquid nitrogen, mixture was incubated for 5 minutes at 37 °C in a water bath. Then 100 µL of cells were transferred into one mL LB media and incubated for 2-4 hours at 28 °C. After the incubation period, the pellet was collected by spinning at 4500 x g for 2 minutes and resuspended in 100-200 µL of LB media. Subsequently, cells were spread on the LB agar plates containing an appropriate antibiotic (Kanamycin) and incubated for 3-6 days at 28 °C for colony formation.

Infiltration of Agrobacterium in N. benthamiana Leaves

Agrobacterium tumefaciens LBA4404 strains containing the plant expression vector (pB110) cloned with PamWRI1 and PamWRI2 or control P19 were grown in LB media with an appropriate antibiotic (Kanamycin) at 28 °C overnight with shaking at 250 rpm. The culture was supplemented with 100 mM acetosyringone and allowed to grow for additional two hours. After incubation, solution was centrifuged at 400 x *g* for 5 minutes to collect the pellet and it was resuspended in an infiltration buffer (5 mM $MgSO_4$, pH 5.7, 100 mM acetosyringone and 5 mM MES). Finally, *Agrobacterium* constructs with an O.D. 600 nm of 0.3 was prepared for infiltration.

N. benthamiana plants were grown in a growth chamber with an overhead lighting of 9:15 light: dark cycle and housed at 24 °C For infiltration, 5/6 days old *N. benthamiana* plants were chosen and three leaves (not too young or too old) were selected. The first two leaves were infiltrated with one of the combinations of LBA4404 strain in infiltration buffer and the last leaf was used for infiltrating both mock and negative control. After infiltration, plants were transferred into the growth chamber and allowed to grow for 6/7 days to express the recombinant protein.

Nile Red Staining

After 6/7 days of *Agrobacterium* infiltration, *N. benthamiana* plant leaf disc was collected and placed in paraformaldehyde in 1X PBS (phosphate-buffered saline). The sample was gently shaken in a rotational shaker (75 rpm) for one hour. After incubation, 1X PBS was used to wash the leaf discs three times. Leaf discs were stained with Nile Red (4 µg/ml in DMSO) in 1X PBS and incubated at room temperature in a rotational shaker (100 rpm) for 15 minutes in a dark place. Then the leaf discs were washed twice with 1X PBS on a rotational shaker and lastly a single wash with ddH_2O. Leaf disc was then mounted in H_2O and

immediately observed under the Leica TCS SP8 Confocal Fluorescence Microscope**.** The excitation and emission wavelength for Nile Red staining were 488 nm and 560-620 nm, respectively.

In this experiment, the total number of lipid droplets (LDs) were counted using ImageJ software. Overall, three panels from each biological replicate: a total of nine panels were taken to quantify the LDs.

Analysis of Plant Lipids

To analyze the plant lipids by thin-layer chromatography (TLC), *N. benthamiana* leaves were collected at 6/7 days after *Agrobacterium* infiltration. Total lipid was extracted from leaves using hexane: isopropanol method (Hara and Radin, 1978). Briefly, 200 mg of plant tissue (FW) was ground in liquid nitrogen and 2ml hot isopropanol was added into the sample for homogenization. The sample was heated for 70 °C for 30 minutes in a water bath to inactivate any internal lipase. After cooling down to room temperature, one mL of chloroform and 250 µL water were added to make the ratio, 2 mL isopropanol: 1 mL chloroform: 0.45 ml water/tissue. The sample was kept overnight at 4 °C. The next day, the supernatant was collected from the sample by vortexing and centrifuged for 5 minutes at 5000 x g. After that, one mL of chloroform and two mL of 1M KCl were added into the sample for phase separation. With 5 minutes centrifugation, upper aqueous phase and the interphase were aspirated by Pasteur pipette. The sample was then washed twice with 2 mL of 1M KCl and the organic phase was collected and dried under nitrogen gas. Dried lipid was reconstituted in ~1mL of chloroform and transferred into a pre-weighed glass vial. After evaporating the chloroform in nitrogen gas, glass vial was weighted again and subtracting the weight, the weight of the total lipid was obtained. Finally, total lipid was dissolved in chloroform and kept at -20 °C for further analysis.

Fatty Acid Analysis by GC-FID

After 6 days of *Agrobacterium* infiltration, *N. benthamiana* plant leaves were collected (200 mg fresh weight) for TAG composition analysis. Before homogenization, an appropriate amount of heptadecanoic acid, 17:0 standard (100 µg) (Sigma-Aldrich) was added to the plant tissue. Using the same protocol mentioned earlier, total lipid was extracted from leaf tissues. Then, extracted lipids dissolved in chloroform were dried under nitrogen gas at 40 °C. To perform fatty acyl methyl esterification (FAME), HCl methanolic acid (1 N) was added into the samples and to complete the transesterification reaction, sample was heated for 2 hours at 85 °C in a water bath. After cooling each sample to room temperature, 1 mL KCl and 1 mL hexane were added to separate the sample into two solvent phases-the organic and aqueous phase. Hexane containing organic phase containing FAME was collected and dried under nitrogen gas at 40 °C. In the sample, hexane was and evaporated subsequently under nitrogen gas at 40 °C and again resuspended in 100 µL of hexane. For GC-FID, 1 µL of FAME sample was injected (SHIMADZU). Using Supelco FAME mix standard, retention time for each fatty was detected. In this assay, fatty acids were quantified relative to 17:0 spiked internal standards.

Statistical Analysis

In this study, statistical analysis was conducted using Minitab software (version 18). Experiments were performed as triplicate and data were expressed as their mean value. Error bar was calculated using standard deviation (SD). To test whether there is any significant difference amongst the dataset, One-way analysis of variance (ANOVA) followed by Tukey's post-test were performed.

Results and Discussion

Ectopic Expression of Pamwri1 and 2 Increases Lipid Droplet Accumulation in N. benthamiana Leaves

In this experiment, we tested the potential of putative avocado WRI1 and 2 to increase the lipid content in vegetative tissues. The avocado *WRI1* and *2* were cloned in a plant expression vector under the control of CaMV 35S promoter (Figure 4.1 A). Agrobacteria containing the vector constructs cloned with either *WRI1* or *WRI2* gene were infiltrated into *N. benthamiana* leaves. Leaves were collected after 6/7 days of infiltration. Subsequent to Nile red staining, LDs were observed (Figure 4.1 B) and quantified. The occurrence of LDs was significantly higher in leaves expressing PamWRI1 (Figure 4.1 C) and PamWRI2 (Figure 4.1 D) compared to control leaves transformed with P19 viral silencing suppressor protein or wild control.

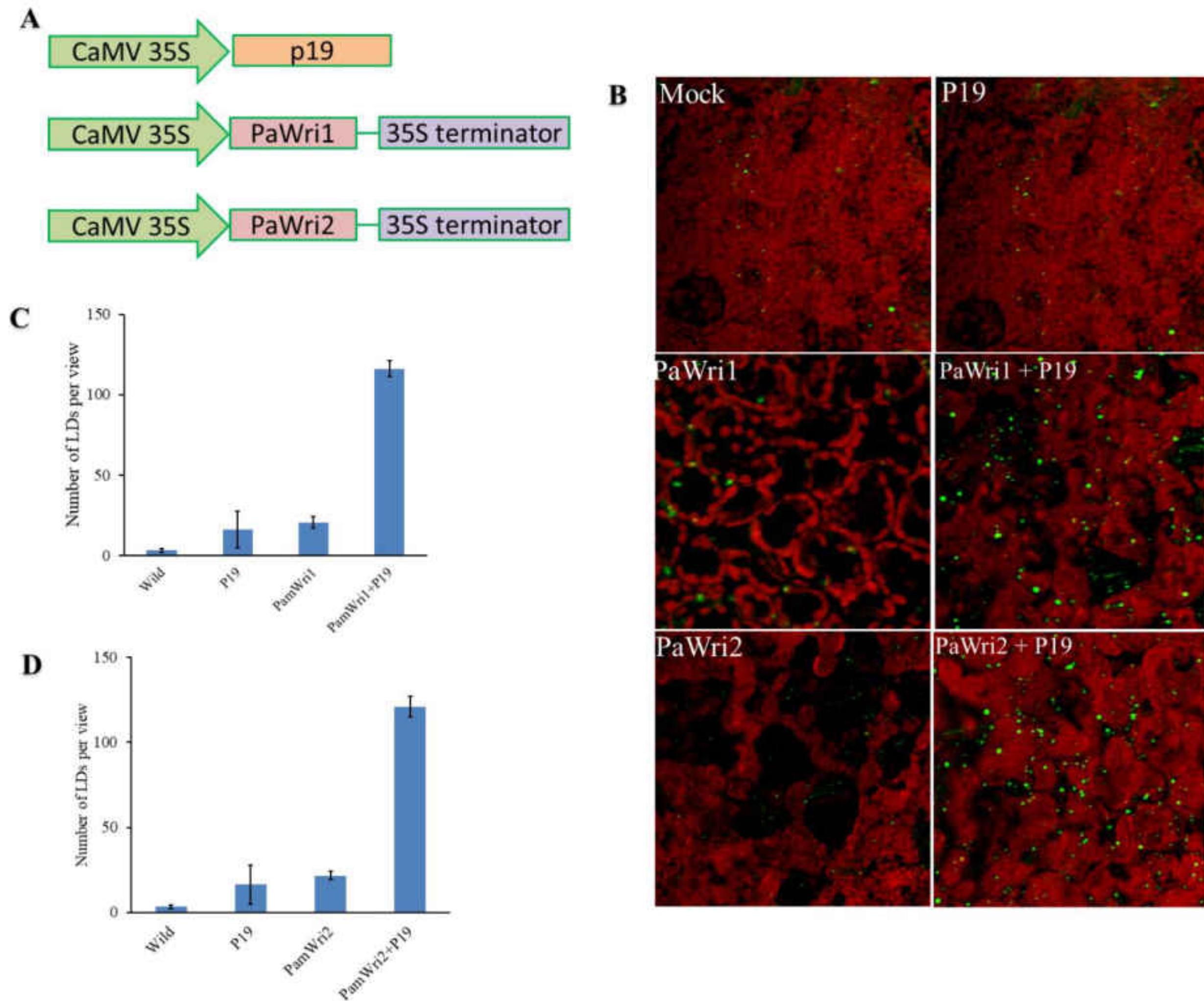

Figure 4.1 Vector constructs, LD visualization and quantification in N. benthamiana leaves expressing PamWRI1 and PamWRI2. Schematic diagram of vector constructs for Agrobacterium transformation of P19, PamWRI1 and PamWRI2 in N. benthamiana leaves (4.1A). Visualization of LDs in different N. benthamiana leaves expressing P19, PamWRI1 and PamWRI2 alone or co-expression of PamWRI1 or PamWRI2 with P19. LDs were stained with Nile Red to visualize under confocal microscopy and shown in green color (4.1B). Quantification of LDs in different N. benthamiana leaves expressing PamWRI1 (4.1C) and PamWRI2 (4.1D). Data represent mean ± SD of three independent experiments and different letters indicate significant differences (P < 0.05), as determined by one-way analysis of variance (ANOVA) with Tukey's post-test.

Overexpression of Arabidopsis *WRI1* increased TAG content by 2.8-fold in its seedling (Sanjaya *et al.*, 2011). Co-expression of *WRI1* from Arabidopsis with *DGAT1* catalyzes the final step in TAG accumulation of Kennedy pathway and, oleosin, stabilizing protein found in LD, resulted in

an accumulation of 15% TAG (Vanhercke *et al.*, 2014). Besides seed tissue, WRI1 is a regulator for oil accumulation in nonseed tissues (Kilaru *et al.*, 2015), WRI1 paralogs other than seed tissues were observed in various nonseed tissues including tallow layer of Chinese tallow, an oilseed producing tree (Divi *et al.*, 2016). In another study, expression of *B. distachyon* WRI1 isolated form nonseed tissue increase the TAG content (Y., Yang *et al.*, 2015). WRI2 gene was detected in the seeds of Arabidopsis (A. To *et al.*, 2012) but it's functional role in TAG accumulation is not clear. It was found that in Arabidopsis WRI2 neither bind with promoter region nor is able to activate the fatty acid biosynthetic gene (To *et al.*, 2012).

PamWRI1 and 2 Increase Lipid Content and Alter the Fatty Acid Profile

Total lipid was quantified from *N. benthamiana* leaves to determine the correlation between the number of LDs and their correspondence lipid amount. The total lipid content was increased by two-fold in *N. benthamiana* leaves expressing either PamWRI1 (Figure 4.2A) compared to the control. The WRI1 is the first and only characterized transcription factor among WRI homologs that stimulates fatty acid biosynthesis in the oleaginous plant tissues (Cernac and Benning, 2004). The overexpression of *AthWRI1* elevated the total lipid content in the seedlings by 2-8-fold (Sanjaya *et al.*, 2011). The TAG content in the *N. benthamiana* leaves was increased 15% of the total lipid with the co-expression of *AthWRI1, AthDGAT1*, and oleosin (Vanhercke *et al.*, 2014). In addition to seed tissues, WRI1 homologs were detected in various nonseed tissues, and overexpression of these *WRI1* homologs in vegetative tissues were resulted in an increased TAG content. For instance, *WRI1* genes were isolated from potato, oat, and nutsedge and ectopic expression of theses into *N. benthamiana leaves* resulted in an elevated TAG content from 0.05% to 2.20% (Grimberg *et al.*, 2015). The fatty acid profile in the transformed *N. benthamiana leaves* with PamWRI1 also showed difference with the control. Among five major fatty acids

(16:0, 16:1, 18:1, 18:2 and 18:3) that were quantified16:0 and 18:1 significantly increased while 18:3 significantly decreased compared with the wild type (Figure 4.2 C). As for example, camelina *CsaWRI1* increase the content of 18:2 and decrease 18:3 when transformed in the *N. benthamiana* leaves (An *et al.*, 2017). In a similar fashion, the total lipid content was increased by near two-fold when *PamWRI2* transformed into *N. benthamiana* leaves compared to wild type (Figure 4.2B). Fatty acid composition analysis revealed that *PamWRI2* increase the amount of 16:0 and 18:0 significantly but significantly decrease the amount of 18:3 significantly compared to with type (Figure 4.2D). This is the first study where we able to show that *PamWRI2* is functional and increase lipid content when transformed in *N. benthamiana* leaves probably by stimulating fatty acid biosynthesis. Previously, *AthWRI2* transformed into *Arabidopsis wri1* mutant but it failed to complement the mutant. Further, yeast one hybrid assay also showed that *AthWRI2* has not any transcriptional activity for fatty acid biosynthesis (Alexandra To *et al.*, 2012). Interestingly, PamWRI2 increased the total lipid content as well by changing the fatty acid profile when expressed in *N. benthamiana* leaves. The enhanced leaf lipid content suggests that PamWRI1 and PamWRI2 can be employed to increase lipid content in nonseed tissues.

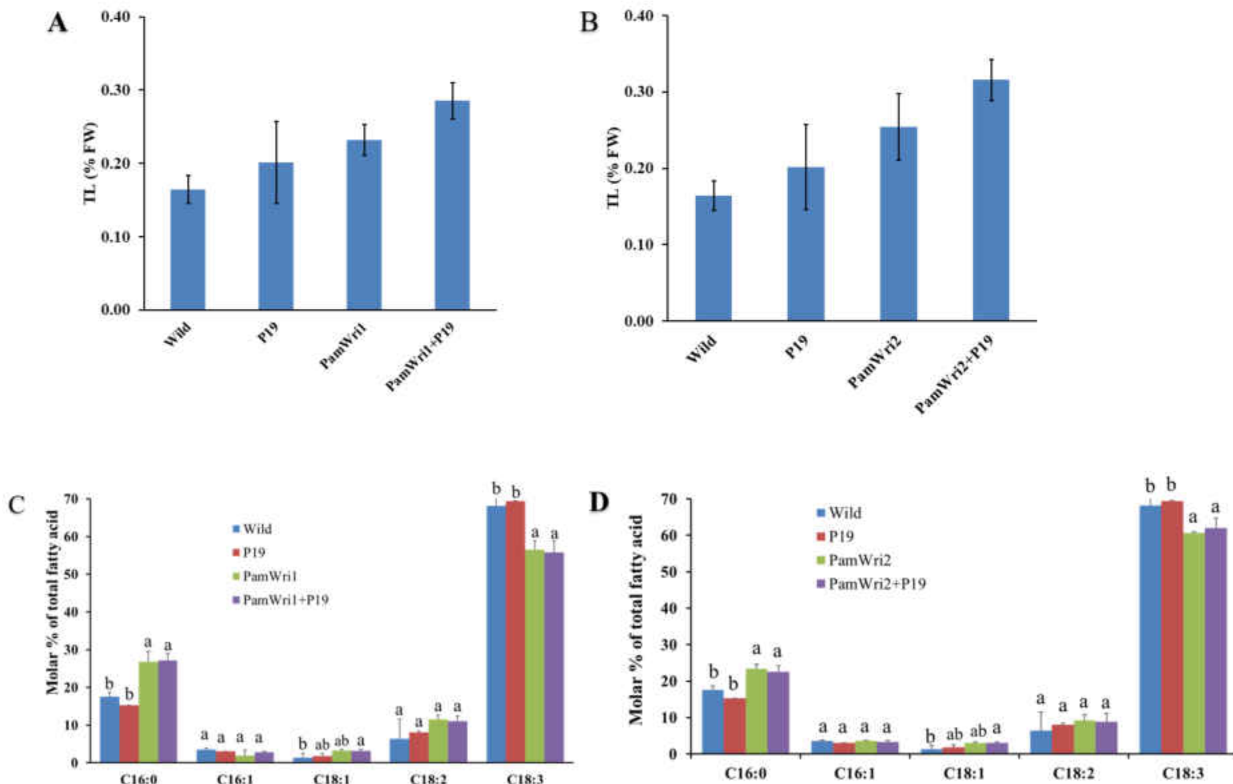

Figure 4.2 Total lipid quantification and analysis of fatty acid of total lipid in N. benthamiana leaves expressing PamWRI1 and PamWRI2. Quantification of total lipid from N. benthamiana leaves expressing PamWRI1 (4.2A) and PamWRI2 (4.2B) respectively. Fatty acids were analyzed and quantified by GC-FID in N. benthamiana leaves expressing PamWRI1 (4.2C) and PamWRI2 (4.2D), respectively. Values represent average and SD of three independent experiments and different letters indicate significant differences (P < 0.05), as determined by one-way analysis of variance (ANOVA) with Tukey's post-test.

Conclusions

In conclusion, in this study we show that PamWRI1 and PamWRI2 transcription factors involve in the increase of total lipid content in leaf tissue of *N. benthamiana* leaves by upregulating the fatty acid biosynthesis. In Arabidopsis, WRI2 is also detected but did not show any transcriptional activity for fatty acid biosynthesis. This is the first study where we show that WRI2 from avocado mesocarp could increase the TAG content by regulating fatty acid biosynthesis in vegetative tissue. Future studies will be required to isolate other WRINKLED

homologs (WRI3 and WRI4) from avocado mesocarp and characterize their function in regulating fatty acid biosynthesis. In addition, it is also necessary to isolate and characterize the transcription regulators for basal fatty acid biosynthesis pathway, if any. Finally, for the complete understanding of the regulation of fatty acid biosynthesis will be required further characterizing of post-translational modification of these transcription factors.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Triacylglycerol biosynthesis is a common metabolic pathway that occurs in all animals, plants, fungi, and some bacteria. The main purpose of TAG biosynthesis pathway is to reserve acyl chains and provide energy and carbon when needed. Regardless of the conserved nature of TAG biosynthesis pathway, current progress in understanding the regulatory mechanisms of TAG biosynthesis in several other aspects has been slow. Plentiful effort has focused on understanding the control of TAG biosynthesis in seed tissue of plants, because, the majority of plants contain TAG is the seed. These include both enzymatic and transcriptional regulations of TAG biosynthesis. For example, the transcription factor of WRI1 has shown to be involved in the regulation of fatty acid biosynthesis by mutational downregulation and overexpression in Arabidopsis seed tissue. Although other WRINKLED homologs (WRI2, WRI3, and WRI4) have been identified in Arabidopsis, only WRI1 has shown regulatory role in fatty acid biosynthesis in the developmental of seed storing TAG (To et al. 2012). WRINKLED homologs such as WRI1, WRI3, and WRI4 have been identified in nonseed tissues. For instance, oil palm WRI1 was involved in the regulation of fatty acid biosynthesis by increasing total oil content in the mesocarp tissue (Subroto et al. 2015). In avocado mesocarp, WRI2 along with WRI1 and WRI3 highly expressed in the all stages of fruit development (Kilaru et al. 2015). Besides transcriptional regulation, numerous studies have been conducted to regulate the enzymatic conversion of DAG to TAG particularly on DGAT as it is a unique enzyme for TAG biosynthesis (Cases et al. 1998). The role of other acyltransferases such as PDAT in TAG biosynthesis remains uncertain. Although in Arabidopsis, PDAT1 has an overlapping function with DGAT1, more studies are needed to understand the specificity of these enzymes in TAG

biosynthesis, particularly in nonseed tissues (Zhang et al. 2009). In this presented study, our hypothesis was PamDGAT and PamPDAT are likely to catalyze the conversion of DAG to TAG and WRINKLED (WRI) transcription factors regulate fatty acid biosynthesis. We have successfully shown that PamDGAT1 is capable of restoring neutral lipid in mutant H1246 yeast strain, which is defective in TAG biosynthesis. *In vitro* enzyme activity revealed that PamDGAT1 has a preference for oleic acid (18:1) compared to palmitic acid (16:0). Our study has also shown that transient expression of PamDGAT1 and PamPDAT1 in tobacco leaves increased total lipid content and altered the fatty acid content by increasing oleic acid (18:1). In addition, the transient expression of PamWRI1 and WRI2 in *N. benthamiana* resulted in an elevation of total lipid content.

Identification of Acyltransferases Sequences from Avocado Transcriptome Database

The putative PamDGAT1 and 2 and PamPDAT1 amino acid sequences were identified from the avocado transcriptome data by *in silico* analysis (Kilaru et al. 2015). Multiple sequence alignment of putative PamDGAT1 and 2 and PDAT1 with other biochemically characterized plants DGAT1 and 2 and PDAT1 proteins revealed high sequence similarity with strong conservation of putative transmembrane domains. Multiple sequence alignment also revealed the conserved domains of PamDGAT1 and 2 and PDAT1 (Zou et al. 1999; Jako et al. 2001; Shockey et al. 2006). In addition, PamDGAT1 has 41 conserved amino acids highlighted by Cao (Cao 2011). *In silico* analyses suggested that PamDGAT1 and 2 belong to DGAT family and PamPDAT1 belongs to PDAT family. Together, these results encourage us to further examine the function of PamDGAT1 and 2 and PamPDAT1 by complementing yeast mutant H1246 and transient expression in *N. benthamiana*.

Acyl-CoA Dependent and Independent Pathways Are Active in Avocado Mesocarp for TAG Biosynthesis

The last step of TAG biosynthesis that is conversion of DAG to TAG in ER is considered one of the committed steps in TAG biosynthesis and probably a rate-limiting step. This step is catalyzed by the acyl-CoA dependent pathway enzyme, DGAT or acyl-CoA independent pathway enzyme, PDAT. Biochemical characterization of avocado DGAT1 and 2 and PDAT1 suggest that only DGAT1 and PDAT1 are involved in TAG accumulation of avocado mesocarp which suggest both acyl-CoA dependent and acyl-CoA independent pathways are active in avocado mesocarp for TAG biosynthesis. Our results are consistent with the transcriptional expression pattern of DGAT1 and PDAT1 (Kilaru et al. 2015). Heterologous expression of avocado DGAT1 in yeast and transient expression in *N. benthamiana* leaves also support the possible role of DGAT1 in TAG biosynthesis in mesocarp tissue.

Avocado DGAT2 did not show any activity either in yeast or plant system. In the yeast expression system, putative PamDGAT2 failed to complement H1246 in a lipotoxicity assay with free fatty acids supplemented in the yeast growth media. Several studies have reported that heterologous expression of native DGAT2 from several plants including *Arabidopsi*s (Aymé et al. 2015), and fungus *Umbelopsis ramanniana* (Lardizabal et al. 2008) showed almost no DGAT activity although, their codon-optimized version of DGAT2s were active. Further, we tried to express a codon-optimized version of PamDGAT2 in *Saccharomyces cerevisiae* mutant strain (H1246) for functional complementation, but the codon-optimized version of PamDGAT2 also failed to complement H1246 yeast mutant strain. Lastly, we transiently expressed PamDGAT2 in *N. benthamiana* leaves but it was unable to increase lipid content in the planta system also. In plants, DGAT2 generally incorporates unusual fatty acids to TAG (Napier 2007) and because

avocado does not accumulate unusual fatty acids, it might be possible that the putative PamDGAT2 is not functional in avocado. In addition, the transcript level of *DGAT2* was very low in all developing stages of avocado mesocarp, relative to *DGAT1* and *PDAT1* (Kilaru et al. 2015) which indicates that maybe DGAT2 is not functional.

Avocado DGAT1 and PDAT1 Dictating the Fatty Acid Composition in Mesocarp Tissue

Avocado mesocarp contains 60-70% lipid on dry weight which mostly comprises of monounsaturated oleic acid (18:1) (Kilaru et al. 2015). Our results from *in vitro* enzyme activity showed that PamDGAT1 preferentially incorporated oleic acid (18:1) into TAG when expressed in the yeast system. Furthermore, *in planta* expression of PamDGAT1 in *N. benthamiana* leaves also changed the fatty acid composition and increased oleic acid (18:1) significantly. A similar result was observed when PamPDAT1 expressed in *N. benthamiana* leaves. DGAT1 from *B. napus* showed a higher preference for 18:1 compared to 16:0 when expressed in the yeast system. The high preference of *Brassica* DGAT1 for 18:1 has a correlation with the seed fatty acid composition which is characterized by a high percentage of 18:1 (Aznar-Moreno et al. 2015).

A Novel Transcriptional Regulator in the Fatty Acid Biosynthesis Pathway

WRI1 was the first positive regulator of fatty acid biosynthesis identified in plants (Cernac and Benning 2004). WRI1 was shown to involve in the regulation of fatty acid biosynthesis both is seed and nonseed tissues. In this study, we showed both WRI1 and WRI2 increase the lipid content when transiently expressed in tobacco. Mutant *wri1* alleles showed defective in lipid biosynthesis and resulted in severe depletion in TAG in mature seeds (Focks and Benning 1998). These mutant alleles also altered the seedling establishment (Cernac et al. 2006), while flowering and other vegetative development were unchanged, suggesting that the

fatty acid production was not significantly changed in nonseed tissues such as flower and leaves. Therefore, the transcriptional activity of fatty acid synthetic genes was not compromised in nonseed tissues of *wri1* mutant plants. These data suggest that there might be some other transcriptional machinery regulating fatty acid biosynthetic pathway, which may contain partially redundant or distinct factors. Besides WRI1, WRI2 has been identified in Arabidopsis but did not show any transcriptional activity for the control of fatty acid biosynthesis (To et al. 2012). Interestingly, WRI2 was highly expressed in all the developing stages of avocado mesocarp (Kilaru et al. 2015), and transient expression of avocado WRI2 under the control of cauliflower mosaic virus 35S promoter in *N. benthamiana* leaves significantly increased the fatty acid content compared to wild type. In this study, we showed the activity of WRI2 for fatty acid biosynthesis. Further investigation will be conducted to complement the *arabidopsis wri1 mutant.*

Future Directions

Future studies will be conducted to understand the role of Avocado WRI1, WRI2, DGAT1, and PDAT1 in seed tissue by complementing Arabidopsis seed mutants. Understanding their role in seed tissue is important because one of our primary goals is to transform these genes into *Camelina sativa* to improve seed TAG content for biodiesel production as well as human consumption. Camelina is a good model plant to alter the TAG content and composition. Because the life cycle of Camelina (80-120 days from planting to harvest) is very short compared to other crop plants such as soybean and corn. Camelina can tolerate drought, cold and requires very less pest control and fertilizer to grow. It can also grow in a marginal land where other crop plants, e.g., corn and soybean do not grow efficiently (Nguyen et al. 2013). Lastly, the transformation method for genetic engineering in Camelina is very simple and can be done by

Agrobacterium through floral vacuum method (Lu and Kang 2008). In addition, these characterized genes from avocado can be transformed in microalgae like *Chlamydomonas reinhardtii*, which is a less complex organism to increase and improve the TAG content. Previously, a DGAT2 from *B. napus* was transformed in *C. reinhardtii* and resulted in an increased of TAG content and composition (Ahmad et al. 2015).

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