Transcriptomic and lipidomic profiling in developing seeds of two *Brassicaceae* species to identify key regulators associated with storage oil synthesis

by

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B.S., Panjab University, 2002
M.S., Panjab University, 2004

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Biochemistry and Molecular Biophysics
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Abstract

In plants including the members of *Brassicaceae* family, such as *Arabidopsis thaliana* and *Brassica juncea*, seed storage reserves, which include lipids and proteins, accumulate in seeds during development. Triacylglycerols (TAG) are the major storage lipids found in the developing seeds, petals, pollen grains, and fruits of plants. In Arabidopsis seeds, acyl-CoA: diacylglycerol acyltransferase 1 (DGAT1) is the major enzyme contributing to TAG biosynthesis. In Arabidopsis, *dgat1-1* mutants retain 60-80% seed TAG content due to the involvement of phospholipid: diacylglycerol acyltransferase (PDAT1) in acyl-CoA independent TAG biosynthesis. My study focuses on the elucidation and functional characterization of novel genes involved in the regulation of the TAG biosynthesis pathway. In developing seeds of the *dgat1-1* mutant, altered fatty acid composition was observed with reduced TAG content and increased polar lipid content as compared to wild type. RNA-Seq of developing Arabidopsis seeds was employed to detect differentially expressed genes in *dgat1-1*. An empirical analysis for differential gene expression revealed a significant number of differentially expressed genes among all developmental stages in *dgat1-1*. Significant changes in gene expression profile were detected in lipid-related genes such as lipases and desaturases. RT-PCR was used to confirm the differential expression of major lipid-related genes including *DGAT1*, *PDAT*, and *FAD2*. Lipid profiling of T-DNA insertion mutants for differentially expressed genes revealed significant changes in lipid content and composition. Mutations in a member of the α, β-hydrolase family, encoded by gene named *PLIP1*, resulted in smaller seed and an altered seed oil phenotype. Also, combining the *dgat1-1* and *plip1-2* mutations resulted in a lethal phenotype, demonstrating the important role of this enzyme in embryo development and TAG biosynthesis.

To identify key components in the regulation of storage lipid biosynthesis, correlation analysis using differential transcript abundance and lipid profile during different stages of seed development from *dgat1-1* and wild type lines of Arabidopsis was performed. Using clustering analysis with Pearson correlation coefficient and single linkage identified one cluster of genes which included *PLIP1, FAD2*, *FAD3*, and *PDCT* . Similar analysis using combined data from the neutral and polar fractions resulted in clustering of lipids containing polyunsaturated fatty acids. To investigate the reduced seed germination phenotype for mature seeds of *dgat1-1* and non-germinating green seed phenotype of *dgat1-1 plip1-2* lines, differential expression (DE) analysis for genes involved in hormone metabolism was performed. Upregulation of expression was observed for genes involved in promoting abscisic acid (ABA) response, which led us to specuate the role of altered hormone metabolism in delayed germination of *dgat1-1* seeds.

Development of allopolyploid *Brassica* species from its diploid progenitors involves duplication, loss, and reshuffling of genes leading to massive genetic redundancy. It leads to selective expression or newly acquired role for duplicated homeologs. Differential expression (DE) analysis for homoeologous genes from A and B subgenomes of allopolyploid *B. juncea* implicated in FA synthesis, acyl editing, and TAG biosynthesis and metabolism was performed. Differential expression (DE) analysis identified the transcriptional dominance of A subgenome homoeologs. Identification of these homoeologs will enable their use in breeding programs directed towards improvement of lipid content and composition in seeds.

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Major Professor
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I dedicate my Ph.D. to my parents, Mrs. Balwinder Kaur Sandhu and Dr. Paramjit Singh Aulakh, whose simple life lessons and encouragement helped me to push forward during difficult times.

Chapter 1 - Review of Literature

1.1 Seed development and maturation

The plant produces oil in the seeds as an energy reserve, with a major portion of storage oil comprised of triacylglycerols (TAG), molecules with long fatty acids esterified to a glycerol backbone. The presence of long chain hydrocarbons in the structure of oil makes it useful for a wide variety of applications including industrial feedstocks and biofuels (Durrett et al., 2008; Dyer and Mullen, 2008). With increasing demands for edible oil, fuels, and industrial feedstocks, oil consumption is expected to double by 2040 (Durrett et al., 2008; Dyer and Mullen, 2008), and thus there is a need for understanding the factors affecting seed oil content. As seeds are of major importance as a source of vegetable oil, it is important to study and understand the mechanism and regulation of the complex pathways for lipid biosynthesis during seed development, maturation and germination. I used Arabidopsis as a model organism because it has short life cycle, is easy to grow in growth chambers, and possesses a relatively small genome which is fully sequences and annotated which can be easily and rapidly manipulated through genetic engineering.

1.1.1 Different phases of seed development

In angiosperms, there are two distinct phases of seed development, embryo morphogenesis and seed maturation (Jenik et al., 2007; Fait et al., 2006; Baud et al., 2002; Goldberg et al., 1994). Double fertilization of the female gametophyte initiates embryo morphogenesis. The haploid egg cell forms the diploid embryo, whereas the homodiploid central cell forms the triploid endosperm, which acts as a nourishing and nutrient source for the developing embryo (Ingram, 2010; Drews and Yadegari, 2002).

During the early stages of seed development, the suspensor cells are the major source of nutrients for the growth of the developing embryo proper (Kawashima and Goldberg, 2010). During the heart stage of embryo development, the endosperm is the major source of nutrients and undergoes an important transition and cellularization (Morley-Smith et al., 2008; Kim et al., 2005; Stadler et al., 2005). Embryo mutants which are arrested at heart stage lack the endosperm cellularization and demonstrate the significance of endosperm cellularization in embryo viability (Hehenberger et al., 2012; Köhler et al., 2003; Grossniklaus et al., 1998; Chaudhury et al., 1997). The rapid growth of embryo and storage reserve accumulation follows endosperm cellularization (Baud et al., 2008; Morley-Smith et al., 2008).

1.1.2 Storage reserve mobilization and utilization during seed maturation

During the maturation phase of seed development in Arabidopsis, there is an observed increase in the production of major storage reserves, which include seed storage proteins (SSP), and storage lipids in the form of triacylglycerol (TAG) (Figure 1.1). SSP and TAG act as the source of nitrogen and carbon respectively. Based on the metabolic profiles and days after flowering (DAF) at each stage, seed development stages are classified into early (0-7 DAF), middle (7-16 DAF) and late stage (17-20 DAF).

During the early stages, there is an increase in the levels of soluble sugars and starch. The middle stage is defined by cellular elongation and differentiation of embryo resulting in decreased levels of starch and increased amounts of storage lipids and seed storage proteins. The final and late stage of development is characterized by a sharp decline in water content from 32% to 10% accompanied by synthesis of seed storage proteins (Baud et al., 2002). This increase in production of reserve compounds results in a substantial increase in seed dry weight and results

in a metabolically quiescent embryo (Baud et al., 2008). In Arabidopsis, based on the percent dry weight, the mature dry seed is comprised of 37% lipids, 30% proteins, 20% carbohydrates, and 13% is made up of other metabolites (Figure 1.2) (Li et al., 2006).

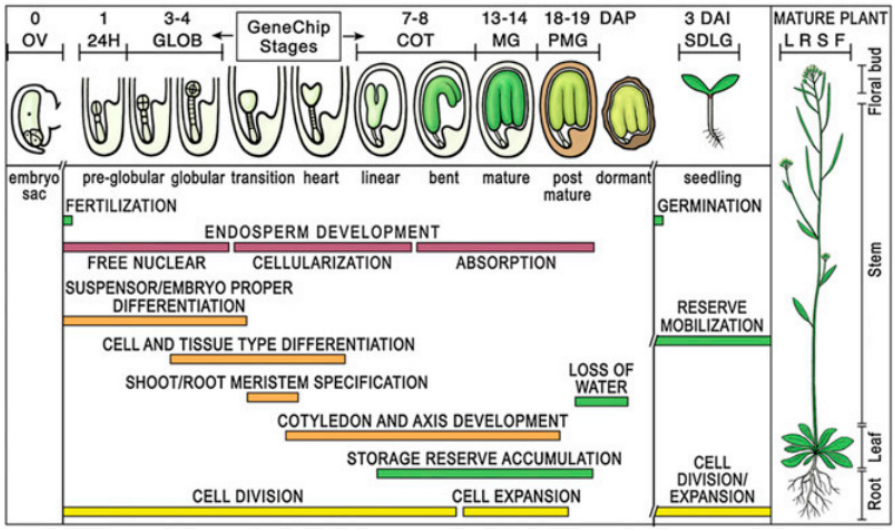


Figure 1.1 Schematic representation of seed development and different stages of the life cycle in Arabidopsis.

Pictures of seed development are not drawn to scale, and numbers on top of seed pictures correspond to days after pollination (DAP) or days after imbibition (DAI). OV, unfertilized ovule; 24H, 24-hr postpollination seed; GLOB, globular-stage seed; COT, cotyledon-stage seed; MG, mature-green-stage seed; PMG, postmature-green-stage seed; SDLG, seedling; L, leaf; R, root; S, stem; F, floral buds. Events of seed development were modified from Goldberg et al., 1994; Image was taken from Le et al., 2010 with permission from Dr. Bob Goldberg. Copyright (1994) National Academy of Sciences.

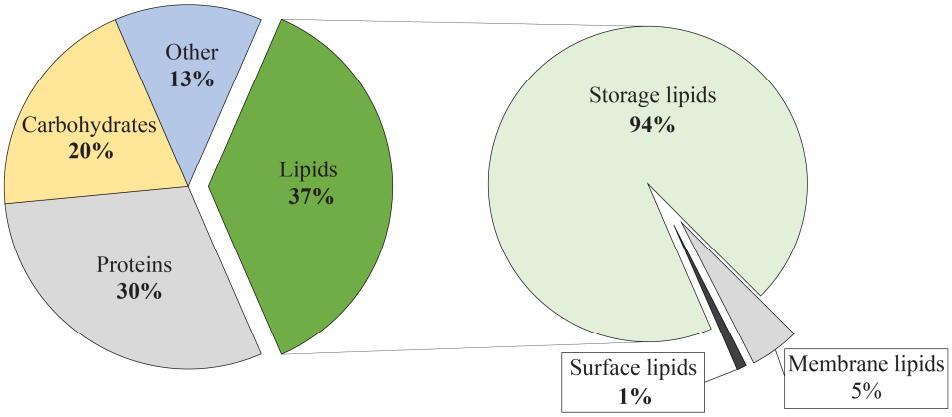


Figure 1.2 Schematic representation of lipid composition in mature seed of Arabidopsis.

The relative contribution of storage lipids and proteins were obtained from Li et al., 2006. Percentage of membrane glycerolipids relative to total lipids is from Ohlrogge and Browse, 1995. The content of surface lipids is from Molina et al., 2006 and Beisson et al., 2007 Figure adapted from Li-Beisson et al., 2013.

1.2 Lipid composition of seed

1.2.1 Classification of seed lipids

The lipid composition of the mature seed is comprised of 94% storage lipids, 5% membrane lipids, and 1% surface lipids (Beisson et al., 2007; Li et al., 2006; Molina et al., 2006). Triacylglycerol (TAG) is the major component of storage lipids; membrane glycerolipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylglycerol (PG), diphosphatidylglycerol (cardiolipin), phosphatidylinositol (PI), sulphoquinovosyldiacylglycerol (SQDG), monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG). Suberin and cutin comprise the majority of seed surface lipids (Molina et al., 2008; Beisson et al., 2007). Based on the structure and cellular localization, the major lipids in seed can be classified into following classes: one class is comprised of glyceroglycolipids and glycerophospholipids both of which are the major fraction of membrane lipids (Figure 1.4; Figure 1.5). The second class is comprised of triacylglycerols (TAG) which form the major fraction of storage lipids (Figure 1.3). Since the glycerol molecule does not have radial symmetry, all three carbon atoms are distinguishable and are classified as *sn*-1, *sn*-2, *sn*-3 (stereochemical numbering) according to the recommendation of IUPAC-IUB Commission of Biochemical Nomenclature (Stymne and Stobart, 1987).

General structure of Triacylglycerol (TAG)

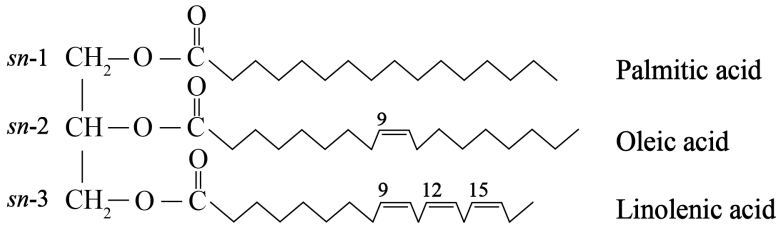


Figure 1.3 The structure of a triacylglycerol molecule.

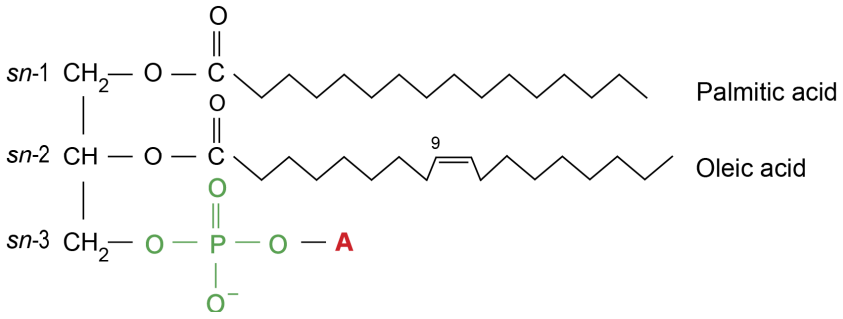
The glycerol backbone is esterified to three different acyl groups, namely palmitic acid, oleic acid, and linolenic acid at *sn*-1, *sn*-2 and *sn*-3 positions respectively. The position of double bonds is numbered from the carboxyl end. s*n* refers to the stereochemical nomenclature system.

1.2.2 Glycerolipids and TAG as a major source of energy for developing seed

Despite the high levels of protein and carbohydrate in seeds, lipids are the major source of energy for the germination of mature seeds. The oxidation of reduced carbons present in the hydrocarbon chain in lipids provides twice as much energy than carbohydrates or proteins (Abbadi et al., 2004).

Glycerolipids are composed of acyl chain esterified to *sn*-1 and *sn*-2 positions and are classified as neutral or polar based on non-polar/polar groups at the *sn*-3 position of the glycerol backbone. Neutral glycerolipids with one, two or three acyl groups esterified to glycerol backbone are named as mono, di or triacylglycerols respectively and accumulate in seeds, in addition to other vegetative tissues like stem and leaves (Stymne and Stobart, 1987). Polar glycerolipids such as phospholipids PC, PE, PS, PI, and PA, are composed of acyl groups esterified at *sn-1* and *sn-2* positions and a phosphate ester at the *sn*-3 position of the glycerol backbone. Glyceroglycolipids are composed of acyl groups at *sn-1* and *sn-2* positions and a carbohydrate headgroup at the *sn*-3 position of the glycerol backbone. The carbohydrate headgroup can be simple or complex and also can be neutral or charged (Gigg, 1980) (Figure 1.5).

General structure of Glycerophospholipids



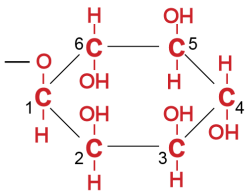
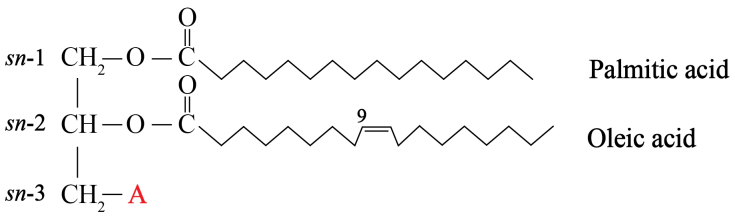
Formula for **A**	Name of lipid
— **H**	Phosphatidic acid
— **$CH_2CH_2N^+(CH_3)_3$**	Phosphatidylcholine
— **$CH_2CH_2NH_3^+$**	Phosphatidylethanolamine
— **$CH_2CH(COO)^-NH_3^+$**	Phosphatidylserine
— **$CH_2CH(OH)CH_2OH$**	Phosphatidylglycerol
— **$CH_2CH(OH)-CH_2-O-P(O^-)-O-CH_2-CH(O-C(=O)-R)-CH_2-O-C(=O)-R$**	Diphosphatidylglycerol
	Phosphatidylinositol

Figure 1.4 The basic structure of glycerophospholipids

The basic structure of glycerophospholipids is shown at the top. The glycerol backbone is esterified to two acyl groups, namely palmitic acid, and oleic acid at *sn*-1 and *sn*-2 positions respectively. The modifications of groups at the *sn*-3 position can be described by the substituents A and B which correspond to the compounds shown in the lower part of the Figure. s*n* refers to the stereochemical nomenclature system. Figure adapted from Chapter 8, Buchanan et al., 2015.

General structure of Glyceroglycolipids



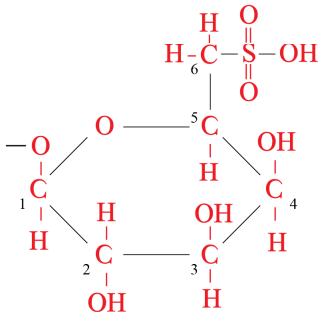
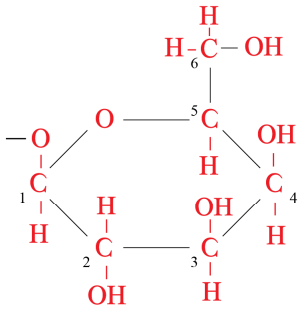
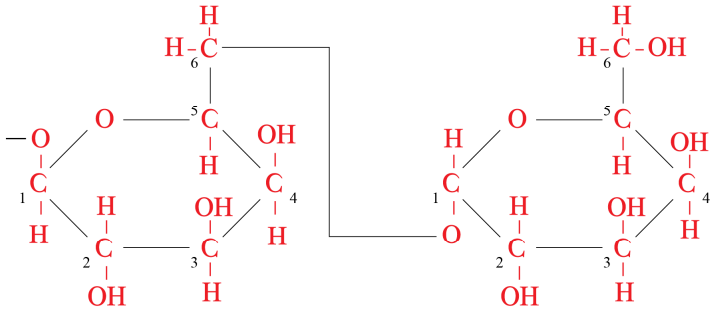
Formula for A	Name of lipid
	Sulphoquinovosyldiacylglycerol (SQDG)
	Monogalactosyldiacylglycerol (MGDG)
	Digalactosyldiacylglycerol (DGDG)

Figure 1.5 The basic structure of glyceroglycolipids

The basic structure of glyceroglycolipids is shown at the top. The glycerol backbone is esterified to two acyl groups, namely palmitic acid and oleic acid at *sn*-1 and *sn*-2 positions respectively. The modifications of groups at *sn*-3 position can be described by the substituents B which correspond to the compounds shown in the lower part of the table. *sn* refers to the stereochemical nomenclature system Figure adapted from Chapter 8, Buchanan et al., 2015.

1.3 Fatty acid synthesis

1.3.1 The fatty acid composition of major oilseed crops

Plants produce more than 200 different types of fatty acids (Van de Loo et al., 1993). Fatty acids vary in carbon chain length between 12C and 22C, and the amount of desaturation varies in either the absence or the presence of *cis* double bonds (Somerville and Browse, 1991). The fatty acid acylation and modification pathways of some plant species have evolved to produce unusual fatty acids in seeds. Seed oil from the *Brassicaceae* (*B. napus*, *A. thaliana*) contains long chain fatty acids (C20-C24), whereas that from the in *Lauraceae* contains short-chain fatty acids (C8-C14). The hydroxylated fatty acid named ricinoleate: (Δ^{9cis} 18:1-OH^{12}) is present in castor bean (*Ricinus communis*) (Baud and Lepiniec, 2010). Soybean, oil palm, rapeseed, and sunflower are considered the world's most agronomically essential oilseed crops and produce about 65% of global vegetable oil, with fatty acid composition primarily consisting of palmitate, oleate, and linoleate (Gunstone, 2000).

1.3.2 Fatty acid synthesis

In plants, the energy requirement for the different metabolic pathways involved in fatty acid and oil synthesis is provided primarily by photosynthesis. The Calvin cycle uses the energy generated by photosynthesis to fix CO_2 and convert them to sugars. Glycolysis converts sugar intermediates into acetyl-CoA, which is further utilized for fatty acid synthesis in the plastid (Buchanan et al., 2015). The rate of fatty acid synthesis varies under different conditions including the type of plant cell, seed development stage, time of day and plant growth rate (Ohlrogge and Jaworski, 1997).

In plants, *de novo* fatty acid synthesis occurs in the plastid (Figure 1.6) (Schultz and Ohlrogge, 2002; Voelker and Kinney, 2001). Following their synthesis, fatty acids are exported to the endoplasmic reticulum (ER) where fatty acids are subjected to elongation and desaturation before being assembled into the TAG.

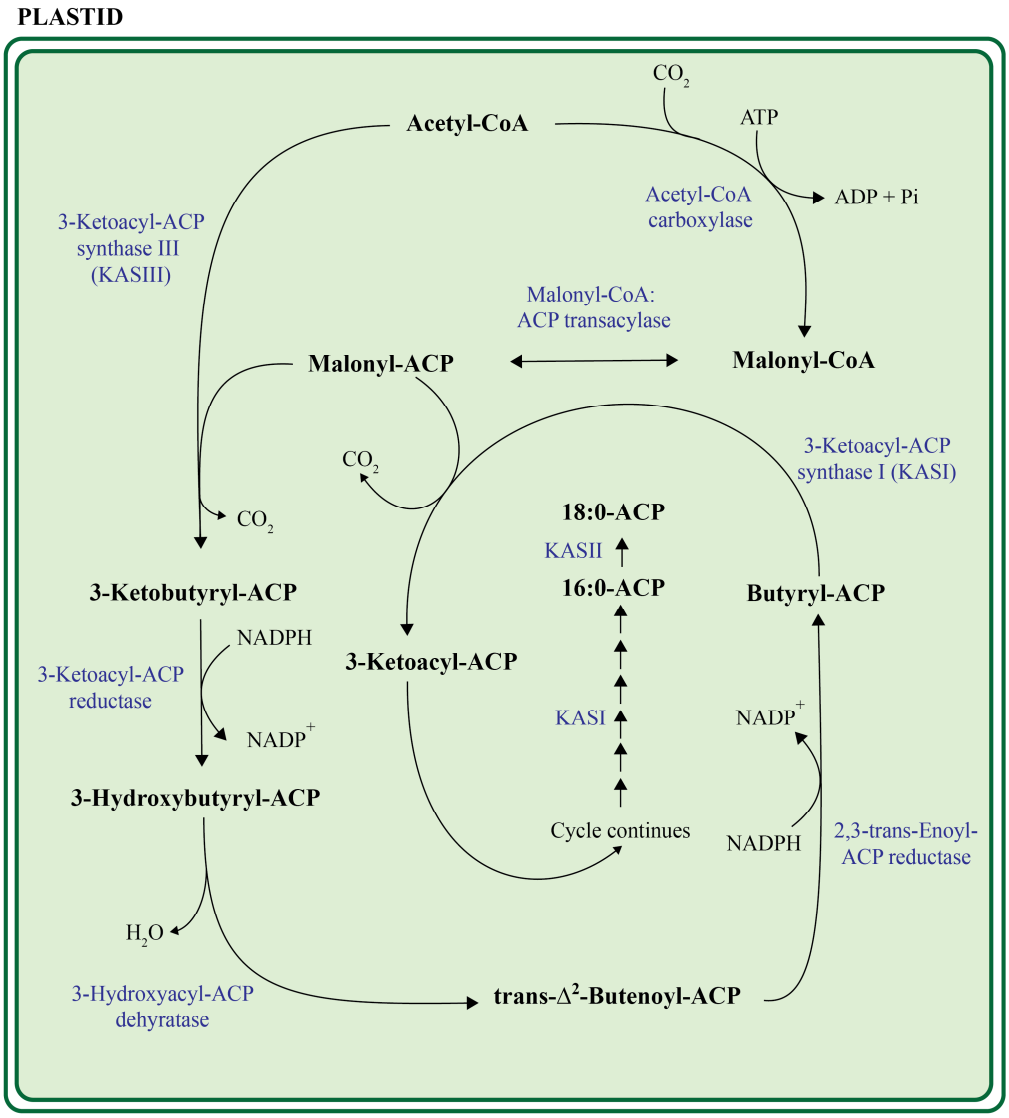


Figure 1.6 Overview of fatty acid synthesis in the plastid.

The synthesis of a C16 fatty acid requires repetition of the cycle seven times. The initial condensation reaction is catalyzed by ketoacyl-ACP synthase (KAS) III, followed by sequential reduction, dehydration and reduction steps. The initiation of the next six cycles are catalyzed by isoform I of KAS. KASII catalyzes the conversion of 16:0-ACP to 18:0-ACP. Figure adapted from Chapter 8, Buchnan et al., 2015.

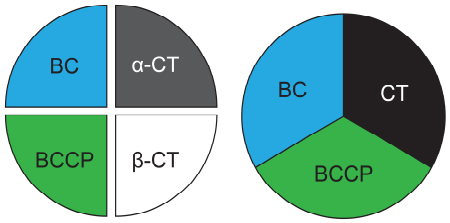
De novo fatty acid biosynthesis is catalyzed by two major enzyme complexes namely, acetyl-coenzyme-A carboxylase (AACase) and fatty acid synthase. The ACCase complex utilizes an ATP molecule and a source of carbon in the form of acetyl-coenzyme-A. In photosynthetic plants, glycolysis is the major source of reducing agents (NADPH and NADH) (Slabas and Fawcett, 1992). In achlorophyllous embryos of sunflower (*Helianthus annuus*), reducing power is generated by the plastidial oxidative pentose phosphate pathway (OPPP) which is regulated by Glc6P dehydrogenase (Kruger and von Schaewen, 2003).

1.3.2.1 Acetyl-Co-A carboxylase (ACCase)

ACCase catalyzes the first committed step of fatty acid synthesis and uses bicarbonate and acetyl-coenzyme-A (acetyl-CoA) as substrates to form malonyl-CoA (Harwood, 1996; Turnham and Northcote, 1983). Since acetyl-CoA cannot be transported into the plastid (Roughan et al., 1978; Weaire and Kekwick, 1975), multiple pathways have been proposed for the synthesis of acetyl-CoA in plastid: 1) Acetyl-CoA-synthetase (ACS) activates free acetate in an ATP-dependent reaction (Zeiher and Randall, 1991; Treede et al., 1986; Kuhn et al., 1981), 2) Pyruvate dehydrogenase complex (PDC) converts pyruvate to acetyl-CoA (Kang and Rawsthorne, 1994; Hoppe et al., 1993; Denyer and Smith, 1988; Liedvogel, 1986; Williams and Randall, 1979; Reid et al., 1977; Reid et al., 1975), 3) Plastidial carnitine acyltransferase transfers an acetate group from acetyl-carnitine to CoA to form acetyl-CoA (Wood et al., 1992). 4) Citrate lyase catalyzes ATP dependent cleavage of citrate to yield acetyl-CoA, oxaloacetate, ADP and orthophosphate (Fatland et al., 2002). There is experimental evidence to support the hypothesis for the synthesis of acetyl-CoA by PDC (Weselake et al., 2009).

In grasses and dicots, based on the differences in sensitivity towards herbicides named aryloxyphenoxypropionates (APPs) and the cyclohexanediones (CHDs), two different types of plastidial ACCases are present (Figure 1.7). Herbicide sensitive Type I ACCase is present in *Poaceae* species, and is encoded by a single polypeptide (Liu et al., 2007; Price et al., 2003). Type I ACCase shares structural similarity with ACCase present in animals and yeast. In plant cells, herbicide insensitive and cytosolic type I ACCase are responsible for elongation of fatty acids to generate very long chain fatty acids and flavonoids (Brown et al., 2009).

Subunit name	Subunit abbreviation	Size (kDa)	Location of encoding gene
Biotin carboxylase	BC	50	Nucleus
Biotin carboxyl carrier protein	BCCP	21	Nucleus
α-carboxyl-transferase	α-CT	91	Nucleus
β-carboxyl-transferase	β-CT	67	Plastid



	Heteromeric ACCase	Homomeric ACCase
Location	Plastid of all plants except grass family (herbicide insensitive)	Cytosol of all plants (herbicide insensitive) Plastid of grass family (herbicide sensitive)
Enzyme complex	Multiple subunits	Multipfunctional single polypeptide

Figure 1.7 Different classes of ACCase in plants.

a) List of subunits which make up the ACCase enzyme. Molecular size and location of encoding genes are listed b) Two different classes of ACCase, namely heteromeric and homomeric based on the number of peptides which form the functional enzyme complex. The subcellular location and differences in herbicide sensitivities are listed below each class of ACCase. Figure adapted from Chapter 8, Buchnan et al., 2015.

Type II ACCase is herbicide insensitive and is present in plants outside the *Poaceae* family, as well as in prokaryotes and algae (Huerlimann and Heimann, 2013). Type II ACCase consists of four subunits: biotin-carboxyl-carrier-protein, biotin-carboxylase, α-carboxyltransferase and β-carboxyltransferase (Sasaki and Nagano, 2004; Alban et al., 1994).

1.3.2.2 Fatty acid synthase

In plants, the fatty acid synthase (FAS) complex is dissociable and is comprised of seven proteins, of which six encode for different subunits having a catalytic function (β-ketoacyl-ACP synthase, β-ketoacyl-ACP reductase, β-hydroxyacyl dehydratase, and enoyl-ACP reductase), and the 7th is the low molecular weight acyl carrier protein (ACP). Saturated fatty acids (C16, C18) are formed by a series of condensation reactions on ACP-linked substrates (Harwood, 1996).

3-ketoacyl synthase III (KAS III) catalyzes the first condensation reaction, by using acetyl-Co-A and malonyl-Co-A as substrates to form four-carbon 3-ketoacyl-ACP (butyryl-ACP). Butyryl-ACP acts as a substrate for sequential reduction, dehydration and reduction steps. The initiation of the next six cycles are catalyzed by isoform I of KAS. Palmitoyl-ACP is elongated further to form stearoyl-ACP by KASII (Chen et al., 2015; Jaworski et al., 1993). Stearoyl-ACP acts as a substrate for desaturation by a soluble stearoyl-ACP desaturase (SAD) to form oleoyl-ACP. This desaturase is unique to plant kingdom in being a soluble enzyme as opposed to other membrane-bound desaturases (Ohlrogge and Browse, 1995).

The fatty acid synthesis reaction is terminated either by fatty acyl-ACP thioesterases (FAT) or plastidial acyltransferases (Ohlrogge and Browse, 1995). If the fatty acid is released from ACP by plastidial acyltransferase, it enters the prokaryotic pathway of glycerolipid synthesis in the plastid (Ohlrogge and Browse, 1995; Somerville and Browse, 1991; Roughan

and Slack, 1982). If the fatty acid is released from ACP by thioesterase, the free fatty acid is exported out of the plastid.

In plants, two distinct but evolutionarily related classes of thioesterases are present and are encoded by genes named *FATA* and *FATB* (Jones et al., 1995). FATA and FATB have a substrate preference for oleoyl-ACP and saturated acyl-ACP respectively (Dörmann et al., 1995; Jones et al., 1995). The unesterified fatty acids are transported from the stroma of the plastid to the outer membrane by ABC transporters (Jouhet et al., 2007; Koo et al., 2004). On the outer plastid membrane, fatty acids are activated by long-chain acyl-CoA synthetases (LACS) to form acyl-CoA (Bates et al., 2013; Zhao et al., 2010; Schnurr et al., 2002). Further, acyl-CoA is esterified to *sn-2* position of phosphatidylcholine (PC) by acyl-CoA: lysophosphatidylcholine acyltransferase (LPCAT) at the plastid envelope (Tjellström et al., 2012) before being exported to ER via membrane contact sites (MCS) (Andersson et al., 2007). Once outside the plastid, free acyl-Co-A is bound to acyl carrier protein (ACP), which renders them more soluble and membrane permeable (Fox et al., 2000; Rasmussen et al., 1994).

The FAS complex primarily produces 16:0-ACP, 18:0-ACP and 18:1-ACP, however in some species like cigar flower (*Cuphea lanceolate*), California bay (*Umbellularia californica*) and other species (Davies, 1993; Dörmann et al., 1993), acyl-ACP thioesterases have evolved to be specific for medium chain acyl-ACP. These thioesterases affect fatty acid synthesis by acting on acylthioesters to produce medium chain fatty acids like capric acid (10:0) and laurate (12:0) (Iskandarov et al., 2017; Knutzon et al., 1992; Pollard et al., 1991).

1.3.3 Prokaryotic pathway and plastidial desaturases

Most higher plants have two separate pathways for glycerolipid synthesis, namely the prokaryotic and eukaryotic pathways which occur in the plastid and ER respectively (Figure 1.8; Figure 1.9). In Arabidopsis, the enzyme acyl-ACP *sn*-glycerol-3-phosphate acyltransferase encoded by *ACT1* gene is required for the first step of the prokaryotic pathway. The lipid composition of the null mutant for *act1* indicates that increased production of membrane lipids via the eukaryotic pathway compensates for the lack of the lipid production by the prokaryotic pathway (Kunst et al., 1988).

The enzymes involved in the prokaryotic pathway have different specificities for substrates, and there exists communication and transport of lipids between the plastid and the ER. In the prokaryotic pathway, two acyltransferases are present in the plastid which acylate glycerol-3-phosphate to form phosphatidic acid (PA) (Frentzen et al., 1983). One of the soluble acyltransferases named glycerol-3-phosphate acyltransferase (Bertrams and Heinz, 1976) has specificity for oleoyl-ACP as a substrate, and another membrane-bound acyltransferase named monoacylglycerol-3-phosphate acyltransferase (Joyard and Douce, 1977) has a preference for palmitoyl-ACP (Joyard et al., 1993). Given the specificity of plastidial acyltransferases for acyl groups, PA produced has 18:1 and 16:0 acyl chains esterified at *sn-1* and *sn-2* position respectively. PA-phosphatases present in the inner envelope of plastid acts on PA to form DAG. DAG acts as a substrate for synthesis of galactolipids which include MGDG, DGDG, and SL (Ohlrogge and Browse, 1995).

In 16:3 plants such as Arabidopsis and spinach, the MGDG and DGDG molecular species are characterized by the presence of 16:3 which distinguishes these plants from other

angiosperms that primarily have acyl group 18:3 present characteristically in galactolipids (Ohlrogge and Browse, 1995). In these plants, the plastidial pathway in addition to the ER pathway provides the DAG substrate for the galactolipid synthesis.

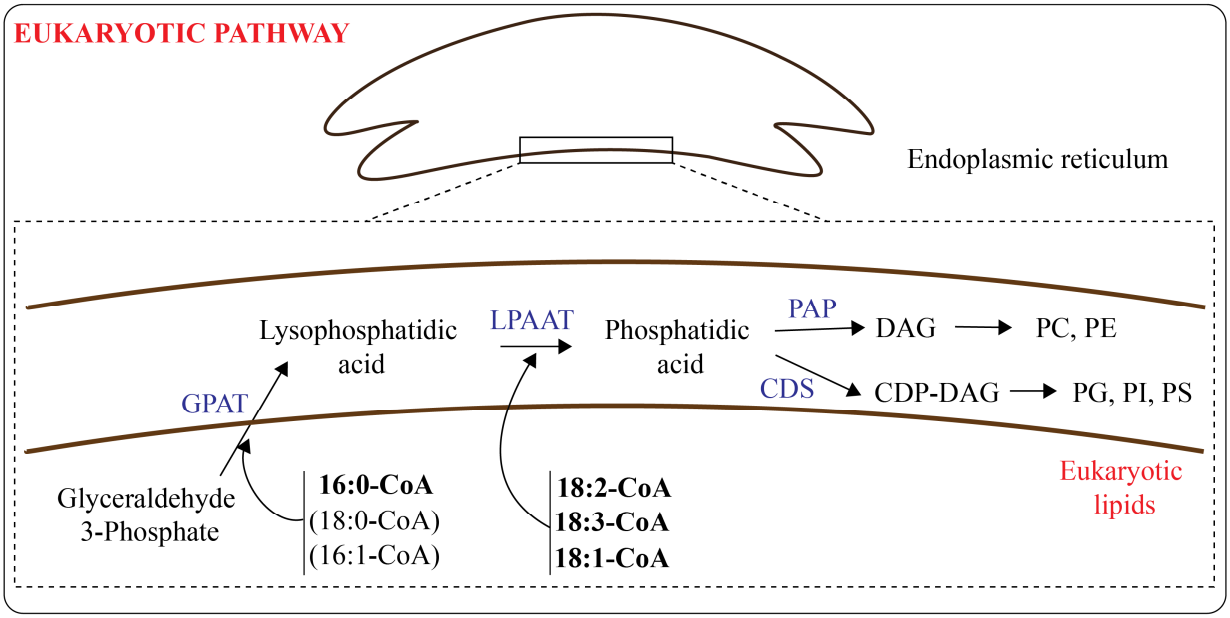


Figure 1.8 The eukaryotic pathway of glycerolipid synthesis.

This pathway occurs primarily in the ER and is characterized by the preferential esterification of C18 acyl groups to the *sn*-2 position of the glycerol backbone. Acyl-CoA substrates are used by membrane-bound enzymes including GPAT, LPAAT, and PAP. Phosphatidic acid (PA) is used as a substrate for the synthesis of other eukaryotic lipids like TAG, PC, PE, PI, PS, and PG. CDP-DAG, cytidine diphosphate-diacylglycerol; DAG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; GPAT, *sn*-glycerol-3-phosphate acyltransferase; LPAAT, acyl-CoA lysophosphatidic acid acyltransferase; PAP, Phosphatidic acid phosphatase; CDS, CDP-diacylglycerol synthase. Figure adapted from Chapter 8, Buchnan et al., 2015.

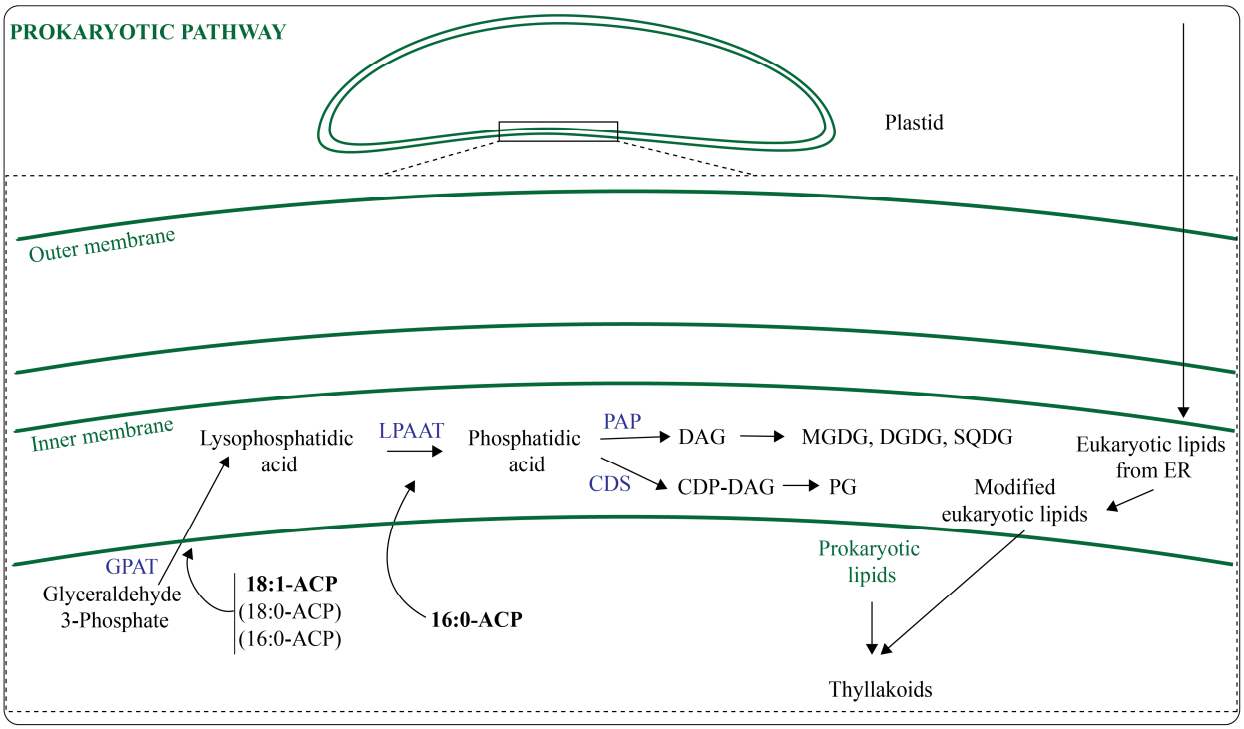


Figure 1.9 The prokaryotic pathway of glycerolipid synthesis.

This pathway is thought to occur in the inner membrane of the plastid and is characterized by the preferential esterification of palmitic acid (16:0) to the *sn*-2 position of the glycerol backbone. Acyl-ACP substrates are used by different membrane-bound enzymes like LPAAT and PAP except for the GPAT enzyme which is a soluble enzyme. Phosphatidic acid (PA) is used as a substrate for the synthesis of other prokaryotic lipids like PG, MGDG, DGDG, and SQDG. Eukaryotic lipids are transported to the inner envelope of plastid where they are acted upon by additional enzymes including desaturase and aid in the synthesis of the prokaryotic lipids. CDP-DAG, cytidine diphosphate-diacylglycerol; DAG, diacylglycerol; PG, phosphatidylglycerol; MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; SQDG, sulfoquinovosyl diacylglycerol; GPAT, *sn*-glycerol-3-phosphate acyltransferase; LPAAT, acyl-CoA lysophosphatidic acid acyltransferase; PAP, Phosphatidic acid phosphatase; CDS, CDP-diacylglycerol synthase. Figure adapted from Chapter 8, Buchnan et al., 2015.

1.3.4 Lipid trafficking between the ER and plastid

In 18:3 plants, ER-localized enzymes synthesize DAG carrying 18C acyl groups at both *sn*-1 and *sn*-2 positions of the glycerol backbone. 18:1-ACP synthesized in the plastid gets exported to the ER, where it gets incorporated into PC. PC linked C18:1 is acted upon by desaturases to form PC linked PUFAs. PC containing modified acyl groups is subsequently converted into DAG by the phosphatidylcholine:1,2-*sn*-diacylglycerol choline

phosphotransferase (PDCT) enzyme. DAG acts as a substrate both for the synthesis of membrane phospholipids including PC, PE, PI, PS, and storage lipid TAG.

When DAG serves as a substrate, the headgroups choline and ethanolamine are activated by cytidine 5'-triphosphate (CTP) to form cytidine 5'-diphosphate (CDP)-choline and cytidine 5'-diphosphate (CDP)-ethanolamine. These activated molecules are substrates for the synthesis of PC and PE respectively. Similarly, UTP-activated headgroups UDP-galactose and UDP-sulfoquinovose act as substrates for MGDG and SQDG synthesis respectively. Additionally, PA reacts with CTP to form activated CDP-DAG which on reaction with headgroups like myo-inositol, serine, and glycerol-3-phosphate lead to the formation of Pl, PS, and phosphatidylglycerol phosphate which is the precursor of PG, respectively (Joyard et al., 1993).

Though most of the polar lipids such as PC, PE, PI, PS are synthesized primarily in the ER, the galactolipids including MGDG, DGDG and sulpholipid SQDG are synthesized and roughly equally contributed by both prokaryotic and eukaryotic pathways (Browse and Somerville, 1991). DAG synthesis mediated by the eukaryotic pathway is followed by the transfer of DAG containing unsaturated FA to the plastid with only minor changes in the overall composition of either of these membranes (Ohlrogge and Jaworski, 1997).

Desaturation of acyl group imparts membrane permeability, flexibility, and adaptation to cold temperature stress (Chen and Thelen, 2013; Zhang et al., 2012; Opekarova and Tanner, 2003; Vance and Vance, 2002; Macartney et al., 1994; Sinensky, 1974). The glycerolipids and membrane lipids are mostly comprised of 16:0 and 18:1 acyl groups, which act as substrates for membrane-bound desaturases both in the plastid and ER (Browse and Somerville, 1991; Heinz and Roughan, 1983). Due to limitations in solubilization and purification of these membrane-

bound enzymes, functional characterization of mutants for different desaturases in Arabidopsis helped in understanding mechanism and regulation of these enzymes (Schmidt et al., 1994; Browse and Somerville, 1991; Somerville and Browse, 1991). Mutations in *FAD2* and *FAD3* loci affect the desaturation of extra-plastidial lipids whereas mutations in *FAD4*, *FAD5*, *FAD6*, *FAD7,* and *FAD8* loci affect the desaturation of plastidial lipids (Table 1.1) (Ohlrogge and Browse, 1995).

Name	Subcellular localization	Substrate	Notes
FAD2	ER	$18:1^{\Delta 9}$	Preferred substrate is phosphatidylcholine (PC)
FAD3	ER	$18:1^{\Delta 9, 12}$	Preferred substrate is phosphatidylcholine (PC)
FAD4	Plastid	16:0	Produces 16:1-trans at sn-2 of phosphatidylglycerol (PG)
FAD5	Plastid	16:0	Desaturates 16:0 at sn-2 of monogalactosyldiacylglycerol (MGDG)
FAD6	Plastid	$16:1^{\Delta 7}$ $18:1^{\Delta 9}$	Acts on all plastidial glycerolipids
FAD7	Plastid	$16:2^{\Delta 7, 11}$ $18:2^{\Delta 9, 12}$	Acts on all plastidial glycerolipids
FAD8	Plastid	$16:2^{\Delta 7, 11}$ $18:2^{\Delta 9, 12}$	Isoenzyme of FAD7 and is induced at low temperature
FAB2	Plastid	18:0	Stromal stearoyl-ACP desaturase

Table 1.1 The list of substrates and subcellular localization of different fatty acid desaturases from Arabidopsis.

Different desaturase enzymes are substrate specific and have differences in subcellular localization. The position of the double bond in different fatty acids is denoted as the carbon number from the carboxyl end.

Plastidial desaturases act on different substrates and stereospecificity. The enzyme encoded by *FAD4* acts on acyl group 16:0 esterified to *sn-2* position of PG, whereas the enzyme encoded by *FAD5* acts on acyl group 16:0 esterified to MGDG and DGDG (Kunst et al., 1989a; Browse et al., 1985). Irrespective of the *sn* position of acyl group on the glycerol backbone or nature of lipid headgroup, the desaturase encoded by *FAD6* acts on 16:1/18:1 (Browse et al.,

1989), whereas two isozymes encoded by *FAD7* and *FAD8* act on 16:2/18:2 (Gibson et al., 1994; McConn et al., 1994; Browse et al., 1986).

1.3.5 Acyl modifications in ER

After export from the plastid, nascent acyl-CoA molecules enter the ER through the ATP-binding cassette (ABC) transporter (Kim et al., 2013). The ER forms a membrane-based network in the cytosol and its associations with non-green plastids has been visualized by laser scanning confocal microscopy (Hanson and Kohler, 2001; Staehelin, 1997). Strong physical associations between ER and plastid has been proposed to be important for transport of plastid-synthesized lipids to ER for the synthesis of membrane and storage lipids (Andersson et al., 2007).

Fatty acids esterified to *sn*-2 position of PC are the main site of FA modification (Van de Loo et al., 1995; Sperling et al., 1993). The ER-localized desaturases encoded by *FAD2* and *FAD3* have a preference for acyl groups 18:1 and 18:2 on the *sn-1* and *sn-2* positions of PC (Browse et al., 1993; Miquel and Browse, 1992). The membrane-bound enzyme complex known as the fatty acid elongation (FAE) complex uses malonyl-CoA as a source of the 2C unit and acyl-CoA as substrates to form a very long chain fatty acid (VLCFA). Elongation of fatty acids is analogous to *de novo* fatty acid synthesis in the plastid and involves four enzymatic reactions catalyzed by FAE complex: 3-ketoacyl-CoA synthase (FAE1), 3-ketoacyl-CoA reductase, 3-hydroxyacyl-CoA dehydrase, and enoyl-CoA reductase (Firth and Patrick, 2008; Wu et al., 2008; Blacklock and Jaworski, 2006; Mietkiewska et al., 2004; Roscoe et al., 2001; Rossak et al., 2001; Millar and Kunst, 1997; James et al., 1995).

1.4 TAG biosynthesis in seeds (Kennedy pathway)

The plant stores energy reserve in the form of oil (triacylglycerols), protein (seed storage protein) and carbohydrate (mostly starch) in the seeds to perpetuate the next generation (Ohlrogge and Browse, 1995). In addition to being a significant storage lipid in developing seeds, TAG can be found in different tissues including pollen, fruits (Murphy, 2001; Murphy et al., 1993), and leaves (Kaup et al., 2002; Sakaki et al., 1990; Cao and Huang, 1986; Martin and Wilson, 1983).

Vegetative tissues such as leaves and stem contain less than 10% lipid by dry weight. Oil content per dry weight of seed varies between different species, from 4% in *Triticum sativum* to over 60% in *Ricinus communis* (Ohlrogge and Jaworski, 1997). In the mature seed of Arabidopsis, oil content varies within different compartments with cotyledons containing 60% of total fatty acid, 30% in radicle and hypocotyl and remaining 10% in the residual endosperm (Li et al., 2006; Penfield et al., 2004).

A major component of seed storage lipids in the mature seed is triacylglycerols (TAG) and is stored in densely packed lipid bodies (Herman, 2017; Murphy, 1993; Huang, 1992). Oil bodies are surrounded by three oil-body-membrane protein families named oleosins, caleosins, and steroleosins (Shimada and Hara-Nishimura, 2010). The TAG molecule is synthesized by a series of acyltransferases and phosphatases which esterify the glycerol backbone. There are acyltransferase enzymes present in different organelles like mitochondria, ER, and plastid, but the most well biochemically characterized acyltransferases are present in ER which form the majority of TAG in developing seeds (Slabas et al., 2001b; Frentzen, 1998). Although most of

the acyltransferases are membrane-bound, in peanut a soluble system for TAG assembly has been reported (Saha et al., 2006; Tumaney et al., 2001).

1.4.1 Enzymes involved in TAG assembly

In Arabidopsis, *de novo* TAG biosynthesis occurs in the ER by a pathway also known as the Kennedy pathway (Stymne and Stobart, 1987; Barron and Stumpf, 1962; Kennedy, 1961; Kennedy and Weiss, 1956). Acyl-CoA: glycerol-3-phosphate acyltransferase (GPAT) catalyzes the first step in the Kennedy pathway. GPAT utilizes the acyl-CoA pool to acylate the *sn-1* position of glycerol-3-phosphate to form phosphatidic acid (PA).

1.4.1.1 Glycerol-3-phosphate acyltransferases (GPAT)

In plants, GPAT enzymes can be found in the mitochondria, plastid and endoplasmic reticulum. The ER-localized GPAT is uniquely involved in TAG biosynthesis via the Kennedy pathway (Snyder et al., 2009). Plant GPATs have broad specificity and utilize both saturated and unsaturated acyl groups (Lisa and Holcapek, 2008; Christie et al., 1991; Brockerhoff and Yurkowski, 1966) suggesting the role of acyl-CoA availability in determining the acyl composition at the *sn-1* position of TAG molecule. In Arabidopsis, the biochemical characterization of enzymes encoded by eight genes *GPAT1-8*, has revealed that at least five genes encode for acyltransferases which are involved in the synthesis of cutin and suberin and are not involved in TAG biosynthesis (Yang et al., 2012; Beisson et al., 2007; Li et al., 2007). However, *GPAT9* which was identified by homology search using mouse and human *GPAT3* (Gidda et al., 2009; Cao et al., 2006), is involved in TAG biosynthesis (Shockey et al., 2016).

1.4.1.2 Lysophosphatidic acid acyltransferase (LPAAT)

Lysophosphatidic acid (LPA) formed by GPAT9 acts as the substrate for LPAAT enzyme, which acylates the *sn-2* position of LPA to form phosphatidic acid (PA). PA is an important signaling molecule and acts as a precursor for other phospholipids (Carman and Henry, 2007). Based on sequence information, genes that encode LPAATs can be classified into two separate classes: A and B (Frentzen, 1998). Members of class A are involved in membrane lipid synthesis, are expressed in all plant tissues and have specificity for 18:1-CoA, whereas members of class B have specificity for both saturated and unsaturated acyl groups and are expressed in storage tissues (Knutzon et al., 1999; Lassner et al., 1995). In Arabidopsis, five putative genes encoding for LPAATs have been identified by using homology search using cytoplasmic LPAAT from maize (Brown et al., 1994) and plastidial LPAAT from *B. napus* (Bourgis et al., 1999). Out of the five putative genes, one gene encodes plastidial LPAAT1 (Bourgis et al., 1999) and other four genes *LPAAT 2-5* encode cytoplasmic LPAATs (Kim and Huang, 2004). The *LPAAT1* gene transcript is expressed at higher levels in leaves as compared to other tissues, whereas *LPAAT2* transcript has similar expression levels and is ubiquitously expressed in all tissues (Kim and Huang, 2004). Complementation studies have shown that the null *lpaat1* mutant phenotype can be functionally complemented by WT copy of *LPAAT1* and cannot be complemented by a truncated copy of the gene, which lacks the plastid localization signal, suggesting LPAAT1 to be localized in the plastid (Kim and Huang, 2004).

1.4.1.3 Phosphatidic acid phosphohydrolase (PAH)

In yeast, cellular levels of PA are regulated by a Mg^{2+}-dependent PA phosphohydrolase called Pah1p/PAH1 (Han et al., 2006). In yeast, *pah1* mutant results in high levels of PA and increased expression of genes involved in phospholipid biosynthesis (Han et al., 2007; Han et al.,

2006; Santos-Rosa et al., 2005). Overexpression of *PAH1* leads to reduced expression of genes involved in phospholipid biosynthesis (Han et al., 2007; O'Hara et al., 2006). In addition to the regulation of PA levels, Pah1p also provides a substrate for membrane and storage lipid production (Carman and Henry, 2007). In yeast, the *pah1* mutant has reduced TAG production biosynthesis (Han et al., 2006). In Arabidopsis, *AtPAH1* and *AtPAH2* have been identified which are similar to yeast *PAH1* (Han et al., 2006) and mammalian *Lipin-1* genes (Péterfy et al., 2001). Transcripts for both *PAH1* and *PAH2* genes are ubiquitously expressed in all tissues, but relative expression is highest in the maturation stage of seed development (Eastmond et al., 2010). Individual *pah1* and *pah2* T-DNA insertional mutants do not have any phenotypic effect on plant growth or lipids. However, double mutant *pah1 pah2* exhibited an increase in phospholipid synthesis and ER membrane biogenesis (Wang et al., 2014).

The final rate-limiting step of acylating DAG at the *sn-3* position to form TAG is catalyzed by an enzyme known as diacylglycerol: acyltransferase (DGAT) which is unique to the TAG biosynthesis pathway (Ohlrogge and Browse, 1995). In addition, another enzyme called phospholipid: diacylglycerol acyltransferase 1 (PDAT1) can also synthesize TAG from DAG (Zhang et al., 2009; Mhaske et al., 2005).

1.4.1.4 DGAT, a rate-limiting step in acyl-CoA dependent mechanism of TAG biosynthesis

The final step of *de novo* TAG biosynthesis is catalyzed by a membrane-bound enzyme known as acyl CoA: diacylglycerol acyltransferase (DGAT) (Ohlrogge and Browse, 1995; Kennedy and Weiss, 1956). This final rate-limiting acylation reaction (Perry et al., 1999; Perry and Harwood, 1993b; Perry and Harwood, 1993a; Ichihara et al., 1988) is the only committed step towards TAG biosynthesis (Cao and Huang, 1987; Cao and Huang, 1986). DGAT catalyzes

the transfer of an acyl group from acyl-CoA pool onto the *sn-3* position of DAG to form TAG (Zou et al., 1999). DGAT plays a vital role in regulating the type and quantity of acyl groups that are incorporated into TAGs (Lung and Weselake, 2006; Sorensen et al., 2005; He et al., 2004). In developing seeds of oleaginous plants, TAG accumulation and DGAT activity are associated with the ER (Lacey and Hills, 1996; Settlage et al., 1995; Frentzen, 1993; Stymne and Stobart, 1987; Cao and Huang, 1986; Stobart et al., 1986). Also in developing seeds, increased DGAT activity corresponds to increase in the TAG production and the DGAT activity rapidly decreases as the seed oil content reaches a plateau (Tzen et al., 1993; Weselake et al., 1993).

1.4.2 DGAT encoding genes

DGAT activity was first reported in chicken liver by (Weiss et al., 1960) and thereafter the genes encoding DGAT enzymes have been identified and studied in different plant species (Zheng et al., 2017). In plants, DGAT encoding genes play an essential role in determining the qualitative and quantitative flow of fatty acids in TAG content (Lung and Weselake, 2006; Sorensen et al., 2005; He et al., 2004). DGAT encoding genes have been utilized for improving oil content and fatty acid composition of oleaginous crops (Andrianov et al., 2010; Lardizabal et al., 2008; Xu et al., 2008; Lung and Weselake, 2006; Slabas et al., 2001a; Settlage et al., 1998). Different types of genes encoding DGATs have been identified, namely: *DGAT1*, *DGAT2*, *DGAT3* and the bifunctional *DGAT/wax ester synthase* (*WS*/*DGAT*) (Turchetto-Zolet et al., 2016).

1.4.2.1 Diacylglycerol acyltransferase 1 (DGAT1)

DGAT1 was first identified in mouse based on sequence homology with a functionally different acyltransferase known as acyl-CoA: cholesterol acyltransferase (ACAT) (Chang et al.,

1993). The ortholog of *DGAT1* gene has been identified in Arabidopsis and is named *AtDGAT1* (Routaboul et al., 1999; Zou et al., 1999; Katavic et al., 1995). *AtDGAT1* transcripts can be detected at variable levels in developing siliques, stem, leaves, flowers, germinating seeds and seedlings (Hobbs et al., 1999; Zou et al., 1999). Overexpression of *AtDGAT1* cDNA in yeast resulted in a 3.5-4-fold increase in microsomal DGAT activity as compared to vector control indicating that *AtDGAT1* is primarily involved in the acylation of DAG molecule to form TAG. Overexpression of *AtDGAT1* in Arabidopsis and *B. napus* resulted in increased seed oil content (Zheng et al., 2003; Jako et al., 2001). Functional characterization of *AtDGAT1* was done using the ethyl methanesulfonate (EMS) induced mutant *dgat1-1* (also referred to as AS11) which was identified as part of screening for mutants having reduced levels of very long chain fatty acids (VLCFA) (Kunst et al., 1992a; Kunst et al., 1992b; Kunst et al., 1989b). Screening for wrinkled and incompletely filled seeds among the Versailles T-DNA insertion mutant collection led to the identification of another mutant allele *dgat1-2* (also referred to as ABX45) for *AtDGAT1* gene (Routaboul et al., 1999).

The *dgat1-1* mutant allele has a 147bp insertion which encompasses part of intron1 and intron2, in addition to duplicated exon 2 (Figure 1.10). This insertion did not affect the reading frame and resulted in the altered transcript and altered protein (Xu et al., 2012). This mutation resulted in a 40-70% reduction in DGAT activity during different stages of seed development (Katavic et al., 1995). The comparative sequence analysis of cDNA from the WT and the *dgat1-2* lines indicates the presence of a deletion of G at position 180 resulting in the formation of truncated protein due to the frameshift mutation (Figure 1.10) (Routaboul et al., 1999). The observed reduction in DGAT1 activity and altered lipid phenotype of the *dgat1-2* line is similar to that observed for the *dgat1-1* mutant (Routaboul et al., 1999).

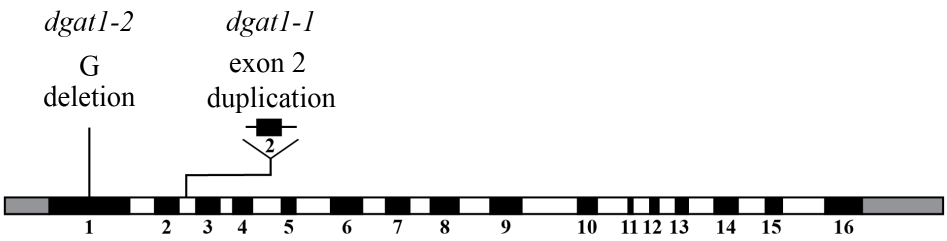


Figure 1.10 Diagrammatic representation of gene structure for the genomic sequence of *dgat1-1* and *dgat1-2* mutant alleles.

The solid boxes represent the exons; open boxes represent the introns and grey fill boxes represent the UTRs. Numbers below the solid boxes represent the exon number.

Analysis of the total lipid content of mature seeds revealed that the *dgat1-1* mutant has 75% of total oil content, whereas *dgat1-2* has 55% of total oil content relative to WT. The fatty acid composition of mature seeds for *dgat1-1* and *dgat1-2* mutant lines is altered and has a 60% decrease in 18:1 and 20:1 acyl groups and a 100% increase in 18:3 as compared to WT (Routaboul et al., 1999; Zou et al., 1999; Katavic et al., 1995).

1.4.2.2 Diacylglycerol acyltransferase 2 (DGAT2)

DGAT2 was first identified in the oleaginous fungus *Umbelopsis ramanniana* (Lardizabal et al., 2001). Since this gene did not share any sequence homology with *DGAT1* or acyl-CoA: cholesterol acyltransferase (*ACAT1/ACAT2*), it was named *DGAT2*. Gene-targeted mutagenesis in mammals revealed that *DGAT2* plays an important role in TAG biosynthesis (Stone et al., 2004; Smith et al., 2000). Functional studies of the *AtDGAT2* mutant line shows that there is no effect of the mutation on the seed oil content. Additionally, a double mutant for *dgat1-1 dgat2* did not aggravate the *dgat1-1* oil phenotype suggesting that it is not involved in TAG biosynthesis in Arabidopsis (Zhang et al., 2009). Transient expression studies done on *N. benthamiana* leaves have shown that overexpression of *AtDGAT2* results in 2-fold higher production of TAG in leaves as compared to *AtDGAT1* (Zhou et al., 2013). Heterologous

expression of *AtDGAT2* in yeast was unsuccessful and resulted in no protein accumulation possibly due to differences in codon usage between plants and yeast (Aymé et al., 2014). *DGAT2* homologs have been identified from other species such as *S. cerevisiae* (*Dga1*), *Y. lipolytica* (*DGA2*), castor bean (*Ricinus communis*), tung tree (*Vernicia fordii*) and Ironweed (*Vernonia galamensis*) (Athenstaedt, 2011; Zhang et al., 2009; Kroon et al., 2006; Shockey et al., 2006; Sandager et al., 2002). Functional characterization of DGAT2 from these species has been shown to have preference for incorporation of unusual fatty acids like ricinoleic (12-OH 18:1*cis*Δ9), a-eleostearic (18:3 *cis*Δ9, *trans*Δ11, *trans*Δ13) and vernolic (12-epoxy, *cis*Δ9 octadecenoic) acid into TAG (Li et al., 2010; Kroon et al., 2006; Shockey et al., 2006).

In lipid metabolism, DGAT1 and DGAT2 are the major enzymes studied in eukaryotes including algae, fungi animals, and plants (Turchetto-Zolet et al., 2016). Comparison of amino acid sequences for DGAT1 and DGAT2 from Arabidopsis, rice (*Oryza sativa*) and tung tree (*Vernicia fordii*) revealed structural and functional differences between DGAT1 and DGAT2. The DGAT1 protein generally is 500 amino acids in length with ten predicted transmembrane domains (TMDs) whereas DGAT2 protein is 320 amino acids in length with 2 predicted TMDs. DGAT1 and DGAT2 enzymes from tung tree have a nonredundant function in storage oil synthesis and localize to distinct and dynamic regions of ER (Shockey et al., 2006). Phylogenetic and evolutionary studies show that DGAT1 and DGAT2 have evolved separately during eukaryotic evolution with functional convergence (Turchetto-Zolet et al., 2011).

1.4.2.3 Diacylglycerol acyltransferase 3 (DGAT3)

A soluble DGAT3 has been identified in different plant species including soybean (*Glycine max*), rice (*Oryza sativa*), and peanut (*Arachis hypogaea*) (Peng and Weselake, 2011;

Saha et al., 2006). DGAT3 has been proposed to be involved in cytosolic TAG synthesis and recycling of TAGs in germinating seedlings (Hernández et al., 2012). Defective Cuticle Ridge (DCR) is a member of the BAHD acyltransferase family which is involved in the synthesis of TAGs enriched with hydroxy fatty acids (Rani et al., 2010; Panikashvili et al., 2009). These soluble acyltransferases share minimal sequence homology with DGATs and suggest that TAG biosynthesis is not limited to membranes but also occurs in the cytoplasm.

1.4.2.4 Bifunctional wax synthase/diacylglycerol acyltransferase (WS/DGAT)

In bacteria, TAG synthesis is catalyzed by a membrane-bound bifunctional enzyme named WS/DGAT which possesses both wax synthase (WS) and TAG biosynthesis activities (Waltermann et al., 2007). Homologs for WS/DGAT have been identified in *Mycobacterium tuberculosis*, *Alcanivorax borkumensis* and gram-positive bacteria *Streptomyces avermitilis* MA-4680 (Kaddor et al., 2009; Kalscheuer et al., 2007; Daniel et al., 2004). Bacterial WS/DGAT synthesizes wax esters and TAGs under limited growth conditions. Heterologous expression of bacterial WS/DGAT in yeast promotes TAG synthesis (Kalscheuer et al., 2004) in addition to exhibiting *in-vitro* acyl-CoA: monoacylglycerol acyltransferase (MGAT) activity (Stöveken et al., 2005). This bacterial WS/DGAT is not related to jojoba wax synthase, DGATs, and PDATs (Liu et al., 2012). In Arabidopsis, a bifunctional WS/DGAT (*WSD1*) (Li et al., 2008) is involved in wax ester synthesis and is homologous to *Acinetobacter calcoaceticus* WS/DGAT (Kalscheuer and Steinbüchel, 2003).

1.4.3 PDAT1 catalyzed acyl-CoA independent mechanism of TAG biosynthesis

The fact that the seed of *dgat1-1* mutant produces only 75% of oil suggests the existence of alternative mechanisms of seed oil biosynthesis besides DGAT1.

1.4.3.1 Yeast PDAT

In mammals, an enzyme named lecithin: cholesterol acyltransferase (LCAT) utilizes an acyl-CoA independent mechanism for transfer of acyl group from PC onto cholesterol to form cholesterol ester (Glomset, 1968). A homolog for HsLCAT has been identified in yeast and is named LRO1. Yeast LRO1 plays an important role in TAG accumulation during the late stationary phase. Even though it is homologous to mammalian LCAT, yeast LRO1 does not have cholesterol ester synthesizing activity. Instead, *in vitro* enzyme assays using microsomes from control and *lro1* mutant from the yeast along with radiolabeled acyl groups at the *sn*-2 position of PC indicated that ScPDAT catalyzes the transfer of acyl group from an *sn*-2 position of PC to DAG to form TAG (Dahlqvist et al., 2000). In yeast, deletion of *LRO1*, results in significant reduction in TAG synthesis, whereas the contribution of *DGA1* towards TAG synthesis varies under different growth conditions. The double mutant strain of yeast which lacks both *DGA1* and *LRO1* results in > 97% loss in TAG production (Oelkers et al., 2002).

1.4.3.2 Arabidopsis PDAT1

A homology search using HsLCAT and ScPDAT as query sequences against the Arabidopsis database identified six homologous sequences. Comparative analysis of the amino acid sequences from the closest homologs to ScPDAT in Arabidopsis, named AtPDAT, and At3g44830 showed 57% sequence identity with each other and 28% and 26% sequence identity

with ScPDAT respectively (Ståhl et al., 2004). Both *AtPDAT* and At3g44830 encoded proteins are predicted to have an N terminal cytoplasmic tail, transmembrane domain and a C terminal ER retrieval motif (McCartney et al., 2004; Krogh et al., 2001). Transcript analysis showed that *AtPDAT* was expressed at similar levels in leaves, roots, flowers and developing seeds, whereas At3g44830 expression was detected at high levels in developing seeds and low expression in flowers and leaves (Ståhl et al., 2004).

The functional role of AtPDAT in acyl-CoA independent mechanism of TAG biosynthesis was validated by comparing enzyme activity of PDAT in microsomal preparations from leaves and roots of control and 35S-*At*PDAT transgenic lines. Radiolabeled PC was used as an acyl donor, and unlabeled DAG was used as a substrate for the assay with microsome preparations and the amount of radiolabeled TAG formed correlated with the transcript levels of the *AtPDAT* gene. The results highlighted the importance of phospholipids as an acyl donor for PDAT activity, irrespective of the substrate used.

Substrate specificity studies of AtPDAT indicated the preference for acyl groups containing double bonds and presence of functional groups like epoxy or hydroxy groups (Ståhl et al., 2004). AtPDAT displays three times higher activity and thus positional specificity for acyl group present at the *sn*-2 position as compared to acyl group present at *the sn*-1 position. Comparison of AtPDAT activity between different phospholipid substrates PC, PE and PA-containing [^{14}C] labeled 18:1 showed a 1.7-fold and 5.6-fold increase in AtPDAT activity with PE as compared to PC and PA respectively (Ståhl et al., 2004). Neither *pdat1-2* mutant nor the *AtPDAT* overexpressing line had any effect on fatty acid content or composition as compared to WT (Mhaske et al., 2005; Ståhl et al., 2004). No change in lipid content or composition of seeds

for *pdat1-2* suggests that AtPDAT1 is not a major determining factor for TAG biosynthesis in seeds (Zhang et al., 2009).

1.4.4 Role of DGAT and PDAT homologs in TAG biosynthesis

The lipid content and composition analysis for *dgat2-like* and *pdat1-like* single mutants did not show any changes as compared to WT (Zhang et al., 2009). To identify genes which have a redundant or complementary role with *AtDGAT1*, double mutants were generated by crossing T-DNA mutant line for each of *DGAT2*, *PDAT1*, *PDAT1-like* genes with the *dgat1-1* mutant. Double mutants *dgat1-1 dgat2-like* and *dgat1-1 pdat1-like* did not show any further decrease in oil content as compared to *dgat1-1* single mutant. The lipid phenotype of single and double mutant lines suggests that *AtDGAT2* and *AtPDAT1-like* do not have a role in TAG biosynthesis in the absence of *AtDGAT1* (Zhang et al., 2009).

The double mutant *dgat1-1 pdat1-2* is lethal and leads to sterile pollen and abnormal embryo development. Additionally, flowers of plants with genotype *dgat1-1*/*dgat1-1 PDAT1*/*pdat1-2* produced sterile, smaller, deformed, and shrunken pollen. A *PDAT1-RNAi* line does not show any changes in lipid content or composition as compared to WT and is consistent with lipid analysis of *pdat1-2* line (Mhaske et al., 2005). However, the average % oil content per dry weight of seed in *dgat1-1 PDAT1 RNAi* line is reduced by 63% as compared to *dgat1-1*. Lipid composition analysis from seeds of *dgat1-1 PDAT1 RNAi* line indicated reduced levels of 18:3 and similar levels of 18:1 and 20:1 as compared to *dgat1-1*. These results indicate the significant role of PDAT1 in TAG biosynthesis in the absence of DGAT1 (Zhang et al., 2009).

1.4.5 Role of PC in TAG synthesis

PC acts as an intermediate for the flux of FAs or DAG or both substrates for TAG production. PC is also a major site for fatty acid modification including desaturation and hydroxylation in the ER. Modified acyl groups are incorporated into TAGs via different mechanisms. First, by reverse action of CPT enzyme, PC is converted back to DAG which is further utilized by DGAT enzyme for TAG assembly (Vogel and Browse, 1996). Secondly, an enzyme called phosphatidylcholine: 1,2-*sn*-diacylglycerol choline phosphotransferase (PDCT) converts PC with modified acyl groups into DAG thereby generating PC derived PUFA-rich DAG pool which is utilized by DGAT for TAG assembly (Lu et al., 2009). Another mechanism involves an enzyme named acyl-CoA: lysophosphatidylcholine acyltransferase (LPCAT) which catalyzes the acylation of lysophosphatidylcholine (LPC) to form PC (Stymne and Stobart, 1984). Acyl groups are released from the *sn-2* position of PC by either the reverse reaction of LPCAT (Stymne and Stobart, 1984) or by the action of phospholipase A_2 (PLA_2) before getting activated by long-chain acyl-CoA synthetases (LACS) (Weselake et al., 2009).

In developing seeds of Arabidopsis, the combined action of PDCT and LPCAT enzymes accounts for 2/3rd of the flux of PUFAs from PC into the TAG. This is supported by a study of acyl editing by metabolic labeling of developing *lpcat1*/*lpcat2* seeds using [^{14}C] acetate. *lpcat1*/*lpcat2* mutant cannot incorporate newly synthesized fatty acids into PC, and the triple mutant *rod1/lpcat1*/*lpcat2* had accumulated only 1/3rd of PUFA as compared to WT (Bates et al., 2012).

1.5 Critical genes involved in seed development, maturation, dormancy, and lipid metabolism

In our study presented in Chapter 2, we are interested in identifying critical regulatory genes which are directly or indirectly involved in response to the low oil content of *dgat1-1* seeds. The following sections will discuss some of the candidate genes identified in our study and their respective roles in seed development and lipid metabolism.

1.5.1 Role of abscisic acid signaling in regulating seed dormancy and germination *dgat1-1*

In the *dgat1-1* mutant, there is a delay in seed germination (Routaboul et al., 1999). Exogenous abscisic acid (ABA) did not affect radicle emergence in *dgat1-1* mutant but had an inhibitory effect on cotyledon emergence and seedling growth as compared to WT. In the *dgat1-1* mutant, there is an increased sensitivity to ABA, osmotic, salt and cold stress (Lu and Hills, 2002).

Seed development precedes chlorophyll degradation, and cell elongation which coincides with the accumulation of storage compounds Seed dormancy suppresses seed germination to favor seed dispersal and time plant growth and reproduction under optimal conditions (Bewley, 1997). Upon seed maturation, the seed acquires desiccation tolerance (DT) which is linked to accumulation of oligosaccharides antioxidants and seed storage proteins including LATE EMBRYOGENESIS ABUNDANT (LEA) and HEAT SHOCK PROTEINs (HSP) (Nguyen et al., 2015; Chatelain et al., 2012; Rajjou et al., 2012; Kotak et al., 2007; Bailly, 2004; Baud et al., 2002; Wehmeyer and Vierling, 2000)

The plant hormone, abscisic acid (ABA) plays a critical role in seed storage accumulation, desiccation tolerance, chlorophyll degradation and induction of dormancy (Finkelstein, 2013; Footitt et al., 2011; Finkelstein et al., 2008; Finch-Savage and Leubner-Metzger, 2006; Phillips et al., 1997). The seeds of mutants including *abi1-1*, *abi2-1*, and *abi3-1*, which lack ABA production or signal transduction of ABA are completely non-dormant (Koornneef et al., 1984; Koornneef et al., 1982). Also, seeds from mutants like *cyp707a2* and *era1*, which over accumulate ABA or are hypersensitive to ABA, have an enhanced dormancy phenotype (Kushiro et al., 2004; Cutler et al., 1996)

In Arabidopsis, levels of ABA increase during seed maturation, and ABA accumulation positively regulates the expression of maturation genes encoding for 2S and 12S globulins (Guerche et al., 1990; Pang et al., 1988), and *LEA* genes which are known to be involved in desiccation tolerance (Hoekstra et al., 2001). The transcription factor ABA-INSENSITIVE3 (ABI3) is considered to be a global regulator of seed maturation and is involved in regulation of *LEA* genes (Nambara et al., 1992). In the *abi3-1* mutant, the expression of *LEA* genes is downregulated as compared to WT. ABA-INSENSITIVE5 (ABI5) is a transcription factor that acts as a positive regulator of ABA response by binding to the ABA-responsive element (ABRE) present in the promoter of the *LEA1* and *LEA6* gene (Reeves et al., 2011; Bensmihen et al., 2002; Carles et al., 2002) to positively regulate their expression. In the *abi5-1* mutant, expression of *LEA1* and *LEA6* is downregulated during seed maturation (Finkelstein, 1994; Finkelstein, 1993; Gaubier et al., 1993). ABI5 acts downstream of ABI3, and their interaction has been shown by a yeast two-hybrid experiment (Nakamura et al., 2001). During seed maturation, ABI5-related bZIP proteins mediate the recruitment of ABI3 proteins to its target genes (Bensmihen et al., 2002).

1.5.2 Role of *DOG1* gene in regulating seed dormancy and germination

DELAY OF GERMINATION 1 (*DOG1*) has been identified as a major QTL for seed dormancy among natural Arabidopsis accessions (Bentsink et al., 2010). There is a positive correlation between seed dormancy and *DOG1* transcript and protein levels (Graeber et al., 2014; Nakabayashi et al., 2012; Footitt et al., 2011; Kendall et al., 2011). The *DOG1* gene is highly conserved in monocots and dicots (Ashikawa et al., 2010; Graeber et al., 2010; Sugimoto et al., 2010). *DOG1* is expressed in seeds, and highest expression is detected during middle stages of seed maturation, with expression reduced in the mature seed. The gene structure of *DOG1* (Figure 1.11) is comprised of 3 exons, and the junction of exon2 and exon3 is subject to alternative splicing, generating five different transcripts designated as *DOG1-α*, *DOG1-β*, *DOG1-γ*, *DOG1-δ* and *DOG1-ε* (Nakabayashi et al., 2015). *DOG1-ε* transcript represents 90-95% of the total transcript level at any stage of seed maturation. The second most abundant transcript is *DOG1-α* which peaks during early stages of seed maturation, whereas *DOG1-δ* is the least abundant isoform which increases towards the end of seed maturation. *DOG1* transcript variants encode three different protein isoforms named as *DOG1-β*, *DOG1-γ*, and *DOG1-ε* all of which encode for the same protein (Nakabayashi et al., 2015). DOG1 is predominantly present in the nucleus, which suggests its possible role as a transcriptional regulator (Nakabayashi et al., 2012).

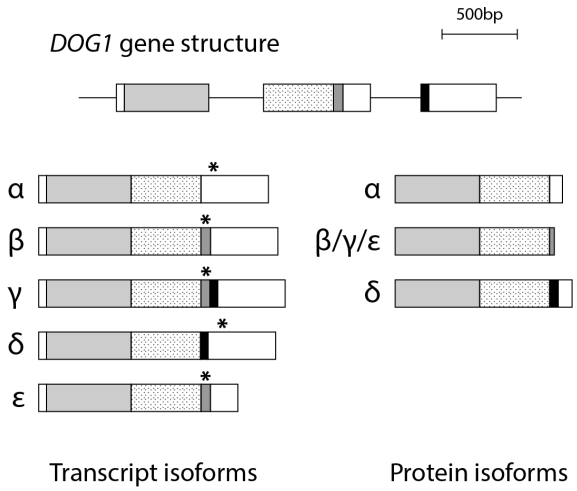


Figure 1.11 Schematic representation of transcript isoforms and protein variants for *DOG1* gene.

The genomic structure of *DOG1* is presented at the top. Exons are shown as boxes, and introns as lines; asterisk represents STOP codon. Different transcripts of *DOG1* and protein isoforms for DOG1 are shown in the lower panel. Adapted from Nakabayashi et al., 2015.

In Arabidopsis, DOG1 belongs to a small family of proteins which contain three conserved domains of unknown function namely: PD870616, PD004114, and PD388003. *DOG1-like* genes do not affect seed dormancy (Bentsink et al., 2006). The *dog1*-1 mutant does not produce full-length DOG1 protein and results in nondormant seeds (Ashikawa et al., 2014). Functional complementation of *dog1-1* mutant using single *DOG1* transcript isoforms expressed under native promoter failed to restore the seed dormancy. Two independent transgenic lines containing two isoforms of DOG1, namely DOG1-β with DOG1-α and DOG1-β with DOG1-δ showed relatively higher levels of seed dormancy as compared to the double transgenic line containing DOG1-α and DOG1-δ isoforms (Nakabayashi et al., 2015).

1.5.2.1 DOG1 and ABA are both required for dormancy, but function in independent pathways

The effect of DOG1 on seed dormancy depends on seed maturation conditions like temperature. Changes in the *DOG1* expression are not affected by changes in the expression of

genes involved in ABA anabolism or catabolism. High levels of DOG1 cannot compensate for the absence of dormancy in the *aba1* mutant, which is defective in ABA biosynthesis. DOG1 therefore acts in parallel to ABA signaling and functions as a timer for the release of seed dormancy (Nakabayashi et al., 2012). *abi3-1*, a weak allele mutant, has non-dormant seeds which are ABA-insensitive (Clerkx et al., 2004; Ooms et al., 1993), whereas the stronger allele *abi3-5* results in green seeds, which are non-dormant and impaired in storage protein accumulation (Nambara et al., 1995; Ooms et al., 1993). The double mutant *dog1-1 abi3-1* resulted in a phenotype similar to *abi3-5*, suggesting that the *dog1-1* mutant allele is an enhancer of a weak *abi3-1* allele (Dekkers et al., 2016). ABI5 acts downstream of DOG1. There is a large set of common genes, which are enhanced in both *dog1-1* and *abi5-7*, suggesting that DOG1 activates ABI5 to induce seed maturation genes and suppress a set of seed germination related transcripts (Dekkers et al., 2016).

ABA affects seed dormancy by reducing the activity of protein phosphatase 2C (PP2C) clade-A proteins such as ABA-INSENSITIVE1 (ABI1) and ABA-INSENSITIVE2 (ABI2) which are involved in the release of seed dormancy (Ma et al., 2009; Merlot et al., 2001). DOG1 controls dormancy by suppressing the action of specific PP2C phosphatases including ABA-HYPERSENSITIVE GERMINATION 1 (AHG1) and ABA-HYPERSENSITIVE GERMINATION 3 (AHG3). This mechanism of action of DOG1 is supported by an observed increase in dormancy of *ahg1 ahg3* double mutant (Nee et al., 2017).

1.5.3 Phosphatidylethanolamine-binding protein (PEBP) family

A family of phosphatidylethanolamine-binding protein (PEBP) is highly conserved among bacteria, animals and plants and functions in different signaling pathways involved in growth and

differentiation (Karlgren et al., 2011; Ahn et al., 2006; Hanzawa et al., 2005; Chautard et al., 2004; Kardailsky et al., 1999; Kobayashi et al., 1999; Yeung et al., 1999). Members of this family include *FLOWERING LOCUS T* (*FT*) and *TERMINAL FLOWER 1* (*TFL1*). In Arabidopsis, four homologs of *FT* and *TFL1* have been identified: *Arabidopsis thaliana CENTRORADIALIS homologue* (*ATC*) (Mimida et al., 2001) , *BROTHER OF FT AND TFL1* (*BFT*), *MOTHER OF FT AND TFL1* (*MFT*), and *TWIN SISTER OF* (*TSF*) (Kobayashi et al., 1999). The members of the PEBP family share 50-60% amino acid sequence similarity but are functionally different (Wickland and Hanzawa, 2015). The phylogenetic relationship between different members was computed by alignment of amino acid sequence (Figure 1.12).

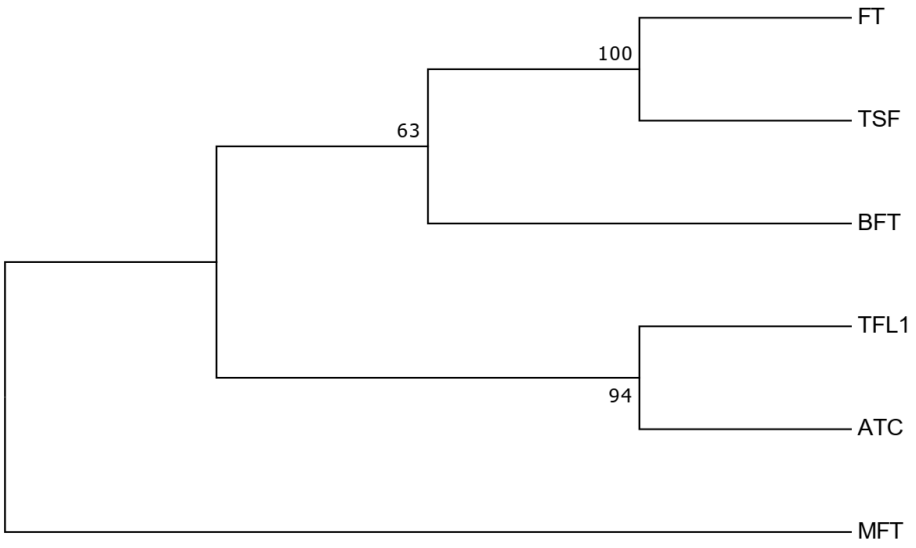


Figure 1.12 Phylogenetic relationship between members of the PEBP family in Arabidopsis.

A phylogenetic tree, constructed by the maximum-likelihood method, for six different members of PEBP family. Bootstrap values are shown at each node of the tree. MEGA 7 software was used to construct the phylogenetic tree.

FT and TFL1 proteins have been reported to function as activators and repressors of flowering respectively (Kardailsky et al., 1999; Kobayashi et al., 1999; Bradley et al., 1997). FT has been reported to bind specifically to phosphatidylcholine (PC) (Nakamura et al., 2014). Structural studies of mammalian PEBPs have identified few conserved residues - Asp70, His86, Tyr120,

and Gly110 - which form a ligand binding pocket that binds to various anionic ligands (Simister et al., 2002; Banfield et al., 1998; Serre et al., 1998). In addition to conserved ligand binding sites, a ligand binding pocket similar to mammalian PEBP has been observed in FT and TFL1, with the presence of Val120 instead of Tyr120 in FT and Phe120 instead of Tyr120 in TFL1 (Ahn et al., 2006). Additionally, the lack of interaction between Tyr85 and Gln140 in FT and interaction between Asp144 and His88 in TFL1 have been proposed to be important in affecting the ligand binding site and thus the functional specificities of FT and TFL1 (Ahn et al., 2006) (Figure 1.13).

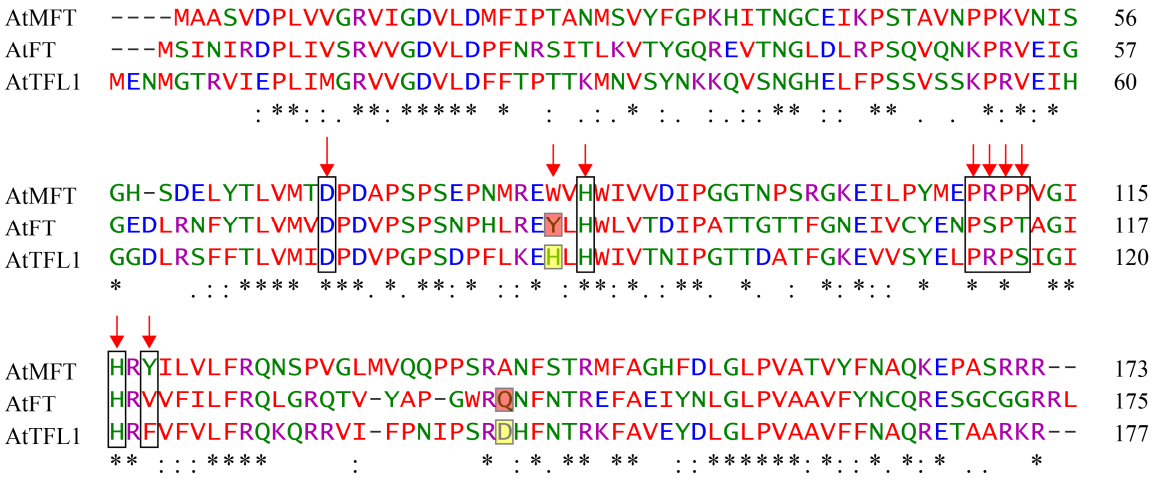


Figure 1.13 Comparison of amino acid sequences for AtMFT, AtFT, and AtTFL1.

Vertical arrows and boxes represent the predicted substrate binding sites. For each sequence, boxes with yellow fill indicates residues which interact with each other and box with red box indicate residues which do not interact. * asterisk indicates positions which have a single, fully conserved residue. : colon indicates conservation between groups of strongly similar properties, and a period indicates conservation between groups of weakly similar properties. Multiple sequence alignment for amino acid sequences was generated using Clustal Omega.

Characterization of other members of PEBP family has suggested functional similarities among FT and TSF in promoting flowering (Yoo et al., 2004) and TFL1 and ATC in repressing flowering (Huang et al., 2012). Even though BFT shares higher amino acid sequence similarity

with FT, it functions in a TFL-like manner and has a role in repressing genes involved in flowering (Yoo et al., 2010).

1.5.4 Role of MFT in seed dormancy and germination

MOTHER OF FT AND TFL1 (MFT) act in response to changes in level of phytohormones like abscisic acid (ABA), gibberellic acid (GA), brassinosteroids (BR) and jasmonic acid (JA) (Dave et al., 2016; Xi et al., 2010; Xi and Yu, 2010). ABA is involved in the regulation of growth, development and physiological processes including seed development, maturation, desiccation, dormancy and germination (Feng et al., 2014; Tahtiharju and Palva, 2001). Different *ABA-insensitive* (*ABI*) genes are involved in ABA signaling (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Finkelstein et al., 1998; Leung et al., 1997; Leung et al., 1994; Meyer et al., 1994; Giraudat et al., 1992). *ABI1* and *ABI2* encode for protein phosphatases PP2Cs (Rubio et al., 2009; Hoth et al., 2002; Merlot et al., 2001; Gosti et al., 1999; Sheen, 1998; Leung et al., 1997; Leung et al., 1994; Meyer et al., 1994) and both are negative regulators of ABA signaling (Sheen, 1998; Sheen, 1996). *ABI3*, *ABI4*, and *ABI5* encode transcription factors containing B3, APETALA2-like, and basic leucine zipper (bZIP) domains respectively and act as positive regulators of ABA signaling (Finkelstein and Lynch, 2000; Finkelstein et al., 1998; Giraudat et al., 1992). Ectopic expression of *ABI3* or *ABI4* results in accumulation of mRNA for *ABI5* (Soderman et al., 2000) and therefore are considered positive regulators for *ABI5* (Chen et al., 2008; Lopez-Molina et al., 2002; Lu et al., 2002). Kinases and phosphatases play an essential role in the activation of various ABA-responsive genes (Hauser et al., 2011; Hirayama and Shinozaki, 2007; Verslues and Zhu, 2007). In Arabidopsis, three kinases named SnRK2.2 (SRK2D), SnRK2.3 (SRK2I), and SnRK2.6 (OPEN STOMATA1/OST1/SRK2E) (Fujii et al., 2007; Mustilli et al., 2002; Yoshida et al., 2002) have

redundant function and are activated by ABA (Hubbard et al., 2010; Boudsocq et al., 2004; Yoshida et al., 2002) and repressed by ABI1 (Xie et al., 2012; Umezawa et al., 2009; Vlad et al., 2009). These kinases act as positive regulators of ABA signaling during early seed development, maturation, and germination (Fujii and Zhu, 2009; Nakashima et al., 2009; Fujii et al., 2007; Umezawa et al., 2004; Mustilli et al., 2002; Yoshida et al., 2002). Another transcription factor called Related to ABI3/VP1 (RAV1) plays an important role in ABA signaling during seed development and seed germination (Feng et al., 2014), and belongs to the A-PETALA2/Ethylene Responsive Factor (AP2/ERF) family (Dietz et al., 2010; Feng et al., 2005; Riechmann et al., 2000). Phospholipase D (PLD) is involved in mediating ABA response (Zhang et al., 2004). In Arabidopsis, 12 different phospholipase D (PLD) encoding genes have been identified (Qin and Wang, 2002). Different PLDs have distinct catalytic and regulatory roles (Wang, 2004; Meijer and Munnik, 2003; Jacob et al., 1999; Ritchie and Gilroy, 1998). In response to ABA, PLDα1 is activated and acts on phospholipids to produce phosphatidic acid (PA) (Figure 1.14). PA binds to ABI1 and anchors it to the plasma membrane, which affects the movement of ABI1 to the nucleus. ABI1 is involved in activation of a homeodomain leucine zipper class I (HD-Zip I) protein named ATHB6, which is a negative regulator of ABA response (Zhang et al., 2004). RAV1 is a negative regulator of ABA response and acts by binding directly to the promoter of *ABI3*, *ABI4,* and *ABI5* (Feng et al., 2014). Further, in response to ABA, the SnRK2 kinase is activated which phosphorylates and inactivates RAV1. Inactivation of RAV1 leads to activation of ABI3, which further activates and acts upstream of ABI5 (Lopez-Molina et al., 2002). In addition, SnRK2 phosphorylates and activates ABI5. ABI3 is a negative regulator of MFT and acts by directly binding the promoter of *MFT* gene. On the other hand, ABI5 is a positive

regulator of MFT and also acts by directly binding the promoter of *MFT*. MFT antagonizes ABA signaling by downregulation of ABI5 by a negative feedback loop (Xi et al., 2010).

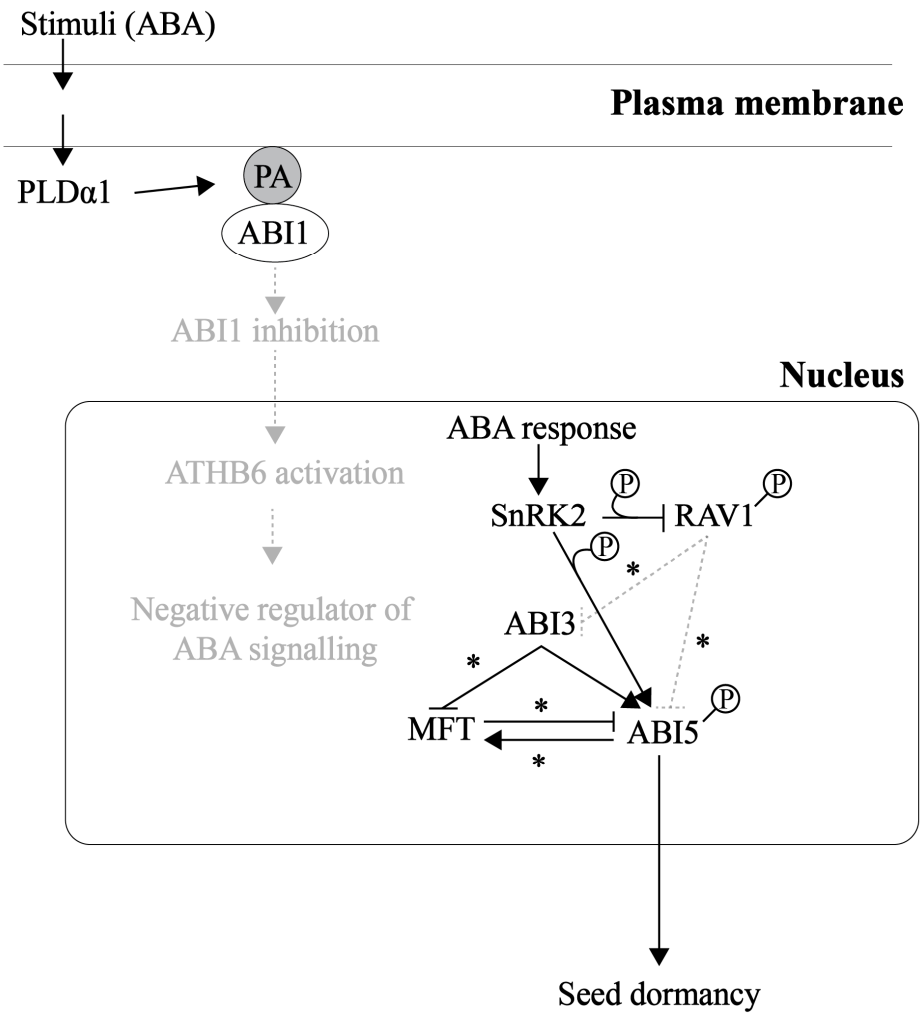


Figure 1.14 A proposed model of seed germination mediated by MFT in response to ABA stimuli.

ABA regulates *MFT* expression through the activation of SnRK2 and suppression of RAV1. SnRK2 further phosphorylates and activates ABI5 which is the promoter of *MFT* expression. Upon activation by ABI5, MFT represses ABI5 by negative feedback regulation. Text and lines highlighted in grey represent suppressed pathway. Ⓟ represents the phosphorylation state. * Asterisk indicates direct transcriptional regulation. Figure adapted from Xi and Yu, 2010; Zhang et al., 2004.

In after-ripened, non-dormant seeds from Arabidopsis, MFT negatively regulates ABA signaling and promotes germination. Germination assay conducted using freshly mature seeds indicated that *mft-2* seeds were significantly less dormant relative to corresponding WT seeds

from two different ecotypes Col-0 and L*er* (Vaistij et al., 2013). In freshly matured dry seeds, ABA levels were almost twice in *mft-2* relative to WT levels, whereas after-ripened seeds of *mft-2* are hypersensitive to exogenous ABA. Moreover, *MFT* expression is promoted by ABI5 and RGL2 which are positive regulators of seed dormancy (Xi et al., 2010). These results propose the role of MFT in promoting dormancy during seed development and germination of after-ripened imbibed seeds via the ABA signaling pathway (Vaistij et al., 2013).

Brassinosteroids (BR) are conserved in all plants and are mainly found in pollen and seeds (Schmidt et al., 1997). Increased ABA sensitivity and reduced male sterility observed in the BR biosynthesis mutant supports the vital role of BR in male fertility, seed development, and germination. One of the mutants, *det2-1* which affects BR biosynthesis results in reduction in plant stature and male fertility (Fujioka et al., 1997; Chory et al., 1991) whereas the double mutant *det2-1 mft-2* has a much more severe phenotypic effect on plant stature and male fertility relative to the single *det2-1* mutant. These results indicate the redundant roles of DET2 and MFT in plant development (Fujioka et al., 1997; Chory et al., 1991).

Jasmonic acid (JA) has an essential role in plant growth and development and helps in regulating different plant responses to both biotic and abiotic stresses (Wasternack, 2014; Santino et al., 2013; Wasternack et al., 2013; Balbi and Devoto, 2008). JA and its precursor 12-oxo-phytodienoic acid (OPDA) are oxylipins derived from α-linolenic acid (Wasternack and Hause, 2013). The *pxa1-1* mutant has disrupted the activity of the ATP binding cassette (ABC) transporter CTS which is involved in the transport of OPDA into peroxisomes for JA synthesis (Footitt et al., 2002; Hayashi et al., 2002; Zolman et al., 2001; Russell et al., 2000). Lower germination rates have been attributed to increased levels of OPDA in the *pxa1-1* mutant (Dave et al., 2011). OPDA and ABA pathways are linked and act synergistically to suppress seed

germination (Dave et al., 2011). Additionally, endogenous and exogenous OPDA levels result in protein accumulation of ABI5 which in turn is a positive regulator of MFT. *MFT* expression is upregulated in developing seeds of *pxa1-1* mutant as compared to WT. Increased expression of *MFT* was also observed when after-ripened seeds were exogenously treated with OPDA. These results suggest that both high levels of exogenous and endogenous OPDA induce the expression of *MFT*. Lower levels of endogenous OPDA was observed in the *mft-2* mutant. Further, when treated with exogenous OPDA, ABA levels in after-ripened seeds of *mft-2* were comparatively lower than WT suggesting that OPDA requires MFT for triggering ABA biosynthesis (Dave et al., 2016).

1.5.5 Phospholipase family

In plants, phospholipases are a critical component of metabolism and intracellular signaling during cellular growth, development and stress response. Phospholipase C (PLC) acts on a phospholipid substrate and generates a diacylglycerol (DAG) molecule and a water-soluble phosphorylated head group (Pokotylo et al., 2013). Based on the substrate specificity PLCs can be sub-divided into phosphatidylcholine-specific phospholipases (PC-LPC) and phosphatidylinositol-specific phospholipases C (PI-LPC). In plants, PI-PLC and PC-PLC generate products like DAG and PA which are further involved in lipid remodeling upon phosphate starvation and lipid signaling (Wang, 2014). DAG and PA intermediates are acted upon by different kinases such as diacylglycerol kinase (DGK) and PA kinase (PAK), and phosphatases like PA phosphatase (PAP), and diacylglycerol pyrophosphate phosphatase (DPP) to form phosphorylated and dephosphorylated derivatives of DAG, PA and diacylglycerol pyrophosphate (DGPP) (Wang, 2014). PI-PLCs have been reported to be involved in growth, development and stress responses (Vossen et al., 2010) whereas PC-PLCs, which are also known

as non-specific phospholipases (NPC), have been proposed to be involved in signaling (Scherer et al., 2002). Based on homology search using bacterial PC-LPC, six genes have been identified in Arabidopsis which are encoded by genes named *AtNPC1* through *AtNPC6* (Nakamura et al., 2005). The general illustration of lipid signaling mediated by phospholipases is shown in Figure 1.15.

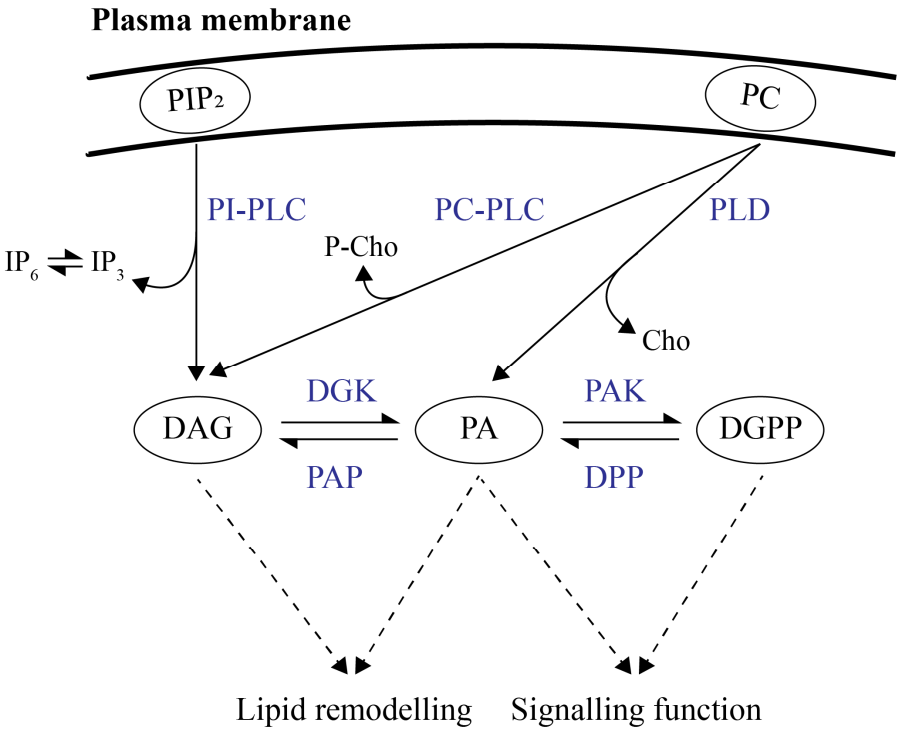


Figure 1.15 Phospholipase C- and phospholipase D-dependent signaling in plants.

A schematic diagram depicts a model of metabolism regulation carried out by plant cell phospholipases. Different pathways demonstrate synergistic interactions between phospholipases and lipid second messenger molecules in excitation of cell responses. PIP_2, phosphatidylinositol 4,5-bisphosphate; PC, phosphatidylcholine; NPC, non-specific phospholipase C; PI-PLC, phosphatidylinositol-specific phospholipase C; PLD, phospholipase D; DAG, diacylglycerol; PA, phosphatidic acid; DGPP, diacylglycerol pyrophosphate; IP3/IP6, inositol 1,4,5-trisphosphate/inositol hexakisphosphate; DGK, diacylglycerol kinase; PAP, phosphatidic acid phosphatase; PAK, phosphatidic acid kinase; DGPPP, diacylglycerol pyrophosphate phosphatase. Figure adapted from Pokotylo et al., 2013.

Amino acid sequence comparison of Arabidopsis AtNPCs with bacterial PLC showed the presence of separate conserved domains in Arabidopsis AtNPCs which are unrelated to known

domains of bacterial LPC (Nakamura et al., 2005). These results indicate that during evolution, the NPC family acquired different sequence features in bacteria, plants, and animals. In plants, features of NPC family include the presence of an N-terminal signal peptide, followed by a short variable region, and a conserved sequence ENRSFDxxxG at the start of phosphoesterase domain. In Arabidopsis, different members of the NPC family differ in their interacting partners and cellular localization which is attributed to the divergent 50-100 amino acids region towards the C terminal. Phylogenetic analysis of NPC genes from distinct plant species revealed that the common ancestor for all seed plants had at least *NPC1*, *NPC2*, and *NPC6*-like genes. These results suggest that among the different members of the NPC family present in seed plants, *NPC1*, *NPC2*, and *NPC6* are highly conserved. Neither of *NPC3*, *NPC4*, and *NPC5* was identified among the available EST sequences of gymnosperms. Further, the signal peptide is missing in *NPC3*, *NPC4*, and *NPC5*. Based on the phylogeny data, one hypothesis states that after the ancestral separation of angiosperms from gymnosperms, *NPC3*, *NPC4*, and *NPC5* originated in angiosperms from a common ancestor *NPC2* (Pokotylo et al., 2013).

Using the model structure of bacterium *F. tularensis* (Felts et al., 2006), a 3D model for Arabidopsis NPC family was generated indicating the presence of negatively charged pocket which is involved in phospholipid substrate binding. Expression of recombinant AtNPC3 protein in *E. coli* has been shown to have lysophosphatase activity which results in the formation of MAG instead of DAG suggesting multivalent functionality of NPCs (Reddy et al., 2010). AtNPC3 protein lacks activity for substrates including PA, PS, PC, LPC, PE, and LPE. Expression of recombinant AtNPC4 protein in *E. coli* has shown activity for PC, PE, PS, PG and negligible activity for PA, PIP_2 (Peters et al., 2010; Nakamura et al., 2005). Expression of

recombinant AtNPC5 protein in *E. coli* has shown activity for PC and PE, with the activity being more than 40-fold lower than AtNPC4 (Gaude et al., 2008).

Subcellular localization experiments showed that AtNPC3 is localized to tonoplast, AtNPC4 is localized to plasma membrane whereas AtNPC5 is present in the cytosol. One theory for the presence of AtNPC5 in the cytoplasm is that it could be transported to the membrane upon stimulation, similar to PLDα in Arabidopsis (Wang et al., 2000). Localization of the other NPCs is not known but is predicted to localize to endomembranes despite the lack of transmembrane domains. Localization of NPCs to endomembranes can be explained by the packaging and transport of proteins from ER to Golgi apparatus by membrane vesicles. Membrane vesicles transport the protein to Golgi apparatus where the protein is further modified by either the glycosylation or phosphorylation which aids in targeting the protein to a specific cellular compartment or the protein is secreted outside the cell.

In Arabidopsis, the transcript levels of all NPC genes were determined in roots, floral organs, siliques and leaves at various stages of development. Spatial analysis of transcript abundance revealed the varied distribution of different NPC genes in Arabidopsis. *AtNPC1* expression was relatively higher in root, stem, leaves and was low in pollen. *AtNPC2* was upregulated in siliques and during early stages of seed development, whereas it was low in roots and leaves. *ATNPC3* had higher expression in root, stem, dry seed, and early germinating seeds. *AtNPC4* and *AtNPC5* expression levels were detected in root and developing floral organs but had very low expression in pollen, but the expression of *AtNPC5* was significantly lower than *AtNPC4*. *AtNPC6* was expressed at higher levels in shoot apical meristem, leaf primordia and floral organs but can also be detected in young leaves (Pokotylo et al., 2013).

Genes encoding for different NPC have an important physiological role. NPC acts on the substrate PC and results in the formation of DAG which is an intermediate in lipid metabolism and acts as an important signaling molecule. DAG can be rapidly phosphorylated to form an important signaling molecule called PA. In the *npc4* mutant, DAG levels are reduced in leaves with no changes in the overall profile of DAG species. Also, lower levels of MGDG and DGDG are observed in the *npc4* mutant (Peters et al., 2010).

1.5.6 Lipase superfamily

1.5.6.1 Role of lipases in seed development and maturation

Lipases/hydrolases are ubiquitously expressed in different tissues of plants. Hydrolases play a significant role in catabolic processes during seed maturation (Graham, 2008) and lipid mobilization during and post seed germination (Quettier and Eastmond, 2009; Iwai et al., 1984). A study investigated the role of *SUGAR-DEPENDENT1* (*SDP1*) and *SDP1-LIKE* (*SDP1L*), both of which encode for lipase and have important role during seed development and germination (Kelly et al., 2011; Eastmond, 2006). *SDP1* is expressed in all tissues, but the expression is relatively higher during seed development. RNAi based suppression of *SDP1* during seed development resulted in an 8% increase in oil yield suggesting its essential role in lipid homeostasis during seed development (Kelly et al., 2013).

Many hydrolases belong to the alpha/beta hydrolase fold superfamily. Members of the ABH superfamily are versatile in function and play an important role in catalytic processes in primary and secondary metabolism. ABH fold-containing proteins belong to different classes including esterases, thioesterases, lipases, proteases, dehalogenases, haloperoxidases, and epoxide hydrolases (De Simone et al., 2001; Nardini and Dijkstra, 1999; Klenk et al., 1998). In

addition to primary role of esterases and peptidases, the ABH family includes members which are involved in breakage of carbon-carbon bonds (Gruber et al., 1999), decarboxylation reactions (Auldridge et al., 2012; Ben-Israel et al., 2009; Yang et al., 2009; Fridman et al., 2005), and cofactor-independent dioxygenation of heteroaromatic rings (Steiner et al., 2010).

1.5.6.2 Conserved domain (catalytic triad) of ABH family

The ABH domain is composed of 8-stranded β sheets surrounded by α helices. The functional diversity of this superfamily is attributed not only to the differences in the number of α-helices and β-sheets but also to the presence of additional structural elements known as lid domains (Nardini and Dijkstra, 1999). Although members of the ABH superfamily share very little homology at the amino acid level, they maintain the overall three-dimensional core architecture (Mindrebo et al., 2016). The Arabidopsis genome contains ~638 genes which contain the ABH fold (Mitchell et al., 2015; Lamesch et al., 2012). The presence of conserved lipase motif (GXSXG) suggests that members of the ABH superfamily play an essential role in lipid metabolism (Lord et al., 2013).

A homolog of human *ABHD11* is present in Arabidopsis. Biochemical studies have reported AtABHD11 to be a lysophospholipase and are supported by *in vivo* and *in vitro* studies. The *abhd11* mutant had an accumulation of galactolipids and phospholipids in leaves and overexpression of *ABHD11* in *E. coli* resulted in the reduction of phospholipids. Comparative transcript analysis from leaf tissue of *abhd11* mutant with WT showed increased expression of genes involved in the biosynthesis of galactolipids (Vijayakumar et al., 2016).

1.5.7 Plastid-localized lipases.

The Arabidopsis genome encodes for ~300 putative lipases, the majority of which are not characterized or have unknown function (Kelly and Feussner, 2016; Li-Beisson et al., 2013; Troncoso-Ponce et al., 2013). In the Chloroplast 2010 project, which aimed at the characterization of all plastid-localized proteins, 46 lipase encoding genes were selected (Ajjawi et al., 2010; Lu et al., 2008). Plastid-localized lipases have been hypothesized to play an important role in the maintenance and signaling of photosynthetic membranes in addition to having a specialized role in developing seeds which have a high demand for lipid metabolism (Wang et al., 2017).

One of the plastid-localized lipases named DEFECTIVE IN ANTHER DEHISCENCE1 (DAD1) has been characterized and belongs to a class of phospholipase called PLA_1. DAD1 catalyzes the initial step of jasmonic acid (JA) production (Ishiguro et al., 2001). A distinct class of lipases called PLASTID LIPASE are localized to the plastid and consists of three members encoded by genes named *PLASTID LIPASE1* (*PLIP1*), *PLASTID LIPASE2* (*PLIP2*), and *PLASTID LIPASE3* (*PLIP3*) (Wang et al., 2018). All three lipases PLIP1, PLIP2, and PLIP3, have been functionally characterized, and share a Lipase 3 domain which is a signature domain for TAG lipases. PLIP1, PLIP2, and PLIP3 localize to different subplastid locations (Wang et al., 2018).

1.5.7.1 PLIP1 contributes to the export of acyl groups from plastid for TAG biosynthesis

PLIP1 is a thylakoid membrane-associated lipase which initiates a mechanism leading to the export of fatty acids derived from thylakoid membrane into the ER for incorporation into the TAG (Wang et al., 2017). *PLIP1* encodes for a 71,734 D protein which contains an N terminal

transit peptide but does not have a predicted transmembrane domain and despite having a Lipase 3 domain is not a TAG lipase. PLIP1 specifically catalyzes the release of a polyunsaturated acyl group from an *sn-1* position of PG, which has 16:1 $^{\Delta 3t}$ at its *sn-2* position (Wang et al., 2017).

A *PLIP1* overexpressing line, named *PLIP1*-OX has smaller rosettes and fewer leaves. *PLIP1*-OX line showed 40-50% increase in seed acyl group content (Wang et al., 2017). There was observed decrease in levels of lipids associated with plastid membrane (MGDG, DGDG, PG), and an increase in levels of lipids associated with ER (PC, PE, PI). The observed lipid phenotype is the result of overproduction of PLIP1, and reduced growth phenotype is attributed to elevated OPDA & JA-Ile levels, increase in expression of JA-responsive genes, production of oxylipins and activation of JA signaling. The *PLIP*-OX line was able to partially reverse the reduced 18:3 phenotype of the *fad3-2* mutant, thus indicating a minor but important role of PLIP1 in the incorporation of 18:3 into the TAG (Wang et al., 2017).

Consistent with the role in TAG production, the *plip1* mutant has 10% reduction in seed acyl group content (Wang et al., 2017). Acyl composition of TAG and PC from dry seeds of *plip1* mutant showed an increase in levels of 18:3 relative to 18:1. The observed increase in 18:3 levels in both *PLIP1*-OX line and *plip1* mutant line, is explained by the reversible compensatory activity of plastidial PLIP1 activity and ER-localized FAD2/3 desaturase activity (Wang et al., 2017).

1.5.7.2 *PLIP2* and *PLIP3* are not involved in seed oil biosynthesis

Comparison of PLIP1 protein sequence against Arabidopsis proteome identified proteins which are closely related to PLIP1. Phylogenetic analysis of top 37 hits, lead to the identification of putative paralogs of PLIP1 named PLIP2 and PLIP3 which clustered together with PLIP1

(Figure 1.16). PLIP2 and PLIP3 only share 57% and 51% amino acid sequence similarity respectively with PLIP1, whereas PLIP2 and PLIP3 are more closely related and share 65% amino acid similarity (Wang et al., 2018).

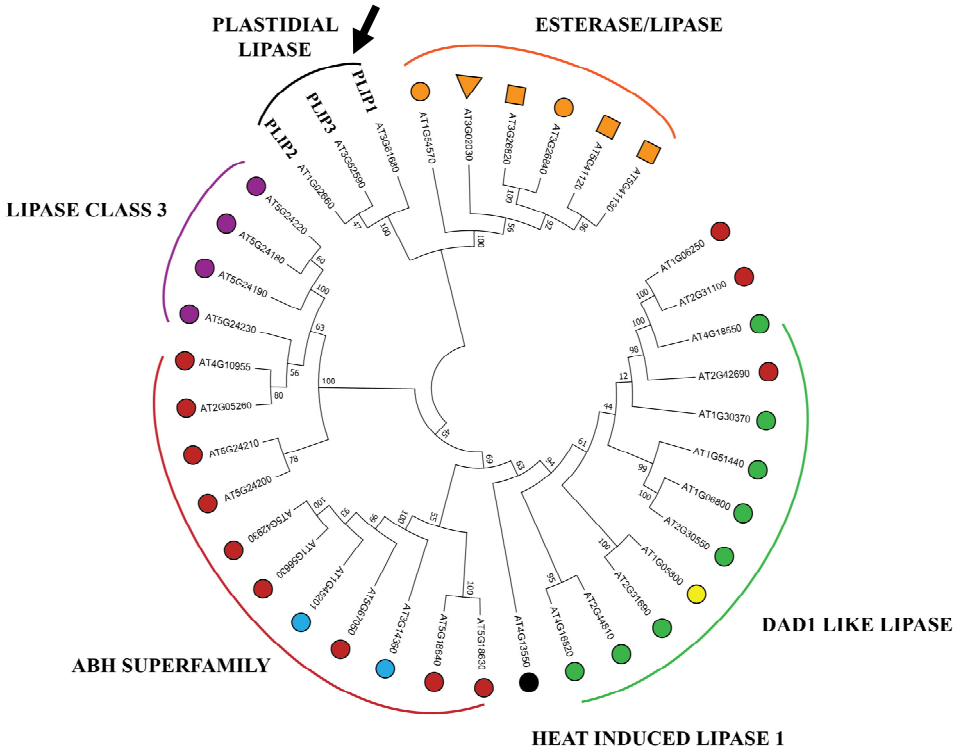


Figure 1.16 Maximum Parsimony Tree of PLIP1-similar protein sequences and other characterized lipase sequences from *A. thaliana*

Sequence alignment and phylogenetic analyses of PLIP1-similar protein sequences and other characterized lipase was done with full-length amino acid sequences using MEGA version 7 (Kumar et al., 2016). The black arrow indicates the position of PLIP1 on the tree.

PLIP2 and *PLIP3* encode for 78,346 D and 73,044 D protein respectively. PLIP2 and PLIP3 are plastid localized and have a similar predicted topology with four transmembrane domains and an N terminal transit peptide. PLIP2 and PLIP3 have distinct subplastid locations. PLIP2 is ubiquitously present in the plastid envelope membranes, stroma, and thylakoids, whereas PLIP3 is membrane protein and is embedded in the plastid envelope membranes and thylakoids. Similar to PLIP1, PLIP2 and PLIP3 have a conserved Lipase 3 domain, and both

belong to the class of PLA_1. Despite the presence of Lipase 3 domain, PLIP2 and PLIP3 lipases hydrolyze plastidial glycerolipids (Wang et al., 2018).

PLIP2 specifically catalyzes the hydrolysis of an acyl group from an *sn-1* position of MGDG. PLIP2 is capable of hydrolyzing both saturated and unsaturated acyl group at the *sn-1* position of MGDG (Wang et al., 2018). PLIP3 is capable of hydrolyzing acyl group at the *sn-1* position of PG. Pulse-chase experiments demonstrated that 18:3 was preferentially present at the *sn*-1 position of MGDG and PG substrates for PLIP2 and PLIP3 respectively. Acyl composition analysis of overexpressing lines for *PLIP2* and *PLIP3* showed a similar result with a decrease in 16:3/18:3 ratio for MGDG and decrease in ratio of 16:1/16:0 for PG respectively. *PLIP2*-OX and *PLIP3*-OX lines displayed reduced rosette size and smaller petioles and were similar to the phenotype observed for *PLIP1*-OX (Wang et al., 2018).

The *PLIP2* transcript is highly expressed in pollen, and *PLIP3* transcript is predominantly expressed in senescent leaf tissues, pollen, and embryos (Wang et al., 2018). Similarities in enzymatic activities for PLIP1, PLIP2 and PLIP3 followed by similar changes in lipid composition in their respective overexpressing lines, suggest that all PLIPs efficiently release and export the polyunsaturated fatty acids from plastid to ER. *PLIP1* and *PLIP3* genes are closely linked on the same chromosome, whereas the function of PLIP2 and PLIP3 is not redundant with PLIP1, as neither *plip2* nor *plip3* mutant display any changes in seed oil content. Lipid analysis of single, double and triple mutant lines generated using *plip1*, *plip2* and *plip3* mutants showed that decreased oil content is genetically exclusively linked to *plip1* mutant (Wang et al., 2018).

1.5.7.3 Regulation of *PLIP* gene expression by abiotic stress

Sequence analysis of upstream sequences for *PLIP1*, *PLIP2* and *PLIP3* show the presence of ABA response elements (ABRE) elements upstream of the transcription start site (TSS) (Figure 1.17). In 2-week old seedlings, expression of *PLIP2* and *PLIP3* but not of *PLIP1* was upregulated in the presence of exogenous 7 µM ABA (Wang et al., 2018). Additionally, based on the transcriptome database, *PLIP2* is induced by cold stress, and *PLIP3* is induced by osmotic stress. As opposed to the WT, failure of *plip* triple mutant lines grown in ABA containing MS medium to produce JA confirmed that PLIPs mediate the ABA-induced JA production. Additionally, the *plip* triple mutant is hypersensitive to ABA. Decreased ABA levels and increased JA levels in *PLIP2*-OX and *PLIP3*-OX lines is explained by a possible feedback regulatory loop by which JA antagonizes ABA anabolism (Wang et al., 2018).

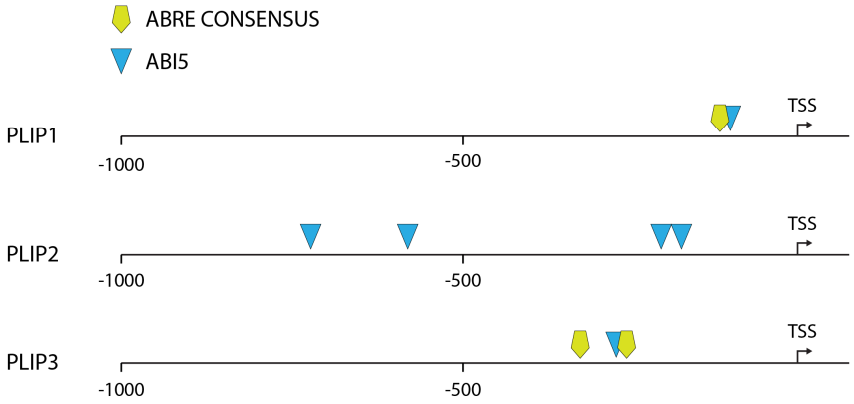


Figure 1.17 *In silico* analysis of cis-regulatory elements in the promoter region for members of PLIP family.

1000bp sequence upstream of the transcription start site of *PLIP1*, *PLIP2* and *PLIP3* were used to screen for cis elements which are targets for ABI genes.

1.6 Research overview

In Chapter 2, a comparative study of whole genome transcript profiling from *dgat1-1* and WT lines of *Arabidopsis thaliana* is described. This led to the identification of novel genes

involved in the regulation of the TAG biosynthesis pathway mediated by PDAT1. Lipid profiling of T-DNA insertional mutant lines for differentially expressed candidate genes *PLIP1*, *MFT,* and *NPC6* is studied with respect to WT. In addition, combining the *dgat1-1* and *plip1-2* mutants resulted in a lethal phenotype, demonstrating the important role of this enzyme in embryo development and TAG biosynthesis.

In Chapter 3, correlation analysis of transcriptomic and lipid profiling of various genes from lipid metabolism, hormone metabolism and seed development pathways at different stages of seed development from *dgat1-1* and WT lines of *Arabidopsis thaliana* is described. Expression profiling of genes belonging to ABA and GA metabolic pathways showed a distinct pattern in *dgat1-1*. Based on the preliminary results of upregulation of *DOG1* and down-regulation of *LEA* encoding genes, further insights into green seed and non-germinating phenotype for *dgat1-1 plip1-2* double mutant can be explored.

In chapter 4, the identification and expression profiling of homoeologs for different genes involved in FA synthesis, acyl editing, and TAG biosynthesis and metabolism from *BjuA* and *BjuB* subgenomes of allopolyploid *B. juncea* is presented. In addition, total lipid content and composition analysis for *B. juncea* from different stages of seed development is described. The identified homoeologs and their expression profiles will help in the selection of subsets of genes for targeting and further manipulations for their use in breeding programs and improving the oil content and composition of lipids in seeds.

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Chapter 2 - Upregulation of a plastid lipase is critical for seed viability in Arabidopsis *dgat1-1* mutant seed

2.1 Introduction

Triacylglycerols (TAG), lipids with three fatty acids esterified to a glycerol backbone, are the major constituent of the seed oil produced by most plants. The highly reduced nature of the fatty acids means that TAGs are considered the most energy-dense products synthesized by plants, making them an ideal energy storage reserve during germination and early stages of seedling development. This high energy density of TAG also means that plant oils have long represented a valuable source of calories for human and animal nutrition. More recently, societal concerns have spurred an interest in obtaining renewable and carbon-neutral fuels and chemicals from plant oils (Durrett et al., 2008; Dyer et al., 2008), expanding the utility and value of TAG. The biosynthesis of TAG in oil seeds, therefore, is important for many aspects of human nutrition and economic activity.

The synthesis and modification of fatty acids and their subsequent incorporation into TAG occurs in both the plastid and the endoplasmic reticulum (ER). In plastids, acetyl-CoA carboxylase (ACC) converts acetyl-CoA to malonyl-CoA, which is then used to synthesize fatty through multiple rounds of condensation, reduction and dehydration reactions. Each round of synthesis adds two carbons to the growing fatty acid chain, which is connected to an acyl carrier protein (ACP). The introduction of the first double bond can also occur in the plastid: while the fatty acid is still conjugated to ACP, the FAB2 Δ9-desaturase converts stearoyl (18:0)-ACP to oleoyl (18:1)-ACP. Acyl-ACP thioesterases then terminate fatty acid synthesis by removing ACP

from the nascent fatty acid, which is then exported from the plastid and conjugated to CoA. Most of these newly synthesized acyl-CoA molecules are used by ER-localized lysophosphatidylcholine acyltransferases (LPCAT) to acylate lysophosphatidylcholine (LPC) to form phosphatidylcholine (PC), a membrane lipid (Wang et al., 2012; Bates et al., 2009). 18:1 can be further desaturated while conjugated to PC through the activity of FAD2, a Δ12-desaturase and FAD3, a Δ15-desaturase, to form the polyunsaturated fatty acids linoleate (18:2) and linolenate (18:3), respectively.

De novo incorporation of fatty acids into glycerolipids also occurs in the ER and involves the sequential acylation of a glycerol-3-phosphate backbone. Glycerol-3-phosphate acyltransferase, encoded by *GPAT9*, adds the first acyl group (Shockey et al., 2016; Singer et al., 2016). The product of this reaction, lysophosphatidic acid (LPA) is further acylated by lysophosphatidic acyltransferases (LPAAT) to form phosphatidic acid (PA). PA phosphatases (PAP) then remove the phosphate group to form diacylglycerol (DAG), a key intermediate for both the synthesis of phospholipids as well TAG. In addition to the de novo DAG formed through this pathway, labeling experiments have suggested the presence of a second, PC-derived DAG pool which could be formed by multiple routes (Bates and Browse, 2011; Bates et al., 2009). For example, phosphatidylcholine: diacylglycerol cholinephosphotransferase (PDCT) transfers a phosphocholine head group between PC and DAG and plays a key role in the flux of polyunsaturated fatty acids into TAG in Arabidopsis seeds (Bates et al., 2012; Lu et al., 2009). In addition, PC can be converted to DAG through phospholipase C or the reverse activity of CDP-choline: diacylglycerol cholinephosphotransferase (CPT) (Vogel and Browse, 1996).

In Arabidopsis seeds, the final acylation of DAG to form TAG occurs primarily through the activity of the acyl-CoA: diacylglycerol acyltransferase DGAT1 (Lung and Weselake, 2006).

Based on mutant phenotypes, DGAT1 plays a key role in the synthesis of TAG in Arabidopsis seeds. Arabidopsis *dgat1-1* mutants possess a 25 to 45% reduction in seed oil content, as well as an altered fatty acid composition, with higher levels of 18:3 and lower levels of eicosenoic acid (22:1) (Routaboul et al., 1999; Zou et al., 1999; Katavic et al., 1995). In addition to DGAT activity, phospholipid:diacylglycerol acyltransferases (PDAT), encoded by *PDAT1* in Arabidopsis, can also synthesize TAG by transferring the acyl group at the *sn*-2 position of phospholipids to the *sn*-3 position of DAG (Ståhl et al., 2004; Dahlqvist et al., 2000). In contrast to *dgat1-1*, *pdat1* mutants do not possess any changes in oil content or fatty acid composition, suggesting an insignificant role in TAG synthesis in wild-type seeds relative to DGAT1 (Mhaske et al., 2005). However, DGAT1 and PDAT1 possess overlapping functions in TAG synthesis in seeds and pollen. A double *dgat1-1 pdat1* mutant genotype in pollen prevented the formation of oil bodies and resulted in abnormal and sterile pollen. Importantly, RNAi silencing of *PDAT1* in the *dgat1-1* background, resulted in a 70 to 80% decrease in oil content, demonstrating that PDAT1 plays a key role in TAG biosynthesis in the absence of DGAT1 function (Zhang et al., 2009). The same study demonstrated that other candidate acyltransferases present in Arabidopsis seed did not possess DGAT activity or lacked any seed oil phenotype when mutated, either individually or in combination with DGAT1.

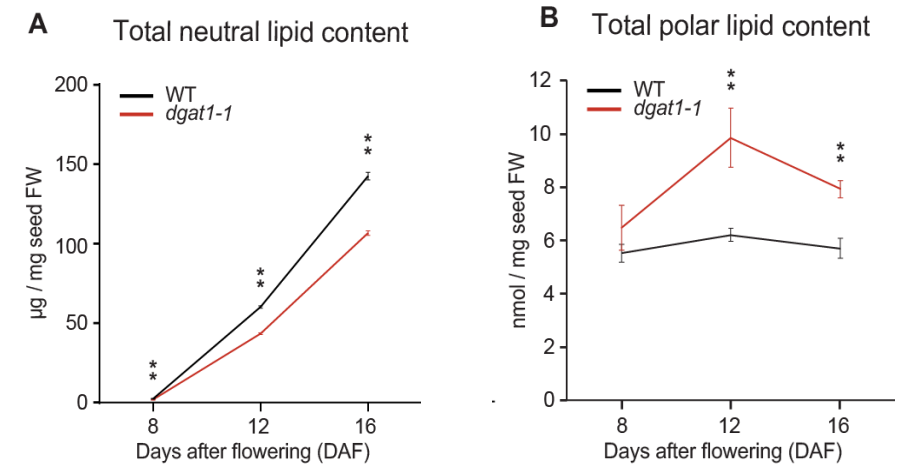
The altered fatty acid composition and the importance of PDAT1 for TAG synthesis in *dgat1-1* seed argue that developing Arabidopsis seed respond to the lack of DGAT1 activity. Given the importance of TAG production, we undertook a transcript profiling approach to better understand how the mutant seeds were responding to the inability to synthesize TAG through DGAT1. The use of RNA-Seq offered advantages over previous microarray studies (Xu et al., 2012), including higher signal to noise ratios, a large dynamic range of expression levels and the

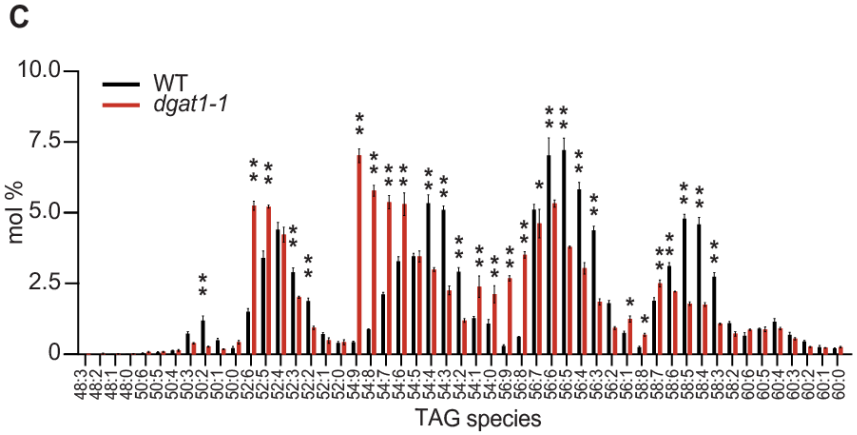
ability to identify novel transcripts (Wang et al., 2009). Through this approach, we identified differentially expressed genes whose altered expression is consistent with the seed phenotypes observed in the *dgat1-1* mutant. Importantly, we were not limited by the choice of transcripts detected by the standard Arabidopsis gene chip, enabling us to identify novel genes associated with TAG biosynthesis in seeds. Mutations in one of these genes that encodes a plastidial lipase resulted in minor changes in oil content, fatty acid composition, and seed size. However, when combined with mutations in *dgat1-1*, these mutations resulted in the seed that failed to germinate. This synthetic lethal phenotype demonstrates the importance of this plastidial lipase in TAG accumulation, particularly when DGAT function is impaired.

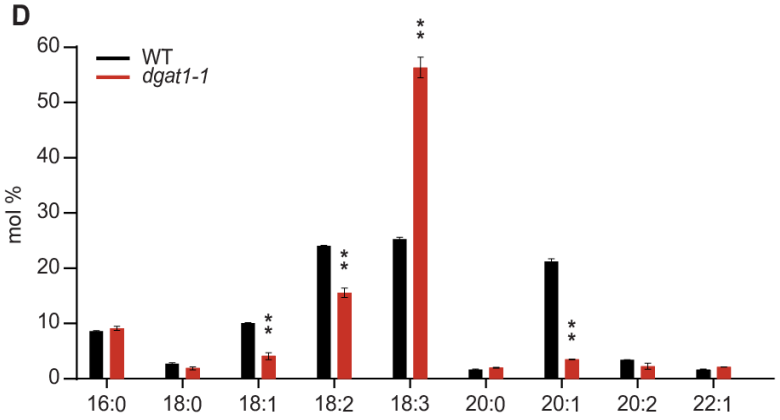
2.2 Results

2.2.1 Altered lipid composition and gene expression in developing *dgat1-1* seeds

To identify differentially expressed genes in *dgat1-1* important for regulating TAG accumulation, we isolated developing seeds during the middle to late embryogenesis when storage lipid synthesis begins (Mansfield and Briarty, 1992). Similar to previous reports (Katavic et al., 1995), we observed a 35% reduction in the neutral lipid content of developing *dgat1-1* seeds relative to those from Col-0 wild-type plants (Figure 2.1A), as well as increased polar lipids (Figure 2.1B). Mutant seed contained more polyunsaturated TAG molecular species and less molecular species with 56 or more acyl carbons (Figure 2.1C), consistent with the increased levels of 18:3 and lower levels of 20:1 characteristic of *dgat1-1* seed (Figure 2.1) (Routaboul et al., 1999; Katavic et al., 1995).


A
Total neutral lipid content
WT
dgat1-1
µg / mg seed FW
Days after flowering (DAF)
B
Total polar lipid content
WT
dgat1-1
nmol / mg seed FW
Days after flowering (DAF)


C
WT
dgat1-1
mol %
TAG species


D
WT
dgat1-1
mol %
16:0
18:0
18:1
18:2
18:3
20:0
20:1
20:2
22:1

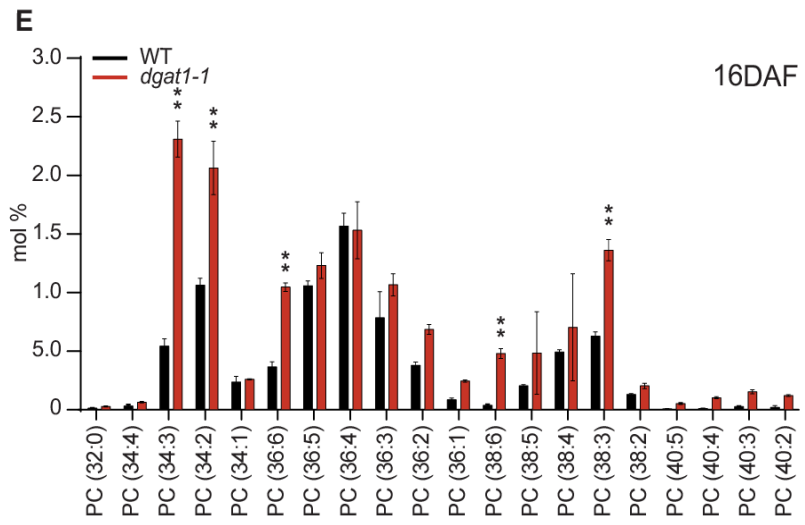


Figure 2.1 Lipid composition of developing wild-type and *dgat1-1* seed.

Total lipids were extracted from wild-type (WT) and *dgat1-1* seed at 8, 12 and 16 days after flowering (DAF), fractionated into neutral (A) and polar lipid (B) fractions which were quantified. (C) TAG molecular species composition of WT and *dgat1-1* seed at 16 DAF. (D) Fatty acid composition of total lipid extracts of WT and *dgat1-1* seed at 16 DAF. (E) PC molecular species composition of WT and *dgat1-1* seed at 16 DAF. Data represents the mean ± SD of three biological replicates. *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test with Holm-Sidak correction for multiple comparisons).

Having confirmed the phenotype of the developing mutant seeds, we extracted RNA from the seeds at 8, 12 and 16 DAF, which corresponded to globular, torpedo and mature embryo stages, respectively (Figure 2.2). Global changes in transcript levels across seed development were then quantified using RNA-Seq. After quality assessment and normalization, more than 95% of the reads mapped to genic regions of the Arabidopsis genome (Table 2.1). We used the EDGE test to identify differentially expressed (DE) genes with greater than 2-fold change ($p<0.05$) that were expressed at levels higher than RPKM of 0.1. With these filtering parameters, the total number of statistically significant DE increased during seed development, with seven-fold more DE genes at 16 DAF compared to 8 DAF (Figure 2.3A). We further classified the genes according to whether they were associated with lipid metabolism using existing detailed annotation (Figure 2.3B) (Li-Beisson et al., 2013).

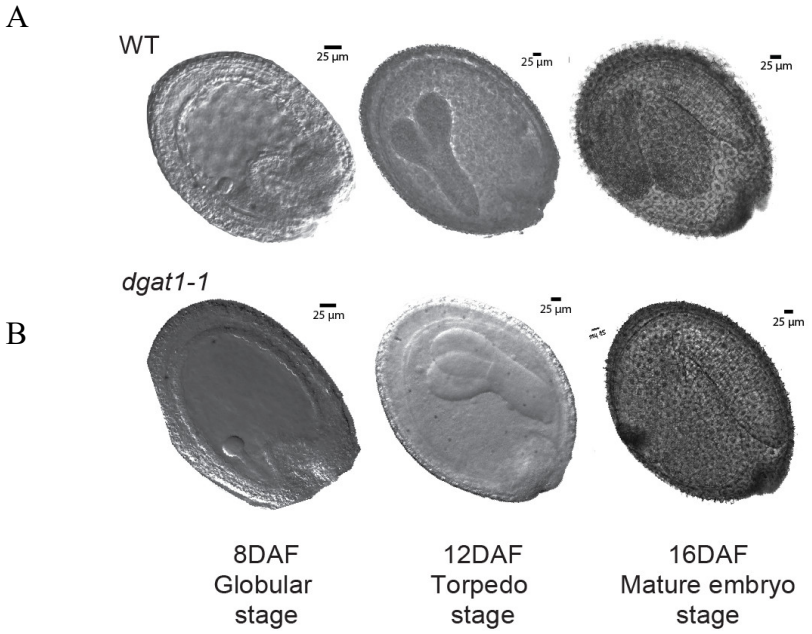


Figure 2.2 Embryo development in wild-type and *dgat1-1* seed.

Representative images of developing wild-type (WT) and *dgat1-1* seed at 8, 12 and 16 days after flowering (DAF). Bars =25 µm.

2.2.2 Desaturases are strongly upregulated in *dgat1-1*

Analysis of genes associated with lipid biosynthetic pathways (Li-Beisson et al., 2013) revealed a striking upregulation of a number of desaturases. For example, the transcript levels of the plastidial stearoyl-ACP desaturase genes *FAB2* and *DES2* were both two-fold higher in *dgat1-1*; expression changes in their relatively uncharacterized homologue *DES6* were even more extreme, with more than 6-fold upregulation in *dgat1-1* evident at 16 DAF (Figure 2.4A).

		Mapping statistics		
Sample	Development stage	Total number of reads	Fraction of mapped reads	Fraction of unmapped reads
WT	8 DAF	195,147,248	99.53	0.47
	12 DAF	205,608,936	99.12	0.88
	16 DAF	186,438,568	99.44	0.56
dgat1-1	8 DAF	188,832,692	99.36	0.64
	12 DAF	168,587,578	99.19	0.81
	16 DAF	218,326,568	99.27	0.73

Table 2.1 Gene-specific mapping of RNA seq reads.

Total reads were trimmed, and quality assessed before mapping to the annotated genome of Arabidopsis (Araport11). Total reads for WT and *dgat1-1* are shown from different development stages (8, 12, 16 DAF), indicating that majority of mapped reads were gene-specific (>95%). Three biological replicates and one technical replicate were used for each genotype per time point.

Interestingly, however, the expression of *FAD4*, *FAD5*, *FAD6*, and *FAD7*, all of which encode desaturases that introduce double bonds in various plastidial glycerolipids, were unaffected in *dgat1-1*. Similar to *FAB2* and *DES2*, the transcript levels of *FAD2* and *FAD3* encoding the ER-localized desaturases responsible for the synthesis of 18:2 and 18:3, were 50% higher in *dgat1-1* at 16 DAF, making them the highest expressed lipid biosynthetic genes in the mutant seed. We confirmed the upregulation of *FAD2* in *dgat1-1* using qRT-PCR, obtaining results highly consistent with those seen using RNA-Seq (Figure 2.5A).

We observed expression changes in genes involved in other lipid biosynthetic pathways, but with the few exceptions of some genes. For example, as exemplified by the expression of genes involved in ACCase or acyl-ACP thioesterase activity, genes encoding components of fatty acid synthesis were expressed about 20% higher at 12 DAF and 40% higher at 16 DAF in *dgat1-1* (Figure 2.4B). The expression profiles of the regulatory proteins PII and WRI1 followed

similar patterns, consistent with their role controlling fatty acid synthesis (Baud et al., 2010; Baud et al., 2007; Cernac and Benning, 2004). Given the lower oil content of *dgat1-1*, we also examined the expression of different enzymes involved in TAG hydrolysis and beta-oxidation. Here, the expression of most genes was unchanged or slightly lower in *dgat1-1* (Figure 2.4E).

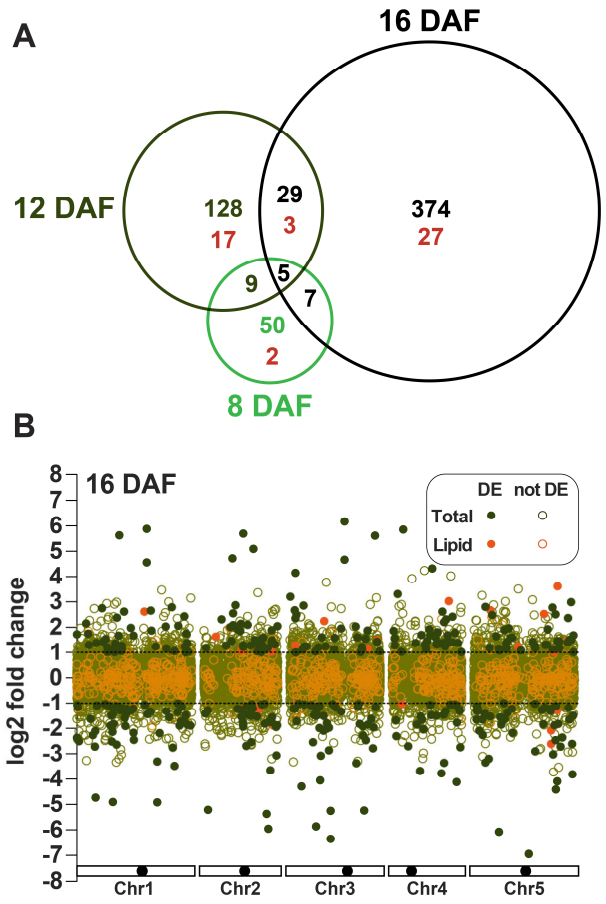


Figure 2.3 Differentially expressed (DE) genes in *dgat1-1* mutant seed.

A. Venn diagram showing the number of DE genes in dgat1-1 relative to Col-0 during different stages of seed development. Circle sizes are proportional to number of genes. Numbers in red and green refer to lipid related and non-lipid related genes, respectively. DAF, days after flowering. B. Manhattan plot with distribution of DE genes across different chromosomes. The y-axis shows $\log_2$ fold change of expression in *dgat1-1* relative to Col-0, and the x-axis indicates the chromosomal position of genes. The horizontal dotted lines represent the threshold for $\log_2$ fold change for selecting DE genes. Green and red colors represent lipid related and non-lipid related genes, whereas filled and open circles represent DE and non-DE genes respectively.

2.2.3 Increased expression of genes important for PC metabolism in *dgat1-1* seed

Of particular interest, we observed no large differences in expression of genes involved in the *de novo* synthesis of DAG in developing *dgat1-1* seeds. *GPAT9* and genes encoding LPAAT activity were slightly upregulated at different time points; combined expression of PAH paralogs was lower in *dgat1-1* seed at 16 DAF (Figure 2.4C). Consistent with previous work (Zou et al., 1999), we were able to detect expression of the mutant *dgat1-1* transcript, which was slightly higher at 12 DAF but otherwise unchanged. Particularly surprisingly, the low transcript levels of *PDAT1* in wild-type seeds were unchanged in *dgat1-1*. Analysis of *PDAT1* expression using qRT-PCR confirmed that the gene was expressed at the same low levels in wild-type and *dgat1-1* seeds (Figure 2.5B).

In contrast to the relative lack of expression change in expression of *de novo* DAG synthesis, *ROD1* encoding PDCT activity that interconverts DAG and PC, was almost two-fold higher in *dgat1-1* at 16 DAF (Figure 2.4D). Similarly, *LPCAT2* was also upregulated in *dgat1-1*, though its closely related homologue *LPCAT1* was significantly down regulated at 16 DAF. Consistent with an increased role of flux through PC, *NMT1* encoding the methyltransferase responsible for the *de novo* synthesis of PC was also strongly upregulated in *dgat1-1*.

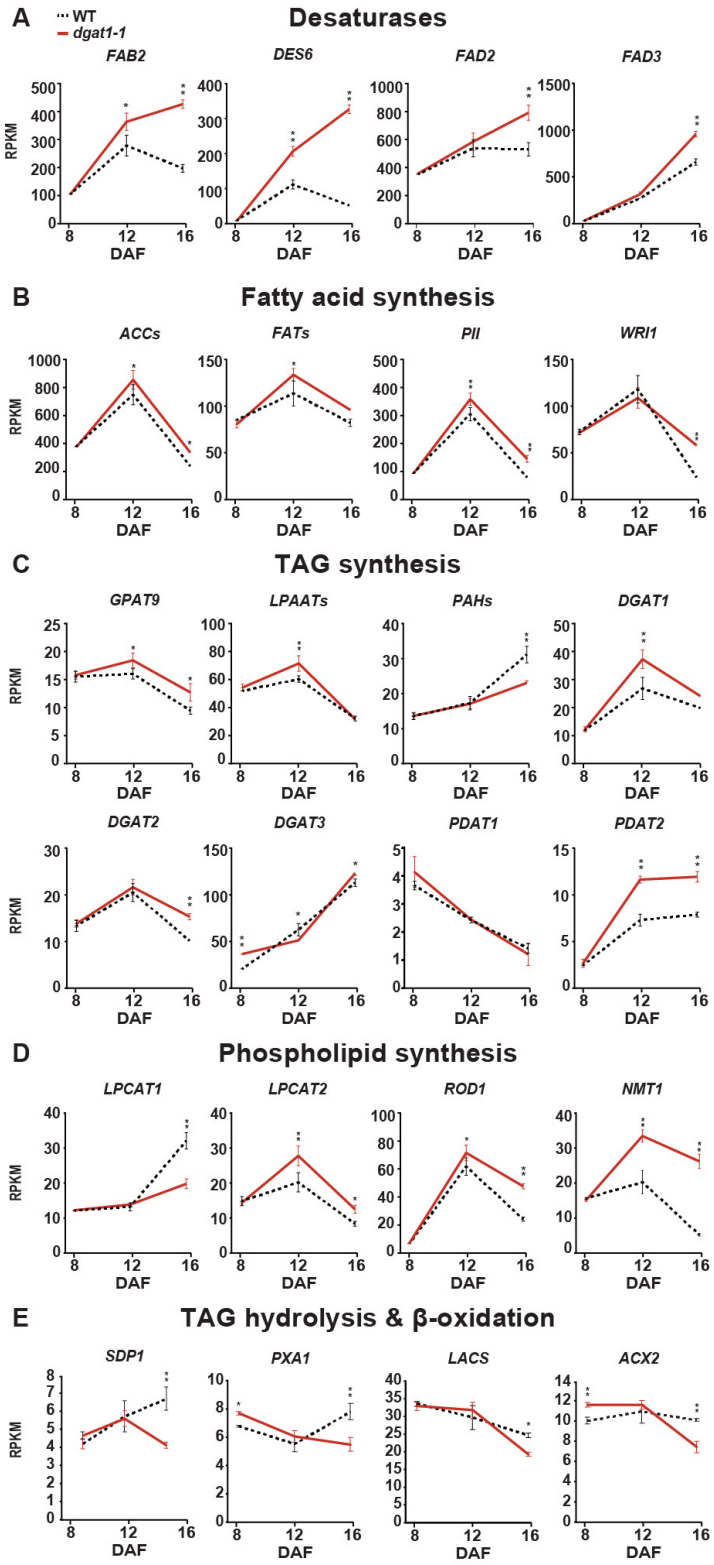


Figure 2.4 Expression profiles of lipid related genes in developing seeds.

Expression levels of selected lipid related genes as determined by RNA-seq in developing wild type (WT) and *dgat1-1* seed at 8, 12 and 16 days afterflowering (DAF). Genes involved in acyl desaturation (A), fatty acid synthesis (B), TAGsynthesis (C), phospholipid synthesis (D), and TAGhydrolysis and β-oxidation (E) are shown. For some enzyme activities, the expression levels of multipleencoding gene isoforms is summed. Data representsthe mean of three biological replicates ±SD. * P <0.05; ** P < 0.01 (Student's t test).

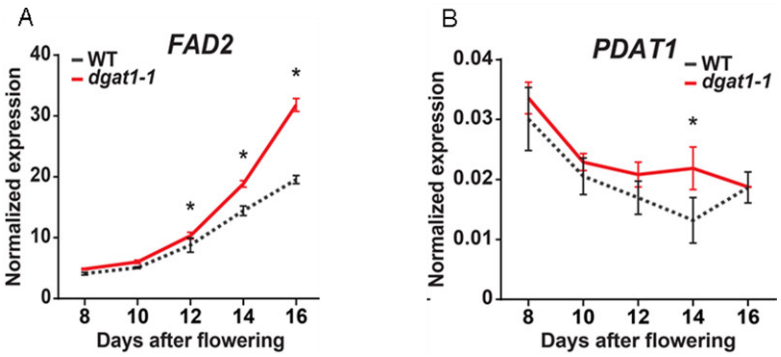


Figure 2.5 Validation of DE genes by qRT-PCR.

A. RNA-seq derived transcript expression levels of the DE genes *FAD2* and *PDAT1* at 8, 10, 12, 14 and 16 days after flowering (DAF). Transcript levels were quantified using real-time PCR and normalized to the reference genes *ASAR1*, *UBC21* and *PP2AA3*. Data presented is the mean ± SD of 3 biological replicates and 2 technical replicates * P <0.05 by Student's t test for significance in difference.

2.2.4 Selection and confirmation of genes upregulated in *dgat1-1* seed

When selecting candidate genes for further characterization, we decided to focus on genes upregulated more than two-fold for an at least one-time point. In addition, the role of the gene product needed to be relatively unknown. With these criteria, we identified four genes of particular interest. Two encoded lipases for which little was known. At3g61680 is a member of the large α,β-hydrolase gene family, of which some members play a role in lipid metabolism (James et al., 2010; Ghosh et al., 2009). Expression of this gene, which here we refer to as *PLIP1*, is remarkably similar in *dgat1-1* and wild-type seeds at 8 and 12 DAF, but is more than two-fold increased in the mutant at 16 DAF (Figure 2.6A). This pattern of expression was confirmed using qRT-PCR (Figure 2.6B). The second lipase is encoded by *NPC6* (At3g48610), a member of the nonspecific phospholipase C gene family. This lipase gene is expressed at lower levels than *PLIP1*. During the course of wild-type seed development, *NPC6* transcript levels decrease from 8 to 12 DAF and then remain stable to 16 DAF. In contrast, after an initial decrease in *dgat1-1* seeds, *NPC6* expression increases at 16 DAF (Figure 2.6A). As with the other genes of interest, qRT-PCR was used to confirm the expression patterns of *NPC6* seen with RNA-Seq (Figure 2.6B).

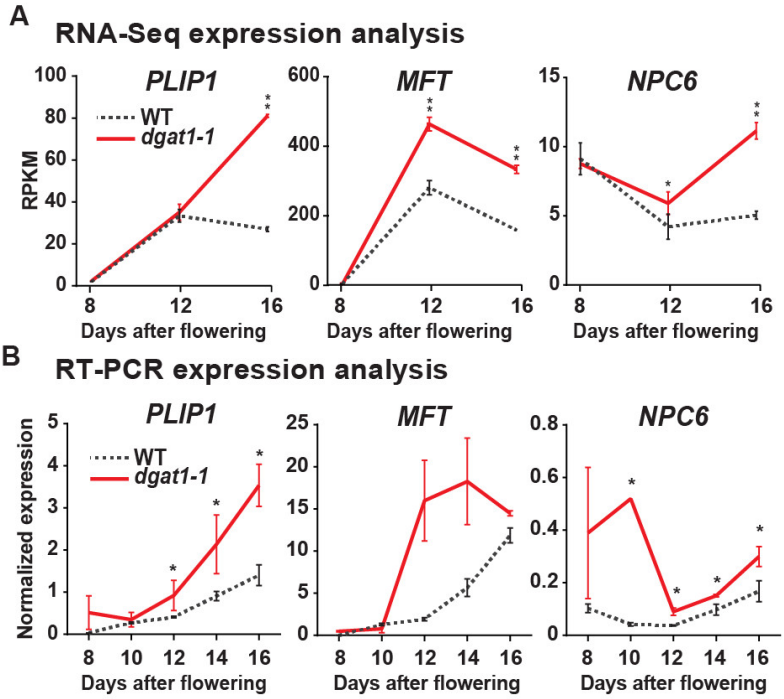


Figure 2.6 Expression levels of differentially expressed genes in developing seeds.

Expression levels of *PLIP1*, *MFT* and *NPC6* in developing wild type (WT) and *dgat1-1* seed were quantified using RNA-seq (A) and qRT-PCR (B). For qRT-PCR, gene expression was normalized to the reference genes *ASAR1*, *UBC21* and *PP2AA3*. Data presented is the mean ± SD of three biological replicates. *, P <0.05 (Student's t test).

We also were intrigued by the expression of *MOTHER OF FT* (*MFT*; At1g18100) which is upregulated more than two-fold at both 12 and 16 DAF in *dgat1-1* (Figure 2.6A). *MFT* has previously been shown to be important for germination (Xi et al., 2010), but a role in TAG accumulation has not yet been demonstrated. As with all the other gene analyzed in this manner, qRT-PCR confirmed the higher expression of *MFT* in *dgat1-1* (Figure 2.6B). In addition, given *NMT1*'s strong upregulation in *dgat1-1* (Figure 2.4D), we were also curious to see if *NMT1* was important for TAG biosynthesis.

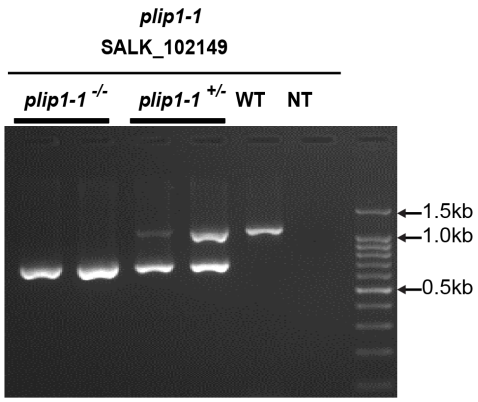
2.2.5 Mutant *plip1-2* seed possess reduced oil content

Homozygous T-DNA mutant lines containing insertions in these differentially expressed genes were obtained (Figure 2.7). For all the candidate genes, we obtained at least two different mutant alleles. Homozygous mutants were then grown alongside wild-type and *dgat1-1* until maturity and seeds collected. The fatty acid content of seeds from both *plip1-1* and *plip1-2* plants

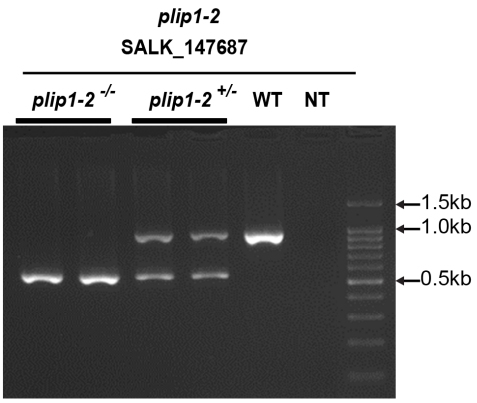
was significantly lower than wild-type seed with levels similar to that of *dgat1-1* plants (Figure 2.8A). The fatty acid composition of both *plip1* mutant lines was also distinct from wild-type and *dgat1-1*, with slightly lower levels of 18:1 and slightly higher levels of 18:3 compared to the wild-type (Figure 2.8B). While the oil content from all three *npc6* mutant lines was lower compared to the wild-type plants, none of the reductions were statistically significant. Similarly, the fatty acid composition of the *npc6* mutant seed was essentially the same as the wild-type (Figure 2.8B). The seed oil content of *mft-2* plants was ~80% that of wild-type; however, that of *mft-3* was essentially the same as that of the wild-type. Both *mft-2* and *mft-3* possessed lower amounts of 18:1 and higher levels of 18:2 compared to wild-type (Figure 2.8B). Finally, both *nmt1* mutant lines produced seed with a 10% reduction in oil, but the fatty acid composition was unchanged (Figure 2.9).

A

AT3G61680 (PLASTIDIAL LIPASE1; PLIP1)



PLIP1-1_F + PLIP1-1_R + LBb1.3



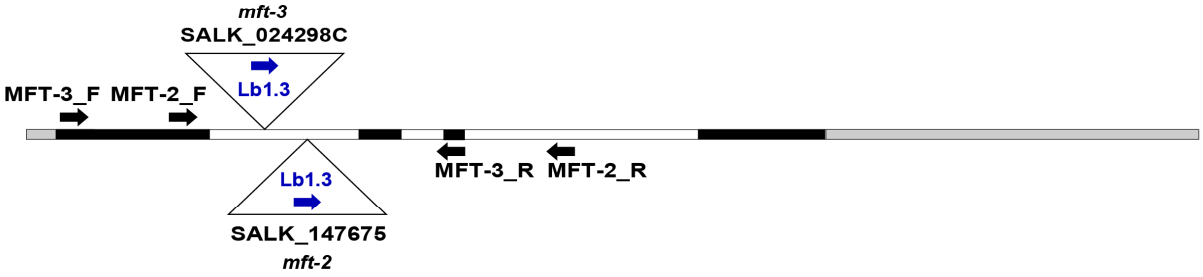
PLIP1-2_F +PLIP1-2_R + LBb1.3

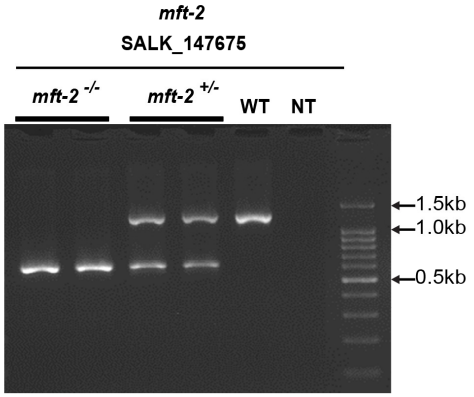
PREDICTED SIZES

Allele	Primer	WT	T-DNA
plip1-1	PLIP1-1_F + PLIP1-1_R	1160	-
	PLIP1-1R + LBb1.3	-	650
plip1-2	PLIP1-2_F + PLIP1-2_R	958	-
	PLIP1-2_F + LBb1.3	-	580

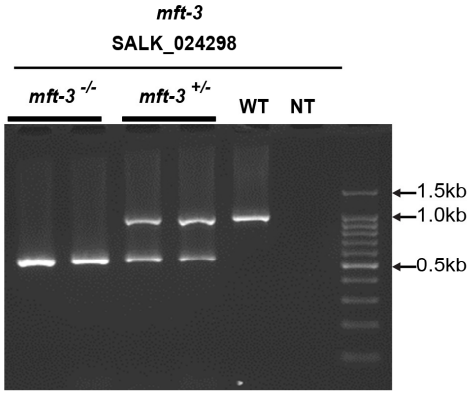
B

AT1G18100 (MOTHER OF FT AND TFL1;MFT)





MFT-2_F + MFT-2_R + LBb1.3



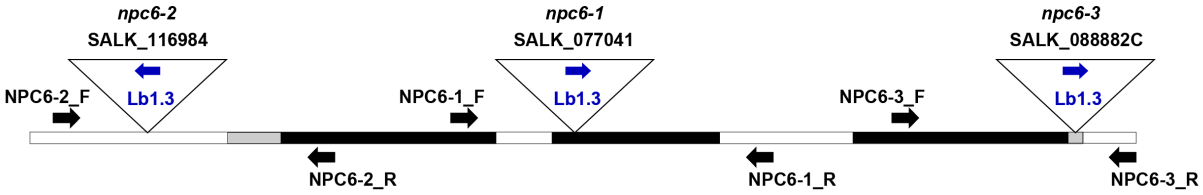
MFT-3_F + MFT-3_R + LBb1.3

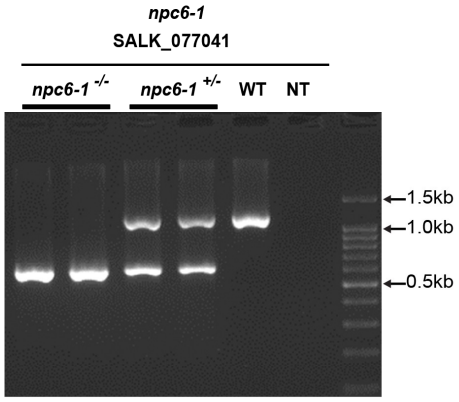
PREDICTED SIZES

Allele	Primer	WT	T-DNA
mft-2	MFT-2_F + MFT-2_R	1118	-
	MFT-2_R + LBb1.3	-	660
mft-3	MFT-3_F + MFT-3_R	980	-
	MFT-3_R + LBb1.3	-	680

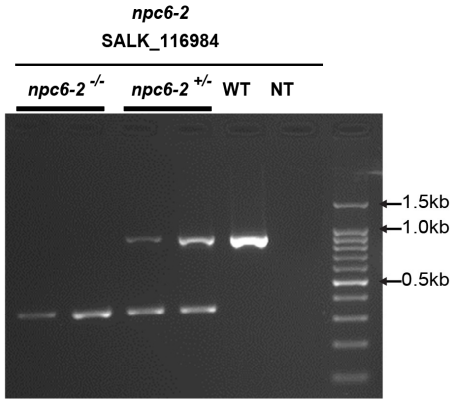
C

AT3G48610 (NON-SPECIFIC PHOSPHOLIPASE C6; NPC6)

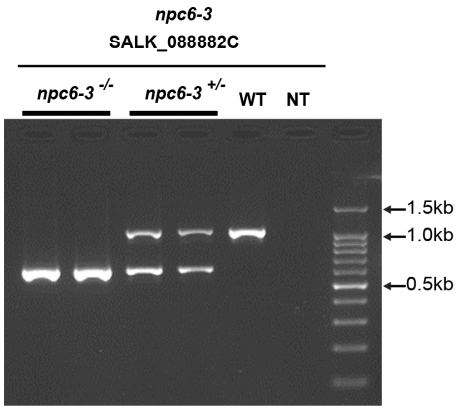




NPC6-1_F + NPC6-1_R + LBb1.3



NPC6-2_F + NPC6-2_R + LBb1.3



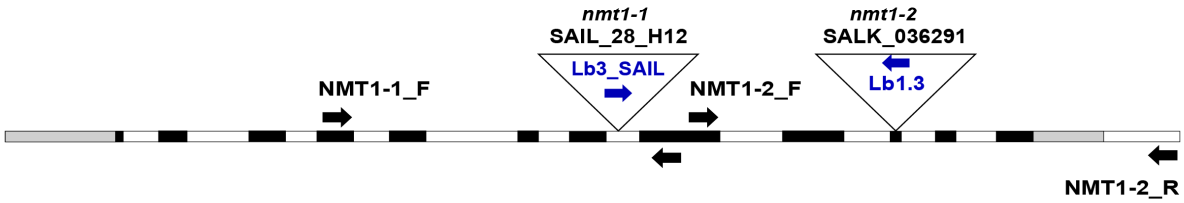
NPC6-3_F + NPC6-3_R + LBb1.3

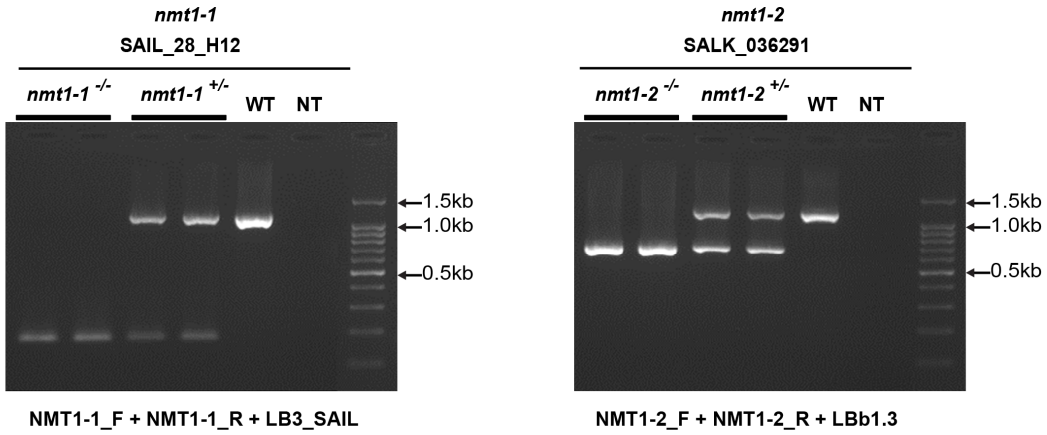
PREDICTED SIZES

Allele	Primer	WT	T-DNA
npc6-1	NPC6-1_F + NPC6-1_R	1139	-
	NPC6-1_R + LBb1.3	-	600
npc6-2	NPC6-2_F + NPC6-2_R	958	-
	NPC6-2_F + LBb1.3	-	380
npc6-3	NPC6-3_F + NPC6-3_R	1039	-
	NPC6-3_R + LBb1.3	-	640

D

AT3G18000 (N-METHYLTRANSFERASE 1; NMT1)





PREDICTED SIZES

Allele	Primer	WT	T-DNA
nmt1-1	NMT1-1_F + NMT1-1_R	1080	-
	NMT1-1_R + LB3_SAIL	-	185
nmt1-2	NMT1-2_F + NMT1-2_R	1264	-
	NMT1-2_F + LBb1.3	-	750

Figure 2.7 T-DNA mutant alleles and genotype confirmation.

T-DNA insertional mutant lines were obtained for *PLIP1* (A), *MFT* (B), *NPC6* (C) and *NMT1* (D). For each, the upper panel depict the gene structure with the position of the T-DNA insertion for each mutant allele. Black, white and grey boxes represent exon, introns and untranslated regions, respectively. The annealing sites for genotyping primers are indicated by arrows. The lower panels show the genotyping results for each mutant allele. WT, Col-0 wild-type control; NT, no DNA template control.

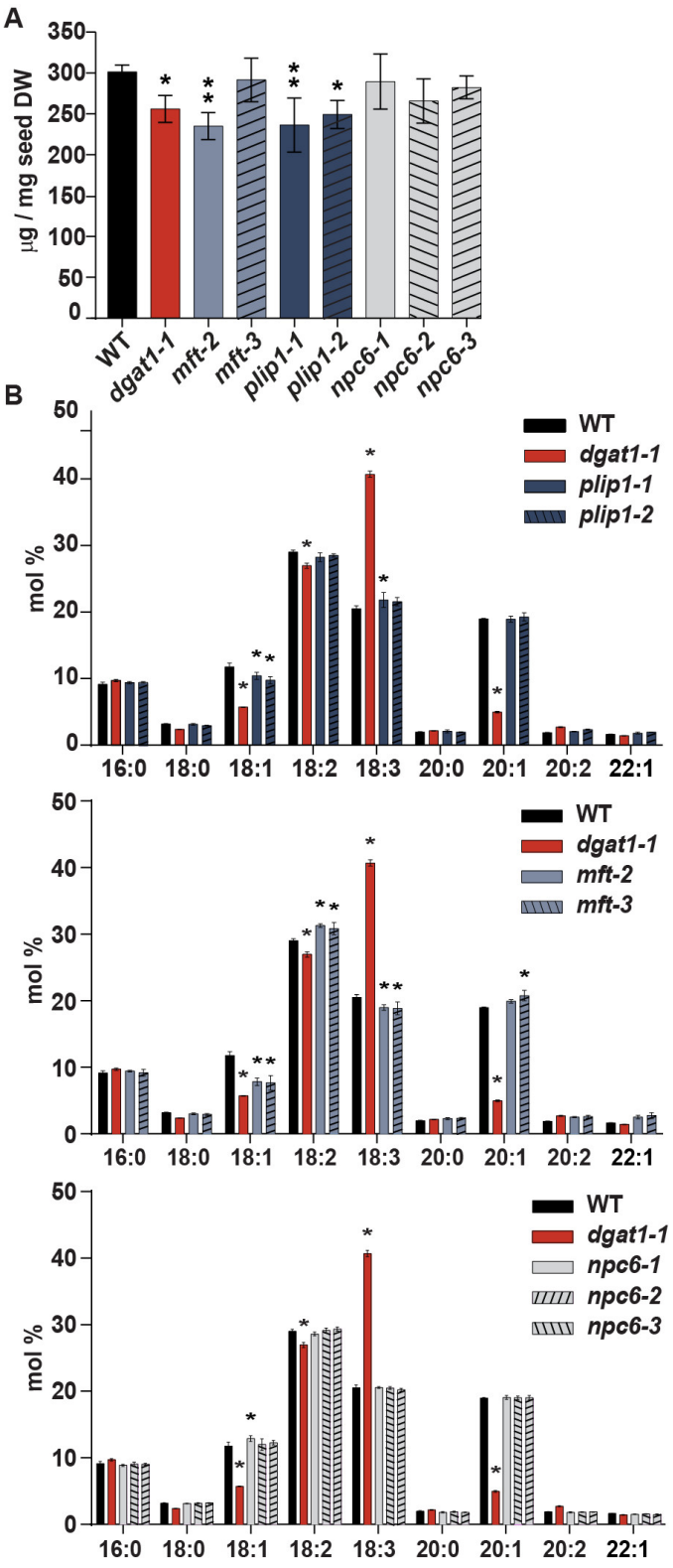


Figure 2.8 Fatty acid content and composition of mutant seed.

Fatty acid content (A) and composition (B) of wild-type (WT) and mutant seed were quantified by whole seed acid-catalyzed transmethylation of dry seeds followed by gas chromatography. Values are the mean ± SD of seeds harvested from three individual plants. * $P < 0.05$ (A: Student's t-test; B: Two-way ANOVA with Holm-Sidak correction for multiple comparisons). The results shown are representative of two independent experiments.

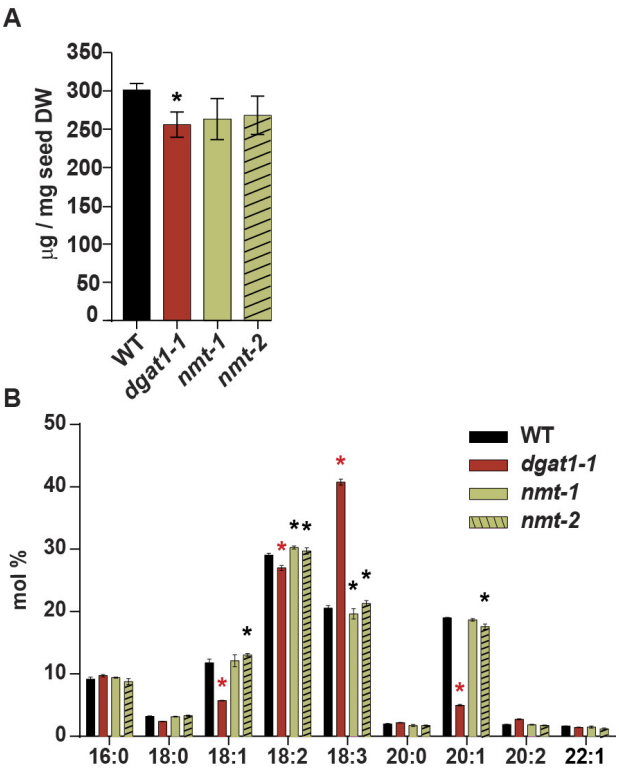


Figure 2.9 Fatty acid content and composition of *nmt1* mutant seed.

Fatty acid content (A) and composition (B) of wild-type (WT), *dgat1-1*, *nmt1-1* and *nmt1-2* seed were quantified by whole seed acid-catalyzed transmethylation followed by gas chromatography. Values are the mean ± SD of seeds harvested from at least three individual plants. * $P < 0.05$ (A: Student's t-test; B: Two-way ANOVA with Holm-Sidak correction for multiple comparisons).

2.2.6 *plip1-2* seeds are smaller relative to WT

While harvesting and analyzing the seeds produced by the different mutant lines, we noticed that a significant proportion of the seeds from *plip1-2* plants were smaller than typical wild-type seed (Figure 2.10A). To quantify these results, we measured the median, minimum seed size and maximum seed size of mature, dry seeds harvested from wild-type, *plip1-2* and *dgat1-1* plants grown together (Figure 2.10B). Under these conditions, wild-type seed had an average size of 111909 µm^2, similar to previous measurements (Herridge et al., 2011). The average seed size of *dgat1-1* mutant seeds was 98325µm^2, in line with previous studies suggesting a smaller size for *dgat1-1* seed (Routaboul et al., 1999). Consistent with our initial observations, the mean seed size of *plip1-2* seeds was 103096 µm^2, with ~70% possessing an area smaller than that of wild-type seed (Figure 2.10B). Interestingly, the size distribution of

plip1-2 seeds was different from *dgat1-1*, with *plip1-2* also able to produce some seeds as large as those from wild-type plants (Figure 2.10C).

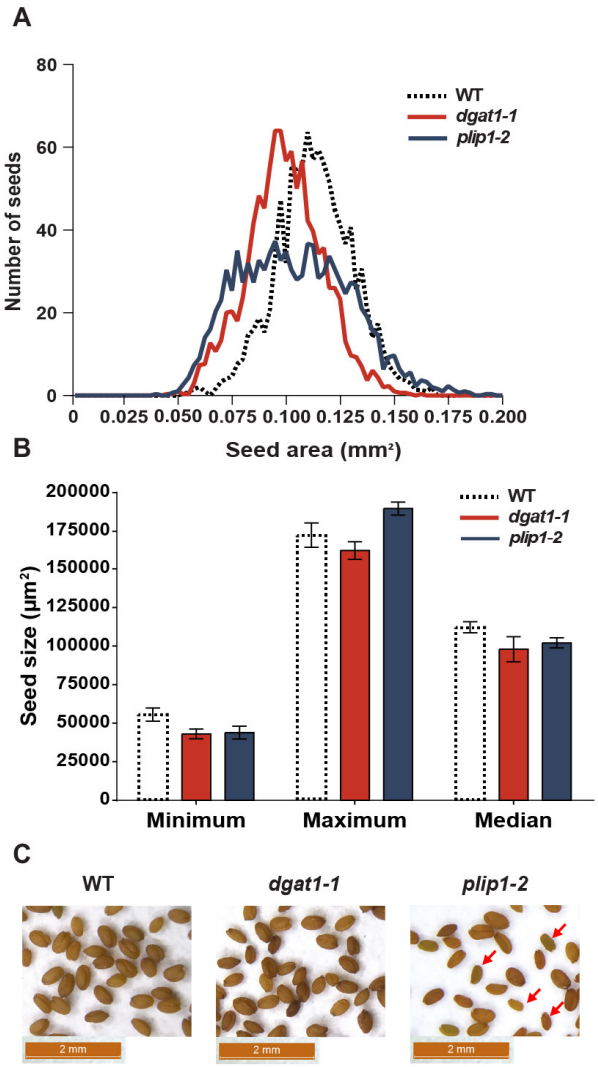


Figure 2.10 The *plip1-2* mutant produces smaller seed than wild-type (WT) plants.

A. Size distribution for seeds harvested from WT, *dgat1-1* and *plip1-2* plants. Data represents the mean of three biological replicates. B. Representative seed from WT, *dgat1-1* and *plip1-2* plants. Bars represent 2mm, arrows indicate smaller or unusual *plip1-2* seed.

2.2.7 *PLIP1* is upregulated in *dgat1-1* seeds

As *PLIP1* transcripts are not detected by the ATH1 GeneChip used by the Arabidopsis eFP Browser (Winter et al., 2007), we quantified the expression of *PLIP1* in different tissues of wild-type plants to determine whether the gene played a role beyond in just seeds. *PLIP1*

transcripts were detected in all tissues examined but were more abundant in flowers, siliques, and roots (Figure 2.11). We also measured *PLIP1* transcript levels in *dgat1-1* tissues to investigate whether the gene was upregulated in different organs of the plant in this mutant background. Here, we observed a more than two-fold increase in *PLIP1* expression in siliques, consistent with results from seeds (Figure 2.6). However, in all the other tissues *PLIP1* expression did not differ between WT and *dgat1-1* (Figure 2.11), suggested that any role for *PLIP1* in *dgat1-1* is seed specific.

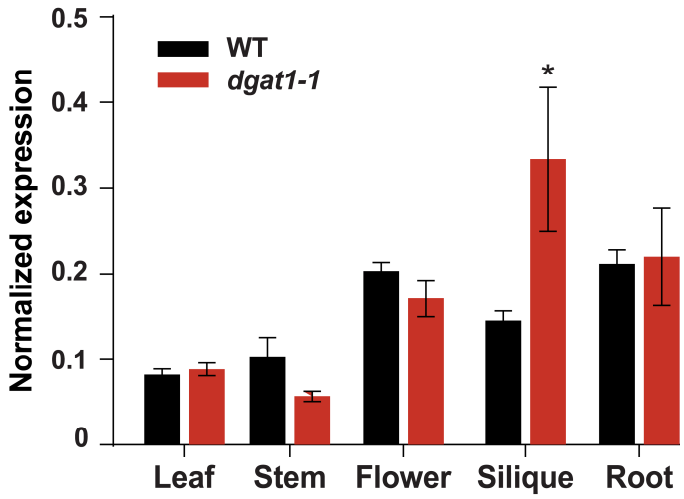


Figure 2.11 *PLIP1* is upregulated *dgat1-1* in siliques.

Expression profile of *PLIP1* in different tissues of wild-type (WT) and *dgat1*-1 plants. The expression of *PLIP1* was quantified using qRT-PCR and normalized to the expression of the reference gene *ASAR1*. Data represents the mean ± SD for three biological replicates. * P <0.05 (Student's t test).

2.2.8 The *dgat1-1 plip1-2* double mutant is synthetically lethal

In addition to quantifying the oil content of mutants in candidate genes, we also crossed some of the mutant lines with *dgat1-1* to determine the role of the upregulated genes in a *dgat1-1* background. Double mutants homozygous for *dgat1-1* and *mft-2*, *mft-3* or *npc6-1* were successfully isolated and the seed fatty acid content quantified. There was no difference in the fatty acid content and composition between these double mutant lines and the *dgat1-1* single mutant (Figure 2.12) indicating that *DGAT1* is epistatic to *NPC6* and *MFT*.

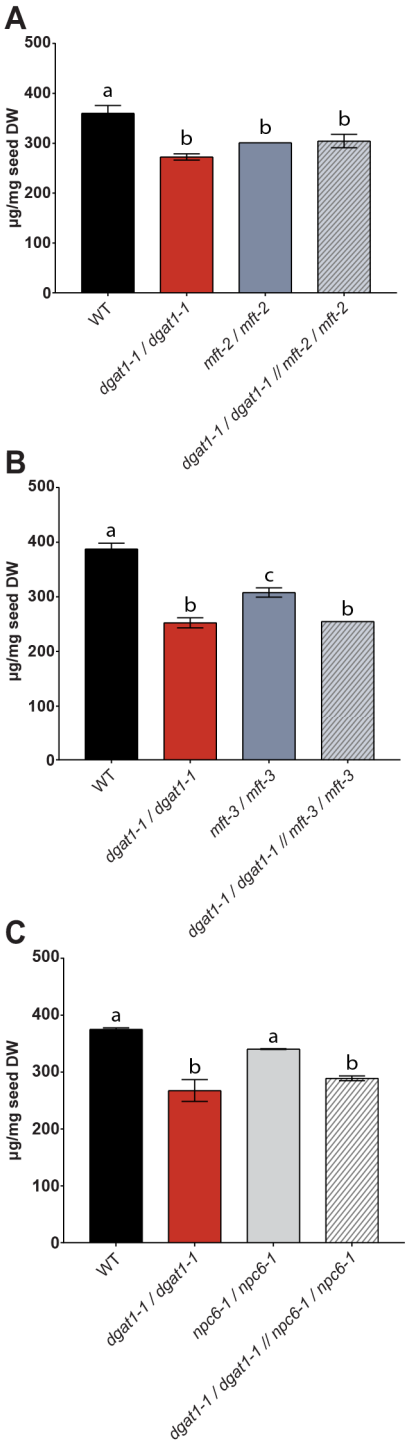


Figure 2.12 Fatty acid content of double mutant seed.

The seed fatty acid content of double homozygous mutants resulting from crossing *dgat1-1* with *mft-2* (A), *mft-3* (B) and *npc6-1* (C). Values represent the mean ± SD for at least three plants of each genotype grown together. ANOVA with Tukey's post-hoc correction was used to test for differences among the means of the different genotypes. Different letters indicate significant difference at $P < 0.05$.

Interestingly, after genotyping 87 plants, we did not obtain any double homozygous *dgat1-1 plip1-2* mutants in the F_2 generation (Figure 2.13). To confirm this observation, we collected seed from *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2* and *DGAT1*/*dgat1-1 plip1-2*/*plip1-2* plants and genotyped the resulting progeny. Again, no double homozygous mutants were obtained from either parental genotype (Figure 2.14). For the plant with genotype *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2*, we observed a segregation ratio close to 1:1.45 for *dgat1-1*/*dgat1-1 PLIP1*/*PLIP1* and *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2* respectively. Similarly, for the plant with genotype *DGAT1*/*dgat1-1 plip1-2*/*plip1-2*, we observed segregation ratio of approximately 1:2.8 for *DGAT1*/ *DGAT1 plip1-2*/*plip1-2* and *DGAT1*/*dgat1-1 plip1-2*/*plip1-2* respectively. These ratios observed for the detected genotypes, and the absence of any double homozygous mutants, provides additional evidence that the combination of *dgat1-1* and *plip1-2* genotypes is lethal.

Interestingly, seed from *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2* plants possessed a lower oil content than *dgat1-1* seed, with higher levels of 18:3 and reduced 18:1 and 18:2 content (Figure 2.15). The oil content of seeds from plants homozygous for *plip1-2* and heterozygous for *dgat1-1* was lower than that of *plip1-2* seed, consistent with the gene dosage effect previously observed for *dgat1-1* heterozygotes.

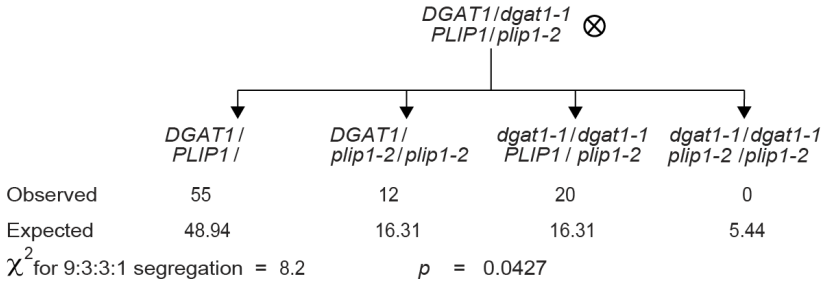


Figure 2.13 The *dgat1-1 plip1-2* genotype is synthetically lethal.

Genotyping results of F_2 progeny derived from self-pollinated *DGAT1*/*dgat1-1 PLIP1/plip1*-2 plants.

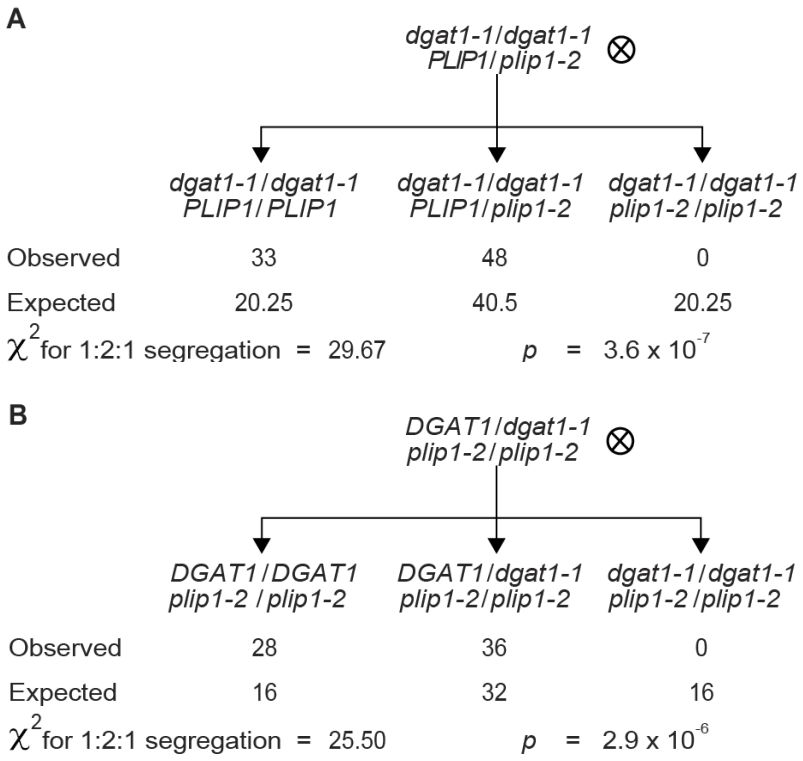


Figure 2.14 A *dgat1-1 plip1-2* genotype is synthetically lethal.

Genotyping results of progeny derived from self-pollinated **A**, *dgat1-1*/*dgat1-1 PLIP1/plip1*-2 and **B**, *DGAT1*/*dgat1-1 plip1-2/plip1*-2 plants.

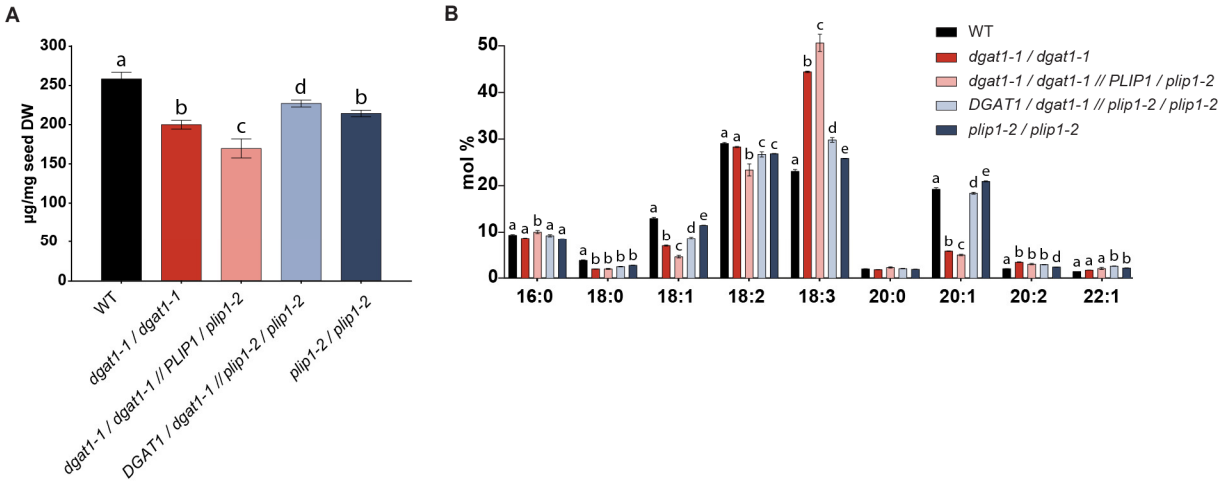


Figure 2.15 Fatty acid content and composition of double mutant *dgat1-1 plip1-2* seed.

The fatty acid content (A) and composition (B) of seed from plants homozygous for *dgat1-1* and heterozygous for *plip1-2* or heterozygous for *dgat1-1* and homozygous for *plip1*-2. Values represent the mean ± SD for at least three plants of each genotype grown together. ANOVA with Tukey's post-hoc correction was used to test for differences among the means of the different genotypes. Different letters indicate significant difference at $P < 0.05$.

2.2.9 Both male and female *dgat1-1 plip1-2* gametophytes are viable

We considered three scenarios that might cause lethality of the *dgat1-1 plip1-2* double mutant: 1) that pollen or egg cells with a *dgat1-1 plip1-2* genotype are non-viable, 2) that *DGAT1* and *PLIP1* are required for normal embryo development or 3) that both genes are required for seed germination. To test the first hypothesis of gametophyte viability, a series of crosses were made with wild-type plants. When wild-type plants were the pollen donor and *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2* was the maternal plant, both the expected genotypes *DGAT1*/*dgat1-1 PLIP1*/*plip1-2* and *DGAT1*/*dgat1-1 PLIP1*/*PLIP1* were obtained in a 1:1 ratio (Figure 2.16A). The *DGAT1*/*dgat1-1 PLIP1*/*plip1-2* genotype could only result from a *dgat1-1 plip1-2* egg cell, thus indicating that a double mutant genotype does not affect the viability of the female gametophyte. Similar results were obtained when *DGAT1*/*dgat1-1 plip1-2*/*plip1-2* plants were used as the maternal plant (Figure 2.16C). When *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2* plants were the pollen donor and wild-type was used as a female receptacle, both the expected genotypes *DGAT1*/*dgat1-1 PLIP1*/*plip1-2* and *DGAT1*/*dgat1-1 PLIP1*/*PLIP1* were obtained in 1:1 ratio (Figure 2.16B). The *DGAT1*/*dgat1-1 PLIP1*/*plip1-2* genotype could only result from *dgat1-1 plip1-2* pollen, thus indicating that the double mutant does not affect pollen viability. Similar results were obtained when *DGAT1*/*dgat1-1 plip1-2*/*plip1-2* was used as the pollen donor, providing additional evidence that *dgat1-1 plip1-2* pollen is viable. (Figure 2.16D). Together these results suggest that the inability to obtain a double homozygous genotype is not the result of non-viable male or female gametophytes.

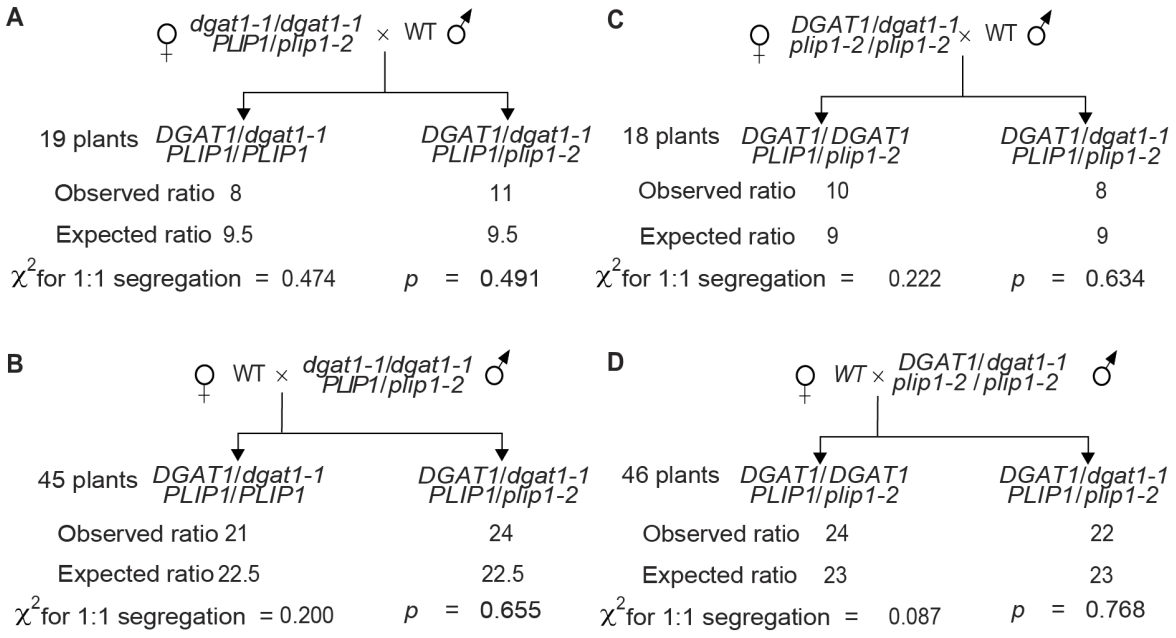


Figure 2.16 Test of genetic transmission of *dgat1-1* and *plip1-2* gametophytes by reciprocal crossing.

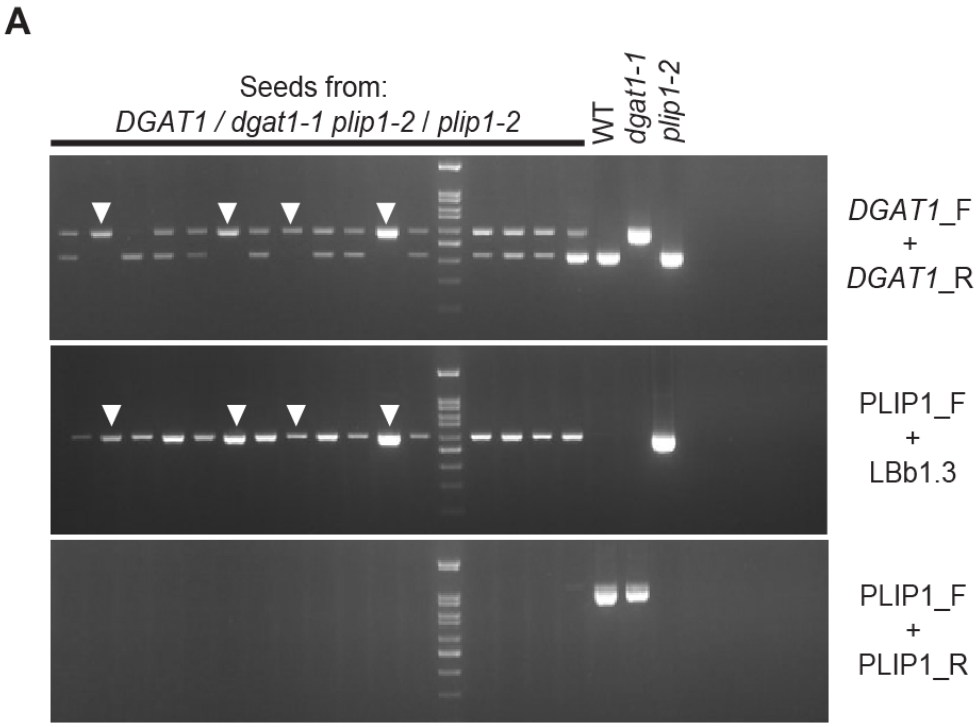
Genotyping results of progeny derived from reciprocal crossing between (A) *dgat1-1*/*dgat1-1 PLIP1/plip1*-2 and wild-type (WT) plants and (B) *DGAT1*/*dgat1-1 plip1*-2/*plip1*-2 and WT plants.

2.2.10 The *dgat1-1 plip1-2* double mutant fails to germinate

No obvious abortion of seeds in siliques was observed, suggesting the inability to obtain a double homozygous mutant is not caused by defects during embryo development. To validate the existence of double mutant seed, we genotyped individual seeds derived from *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2* or *DGAT1*/*dgat1-1 plip1-2*/*plip1-2* plants. For both parental genotypes, we could identify seed homozygous for both mutations, further confirming that such seeds are produced (Figure 2.17A).

We, therefore, tested the ability of the double homozygous mutant seeds to germinate. When seeds from *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2* or *DGAT1*/*dgat1-1 plip1-2*/*plip1-2* genotypes were germinated on plates, approximately one-quarter of the seeds failed to germinate (Figure 2.17B). No defects were noted in the germination of *plip1-2* and *dgat1-1* seed. Genotyping of the

resulting seedlings from *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2* resulted in a 1:2 ratio of *dgat1-1*/*dgat1-1 PLIP1*/*PLIP1* and *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2* respectively. Likewise, genotyping of the resulting seedlings from *DGAT1*/*dgat1-1 plip1-2*/*plip1-2* resulted in 1:2 ratio of *DGAT1*/*DGAT1 plip1-2*/*plip1-2* and *DGAT1*/*dgat1-1 plip1-2*/*plip1-2* respectively. Despite repeated attempts, we were unable to extract DNA of sufficient quality from non-germinating seeds to confirm a homozygous *dgat1-1 plip1-2* mutant phenotype.



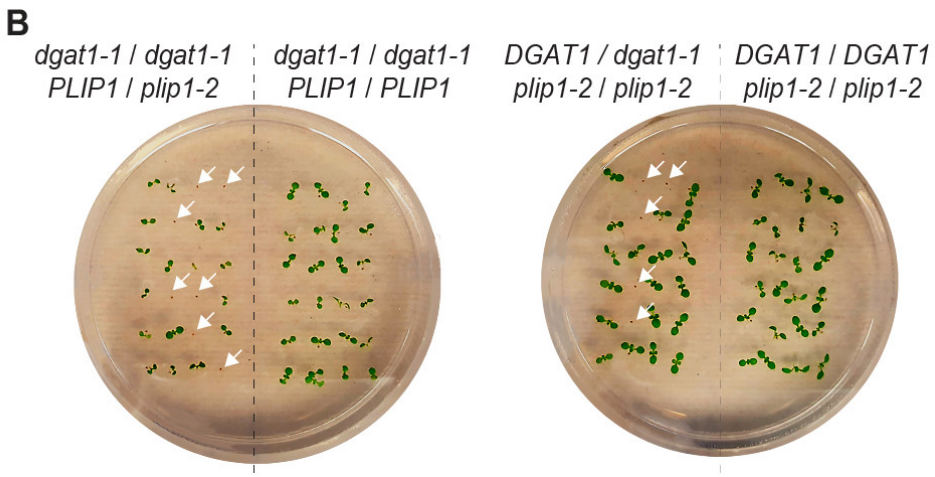
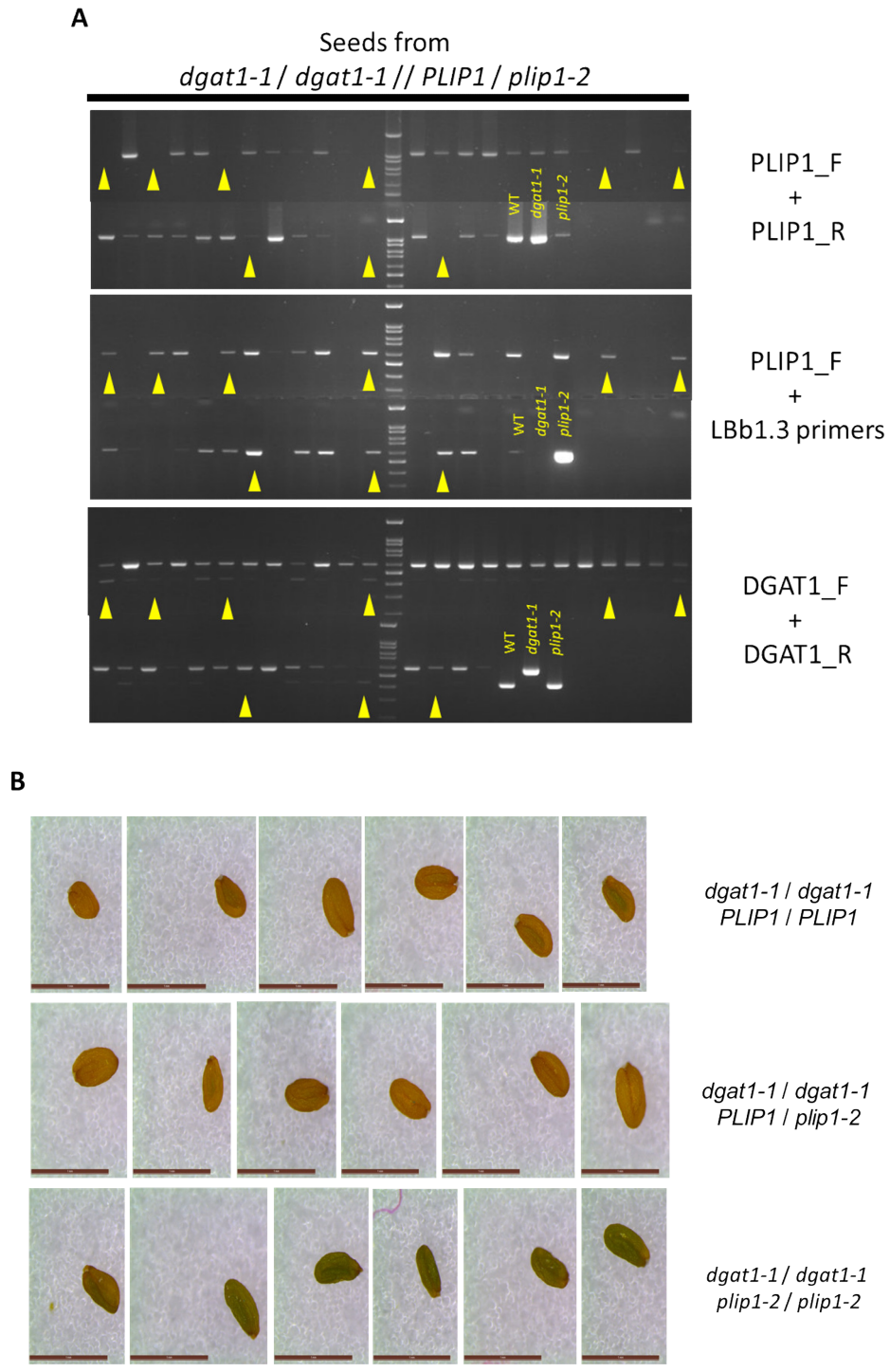


Figure 2.17 Double mutant *dgat1-1 plip1-2* seed is germination defective.

A. Genotyping of individual seeds from a self-pollinated *DGAT1*/*dgat1-1 plip1-2*/*plip1*-2 plant. White arrowheads indicate double homozygous mutant *dgat1-1*/*dgat1-1 plip1-2*/*plip1-2* genotypes. B. Seeds from self-pollinated *dgat1-1*/*dgat1-1 PLIP1*/*plip1*-2, *dgat1-1*/*dgat1-1 PLIP1*/ *PLIP1* plants, *DGAT1*/*dgat1-1 plip1*-2/*plip1*-2 and *DGAT1*/ *DGAT1 plip1*-2/*plip1*-2 plants were germinated on plates. Arrows indicate seed that failed to germinate.

2.2.11 The *dgat1-1 plip1-2* double mutant produces green seed

We observed a green seed phenotype for one-quarter of seeds collected from *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2* or from *DGAT1*/*dgat1-1 plip1-2*/*plip1-2* plants. Seed pictures were taken for individual seeds harvested from these plants prior to testing for germination. . Despite repeated attempts, we were unable to extract DNA of sufficient quality from non-germinating seeds grown on MS plates to confirm a homozygous *dgat1-1 plip1-2* mutant phenotype. Instead, individual seeds harvested from *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2* and *DGAT1*/*dgat1-1 plip1-2*/*plip1-2* plants were photographed and then genotyped. We observed that all the seed with double homozygous mutant genotype *dgat1-1*/*dgat1-1 plip1-2* /*plip1-2* had green seed phenotype (Figure 2.18).


A
Seeds from
dgat1-1 / dgat1-1 // PLIP1 / plip1-2
WT
dgat1-1
plip1-2
PLIP1_F
+
PLIP1_R
PLIP1_F
+
LBb1.3 primers
DGAT1_F
+
DGAT1_R
B
dgat1-1 / dgat1-1
PLIP1 / PLIP1
dgat1-1 / dgat1-1
PLIP1 / plip1-2
dgat1-1 / dgat1-1
plip1-2 / plip1-2

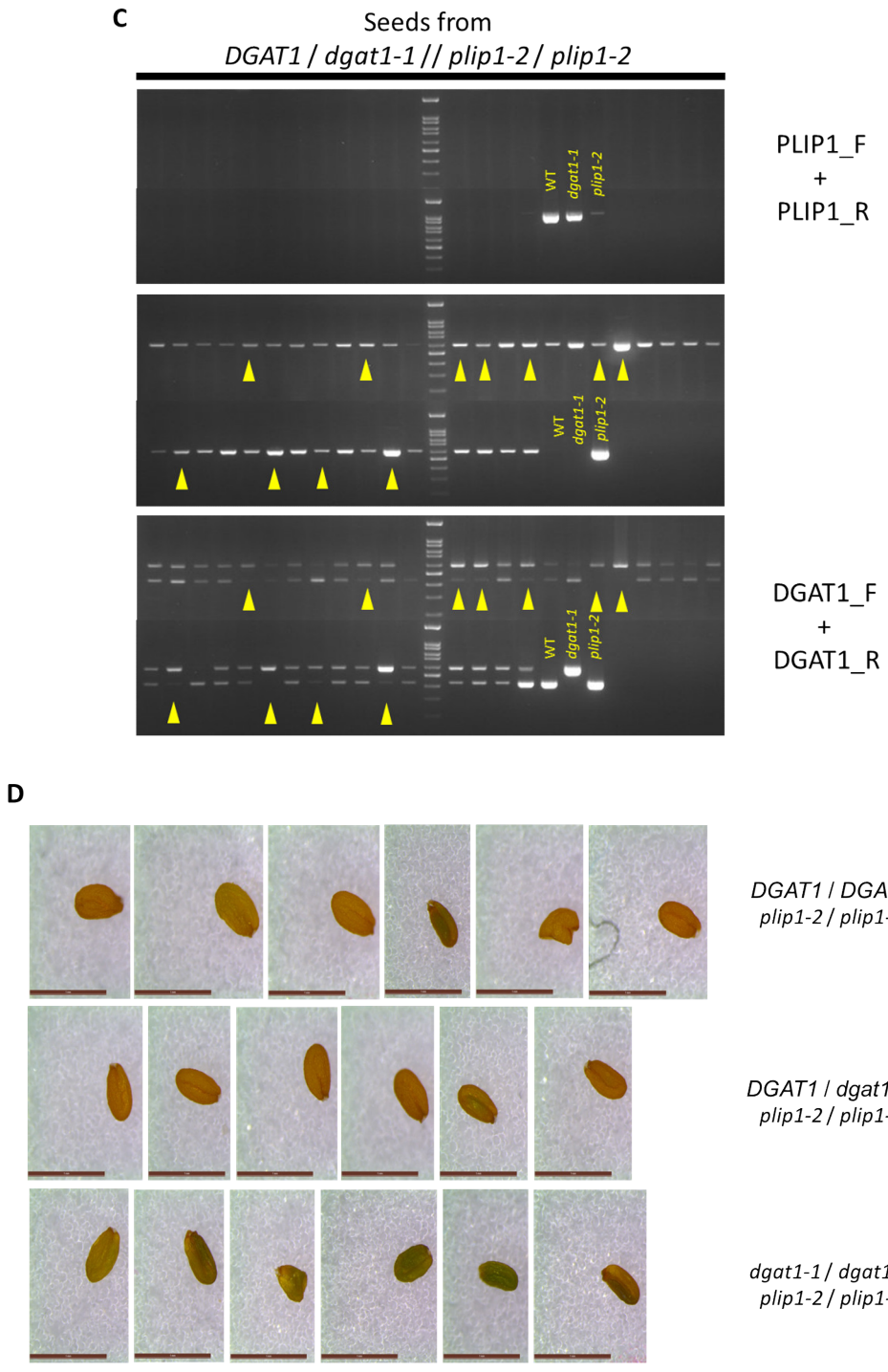
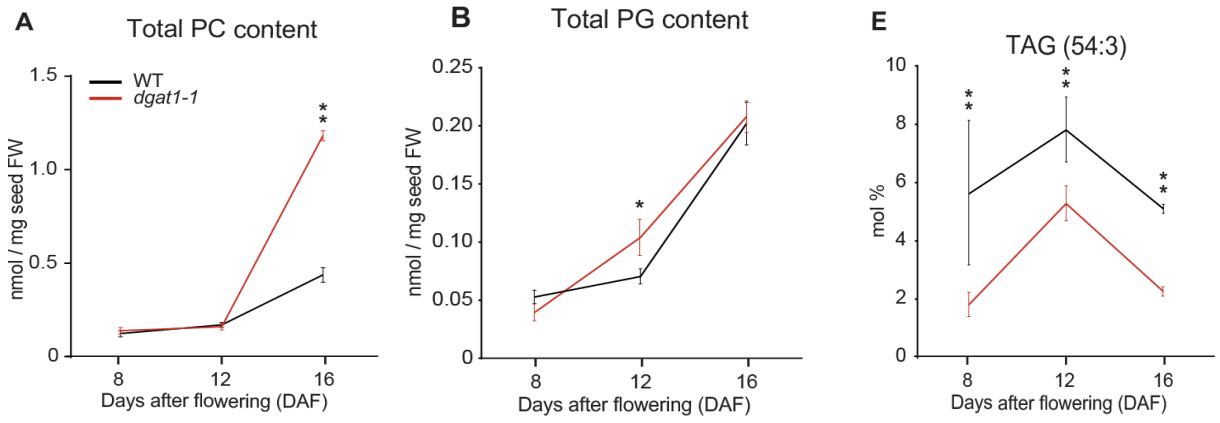


Figure 2.18 Genotypic and phenotypic data for individual seeds harvested from *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2* and *DGAT1*/*dgat1-1 plip1-2*/*plip1-2* plants.

Genotyping of DNA extracted from single seeds harvested from *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2* and *DGAT1*/*dgat1-1 plip1-2*/*plip1-2* plants. Results from PCR using different primer combinations A, seeds from plants with genotype *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2* C, seeds from plants with genotype *DGAT1*/*dgat1-1 plip1-2*/*plip1-2*. B, D Single seed pictures for corresponding genotypes mentioned in the picture. Yellow arrowheads indicate the double mutant genotype *dgat1-1*/*dgat1-1 plip1-2*/*plip1-2.* WT, *dgat1-1,* and *plip1-2* were used as PCR controls.

2.2.12 The lipid phenotype of *dgat1-1* reflects increased PLIP1 activity

During seed development in *dgat1-1*, we observed an overall increase in polar lipid content (Figure 2.1B). Although no change in total PG content was observed across seed development in *dgat1-1* (Figure 2.19B), analysis of the lipid composition in different PG molecular species indicated a reduction in PG 34:4 (Figure 2.19D), consistent with the upregulation of *PLIP1* and the substrate specificity of the encoded lipase. Lipid composition analysis of different PC species indicates an increase in content of PC36:6 species which coincides with the incorporation of 18:3 from PG34:4 into PC as previously reported (Wang et al., 2017). Based on the increased content of 18:3 acyl group in lipids extracted from seeds at 16DAF (Figure 2.1D), elevated levels of 18:3 containing TAG54:9 species and reduction in 54:3 species at 16DAF, we hypothesize that in the absence of DGAT1, PLIP1 provides substrate for PDAT1 mediated TAG biosynthesis in developing seeds of Arabidopsis.



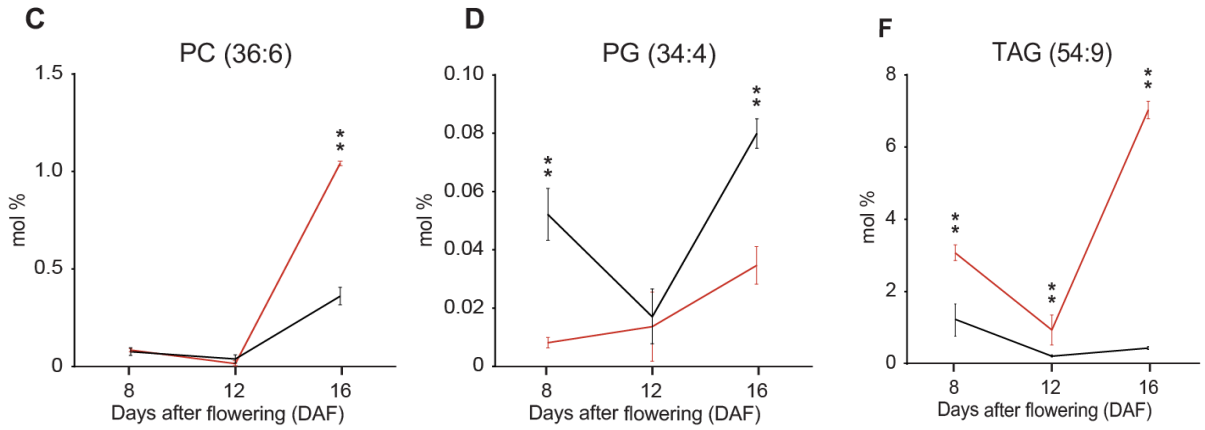


Figure 2.19 Lipid profile for different lipid species across seed development.

Levels of the total PC (A), total PG (B), molecular species PC 36:6 (C), PG 34:4 (D), TAG 54:3 (E) and TAG 54:9 (F) were quantified in developing seeds at 8, 12 and 16DAF. Total PC levels were quantified using neutral loss scan of respective headgroups. Data is normalized using seed fresh weight (FW). For PG and PC mol% was calculated from total polar lipid content, and for the TAG, mol% was calculated from the total neutral lipid contents. Data presented are from three biological replicates. Bars indicate ±SD. **P* <0.05 and ***P*<0.01 by Student's t-test for significance in the difference.

2.3 Discussion

The synthesis of TAG in developing seeds is a complex system that involves the movement of substrates between different pathways and organelles. Not only is TAG biosynthesis valuable for agricultural production, but it also appears intrinsically connected with seed development (Zhang et al., 2009). Given the importance of TAG synthesis at these multiple levels, we used RNA-Seq to obtain global gene expression profiles to gain insights into how developing *dgat1-1* mutant seeds respond to reduced levels of these important storage lipids.

2.3.1 Altered pathways provide substrate for PDAT

Our results demonstrate that in the absence of functional DGAT1, the synthesis of TAG is adjusted to provide increased substrate, particularly PC, so that PDAT1 can now synthesize the TAG found in the *dgat1-1* seed. The absence of any lipid phenotype in *pdat1* mutant seed implies that the enzyme plays a minor role in TAG accumulation when DGAT1 is present

(Mhaske et al., 2005). In contrast, the lethal phenotype of *dgat1-1 pdat1* double mutants argues that PDAT1 is responsible for TAG synthesis in *dgat1-1* mutants (Zhang et al., 2009). In *dgat1-1* mutant seed, therefore, the expression of specific genes is upregulated to increase the supply of substrate for PDAT1 to compensate for the lack of DGAT1. In particular, we observed increased expression of genes involved in the formation of PC, which acts as the acyl donor for the formation of TAG by PDAT, from various sources. These include *LPCAT2*, whose gene product regenerates PC from LPC, one of the products of the PDAT reaction (Figure 2.4D). The importance of LPCAT2 in regenerating PC for PDAT1 has been previously demonstrated, with seed from *dgat1-1 lpcat2* double mutants possessing an oil content 35% of seed from wild-type plants (Xu et al., 2012). The related LPCAT1 acyltransferase appears to play a less important role in this PC generation for PDAT compared to LPCAT2. Our results indicate that the encoding gene is downregulated in *dgat1-1* at 16 DAF (Figure 2.4D). This result is generally consistent with the fact that the oil content of the *dgat1-1 lpcat1* double mutant is the same as that of *dgat1-1* but differs the microarray results in the same study which showed an upregulation of *LPCAT1* in *dgat1-1* seed (Xu et al., 2012).

Other genes upregulated in developing *dgat1-1* seed also play a role in the synthesis of PC, consistent with the increased levels of this phospholipid in the mutant. For example, all three Arabidopsis phosphoethanolamine N-methyltransferases (*PEAMTs*) which methylate phosphoethanolamine to form the PC precursor phosphocholine (Chen et al., 2018), are upregulated, with *NMT1* expression increased almost 5-fold in 16 DAF *dgat1-1* seed (Figure 2.4D). Likewise, *ROD1*, which encodes a phosphatidylcholine:diacylglycerol cholinephosphotransferase that enables the interconversion of DAG and PC through the transfer of the choline headgroup (Lu et al., 2009).

In addition to increasing synthesis of PC for PDAT, *dgat1-1* seeds also increase the proportion of lipid molecular species containing polyunsaturated fatty acids (Figure 2.1C,D), the preferred substrates of PDAT1 (Ståhl et al., 2004). These increased levels of PUFA PC are consistent with the strong upregulation of different desaturases (Figure 2.1E). These include FAD2 and FAD3 which add double bonds to 18:1 and 18:2, respectively, conjugated to PC and other phospholipids found in the ER. Plastidial desaturases such FAB2 and DES2 that add double bonds to fatty acids destined for the eukaryotic pathway are also upregulated (Figure 2.4A), thus providing a more monounsaturated substrate for FAD2 (Figure 2.20). Interestingly, *DES6*, a homolog of *FAB2* and *DES2* was upregulated to an even greater extent than these genes (Figure 2.4A). The function of this enzyme is relatively unknown, with one study suggesting a role to increase levels of unsaturated fatty acids in *Agrobacterium*-derived crown galls under hypoxic and drought conditions (Klinkenberg et al., 2014). Our results imply a role in TAG synthesis, though additional work is needed to demonstrate this. Importantly, desaturases such as *FAD4* that catalyze the synthesis of PUFA in plastidial glycerolipids are not upregulated. Instead, increased desaturase activity appears focused on lipids in, or destined for, the eukaryotic pathways. In this manner, the concomitant upregulation of *FAB2*, *FAD2,* and *FAD3* leads to overall higher levels of PUFA-PC, enabling increased synthesis of TAG by PDAT1 (Figure 2.20)

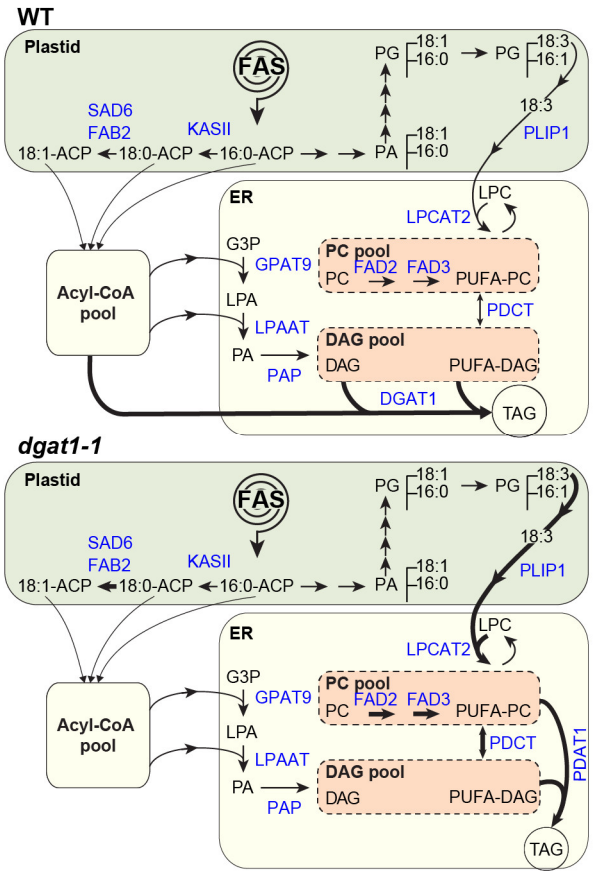


Figure 2.20 The role of *PLIP1* in TAG biosynthesis during development of wild-type (WT) and *dgat1-1* seed.

The top panel depicts an overview of TAG biosynthesis in WT seed, the bottom panel depicts the modified pathway in *dgat1-1* seed. The bold arrows indicate increase in flux in the different seed. In WT seed, TAG is synthesized primarily through the acyl-CoA dependent acylation of DAG by DGAT1. Polyunsaturated fatty acids (PUFA) are synthesized by FAD2 and FAD3 in the ER and incorporated into DAG through the action of PDCT. The plastid-localized lipase PLIP1 plays a minor role in the incorporation of PUFA into PC by releasing 18:3 from 18:3/16:1-PG. In *dgat1-1* seed, PDAT1 is responsible for the synthesis of TAG in the absence of DGAT1. Increased expression of the desaturases SAD6, FAB2, FAD2 and FAD3 result in more polyunsaturated substrate for PDAT1. Upregulation of LPCAT2 also allows the regeneration of PC for the synthesis of TAG. In addition, *PLIP1* is upregulated to increase the flow of 18:3 to PUFA-PC pool, thereby increasing the flux of PUFAs for incorporation into TAG.

Taken together, the alteration of gene expression in developing *dgat1-1* seed is consistent with an increased role for PDAT activity in the absence of DGAT function. Somewhat surprisingly, *PDAT1* mRNA expression was not increased in developing *dgat1-1* mutant seed relative to that in wild-type seeds, both when quantified using RNA-Seq or qRT-PCR (Figure 2.4C, Figure 2.5B). Previous work has demonstrated that many other DAG acyltransferases, including DGAT2 and PDAT2, do not contribute to TAG synthesis in Arabidopsis seed, even in the absence of DGAT1 function (Zhang et al., 2009). In addition, while the *dgat1-1* allele produces an aberrant transcript, this has been proven to encode a non-functional enzyme, thus demonstrating that the mutant is a complete knock out (Xu et al., 2012). The obvious implication is that increases in PDAT1 activity occur post-transcriptionally in the *dgat1-1* seed. Further work

is therefore needed to confirm this hypothesis and determine the nature of the regulation of PDAT1.

2.3.2 *PLIP1* is essential for TAG synthesis in developing *dgat1-1* seed

One of the most highly upregulated lipid metabolism genes in developing *dgat1-1* seeds is a gene we named *PLIP1* as it encodes a member of the ABH lipase family (Figure 2.6). We also demonstrated that *MFT* and *NPC6*, genes which have not been previously shown to play a role in TAG biosynthesis, were also upregulated. However, we focused on *PLIP1* due to its expression pattern and phenotype of mutant alleles, particularly in combination with mutations in *DGAT1*. In wild-type Col-0 plants, *PLIP1* expression was not confined to seeds as transcripts were detected in other tissues (Figure 2.11). However, *PLIP1* was not upregulated in these other tissues in *dgat1-1* plants, consistent with the fact that *DGAT1* is primarily expressed in developing seeds and suggesting that the role of *PLIP1* in *dgat1-1* mutants is seed specific. Seed from *plip1-2* mutants possessed slightly reduced fatty acid levels compared to wild-type seed (Figure 2.8A). The seed fatty acid composition was also slightly changed in the mutants, with higher levels of 18:3 and lower levels of 18:1 and 18:2. A large portion of mutant *plip1-2* seeds was smaller than wild-type or even *dgat1-1* mutant seed, providing additional support for the role of *PLIP1* in seed embryo development. Importantly, we were unable to recover homozygous double *dgat1-1 plip1-2* plants, consistent with *PLIP1* playing an important role in the seed of *dgat1-1* (Figure 2.14). While this synthetic lethal phenotype is reminiscent to that of the *dgat1-1 pdat1* double mutant, the latter is caused by non-viable pollen cells (Zhang et al., 2009). In contrast, reciprocal crossing experiments demonstrated that both gametophytes are viable (Figure 2.16). Instead, the *dgat1-1 plip1-2* double mutant seed is formed but fails to germinate (Figure

2.17B), suggesting the importance of *PLIP1* in seed development, particularly in the *dgat1-1* background.

2.3.3 *PLIP1* provides substrate for PDAT

A recent report has demonstrated that *PLIP1* encodes a plastid-localized lipase, PLIP1, that releases polyunsaturated fatty acids from PG (Wang et al., 2017). These released fatty acids are incorporated into PC and eventually into TAG. Our results suggest that this PUFA-PC is a preferred substrate for PDAT1, providing an additional pathway for the incorporation of PUFA into TAG. Indeed, results from other studies suggest other routes besides acyl editing for the incorporation of PUFA into TAG. In *dgat1-1 lpcat2* mutants, where the acyl editing cycle to regenerate PC for PDAT1 is disrupted, TAG is still synthesized and contains high levels of 18:3 (Xu et al., 2012). Likewise, the seed from *lpcat1 lpcat2 rod1* triple mutants still contains appreciable quantities of 18:3 (Bates et al., 2012). In wild-type seeds, PDAT1 makes only a minor contribution to the overall synthesis of TAG (Mhaske et al., 2005). Thus, mutations affecting PLIP1 activity likewise only have a small effect on TAG synthesis (Figure 2.8A), as a substrate for PDAT1 is not needed. However, when DGAT function is eliminated, PDAT1 activity becomes important for TAG synthesis. Elimination or reduction of both activities results in non-viable seed with oil content only 20% of wild-type levels (Zhang et al., 2009). In *dgat1-1* seed, therefore, *PLIP1* is upregulated to provide more PUFA-PC for PDAT which is then incorporated into TAG. Consistent with this idea, overexpression of *PLIP1* results in higher levels of 18:3 in PC and in TAG (Wang et al., 2017). When TAG is dependent on PDAT activity (as in *dgat1-1* seed), elimination of the source of substrate for PDAT1 results in non-viable seed, similar to previous work knocking down *PDAT1* expression in a *dgat1-1* background (Zhang et al., 2009).

2.3.4 Mutant *dgat1-1 plip1-2* seeds develop but fail to germinate

The most obvious explanation for the inability of *dgat1-1 plip1-2* seed to germinate is their inability to accumulate energy reserves in the form of TAG. This conclusion is consistent with the observation that seeds with the expression of both *DGAT1* and *PDAT1* disrupted through a combination of mutation and RNAi also fail to germinate (Zhang et al., 2009). However, this explanation might be simplistic, as it is quite well known that seeds even with dramatically low levels of seed oil can germinate, albeit less efficiently. Further, it seems surprising that given the strong upregulation of *FAD2* and *FAD3*, as well genes involved in PC synthesis, that there is not enough PUFA substrate (PC or DAG) for PDAT to function, even in the absence of *PLIP1*. Germination delays or defects for both *dgat1-1* and *plip1-2* mutants have been noted (Wang et al., 2017; Routaboul et al., 1999) and it is possible these might be exacerbated in the double mutant. Here, the upregulation of genes important for regulation of seed germination, such as *MFT* in the *dgat1-1* seed is also particularly interesting (Figure 2.6). Future work quantifying the flux of fatty acids through *PLIP1*, particularly in *dgat1-1* seed, will be helpful in determining the importance of the multiple different routes for PUFA incorporation into TAG.

2.4 Material and Methods

2.4.1 Plant Materials and Growth Conditions

T-DNA insertion mutant lines from the SALK (Alonso et al., 2003), SAIL (Sessions et al., 2002) and GABI-Kat (Kleinboelting et al., 2012) collections were obtained from the Arabidopsis Biological Resource Center at the Ohio State University. Seeds were cold stratified for two days at 4˚C before growing on soil mixture (4:2:1 mixture of Metromix Professional

Growing Mix: vermiculite: perlite) in growth chambers at 23 ˚C under long-day conditions (16 h light / 8 h dark). For growth on plates, seeds were surface sterilized before plating on half-strength Murashige and Skoog media with 5% (w/v) phytoblend agar and 1% (w/v) sucrose. When making crosses, immature flower buds were emasculated and manually cross-pollinated with pollen from the flower of the donor parent. To isolate seeds at different developmental stages, emerging flowers were tagged with the cotton thread at specific time intervals. Whole siliques were flash frozen in liquid nitrogen and stored at -80 ˚C. Seed harvesting from siliques was done on dry ice (Bates et al., 2013).

2.4.2 Microscopy

Pictures of mature seeds was taken using a stereo microscope Leica M165 FC and Leica application suite V4.1. Seed size distribution was done using 1000 seeds and area of seed size was quantified using ImageJ particle analysis software (Herridge et al., 2011).

2.4.3 Clearing and imaging of developing seeds

Freshly harvested seeds from different stages of development were collected from wild type and *dgat1-1* plants and fixed for 1 hour in ethanol:acetic acid (6:1, v/v) at room temperature. Samples were washed using 70% ethanol, mounted in chloral hydrate:glycerol:water (8:1:2, v/v/v) and cleared for about 1 hour at room temperature (Berleth and Jurgens, 1993; Anderson, 1954). Cleared samples were visualized using Zeiss Axioplan 2 upright microscope equipped with a Lumenera Infinity 3S-1UR monochrome CCD camera.

2.4.4 Genotyping of T-DNA lines

Genomic DNA was extracted from leaves and seed as previously described (Edwards et al., 1991). Primers used for the PCR-based genotyping of the SALK and SAIL mutant lines were designed using the SIGnAL T-DNA Express Arabidopsis Gene Mapping Tool (Alonso et al., 2003); primers for the GABI-Kat lines were designed using the GK primer tool (Kleinboelting et al., 2012). All genotyping primers used in this study are listed in Table 2.2.

Gene	T-DNA line/Germplasm	Primer name	Primer sequence
T-DNA primer (SALK lines)	SALK	LBb1_v2	5'-GGACCGCTTGCTGCAACT-3'
		LBb1.3	5'-ATTTTGCCGATTTCGGAAC-3'
T-DNA primer (SAIL lines)	SAIL	LBb3_SAIL	5'-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3'
T-DNA primer (GABI-Kat lines)	GABI-Kat	T-8474	5'-ATAATAACGCTGCGGACATCTACATTTT-3'
AT2G19450 (DGAT1)	CS3861	DGAT1-1_F	5'-GCGGAGAAGGAAGAGGAAAC-3'
		DGAT1-1_R	5'-TCGCAGCGATCTTGAACTAA-3'
AT3G18000 (NMT1)	SAIL_28_H12 (CS801444)	NMT1-1_F	5'-GTGCTGATGTTACATCCCCTGAC-3'
		NMT1-1_R	5'-CCAATGCCACACCCAACATCTAAG-3'
	SALK_036291.55.50.x (4546439)	NMT1-2_F	5'-CGCATTGGAACGTGCTATTGGAC-3'
		NMT1-2_R	5'-ACCGCTGAATCAAACACAATCCC-3'
AT1G18100 (MFT)	SALK_147675.50.50.x (CS60000)	MFT-2_F	5'-CTAACCGAAAGCACCATGTCC-3'
		MFT-2_R	5'-GCCATTCCGATGAGCTTTACAC-3'
	SALK_024298.32.55.x (CS27942)	MFT-3_F	5'-CTCTTGAGGGATTTGTGCCTCC-3'
		MFT-3_R	5'-GCGGCTTCTGTTGATCCTTTGG-3'
AT3G48610 (NPC6)	SALK_077041.54.50.x (CS27941)	NPC6-1_F	5'-CCATGTGATTTGGTTTTTGCAGCC-3'
		NPC6-1_R	5'-GACCGGTGGTTTTCTTCAATTCCC-3'
	SALK_116984.37.75.n (4794543)	NPC6-2_F	5'-GTCATCTCTCTGACTCCGACGAC-3'
		NPC6-2_R	5'-CTCGAACCGATTGTCCCATGAC-3'
	SALK_088882C (CS27943)	NPC6-3_F	5'-GGTGGGCTCGATATGTAGAAGAAG-3'
		NPC6-3_R	5'-GTCGCATAATCACGAAAAGTTGGC-3'
AT3G61680 (PLIP1)	SALK_102149.54.50.x (CS60000)	PLIP1-1_F	5'-GTGGTGGTGAGAAGATTCTAGCGG-3'
		PLIP1-1_R	5'-CGCCTGGTTTAATTAGTGGGCC-3'
	SALK_147687.55.25.x (CS27942)	PLIP1-2_F	5'-GCAACGCTGTTTTTGATCCTCC-3'
		PLIP1-2_R	5'-CGATCTCCGTATACCACCGC-3'

Table 2.2 List of primers for genotyping of T-DNA lines

2.4.5 Lipid extraction and quantification

Total lipids were extracted from developing WT and *dgat1-1* seed using a hexane-isopropanol method (Li et al., 2006) with di15:0 PC and tri 13:0 TAG as internal standards. Total

lipids were loaded onto a small silica column and neutral lipids eluted with 5ml 99:1 (v/v) chloroform: methanol. Polar lipids were then recovered with 5 ml methanol. These lipid extracts were then analyzed using electro-spray ionization mass spectrometry (ESI-MS) as described previously (Bansal and Durrett, 2016). Seed fatty acids were quantified by transmethylating intact seeds (Li et al., 2006) using tripentadecanoin (Nu-Chek Prep) as an internal standard. The resulting fatty acid methyl esters were quantified on a Shimadzu GC-2010plus gas chromatograph equipped with a 30 X 0.25mm DB-23 column (Agilent Technologies) as described previously (Aznar Moreno and Durrett, 2017).

2.4.6 RNA isolation and sequencing

Total RNA was extracted from the pooled seeds of 30-40 siliques using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions, followed by additional purification with sodium citrate to improve RNA quality (Nybo, 2011). We obtained total RNA yields in the range of 1-3ug for ~50mg seeds. Using an Agilent Bioanalyzer, we confirmed that the RNA Integrity Number (RIN) for all samples was greater than 9. Total RNA (500 ng) was sequenced at the Genomics Core at the University of Kansas Medical Center. The mRNA fraction was enriched with oligo dT capture, sized, reverse transcribed into cDNA and ligated with the appropriate indexed adaptors using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina). Following Agilent Bioanalyzer QC of the library preparation and library quantification using the Roche Lightcycler96 with KAPA SYBR Fast Universal qPCR kit (KAPA Biosystems), the RNA-Seq libraries were adjusted to a 4nM concentration and pooled for multiplexed sequencing. Libraries were denatured and based on qPCR results, diluted to the appropriate concentration, followed by clonal clustering onto the sequencing flow cell using the TruSeq Paired-End (PE) Cluster Kit v3-cBot-HS (Illumina). The clonal clustering procedure was

automated using the Illumina cBOT Cluster Station. The clustered flow cell was sequenced on an Illumina HiSeq 2500 Sequencing System using the TruSeq SBS Kit v3-HS (Illumina) to obtain 100bp pair-end reads, which were trimmed and quality assessed before assembly against the Arabidopsis reference genome (Araport 11) using CLC genomics workbench (v 7.5.1). To identify differentially expressed genes, the EDGE test (empirical analysis of differentially expressed genes) was used which implements the "Exact test" for two-group comparisons. This test assumes that all samples have negative binomial distribution and accounts for the biological variability of replicates.

2.4.7 Quantitative Real-Time PCR (qRT-PCR)

cDNA was synthesized from 1µg total RNA using the Quantitect Reverse transcription kit (Qiagen). Relative mRNA levels were quantified with iTaq universal SYBR green supermix (Bio-rad) on a CFX-96 real-time PCR system (Bio-rad) using ASAR1 (*AtSAR1*) as a reference gene control. Relative quantification of transcript levels was done using delta Ct method, which assumes PCR efficiency of 100% for primers. Primers used for RT-PCR are listed in Table 2.3

Gene	Primer name	Primer sequence
AT2G19450 (DGAT1)	DGAT1-RT_F	5'-CTGCTTCGGGGATCGTGAAT-3'
	DGAT1-RT_R	5'-TGGCGAGTGTCTTTGGTATCT-3'
AT5G13640 (PDAT1)	PDAT1-RT_F	5'-GTTGCAGTTGCCAGAGCGATTG-3'
	PDAT1-RT_R	5'-GAGTCCCATGTGCGTGTCATTCTC-3'
AT3G61680 (PLIP1)	PLIP1-RT_F	5'-GCGGCAAGGGAGTTACAG-3'
	PLIP1-RT_R	5'-GGACTAATACATCTGTGTCCTCCTC-3'
AT1G18100 (MFT)	MFT-RT_F	5'-TGTTCATCCCAACCGCCAAT-3'
	MFT-RT_R	5'-GGTCAGTCATCACGAGAGTGTAA-3'
AT3G48610 (NPC6)	NPC6-RT_F	5'-TGTGGATACCCTTGAGCGAG-3'
	NPC6-RT_R	5'-TTAGGCCTGAAGCTGTTCCG-3'
AT3G12120 (FAD2)	FAD2-RT_F	5'-CCATCTCCAGAAACATGGGTGCAG-3'
	FAD2-RT_R	5'-GGTTTCTCGCACGGCACAC-3'
AT4G02080 (ASAR1)	ASAR1-RT_F	5'-CTATGCTAAGGTGGACGCTGTGG-3'
	ASAR1-RT_R	5'-CGTCTGAGAGAAGTGCATCAAGTTCC-3'
AT5G25760 (UBC21)	UBC21-RT_F	5'-CTGCGACTCAGGGAATCTTCTAAGATC-3'
	UBC21-RT_R	5'-CCTTTCTTAGGCATAGCGGCG-3'
AT1G13320 (PP2AA3)	PP2AA3-RT_F	5'-GGAGCCAACTAGGACGGATCTG-3'
	PP2AA3-RT_R	5'-CTTGGTAACTTTTCCAGCAGCTGC-3'
AT5G45830 (DOG1)	DOG1_F	5'-GAGCTGATCTTGCTCACCGATGTAG-3'
	DOG1_α_R	5'-CCACTATTCACAGTTGTACATGCATCGAATATTACTTC-3'
	DOG1_ε_R	5'-CACATACGGGTTATATATCCCTAGAATATATGCCA-3'

Table 2.3 List of primers for qRT-PCR experiment

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Chapter 3 - Genome-wide differential gene expression analysis identifies loci associated with altered lipid composition, seed development and maturation for the *dgat1-1* mutant in *Arabidopsis thaliana*

3.1 Introduction

Cellular metabolism has been divided into well-characterized discrete pathways which operate as a highly integrated network (Serin et al., 2016). Changes in gene expression associated with different metabolic pathways during seed development underscore the dynamic nature of metabolism (Allen et al., 2015). An operational network of metabolic pathways communicate with each other to accommodate cellular demands in response to a different set of conditions including environmental stress and plant development. In Arabidopsis, around 600 genes have been annotated as playing a role in lipid catabolism and anabolism (Li-Beisson et al., 2013). In vivo characterization of 115 independent mutant lines for different genes, have clearly demonstrated their role in lipid metabolism (McGlew et al., 2015).

Based on different cellular demands, including lipid production and turnover of storage lipids during seed development and maturation, there are observed changes in the flux of different lipid species to maintain homeostasis (Bates and Browse, 2012; Allen et al., 2009). Storage oils in seeds which are mainly comprised of triacylglycerols (TAG) act as an energy reserve and provide a source of carbon and ATP for growth until the start of autotrophic metabolism (Theodoulou and Eastmond, 2012).

3.1.1 A network of metabolic pathways across seed development

In developing seeds, the source of carbon, energy and reducing equivalents are derived from coordination between various metabolic pathways including the pentose phosphate pathway, glycolytic pathway, TCA cycle, and amino acid biosynthesis. During early stages of seed development, the embryo accumulates starch and oil, for which most of the carbon derived from Glucose 6-phosphate is utilized for starch synthesis and the remainder of carbon is used for fatty acid synthesis and the pentose phosphate pathway. As the seed progresses towards maturity, there is increase in activity for enzymes involved in lipid and carbohydrate degradation (Graham, 2008). Lipases are involved in mobilization of storage lipids and the storage reserve mobilization process further involves a reduction in the flux of Glucose 6-phosphate to starch and fatty acids and increase in the flux of Glucose 6-phosphate to oxidation via pentose phosphate pathway (Rawsthorne, 2002). Proteins such as LACS (Acyl-CoA synthetase), ACX (Acyl-CoA oxidase), MFP2 (Multifunctional protein 2) and KAT2 (3-keto-acyl-CoA thiolase) have an important role in β-oxidation. Futher, proteins which are involved in glyoxylate cycle are citrate synthase (CSY), isocitrate lyase (ICL), malate synthase (MLS) and phosphophoenolpyruvate carboxykinase (PCK1) (Graham, 2008).

The involvement of various metabolic pathways in fatty acid biosynthesis in seeds is demonstrated by ^{13}C-metabolic flux analysis of *wri1* mutant lines (Masterson et al., 1990). WRINKLED1 (WRI1) is an important regulator of storage lipid biosynthesis in developing seed (Cernac and Benning, 2004). Reduced oil content in *wri1* was attributed to decreased flux from sucrose (Allen et al., 2015), and these findings were consistent with the reduction in transcript levels of genes involved in glycolysis (Baud and Graham, 2006) and reduction in transcript levels of genes involved in fatty acid metabolism (Ruuska et al., 2002; Focks and Benning,

1998). Plastidial phosphoglucomutase (PGM1) is an important enzyme which is involved in the carbon partitioning between starch synthesis and carbohydrate oxidation during storage lipid biosynthesis in the seed. A 40% reduction in total lipid content was observed in the embryos of the *pgm-1* mutant (Periappuram et al., 2000) which lacks the plastidial phosphoglucomutase (Caspar et al., 1985).

Differential expression (DE) analysis helps in the identification of statistically significant changes in expression of genes with respect to variation factor involved. Variation factors can be due to the presence of any mutation or experimental treatment. Clustering and co-expression analysis finds groups of genes which have similar expression pattern with different variables such as environment, different time points, different tissue-specific, presence or absence of mutation and disease (Windram et al., 2012; Cai et al., 2010).

In addition to DE analysis, the clustering and correlation-based approaches help in better understanding of complex traits. There have been studies for the co-expression for various genes in lipid metabolism, hormone metabolism and biochemical pathways (Bernard and Joubès, 2013; Avin-Wittenberg et al., 2011; Baud and Lepiniec, 2010; Loraine, 2009; Obayashi et al., 2006; Ruuska et al., 2002). The changes in content and composition of various lipid species in samples can also be correlated with differential expression of genes. Studies combining expression and metabolic profiles have found a correlation with different phenotypes and change in environmental conditions (Djanaguiraman et al., 2018; Redestig et al., 2018; Marla et al., 2017; Vu et al., 2012).

The correlation studies involve combining data from the transcript expression of genes involved in lipid metabolism and associated changes in lipid composition followed by the

analysis of the extent to which changes in lipid composition are reflected by changes in gene expression and vice versa. This approach helps in the identification of new genes and their putative role. Further, genetic and biochemical studies for candidate genes help in determining their role in changes in lipid composition and the observed phenotype.

In this study, both the temporal transcriptome data and lipid data from various stages of seed development in *dgat1-1* and WT seeds from Arabidopsis was investigated. It helps in identification of many uncharacterized genes in close clusters with other known genes which have been previously characterized to be key regulators in seed development and maturation.

3.2 Results and Discussion

3.2.1 The overview of changes in transcriptome during seed development

To examine the changes in transcriptome distribution, we conducted a comparative analysis of the Reads Per Kilobase Million (RPKM) values for all genes across different stages of seed development for WT and *dgat1-1*. The gene expression value was estimated by calculating the RPKM values. RPKM is obtained by normalization of mapped reads to the total read count and gene length. Based on the RPKM value, gene expression was designated as low (<0.1), medium (>=0.1 <=10), high (>=10 <=100) and very high (>100) (Figure 3.1). Consistent with the sequential activation of the zygotic genome during later stages of seed development, there is an increase in the total number of DE genes as the seed progresses towards maturity. For the total DE genes identified for each seed development stage, we further calculated the distribution of genes with low, medium, high and very high expression values. We observed a unimodal distribution of expression values at different stages of seed development for both WT and *dgat1*-1 (Figure 3.1). In *dgat1-1*, as compared to WT we observed a relative increase in a

number of genes with high expression and reduction in the number of genes with medium expression at 8DAF. A similar distribution of expression values was observed between WT and *dgat1-1* at 12DAF and 16DAF (Figure 3.1). Consistency in distribution of RPKM values especially during later stages of seed development in WT and *dgat1-1* samples eliminates the possibility of bias in expression data caused by increase in proportion of genes with high RPKM values.

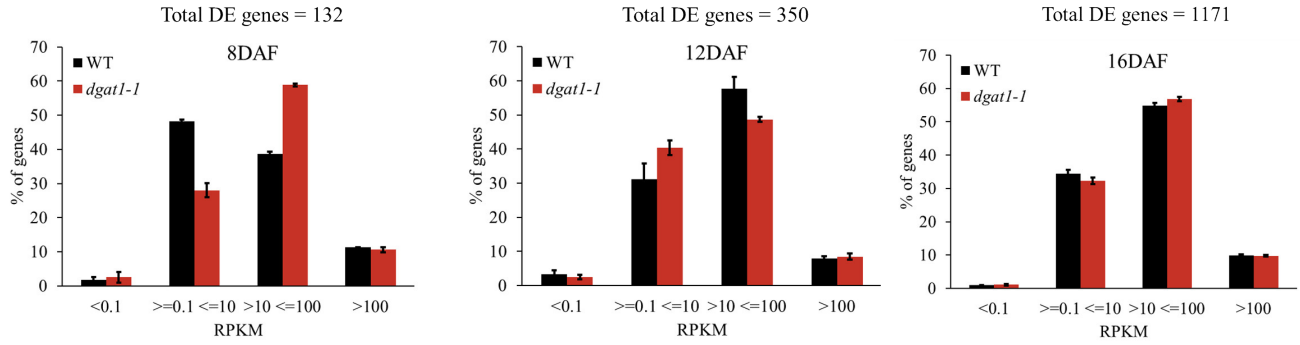


Figure 3.1 Distribution of RPKM values for DE genes across seed development

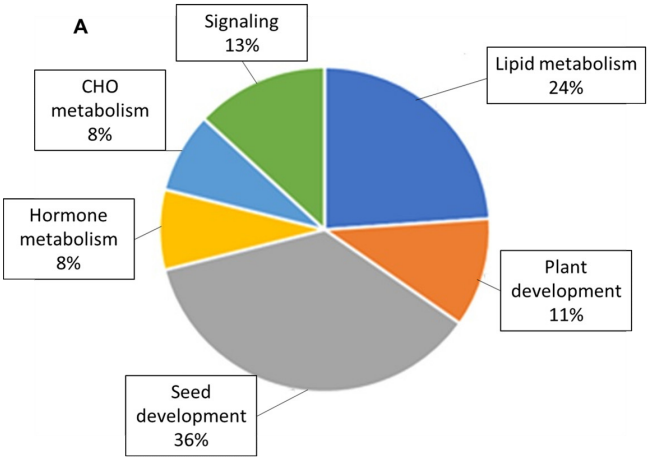
The % distribution of RPKM values was calculated for DE genes. Total DE genes at each stage are indicated on top of the graph. Based on the RPKM value, a set of genes were assigned to each bin. Gene expression values are in represented as RPKM. Results are the mean of 3 biological replicates for each stage of seed development, and the bars represent the ±SD.

3.2.2 Functional classification of DE genes in *dgat1-1* at 16DAF

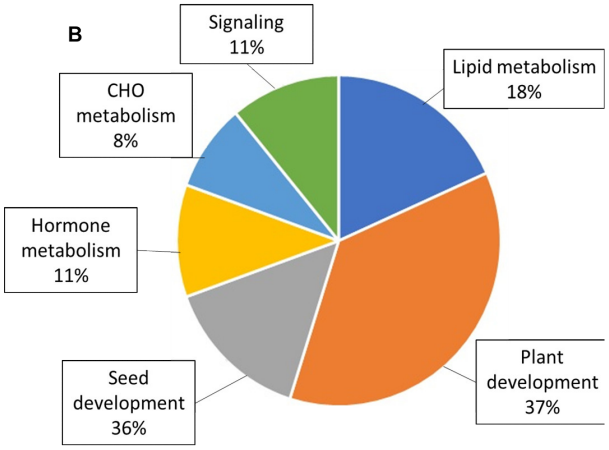
To determine the class of genes which are differentially expressed, gene ontology (GO) enrichment analysis was performed using protein annotation through evolutionary relationship (PANTHER) classification (Mi et al., 2016). From the list of various GO classes, a few important classes were selected based on their direct or indirect role in lipid metabolism. In each GO class, the number of up-regulated genes was greater than the down-regulated genes.

Among the upregulated genes, the major GO categories which belong to the biological process were seed development (36%), lipid metabolism (24%), signaling (13%) and plant development (11%) (Figure 3.2A). Similarly, among the down-regulated genes, major GO categories which belong to the biological process were plant development (37%), lipid metabolism (18%), seed development (15%) and hormone metabolism (11%) (Figure 3.2B). Further, the functional annotation of genes within each GO category, identified genes which belong to different cellular processes. For seed development category, within the down-regulated genes, 42% were seed storage proteins and 58% belong to miscellaneous categories, whereas within the up-regulated genes during seed development, 18% of genes belong to lipid metabolism, 12% belong to glycolysis, 12% belong to photosynthesis, and 58% belong to miscellaneous categories (Figure 3.2C). In lipid metabolism category, among the 69% of up-regulated genes, the majority of genes belong to fatty acid synthesis and elongation. Within the down-regulated genes in lipid metabolism category, the majority of genes are associated with lipid degradation (Figure 3.2D). In plant development process category, all of the up-regulated genes were unspecified, whereas within the down-regulated genes, 40% belong to late embryogenesis abundant (LEA) and 40% belong to unspecified category (Figure 3.2E). In hormone metabolism category, within the upregulated genes, 64% belong to brassinosteroid (BR) degradation pathway, 18% to gibberellic acid (GA) degradation and 9% to abscisic acid (ABA) induced pathways. Within the down-regulated genes in hormone metabolism, 34% belong to ABA-induced pathway, and 33% belong to GA induced pathway (Figure 3.2F).

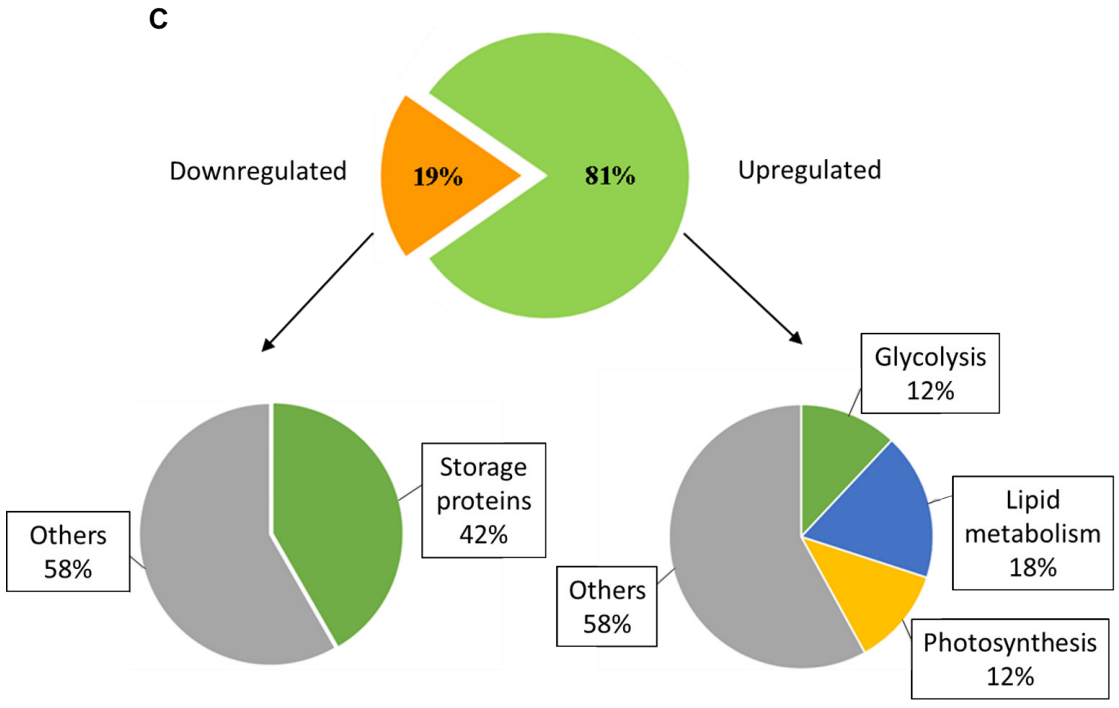
Upregulated genes



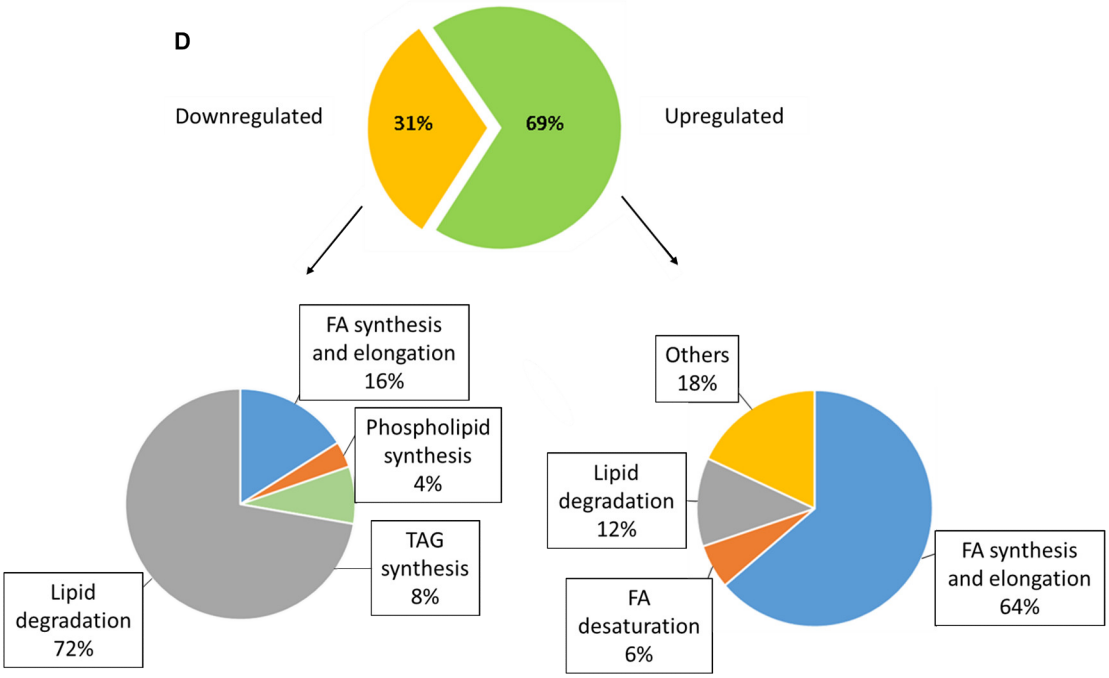
Downregulated genes



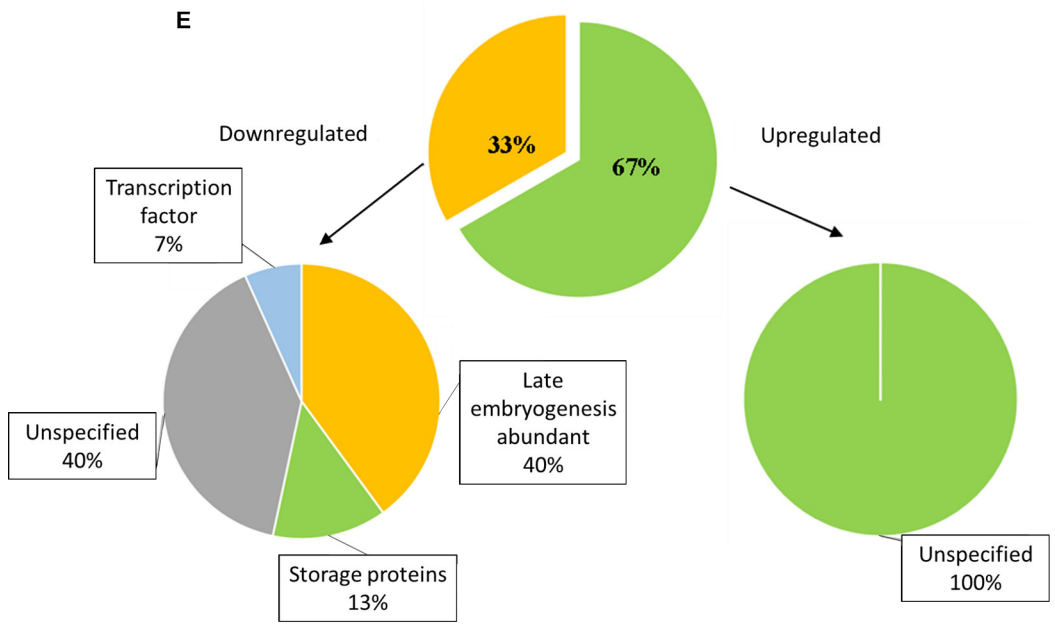
Seed development



Lipid metabolism



Plant development



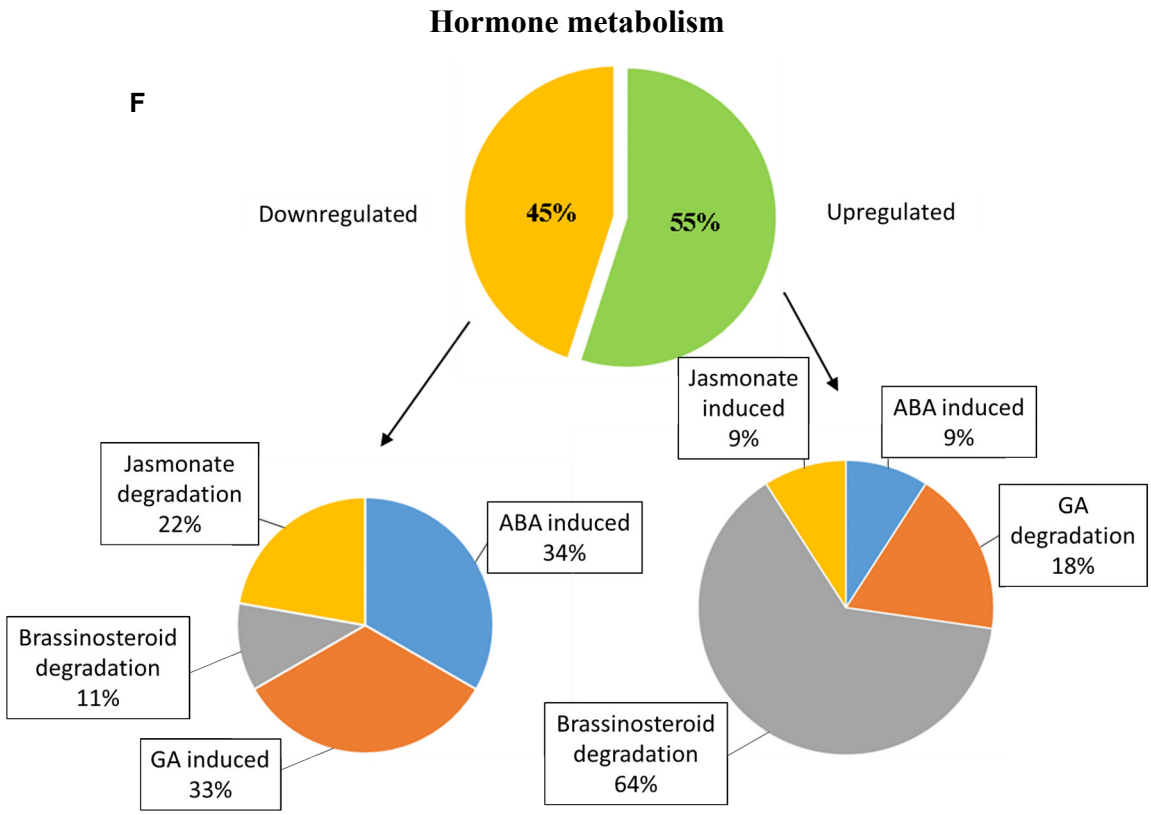


Figure 3.2 Functional classification of differentially expressed genes at 16 DAF in *dgat1-1* Arabidopsis

A, B- Functional classification of the up-regulated and down-regulated genes based on biological processes and metabolic function. GO enrichment analysis was performed using protein annotation through evolutionary relationship (PANTHER) classification system. In C, D, E, F the central pie chart represents the percentage of genes for up-regulated and down-regulated, further GO enrichment and classification of genes either down-regulated or upregulated are shown on the left and right side respectively

In *dgat1-1* seeds, there is a 45% reduction in total oil content relative to WT. The altered fatty acid composition in *dgat1-1* includes a 60% decrease in 18:1 (Routaboul et al., 1999; Zou et al., 1999; Katavic et al., 1995). In plants *de novo* fatty acid synthesis occurs in plastids. During later stages of seed development in *dgat1*-1, we observed a marginal increase in expression for genes involved in fatty acid synthesis (Figure 2.4B). Thus we were interested in the identification of differentially expressed which are localized to the plastid. Among these genes, 67% are up-

regulated, and 33% are downregulated. Further functional classification based on molecular function using PANTHER, identified that a majority of genes encoded proteins with catalytic activity.

Among the downregulated genes in the plastid, categorization of protein classes within the catalytic activity category identified 32% of genes encoding for a protein with hydrolase activity and 36% of genes encoding for a protein with oxidoreductase activity (Figure 3.3). Protein classification of upregulated genes within the catalytic activity identified 39% of genes encoding for a protein with transferase activity followed by 33% of genes encoding for a protein with oxidoreductase activity (Figure 3.3).

3.2.3 Genes positively/negatively regulated to the *PLIP1*

To determine the co-expression network for DE genes in *dgat1-1*, we conducted a clustering analysis using normalized RPKM values with Spearman rank correlation as a measure of distance. Clustering results identified two major clusters (Figure 3.4), with the majority of DE genes encoding for proteins with molecular functions including nucleic acid binding, protein binding, hydrolase activity, transferase activity, and oxidoreductase activity (Figure 3.5). One of the most highly upregulated lipid metabolism genes in developing *dgat1-1* seeds is a gene we named *PLIP1* as it encodes a member of the ABH lipase family.

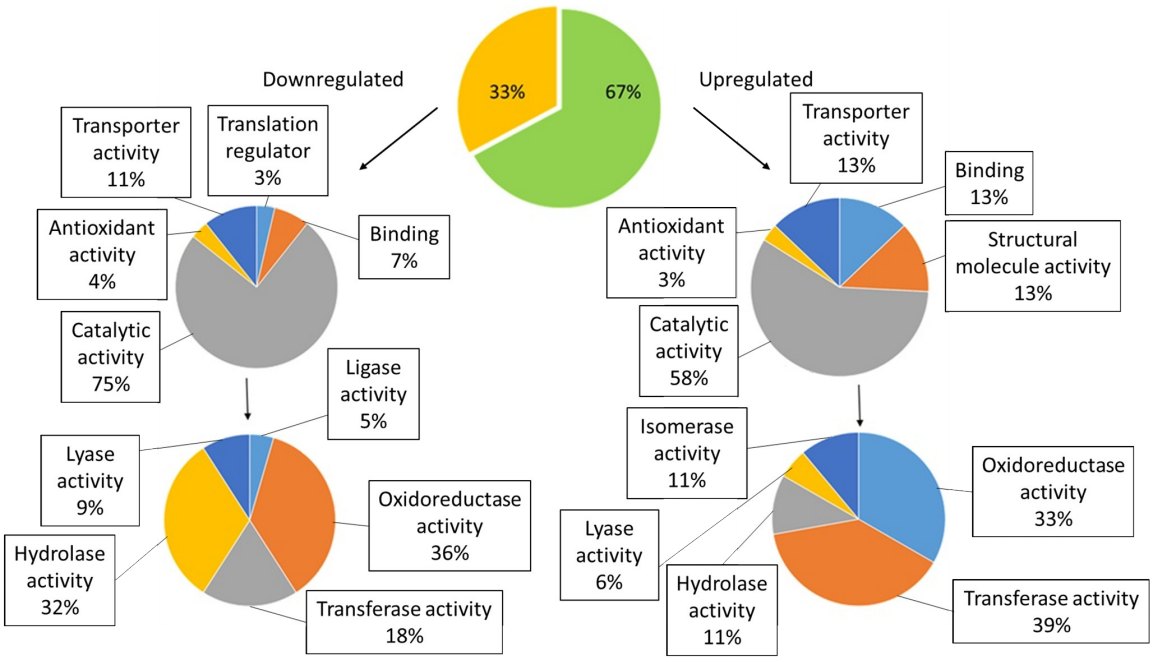


Figure 3.3 Functional classification of plastid localized differentially expressed genes at 16 DAF in *dgat1-1* Arabidopsis

The central pie chart represents the percentage of up-regulated and down-regulated plastid localized genes, further GO enrichment and classification of genes for down-regulated and upregulated are shown on the left and right side respectively. GO enrichment analysis was performed using protein annotation through evolutionary relationship (PANTHER) classification system. Functional classification was based on molecular function.

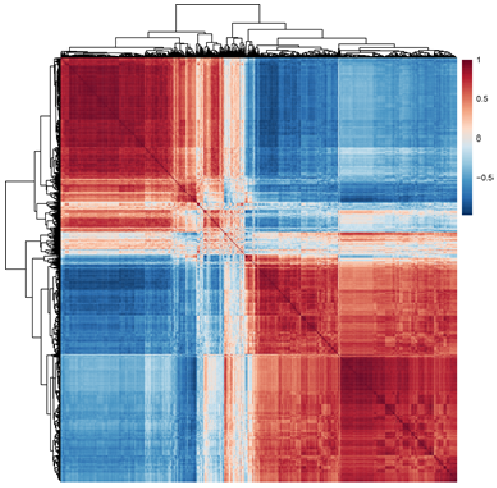


Figure 3.4 The hierarchical clustering analysis

The hierarchical clustering analysis for all annotated genes generated using the MetaboAnalyst. Data presented is for top 1000 significant genes which were clustered using Spearman correlation with single linkage. Bar represents the correlation coefficients with the highest correlation coefficient of 1, represented as red color to a lowest correlation coefficient of -1 represented as blue color.

The expression of *PLIP1* is upregulated in *dgat1-1* and its importance in the incorporation of PUFAs into TAG is discussed in Chapter 2. Further, we identified the genes which show a positive correlation with *PLIP1* expression (Table 3.1).

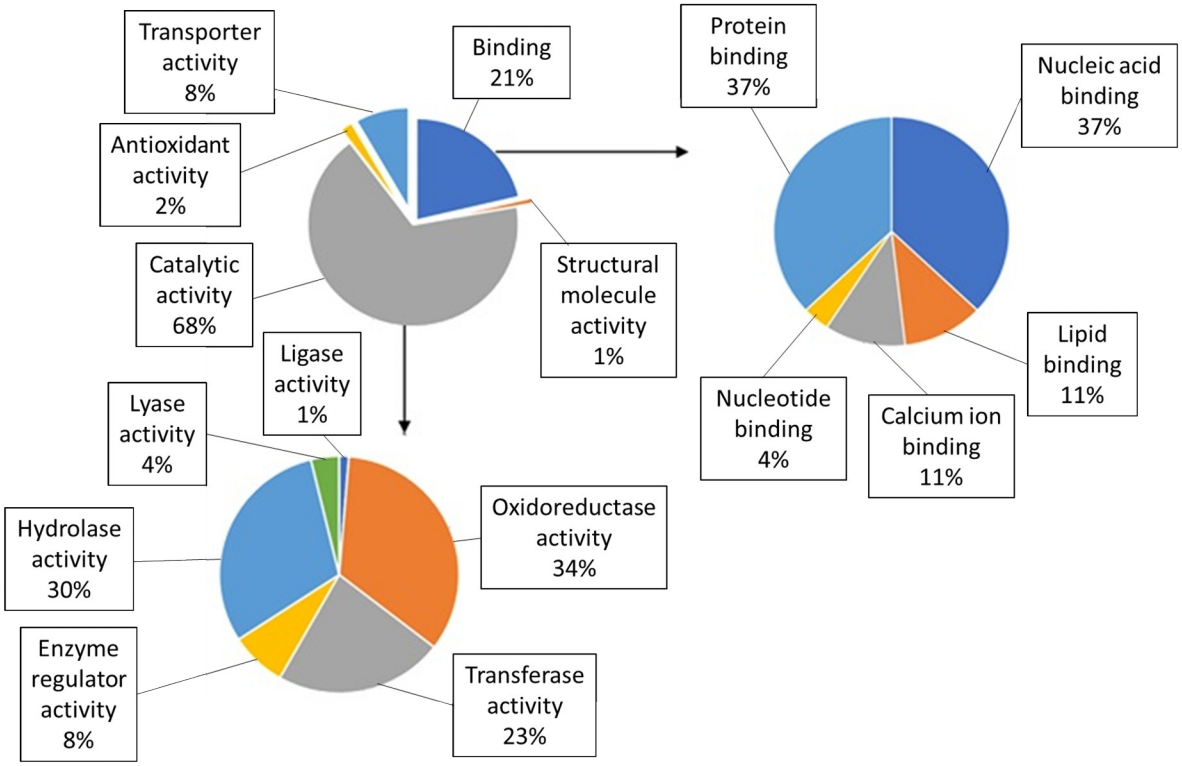


Figure 3.5 Functional classification of statistically significant genes obtained by clustering at 16 DAF in *dgat1-1*

GO enrichment analysis was performed using protein annotation through evolutionary relationship (PANTHER) classification system. Functional classification was based on molecular function.

Gene ID	Abbreviation	correlation coefficient	p-value
AT3G61680	*PLIP1*	1.000	1.06E-05
AT3G12120	*FAD3*	0.936	0.00E+00
AT2G26040	*RCAR14*	0.825	2.52E-05
AT2G29980	*FAD2*	0.804	7.70E-05
AT3G15820	*ROD1*	0.802	8.50E-05
AT4G27030	*FAD4*	0.573	1.46E-02
AT4G30950	*FAD6*	-0.102	6.86E-01
AT3G11170	*FAD7*	-0.021	9.34E-01
AT5G05580	*FAD8*	-0.337	1.71E-01

Table 3.1 List of genes positively correlated with *PLIP1*

Correlation coefficients were calculated by selecting *PLIP1* gene in the pattern hunter in Metaboanalyst. The correlation coefficient was calculated using Spearman correlation with single linkage. The genes associated with increased PUFAs in the prokaryotic and eukaryotic pathway were selected. P value is reported for the calculated correlation coefficient for each gene.

Expression of genes which showed the highest correlation with *PLIP1* gene expression included ER-localized desaturases namely *FAD2*, and *FAD3* in addition to *ROD1* gene. In land plants, the plastidial FAD4 desaturase has been proposed to have coevolved with PLIP1 lipase (Wang et al., 2017). *FAD4* is involved in the introduction of the *trans* double bond on 16:0 located on the *sn*-2 glyceryl position to form $16:1^{\Delta 3t}$-PG. Other plastidial desaturases including *FAD6*, *FAD7*, and *FAD8* are involved in desaturation of 18:1 at the *sn*-1 position to form 18:3. In *dgat1-1*, the expression of *FAD4* is reduced during later stages of seed development and the expression is not correlated with *PLIP1* expression, whereas expression of genes encoding *FAD6*, *FAD7*, and *FAD8* are not differentially expressed in *dgat1-1* during seed development. In *dgat1-1*, the negative correlation of *PLIP1* expression with the expression of genes encoding plastidial desaturases, namely FAD7 and FAD8, is supported by a reduction in mol% of PG (34:4) species which is the substrate for *PLIP1* (Figure 2.19D).

One of the genes involved in ABA signaling named *RCAR14* encodes for a regulatory component of the ABA receptor. In Arabidopsis, seedlings of transgenic lines overexpressing RCAR14 exhibit increased ABA sensitivity and display ABA induced inhibition of seed germination, and reduced cotyledon greening (Li et al., 2018). The relative increase in gene expression for *RCAR14* at 16DAF (Figure 3.6) in *dgat1-1* is consistent with the increased ABA sensitivity of *dgat1-1* during seed development and germination (Lu and Hills, 2002).

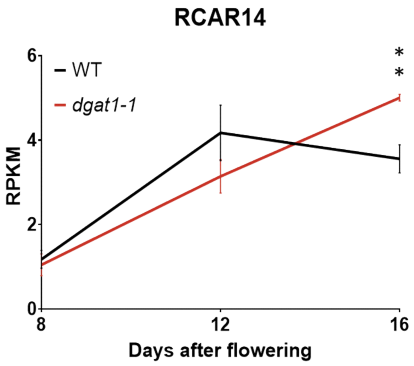


Figure 3.6 Expression profile of *RCAR14* gene in *dgat1-1* relative to WT

Data presented are mean of three biological replicates ±SD. *P <0.05 and **P<0.01 by Student's t-test for significance in the difference.

3.2.4 Reduced seed germination of *dgat1-1*

3.2.4.1 Altered expression of genes involved in hormone metabolism

Seed dormancy and germination is primarily regulated by phytohormones including abscisic acid (ABA), and gibberellic acid (GA) (Koornneef et al., 1984). ABA is involved in the regulation of growth, development and physiological processes including seed development, maturation, desiccation, dormancy and germination (Feng et al., 2014; Tahtiharju and Palva, 2001). ABA promotes seed dormancy and inhibits seed germination, whereas GA has an antagonistic role in breaking seed dormancy and promoting germination (Steber and McCourt, 2001). Brassinosteroids (BR) are involved in a variety of physiological responses including reduced root elongation, cell elongation, pollen tube growth, thermotolerance, and induction of ethylene biosynthesis (Dhaubhadel et al., 1999; Salchert, 1998; Arteca et al., 1996; Mandava, 1988). BR plays an important role in stimulating seed germination. Mutant lines lacking BR biosynthesis and response show increased sensitivity to ABA compared to WT (Li et al., 2001; Steber and McCourt, 2001; Clouse et al., 1996)

Brassinosteroid acts antagonistically to ABA by inducing the degradation of ABI5, which is the key regulator of ABA response during seed germination (Hu and Yu, 2014). BRASSINOSTEROID INSENSITIVE2 (BIN2), acts as an inhibitor of BR signaling and promotes ABA response during seed germination. BIN2 physically interacts with ABI5, and in the presence of ABA, BIN2 phosphorylates and stabilizes the ABI5 protein (Hu and Yu, 2014). GO annotation of DE genes which belong to hormone metabolism have shown that majority of upregulated genes belong to brassinosteroid degradation (Figure 3.2F) Additionally, upregulationof gene expression for *BIN2* at 16DAF in *dgat1-1* (Figure 3.7) could explain the observedincreased ABA sensitivity and delay in seed maturation and germination in *dgat1-1* seeds (Luand Hills, 2002; Routaboul et al., 1999).

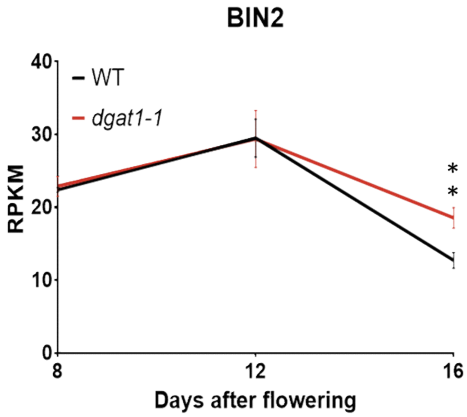


Figure 3.7 Expression profile of *BIN2* gene in *dgat1-1* relative to WT

Data presented are mean of three biological replicates ±SD. *P <0.05 and **P<0.01 by Student's t-test for significance in the difference.

The reduced germination rate of mature *dgat1-1* seeds could be explained by increased expression of genes involved in ABA metabolism including *ABI3* and *ABI5*; genes encoding proteins involved in desiccation tolerance including seed storage proteins LEA1 and LEA6 and a major gene involved in seed dormancy *DOG1*.

Increased expression of *ABI3* in *dgat1-1* (Figure 3.8A) indicates the positive regulation of ABA pathway which negatively regulates seed development and germination. *ABI3* positively regulates the transcript level of *ABI5*. *ABI5* is downregulated in *dgat1-1* (Figure 3.8B), which is explained by negative feedback regulation of *ABI5* by increased expression of *MFT* (Figure 3.8C, Figure 1.14).

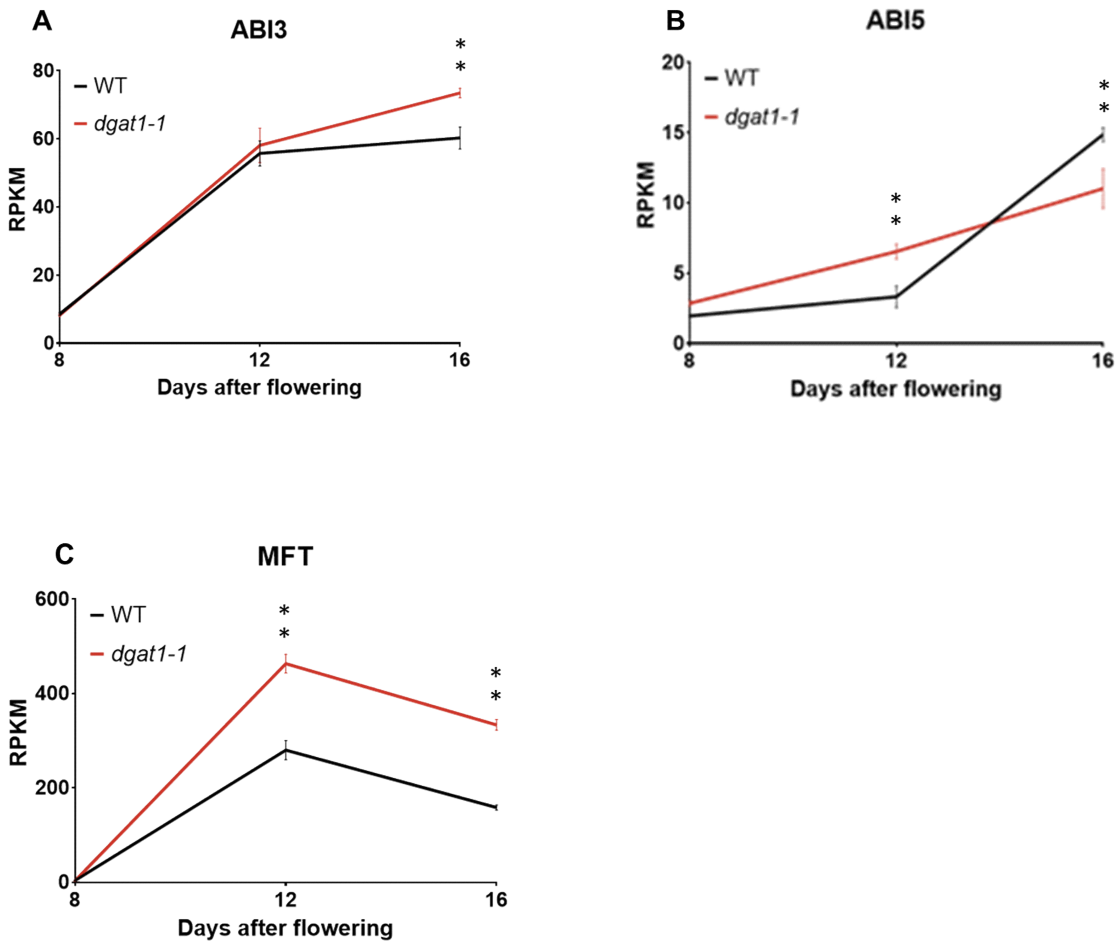


Figure 3.8 Expression profiles of *ABI3, ABI5, MFT* genes in *dgat1-1* relative to WT

Data presented are mean of three biological replicates ±SD. *P <0.05 and **P<0.01 by Student's t-test for significance in the difference.

The gene expression for positive regulators of desiccation tolerance namely LEA1 and LEA6 is downregulated in *dgat1-1* (Figure 3.9A, B) indicating the negative regulation of desiccation tolerance in *dgat1-1* which leads to reduced germination of mature seed.

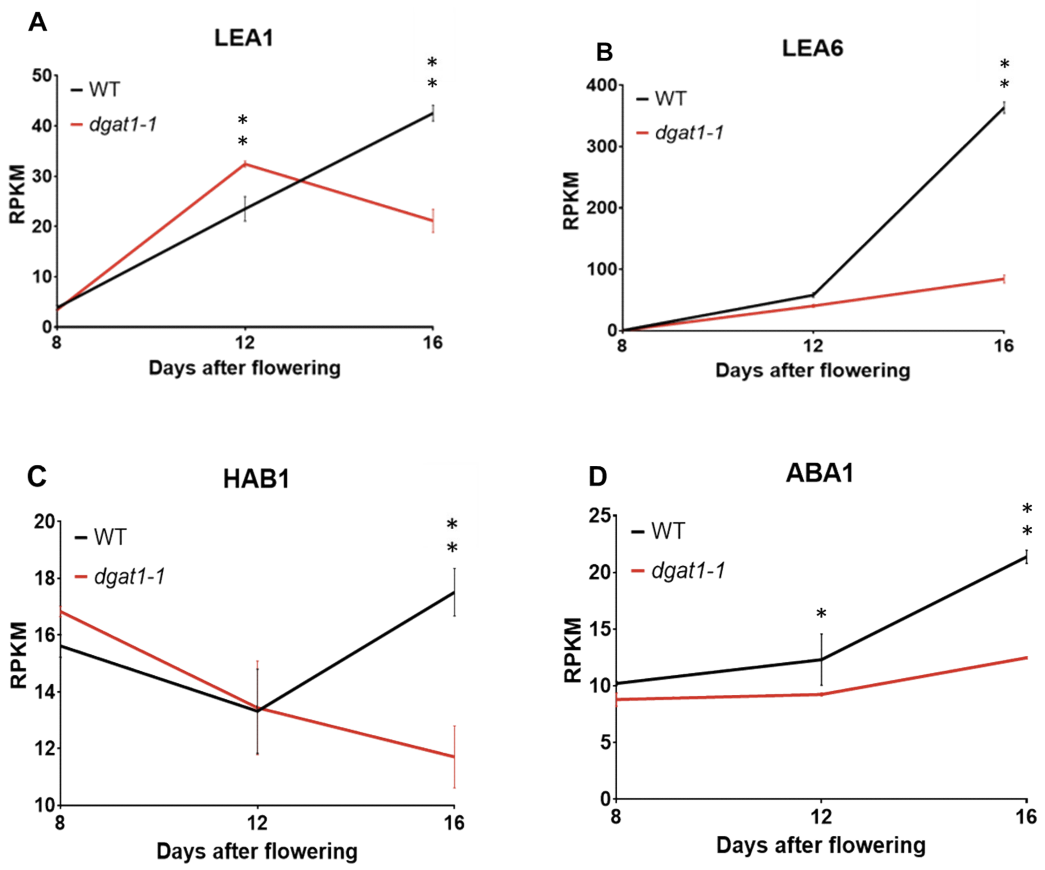


Figure 3.9 Expression profiles of *LEA1, LEA6, HAB1* and *ABA1* genes in *dgat1-1* relative to WT

Data presented are mean of three biological replicates ±SD. *P <0.05 and **P<0.01 by Student's t-test for significance in the difference.

3.2.4.2 DOG1 also regulates seed germination of *dgat1-1* seed

Slow germination rates are observed for *dgat1-1* seeds, despite the downregulation of *ABA1*, an important gene that encodes for zeaxanthin epoxidase that functions in the first step of the biosynthesis of abscisic acid (ABA) (Figure 3.9D). The differential expression and transcript regulation of *ABI3* and *ABI5* alleles in *dgat1-1* suggest that the seed maturation, dormancy, and germination is regulated by different components of ABA signaling and response elements. In

Arabidopsis, one of the regulatory genes involved in ABA signal perception is named *HAB1*. *HAB1* regulates the activation of SnRK2 kinases which are positive regulators of ABA signaling during early seed development, maturation, and germination (Fujii et al., 2009; Nakashima et al., 2009; Fujii et al., 2007; Umezawa et al., 2004; Mustilli et al., 2002; Yoshida et al., 2002). In *dgat1-1*, the expression of *HAB1* is downregulated (Figure 3.9C) and mutant line for *hab1* displays increased hypersensitivity to ABA which is consistent with reduced germination phenotype of *dgat1-1*. The direct role of *HAB1* in reduced seed germination phenotype of *dgat1-1* seeds can be tested by complementing the reduced expression of *HAB1* in *dgat1-1* by overexpressing *HAB1* in seeds, for example, by using the strong seed specific soybean glycinin promoter.

DELAY OF GERMINATION 1 (DOG1) has been identified as a major QTL for seed dormancy among natural Arabidopsis accessions (Bentsink et al., 2010). There is a positive correlation between seed dormancy and DOG1 transcript and protein levels (Graeber et al., 2014; Nakabayashi et al., 2012; Footitt et al., 2011; Kendall et al., 2011). The *DOG1* expression is not affected by changes in the expression of genes involved in ABA anabolism or catabolism. DOG1 acts in parallel to ABA signaling and acts as a timer for the release of seed dormancy (Nakabayashi et al., 2012). *abi3-1*, a weak allele mutant, has non-dormant seeds which are ABA-insensitive (Clerkx et al., 2004; Ooms et al., 1993), whereas the stronger allele *abi3-5* results in green seeds, which are non-dormant and impaired in storage protein accumulation (Nambara et al., 1995; Ooms et al., 1993). The double mutant *dog1-1 abi3-1* resulted in a phenotype similar to *abi3-5*, suggesting that the *dog1-1* mutant allele is an enhancer of a weak *abi3-1* allele (Dekkers et al., 2016).

As discussed in chapter 1, *DOG1* isoforms *DOG1-α* and *DOG1-ε* are predominantly expressed in mature seeds. Preliminary data from RT-PCR indicates an increase in expression of *DOG1* isoforms *DOG1-α* and *DOG1-ε* in mature seeds of *dgat1-1* (Figure 3.10), we hypothesize future experiments to further identify the key regulator for non-germinating green seed phenotype of double mutant *dgat1-1*/*dgat1-1 plip1-2* /*plip1-2*.

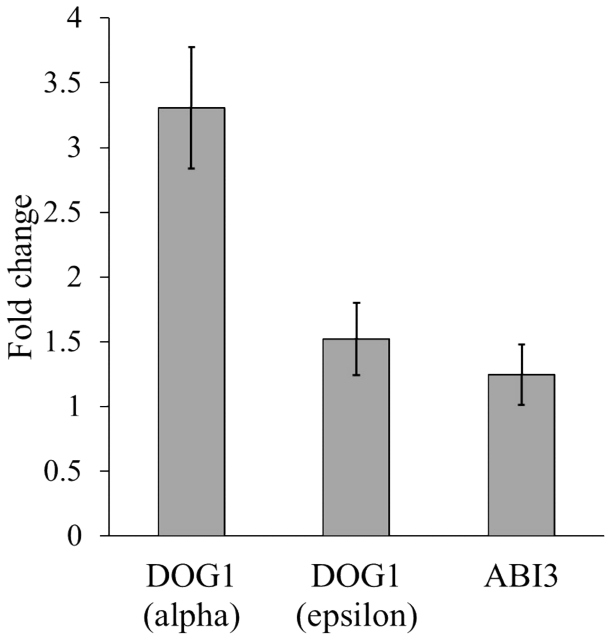


Figure 3.10 Validation of DE genes by qRT-PCR

Quantitative expression analysis of *DOG1* isoforms *DOG1-α*, *DOG1-ε*, *ABI3* in mature seeds. Fold change of expression in *dgat1-1* relative to WT was normalized to the reference gene (*DGAT1*). Data presented are mean of 3 biological replicates and two technical replicates ±SD.

3.3 Future directions

Experiment 1: The objective of this experiment is to determine the effect of different phytohormones on seed maturation and to test the correlation of non-germinating green seed phenotype of double mutant *dgat1-1*/*dgat1-1 plip1-2* /*plip1-2* with endogenous phytohormone levels.

We hypothesize that the non-germinating, green seed phenotype of the double mutant *dgat1-1*/*dgat1-1 plip1-2* /*plip1-2* is either due to the accumulation of endogenous ABA levels or reduced levels of GA. In case of high endogenous ABA, ABA inhibitors such as fluridone should be able to rescue the nongerminating phenotype of the seed. Similarly, in case endogenous GA levels of seed are low, the exogenous supply of GA should complement the non-germinating phenotype of seeds.

To test our hypothesis, quantification of endogenous levels of phytohormones including ABA, and GA will be done for different genotypes including WT, *dgat1-1*, *plip1-2*, and *dgat1-1*/*dgat1-1 plip1-2* /*PLIP1* during different stages of seed development. To test the rescue of non-germinating double mutant *dgat1-1*/*dgat1-1 plip1-2* /*PLIP1* seed, independent experiments would be conducted using different concentrations of ABA, GA, and ABA inhibitors. Different hormones and ABA inhibitors would be supplemented in the growth media, and the sensitivity of seed germination would be tested for different genotypes including WT, *dgat1-1*, *plip1-2*, and *dgat1-1*/*dgat1-1 plip1-2* /*PLIP1*.

Experiment 2: The objective of this experiment is to determine the transcriptional regulation of seed maturation and dormancy in the non-germinating green seed phenotype of double mutant *dgat1-1*/*dgat1-1 plip1-2* /*plip1-2*.

We hypothesize that the upregulation of in transcript levels of genes involved in promoting seed dormancy and regulation of gene expression for genes involved in seed desiccation tolerance would explain the defects in seed maturation and seed germination in the double mutant *dgat1-1*/*dgat1-1 plip1-2* /*plip1-2*.

Major genes involved in promoting seed dormancy and regulating desiccation tolerance include *DOG1*, *ABI3*, *ABI5,* and LEA encoding genes. Abscisic acid (ABA) plays a critical role in seed storage accumulation, desiccation tolerance, chlorophyll degradation and induction of dormancy (Finkelstein, 2013; Footitt et al., 2011; Finkelstein et al., 2008; Finch-Savage and Leubner-Metzger, 2006; Phillips et al., 1997). The seeds of mutants which lack the ABA production or signal transduction of ABA including *abi1-1*, *abi2-1*, and *abi3-1*, are completely non-dormant. The stronger allele *abi3-5* results in green seeds, which are non-dormant and impaired in storage protein accumulation (Nambara et al., 1995; Ooms et al., 1993). DOG1 acts in parallel to ABA signaling and acts as a timer for the release of seed dormancy (Nakabayashi et al., 2012). The double mutant *dog1-1 abi3-1* displays green seed phenotype suggesting that *dog1-1* is an enhancer of the weak *abi3-1* phenotype. DOG1 activates the transcription of *ABI5* to induce seed maturation genes and suppress a set of seed germination related transcripts (Dekkers et al., 2016).

In our study, we observed upregulation of transcript levels for *DOG1* and *ABI3* and downregulation of LEA encoding genes during seed development in *dgat1-1* line as compared to WT. Differential expression of these genes explains the delayed seed maturation and germination phenotype of mature *dgat1-1* seed. The mature seed of *dgat1-1* line does not display a green seed phenotype.

To test our hypothesis, quantification of transcript levels for genes including *DOG1*, *ABI3*, *ABI5* and *LEA* during different stages of seed development would provide insights into transcriptional regulation of seed maturation and dormancy for WT, *dgat1-1*, *plip1-2*, and *dgat1-1*/*dgat1-1 plip1-2* /*plip1-2* grown under similar conditions.

Experiment 3:

The objective of this experiment is to determine whether the incorporation of PUFAs into the TAG by *PLIP1* is solely dependent on PDAT1 mediated TAG biosynthesis.

PLIP1 is a member of alpha beta hydrolase family and encodes for a thylakoid associated lipase which is involved in FA export from thylakoid membrane lipid pool into the TAG in the seed. PLIP1 specifically acts on molecular species PG ($18:3/16^{\Delta 3t}$) and polyunsaturated acyl group from the *sn*-1 position is incorporated into PC (Wang et al., 2017).

Phosphatidylcholine (PC) is an important precursor for TAG biosynthesis in seeds. Multiple pathways exist for the incorporation of PUFAs into the TAG and have been discussed in Chapter 1. In addition to the mentioned mechanisms of PUFA incorporation into the TAG, other compensatory mechanisms exist involving *PLIP1* or *PDAT1*. This is supported by the 50% reduction of 18:3-containing TAGs in the triple mutant *lpcat1 lpcat2 rod1* which is mutant for major loci involved in acyl editing and headgroup exchange. Overexpressing line *PLIP1-OX* has increased seed oil content, reduced plant growth and decreased overall seed yield (Wang et al., 2017).

Additionally, overexpressing line *PLIP1-OX* can partially compensate for the low 18:3-PC phenotype for the *fad2-3* mutant. Increases in 18:1 and decreases in 18:2 in PC for lines overexpressing *PLIP1*, suggests that PLIP1 out competes the FAD2/3 pathway for the incorporation of PUFAs into the TAG (Wang et al., 2017). For the *plip1* mutant lines, an observed increase in levels of 18:3 and decrease in levels of 18:1 in PC and TAG is explained by the compensatory mechanism of FAD3 to counteract reduced 18:3 export from plastid. FAD3 is,

however, unable to compensate for the loss of plastid-derived acyl flux, which results in lower seed oil content for the *plip1* mutant (Wang et al., 2017).

In our study, we observed a significant reduction in the fatty acid content of seeds from both *plip1-1* and *plip1-2* plants as compared to WT seed (Figure 2.8A). Further, the fatty acid composition of the *plip1-1* mutant line was distinct from wild-type and *dgat1-1*, with slightly lower levels of 18:1 and slightly higher levels of 18:3 compared to the wild-type (Figure 2.8B).

Hypothesis: Role of PLIP1 in TAG biosynthesis is independent of PDAT1

The lipid content and composition phenotype of *pdat1* mutant does not show any change from WT (Mhaske et al., 2005). We hypothesize that PLIP1 also acts independently of PDAT1 in exporting acyl groups to ER for incorporation into the TAG. To test our hypothesis, the transcript expression of *PLIP1* would be measured in *pdat1* mutant relative to WT. The unaltered expression of *PLIP1* in *pdat1* mutant would validate the contribution of *PLIP1* in DGAT1 mediated TAG biosynthesis. To validate the null mutant phenotype of *pdat1* mutant, the enzyme activity of PDAT1 will be compared using microsomal preparations from leaves and roots of WT, 35S-AtPDAT1 transgenic line and *pdat1* mutant line. Radiolabeled PC will used as acyl donor, and unlabeled DAG as a substrate for the assay with microsome preparations and the amount of radiolabeled TAG formed will correlate with the transcript levels of the *AtPDAT1* gene.

Based on the inability to obtain a null mutant line for *plip1-1* (Wang et al., 2017), suggests that *PLIP1* has an essential role in embryo development in addition to playing an important role in TAG biosynthesis. We hypothesize that the upregulation of *PLIP1* in *dgat1-1*

compensates for the loss of seed storage oil synthesis and has a direct or indirect role in embryo development and formation of normal seed.

3.4 Material and Methods

3.4.1 Plant materials and growth conditions

T-DNA insertion mutant lines from the SALK (Alonso et al., 2003), SAIL (Sessions et al., 2002) and GABI-Kat (Kleinboelting et al., 2012) collections were obtained from the Arabidopsis Biological Resource Center at the Ohio State University. Seeds were cold stratified for two days at 4˚C before growing on soil mixture (4:2:1 mixture of Metromix Professional Growing Mix: vermiculite: perlite) in growth chambers at 23 ˚C under long-day conditions (16 h light / 8 h dark). For growth on plates, seeds were surface sterilized before plating on half-strength Murashige and Skoog media with 5% (w/v) phytoblend agar and 1% (w/v) sucrose. When making crosses, immature flower buds were emasculated and manually cross-pollinated with pollen from the flower of the donor parent. To isolate seeds at different developmental stages, emerging flowers were tagged with the cotton thread at specific time intervals. Whole siliques were flash frozen in liquid nitrogen and stored at -80 ˚C. Seed harvesting from siliques was done on dry ice (Bates et al., 2013). The size of mature seeds was quantified by photographing approximately 1000 seeds under a light microscope and then using ImageJ particle analysis software to process the images (Herridge et al., 2011)

3.4.2 Single seed genotyping of T-DNA lines

Genomic DNA was extracted from single seed as previously described (Edwards et al., 1991). Primers used for the PCR-based genotyping of the SALK and SAIL mutant lines were

designed using the SIGnAL T-DNA Express Arabidopsis Gene Mapping Tool (Alonso et al., 2003); primers for the GABI-Kat lines were designed using the GK primer tool (Kleinboelting et al., 2012). All genotyping primers used in this study are listed in Table 2.2.

3.4.3 RNA isolation and quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from the single seeds using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions, followed by additional purification with sodium citrate to improve RNA quality (Nybo, 2011). We obtained total RNA yields in the range of 10-30ng for each genotype. cDNA was synthesized from 10ng RNA using the Quantitect Reverse transcription kit (Qiagen). Relative mRNA levels were quantified with iTaq universal SYBR green supermix (Bio-rad) on a CFX-96 real-time PCR system (Bio-rad). Relative quantification of transcript levels was done using the delta Ct method, which assumes a PCR efficiency of 100% for primers. Primers used for RT-PCR are listed in Table 2.3

3.4.4 Microscopy

The dry seeds of WT, *dgat1-1*, *plip1-2*, and progeny of *dgat1-1*/*dgat1-1 PLIP1*/*plip1-2* and *DGAT1*/*dgat1-1 plip1-2*/*plip1-2* plants were examined under the stereo microscope for seed phenotype including color and size. Pictures of mature seeds was taken using a stereo microscope Leica M165 FC and Leica application suite V4.1.

3.4.5 Data processing

Hierarchical clustering and statistical analysis was done using MetaboAnalyst (Chong et al., 2018). The single-linkage hierarchical algorithm was based on Spearman's correlation coefficient, ρ.

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Chapter 4 - Genome-specific differential gene expression analysis using RNA-seq for genes involved in lipid metabolism in the allopolyploid *Brassica juncea*

4.1 Introduction

With the increase in population and limited arable land, water, and fuel there is a need to improve production of oilseed and vegetable crops for more oil as food and fuel (Heinberg and Lerch, 2010). One of the approaches to bridge the demand-supply gap is to increase the yield of seed oil per unit area of cultivated land by utilizing the information of homoeolog expression in allotetraploid *Brassica* species for trait improvements (Yang et al., 2016).

The *Brassica* genus accounts for 12% and 10% of the world's edible vegetable oil and vegetable production respectively (Labana and Gupta, 1993). Among the *Brassica* species, *B. rapa* and *B. oleracea* are mainly utilized as vegetable crops whereas *B. rapa*, *B. napus*, *B. juncea* and *B. carinata* are grown as oilseed crops.

The *Brassica* genus is widely considered as a classical model to study polyploidy of genome during evolution. The widely cultivated diploid genome species are *B. rapa*, *B. nigra* and *B. oleracea* and their genomes are designated by AA, BB, and CC respectively. Natural hybridization of diploid species gave rise to the allotetraploid species namely *B. juncea*, *B. napus* and *B. carinata,* and their genomes are designated as AABB, AACC and BBCC respectively. The evolutionary relationships of diploid and allotetraploid *Brassica* species are depicted by the 'triangle of U' model (Figure 4.1) (Nagaharu, 1935). No natural allohexaploid with AABBCC

genome has been observed, though hexaploid hybrids have been generated (Pradhan et al., 2010; Li et al., 2005).

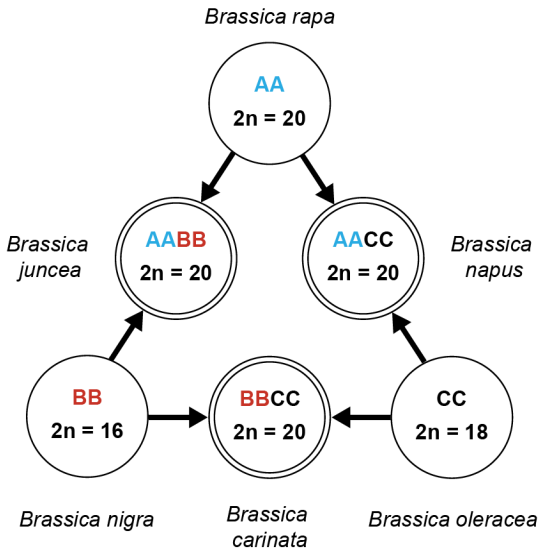


Figure 4.1 *Brassica* species within the triangle of U (adapted from Nagaharu U,1935).

Although the annotated genes of *A. thaliana* share similarity with other members of the *Brassica*ceae family, the oil composition of *B. juncea*, *B. napus* and *B. rapa* is different. Lipid composition in mature seeds of *B. juncea*, contain reduced amount of saturated fatty acids (<10%; SFA), moderate amount of monounsaturated fatty acids (10-30%) and increased amounts (~50%; MUFA) of very long chain polyunsaturated fatty acids (VLCUFAs; principally erucic acid C22:1) (Sinha et al., 2007). Breeding programs have resulted in reduced erucic acid and increased oleic acid content (78-88%) for rapeseed soil which is beneficial to human health (Xiao et al., 2014). The presence of the *B. nigra* diploid genome species in *B. juncea* imparts important traits like seed oil quality, resistance towards diseases, and tolerance to salinity and drought (Gunasekera et al., 2009; Chevre et al., 1997; Roy, 1984).

The members of *Brassica* genus are close relatives to the model plant species, *Arabidopsis* thaliana, which belongs to the *Brassica*ceae family as well. Comparative studies between *A. thaliana* and *Brassica* species have shown around 85 to 90% nucleotide sequence identity in coding regions (Schmidt, 2002; Cavell et al., 1998). The genome size of *A. thaliana* is ~125 Mb. The diploid *Brassica* genomes are three to five times larger than that of *A*. *thaliana*, with 470 Mb for *B*. *nigra*, 529 Mb for *B*. *rapa* to 696 Mb for *B*. *oleracea*. The development of allopolyploid *Brassica* species from its diploid progenitors involves loss and reshuffling of genes (Navabi et al., 2013; Mun et al., 2009; Town et al., 2006; Parkin et al., 2005; Bowers et al., 2003; O'Neill and Bancroft, 2000). The polyploid plant genomes typically have high repetitive elements (Michael and VanBuren, 2015). The recurring genome duplications and triplication events have created massive genetic redundancy which leads to selective expression or newly acquired role for duplicated or triplicated homoeologs (Rastogi and Liberles, 2005; Force et al., 1999). The diversifying selection within a *B*. *juncea* species giving rise to sub-varieties with different morphological features might be due to the presence of genetic redundancy leading to expression bias or differential expression of homoeologs. The conserved synteny and collinearity between Arabidopsis and *Brassica* species have been shown by mapping genetic markers of the *Brassica* genomes onto the Arabidopsis reference genome (Qiu et al., 2009; Panjabi et al., 2008; Parkin et al., 2005; Li et al., 2003; Lukens et al., 2003).

In our study, the subgenome A and subgenome B of *B. juncea* have been designated as *BjuA* and *BjuB* respectively. The identification and expression profiling of homoeologs for different genes involved in FA synthesis, acyl editing, and TAG biosynthesis and metabolism will provide insights into the unexplored transcriptional regulation involved in membrane remodeling and storage lipid synthesis in *B. juncea*.

Although the shotgun sequencing and assembly of *B. juncea* genome was reported by (Yang et al., 2016) neither the whole genome reference assembly nor the expression profiling for various genes in lipid biosynthesis is available yet. We found that the *BjuA* homoeologs were predominantly expressed at higher levels compared with the *BjuB* homoeologs in the allopolyploid *B. juncea*. Identification of homoeologs which have dominance expression for various genes involved in FA synthesis, acyl editing, and TAG biosynthesis and metabolism will help in the selection of subsets of genes for targeting and further manipulations for their use in breeding programs and improving seed oil content and composition.

4.2 Results

4.2.1 Total lipid content and fatty acid composition across different stages of seed development in *B. juncea*

We collected seeds at different stages of seed development and observed an increase in fresh weight (FW)/seed from 10 to 30 days after flowering (DAF) after which there was a decline in FW/seed until maturity (Figure 4.2A). This rapid decline in seed FW/seed towards maturity suggests the onset of seed desiccation which has an important role in the transition of seed from the development to germination phase (Jiang and Kermode, 1994; Kermode and Bewley, 1985; Dasgupta et al., 1982). Analysis of total lipid content from different stages of seed development indicated an increase in total lipid content from 20 DAF until 50 DAF, with a slight decline towards maturity (Figure 4.2B). The observed fatty acid content of 363 µg/mg FW for mature seeds is consistent with previous reports (Munshi et al., 1990).

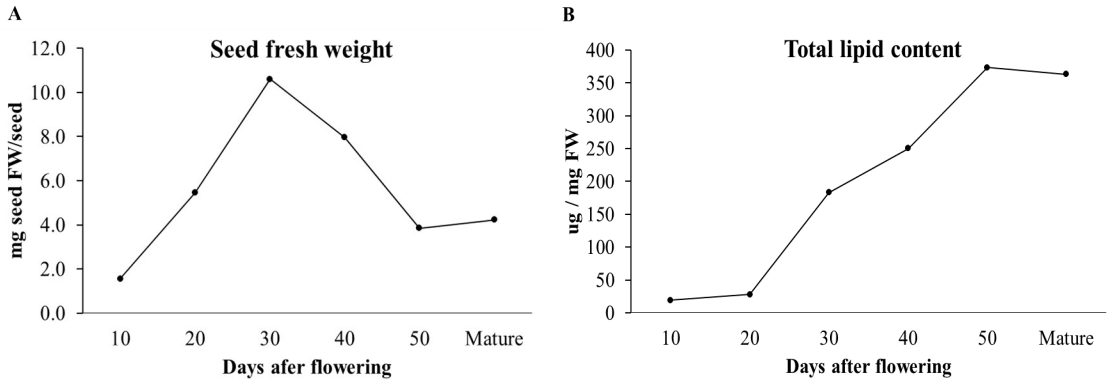


Figure 4.2 Quantitative analysis of seed fresh weight and total lipid content during seed development

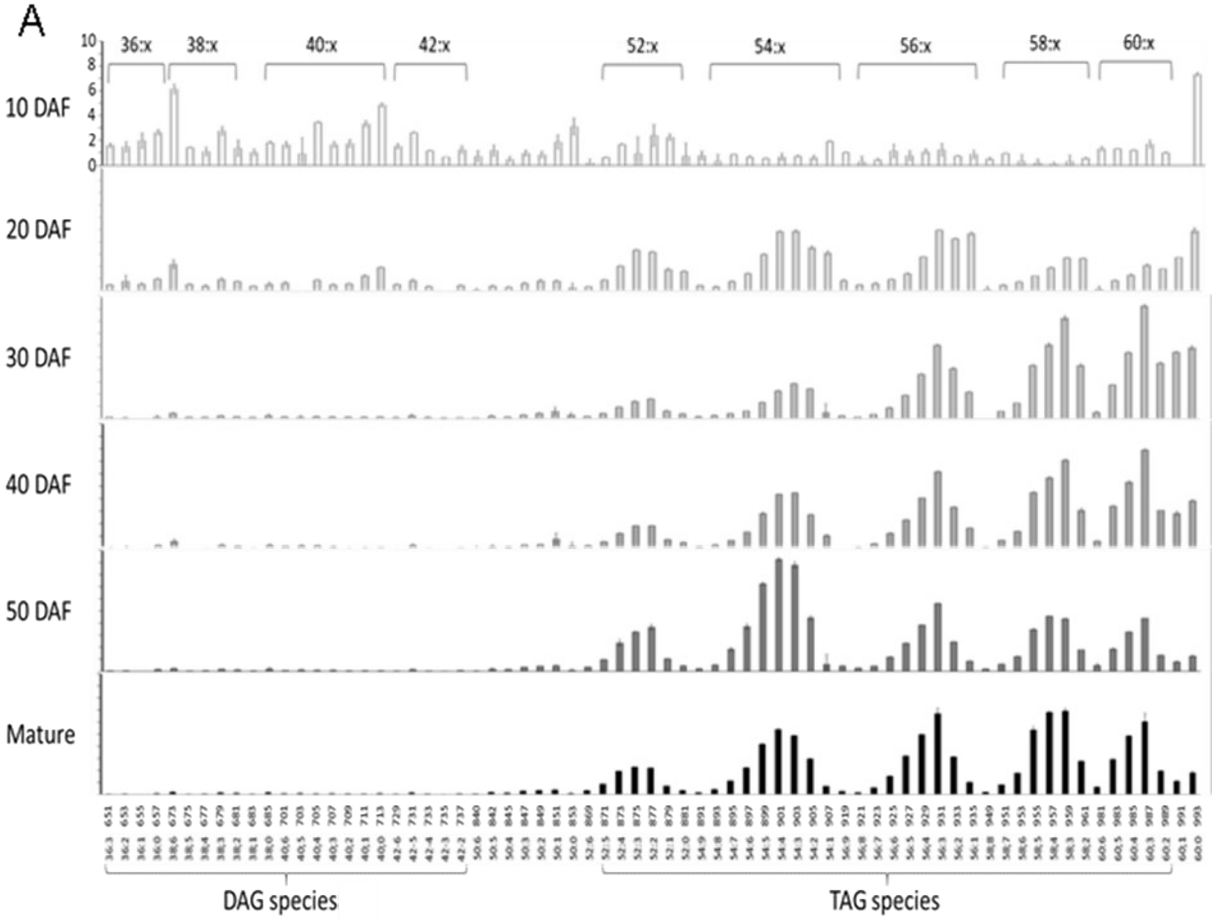
A, The fresh weight of seeds during seed development B, Total lipid content of seeds during seed development for stages 10 DAF through 40 DAF. Three seeds were pooled for each stage ranging from 10-40 DAF; Four seeds were pooled for 50 DAF and mature seed stage.

Storage lipids account for the majority of total lipid content in the seed, and we observed an increase in the storage lipid content (µg/mg seed FW) as the seed progressed during development. The observed neutral lipid content in mature seed is 273.75µg/mg seed FW. Total polar lipids increased up to 30 DAF followed by a sharp decline until seed reached maturity, with polar lipid amount of 0.23 nmol/mg seed FW at seed maturity.

Lipid profiling of storage lipids during seed development indicates the differences in the relative distribution of different triacylglycerol (TAG) species. From early stages of seed development until maturity, there is a decrease in diacylglycerol (DAG) species 36:x, 38:x, 40:x and 42:x and an overall increase in TAG species including 52:x, 54:x, 56:x, 58:x, and 60:x (Figure 4.3A).

In the polar lipid fraction, levels of phosphatidylcholine (PC) declined until 30 DAF followed by a slight increase towards seed maturity. The levels of phosphatidylethanolamine (PE) did not change until 40 DAF when its level increases, followed by a decrease in levels at

seed maturation stage. The levels of phosphatidic acid (PA) show a peak at 20 DAF, whereas levels of phosphatidylinositol (PI) were highest at 50 DAF. The levels of monogalactosyldiacylglycerol (MGDG) increased until 30 DAF and then displayed a sharp decline in levels towards the end of seed development. The levels of digalactosyldiacylglycerol (DGDG) remain unchanged across seed development (Figure 4.3B).



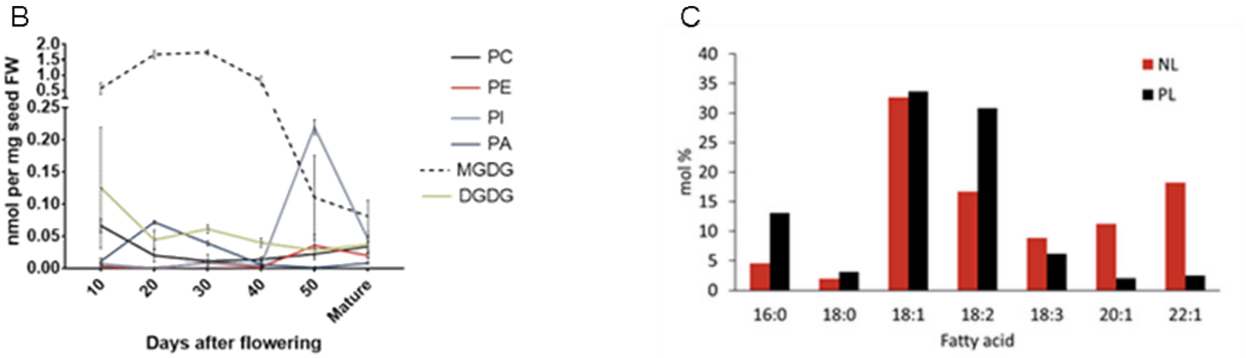


Figure 4.3 Comparative analysis of lipid content and composition during seed development in *B. juncea*

A**,** Comparison of MS/MS mass spectra of neutral lipids extracts within the mass range of 600–1000 m/z obtained from *B. juncea* seeds during different stages of seed development. Data is normalized against the internal standard tripentadecanoin (Tri15:0) TAG. B, Quantification, and comparison of different polar lipid species: Glycerophospholipids PC, PE, PI, PA, and Glyceroglycolipids MGDG and DGDG. C, Comparative analysis of fatty acid composition for neutral and polar lipid fractions at seed maturity.

4.2.2 Changes in fatty acid profile during oil accumulation phase of seed development

In the neutral fraction of total lipids extracted from mature seeds, the most abundant fatty acid is 18:1 followed by 22:1, 18:2, 18:3 and 16:0. In the polar lipid fraction from mature seeds, the most abundant fatty acid is 18:1 followed by 18:2, 16:0, 18:3 and 22:1 (Figure 4.3C). The seed development stages 20, 30 and 40 DAF displayed a sharp increase in storage oil synthesis.

For the neutral lipid (NL) fraction, we observed an overall reduction for 18:0 across 20-40 DAF stages of seed development. For NL fraction, there is a decrease in levels of 18:1 at 30 DAF which stabilized at 40 DAF and a corresponding increase in 20:1 levels at 30 DAF followed by a sharp decline at 40 DAF. The 22:1 levels increased from 20-40 DAF. For PUFAs in the NL fraction, 18:2 levels decreased from 20-40 DAF whereas 18:3 levels relatively remained unchanged (Figure 4.4). For the polar lipid fraction, 18:1 levels increased across 20-40 DAF with little to no change in levels of 20:1 and 22:1 does not change across 20-40 DAF; 18:2

levels remain unchanged whereas 18:3 levels showed an increase at 30 DAF followed by a sharp decline in levels at 40 DAF (Figure 4.4).

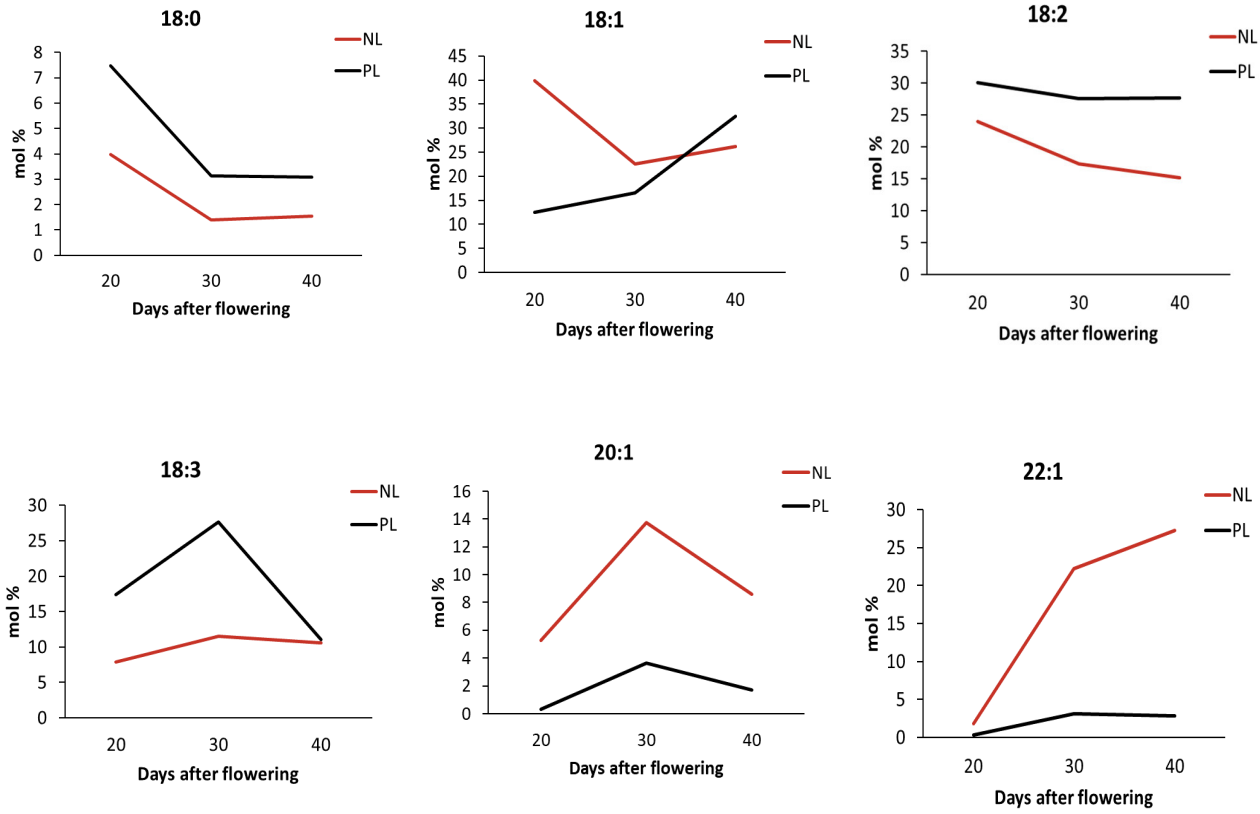


Figure 4.4 Changes in fatty acid profiles in neutral and polar fractions at different stages of seed development in *B. juncea*. The composition analysis was determined using data from GC-FID.

4.2.3 Transcriptome sequencing

Seeds at 20, 30, and 40 DAF were selected for sequencing based on the storage lipid accumulation profile. Samples at 20, 30 and 40 DAF produced 107,860,702; 143,702,754 and 153,723,174 reads respectively. Adapter sequences were removed, and quality testing of sequencing reads showed ~96% of sequences had PHRED score of >= 30; GC content ~ 50%

and low ambiguous base content <0.01% suggesting high sequence quality to be used for further analysis (Figure 4.5A).

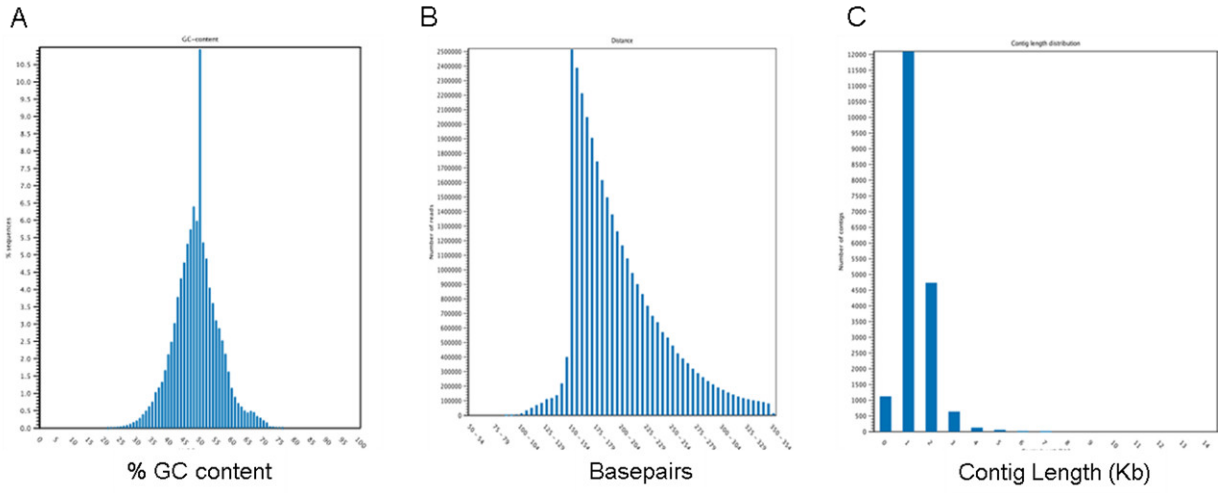


Figure 4.5 Histograms depicting % GC content, the size distribution of transcriptomic reads and contigs after assembly

A, % GC content of trimmed reads B, Distribution of read lengths for trimmed sequences C, Distribution of contig lengths obtained after de novo assembly of trimmed reads. Data shown is for 40 DAF time point and was similar for 20 DAF and 30 DAF.

After the removal of adapter sequences and exclusion of short reads, the high-quality reads with lengths ranging from 100bp to 350bp (Figure 4.5B) were used for *de novo* assembly and resulted in the formation of 17,312 contigs with an average length of 1.5kb (Figure 4.5C).

4.2.4 Development of transformation constructs for the production of reduced-viscosity oil in *B. juncea* seed

The goal of this work is to modify the TAG biosynthesis pathway in the oil seed crop *Brassica juncea* to produce seed oil with reduced viscosity. The EaDAcT enzyme isolated from *Euonymus alatus* produces 3-acetyl-1,2-diacylglycerols (acTAGs) in its seeds (Durrett et al., 2010). Another related acyltransferase named EfDAcT isolated from *Euonymus fortunei*

possesses higher acyltransferase activity and produces a higher amount of acTAG than EaDAcT in vivo and in vitro (Tran et al., 2017). Both *Euonymus alatus* and *Euonymus fortunei* belong to the *Celestraceae* plant family. AcTAG molecules possess an *sn*-3 acetate group on their glycerol backbone which confers them with different chemical and physical properties compared to long chain TAGs. The acTAGs from *Euonymus* possess a 30% reduction in their viscosity compared to oils from other plant species in addition to lower kinematic viscosity and crystallize at lower temperatures, which makes them useful for different applications including direct-use biofuels (Liu et al., 2015a; Liu et al., 2015b; Durrett et al., 2010). Enhanced levels of acTAG in *B. juncea* seed will be achieved by suppressing endogenous long chain TAG production, using RNA interference (RNAi) based silencing of mRNA expression for both *DGAT1* and *PDAT1* (Liu et al., 2015a). *De novo* assembly of reads obtained from *B. juncea* transcriptome resulted in 17, 312 contigs. The homology search was done using the coding sequence for *AtDGAT1* and *AtPDAT1* to identify matching contigs in the *de novo* assembly. Using *AtDGAT1,* we identified two overlapping contigs named Ctg1594 and Ctg12608 (Figure 4.6A) and using *AtPDAT1* a matching contig named Ctg10826 was identified (Figure 4.6C).

A


500
1,000
1,500
2,000
c At DGAT1
c B.rapa DGAT1.1 NCBI
c B.rapa DGAT1.2 NCBI
Contig 12608RC
Contig 1594RC
BjDGAT1-RNAi
1563
1515
1512
1364
1429
294

B


c At DGAT1 TGTGGCTAAA GTTGGTTTCT TATGCTCATA CTAGCTATGA CATAAGATCC CTAGCCAATG CAGCTGATAA 812
c B.rapa DGAT1.1 NCBI TGTGGCTGAA GTTGGTTTCT TACGCTCATA CTAACTATGA CATAAGAACC CTAGCTAATT CATCTGATAA 764
c B.rapa DGAT1.2 NCBI TGTGGCTGAA GTTGGTTTCT TACGCTCATA CTAGCTACGA CATAAGAACC CTGGCCAATT CAGCTGATAA 761
Contig 12608RC TGTGGCTGAA GTTGGTTTCT TACGCTCATA CTAGCTACGA CATAAGAACC CTGGCCAATT CAGCTGATAA 1139
Contig 1594RC TGTGGCTGAA GTTGGTTTCT TACGCTCATA CTAACTATGA CATAAGAACC CTAGCTAATT CATCTGATAA 341
BjDGAT1-RNAi ---------- ---------- ---------- -TAGCTACGA CATAAGAACC CTGGCCAATT CAGCTGATAA 39
c At DGAT1 GGCCAATCCT GAAGTCTCCT ACTACGTTAG CTTGAAGAGC TTGGCATATT TCATGGTCGC TCCCACATTG 882
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Contig 12608RC GGTCGATCCT GAAATCTCCT ACTATGTTAG CTTGAAGAGC TTGGCGTATT TCATGGTTGC TCCCACACTG 1209
Contig 1594RC GGCCAATCCT GAAGTCTCCT ACTATGTTAG CTTGAAGAGC TTGGCGTATT TCATGCTTGC TCCCACATTG 411
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c At DGAT1 TGTTATCAGC CAAGTTATCC ACGTTCTGCA TGTATACGGA AGGGTTGGGT GGCTCGTCAA TTTGCAAAAC 952
c B.rapa DGAT1.1 NCBI TGTTATCAGC CGAGCTATCC ACGTTCTCCT TGTATCCGGA AGGGTTGGGT GGCTCGTCAA TTTGCAAAGC 904
c B.rapa DGAT1.2 NCBI TGTTATCAGC CAAGCTATCC ACGTTCTCCA TGTATCCGGA AGGGTTGGGT GGCTCGTCAA TTTGCGAAAC 901
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C


500
1,000
1,500
2,000
c At PDAT
c B.rapa PDAT1.1 NCBI
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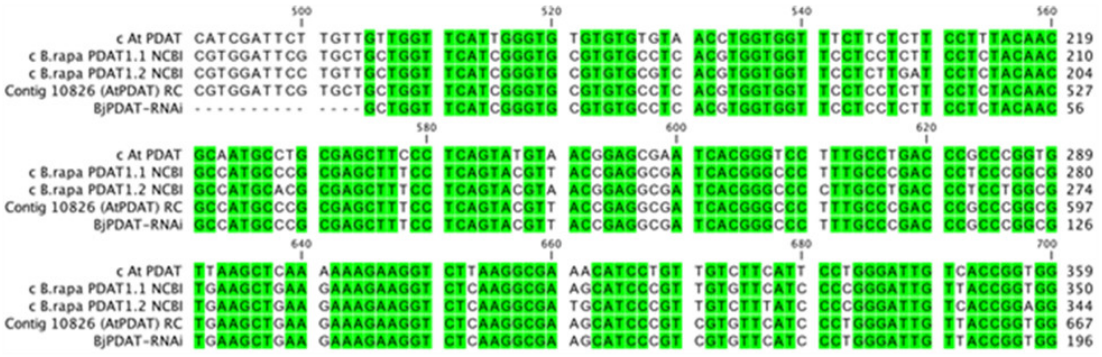
Contig 10826 (AtPDAT) RC
BjPDAT-RNAi
2016
2007
2001
1108
196

D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Contig 10826 (AtPDAT) RC CGTGGATTCG TGCTGCTGGT TCATCGGGTG CGTGTGCCTC ACGTGGTGGT TCCTCCTCTT CCTCTACAAC 527
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Contig 10826 (AtPDAT) RC TGAAGCTGAA GAAAGAAGGT CTCAAGGCGA AGCATCCCGT CGTGTTCATC CCTGGGATTG TTACCGGTGG 667
BjPDAT-RNAi TGAAGCTGAA GAAAGAAGGT CTCAAGGCGA AGCATCCCGT CGTGTTCATC CCTGGGATTG TTACCGGTGG 196

Figure 4.6 Sequence alignment was done using coding sequences (CDS) from Arabidopsis, *B. rapa*, and contigs identified using *de novo* assembly

Numbers on the right of sequences represent the length of the sequence in bp. A, Sequence alignment using CDS sequences of *AtDGAT1*, contig sequences and CDS sequences for *DGAT1* homologs in *B. rapa*. *Bj*DGAT1-RNAi sequence at the bottom was selected as the region for the RNAi construct. B, Sequence alignment showing the sequence selected for *Bj*DGAT1-RNAi construct. Text highlighted in green shows degree of similarity between sequences. C, Sequence alignment using CDS sequences of *AtPDAT1*, contig sequences and CDS sequences for *PDAT1* homologs in *B. rapa*. *Bj*PDAT1-RNAi sequence at the bottom was selected as the region for construction of RNAi construct. D, Sequence alignment showing the sequence selected for *BjPDAT1*-RNAi construct.

Using the sequence information of RNAi target sequences derived from the *de novo* assembly, I then constructed RNAi vectors for suppression of endogenous TAG in *B. juncea* (Figure 4.7).

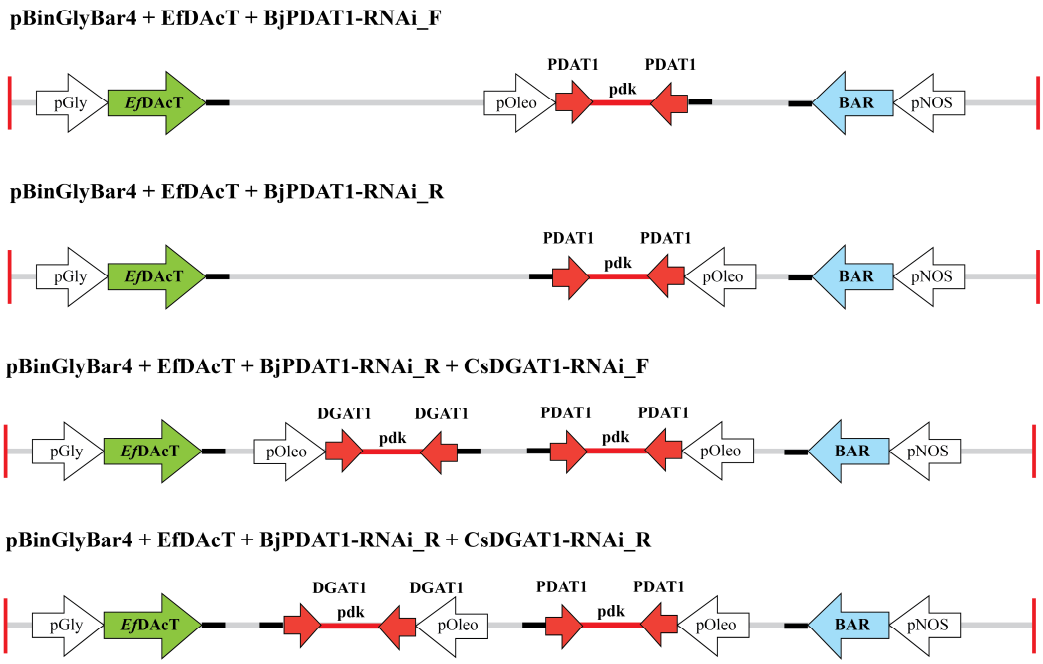


Figure 4.7 The diagrammatic representation of RNAi constructs used for suppression of endogenous TAGs and production of acTAGs in *B. juncea*

Promoters are indicated by white arrows; terminator sequences are represented by black lines. The constructs are flanked by the left and right border of T-DNA shown as vertical red lines; BAR, basta resistance; native EfDAcT coding sequence; DGAT1, portion of CsDGAT1; PDAT1, portion of BjPDAT1; pdk, pdk intron from pHANNIBAL; pGly, Soybean glycinin promoter; pOleo, *Brassica napus* oleosin promoter; pNOS, nopaline synthase promoter.

4.2.5 Role of DGAT1 and PDAT1 in TAG biosynthesis

In Arabidopsis, DGAT1 is the major enzyme responsible for TAG biosynthesis ((Lung and Weselake, 2006), and homology search in BRAD database led to the identification of two *AtDGAT1* homologs from the *BjuA* subgenome named Bra039003 & Bra036722 and two *AtDGAT1* homologs from the *BjuB* subgenome named BniB025147 & BniB009853 (Figure 4.8A, B). Phylogenetic analysis suggested that the two homoeologs named Bra039003 and BniB025147 were closely related to each other. Similarly, the other two homoeologs named Bra036722 and BniB009853 were closely related to each other (Figure 4.8A, B).

The comparative analysis of transcript expression for *BjuA* and *BjuB* homoeologs across the stages of seed development, 20-40 DAF, indicated that the expression of *BjuA* homoeologs is higher than the expression of *BjuB* homoeologs (Figure 4.8, Figure 4.9). These differences in transcript expression suggest that one of the highly expressed *BjuA* homoeologs, Bra039003 is primarily responsible for the increased DGAT1 activity, and the expression profile of Bra039003 is consistent with the accumulation of storage lipids during 20-40 DAF.

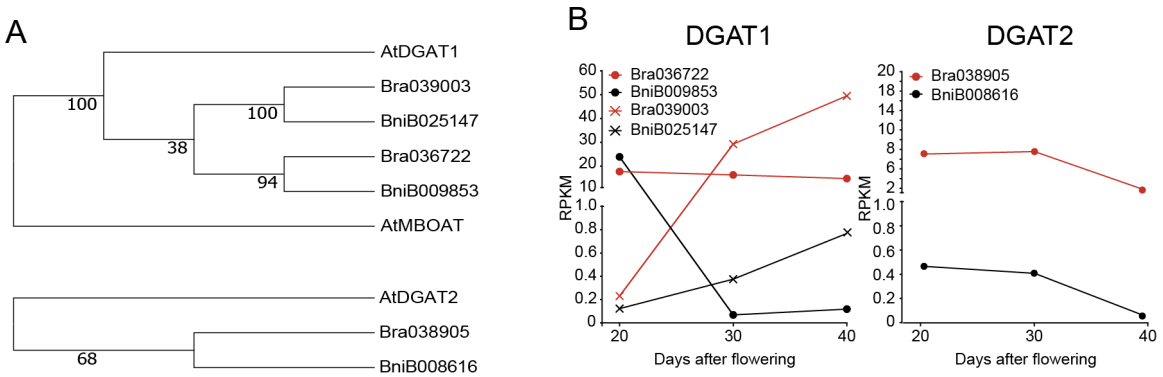
TAG synthesis is also catalyzed by an acyl-CoA–independent enzyme named phospholipid: diacylglycerol acyltransferase (PDAT). AtPDAT1 catalyzes the transfer of acyl group from *sn*-2 position of PC to *sn*-3 position of DAG to form TAG (Dahlqvist et al., 2000). Quantitative analysis of transcript level for *AtPDAT1* homologs identified from *BjuA* and *BjuB* show low transcript expression at 40 DAF relative to *AtDGAT1* homologs, indicating the minor role of PDAT1 in the presence of DGAT1 in *B. juncea* (Figure 4.8D, Figure 4.9B). *AtPDAT-like* shares 69% sequence identity with AtPDAT1 and 58% identity at amino acid level. The same homologs were identified from *BjuA* and *BjuB* when either *AtPDAT1* or *AtPDAT2* were used as a

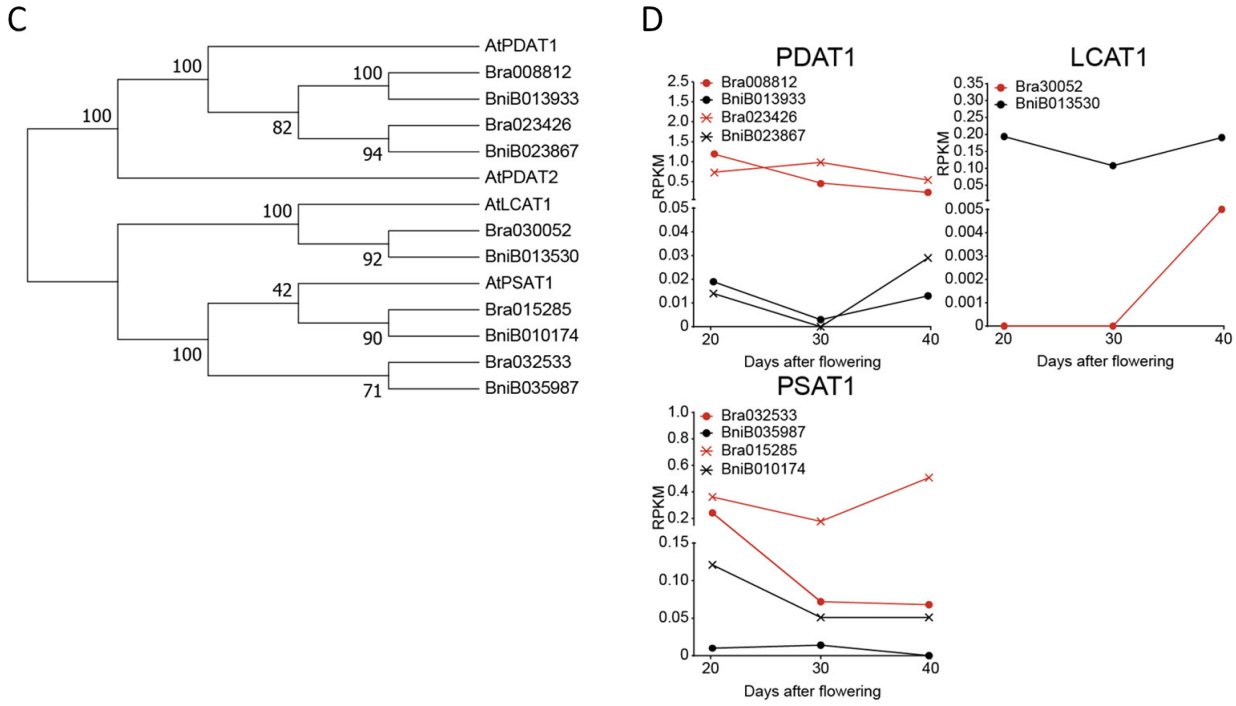
query for BLASTn. Comparative analysis using CDS sequences from *AtPDAT1*, *AtPDAT-like* and the respective homologs showed that the four homologs identified were more closely related to *AtPDAT1* (Figure 4.8C). Closely related homologs of *AtPDAT1* named as *AtLCAT1* and *AtPSAT1* were used as a query for identification of respective homologs in *B. juncea*. Differential expression analysis among the *BjuA* and *BjuB* homoeologs identified using *AtLCAT1* had very low expression relative to *AtPDAT1* homologs (Figure 4.8C, D). Among the *B. juncea* homologs identified for *AtLCAT1*, the expression of *BjuB* homoeolog was higher than *BjuA* homoeolog as opposed to that observed for *AtPDAT1* homologs where the expression of *BjuA* homoeologs was higher than *BjuB* homoeologs. Differential expression analysis of *BjuA* and *BjuB* homoeologs identified using *AtPSAT1* had comparable expression relative to *AtPDAT1* homologs. Among the *B. juncea* homologs identified for *AtPSAT1*, the expression of *BjuA* homoeologs was higher than *BjuB* homoeologs (Figure 4.8C, D).

4.2.6 Role of FAD2, FAD3, FAE1, and PDCT in TAG biosynthesis

The transcript expression of *BjuA* and *BjuB* homoeologs identified using Arabidopsis desaturases including *AtFAD2* and *AtFAD3* is downregulated at 40 DAF (Figure 4.8F, Figure 4.9C), indicating a possible explanation for the low levels of 18:2 and 18:3 at 40 DAF. Phosphatidylcholine: 1,2-*sn*-diacylglycerol choline phosphotransferase (PDCT) catalyzes the conversion of PC with modified acyl groups into DAG thereby generating PC derived PUFA-rich DAG pool which is utilized by DGAT for TAG assembly (Lu et al., 2009). The expression of PDCT is reduced at 40 DAF and correlates with low levels of 18:2 and 18:3 levels in the TAG at 40 DAF (Figure 4.8J, Figure 4.9B).

The different members of the 3-ketoacyl-CoA synthase family are involved in the synthesis of long chain fatty acids. *AtKCS18* is seed-specific and is expressed at higher levels relative to other members of the 3-ketoacyl-CoA synthase family. *BjuA* and *BjuB* homoeologs for *AtFAE1* (KCS18) are downregulated at 40 DAF. Observed lipid phenotype shows an increase in the levels of 22:1 at 40 DAF (Figure 4.8H, Figure 4.9A). In *B. juncea*, the expression of *BjuA* homoeologs was higher than *BjuB* homoeologs identified using AtKCS4 and AtKCS9 (Figure 4.8H, Figure 4.9A). The *BjuA* and *BjuB* homoeologs identified for AtKCS4 and AtKCS9 had low expression relative to those observed for AtKCS18 homologs

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AtPDAT2
AtLCAT1
Bra030052
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BniB010174
Bra032533
BniB035987
100
100
100
82
94
100
100
92
42
90
100
71
D
PDAT1
LCAT1
PSAT1
Bra008812
BniB013933
Bra023426
BniB023867
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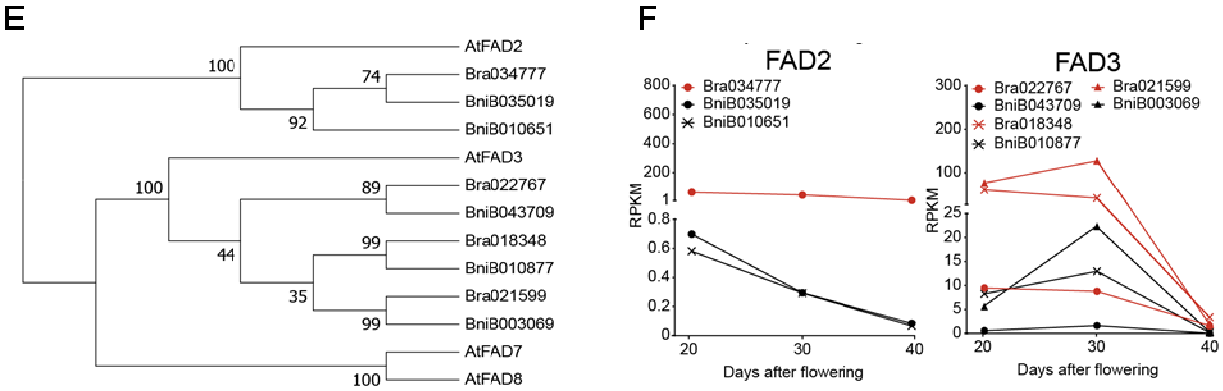
BniB035987
Bra015285
BniB010174
RPKM
Days after flowering
20
30
40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AtFAD3
Bra022767
BniB043709
Bra018348
BniB010877
Bra021599
BniB003069
AtFAD7
AtFAD8
100
74
92
100
89
44
99
35
99
100
F
FAD2
FAD3
Bra034777
BniB035019
BniB010651
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RPKM
Days after flowering
20
30
40

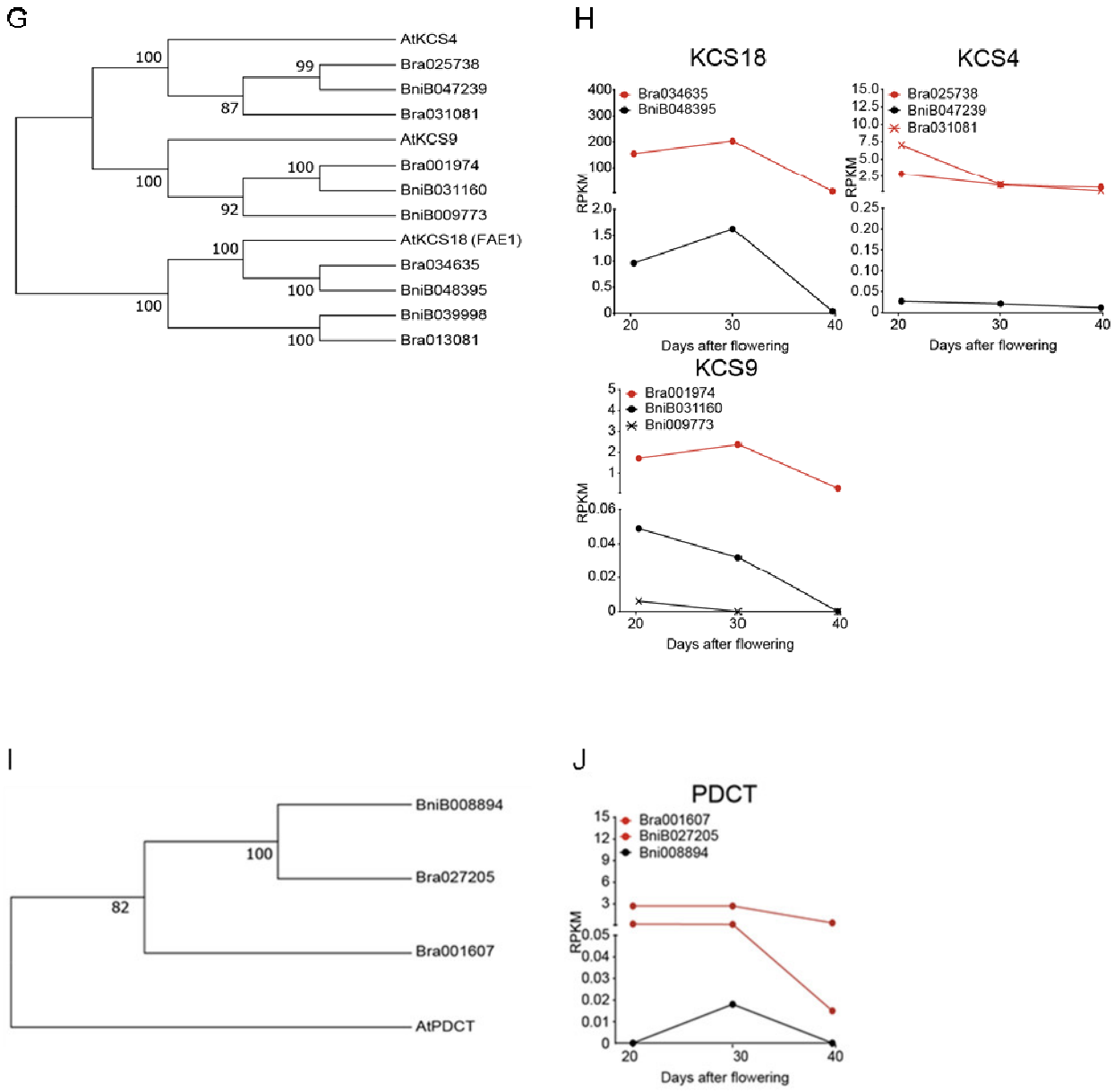


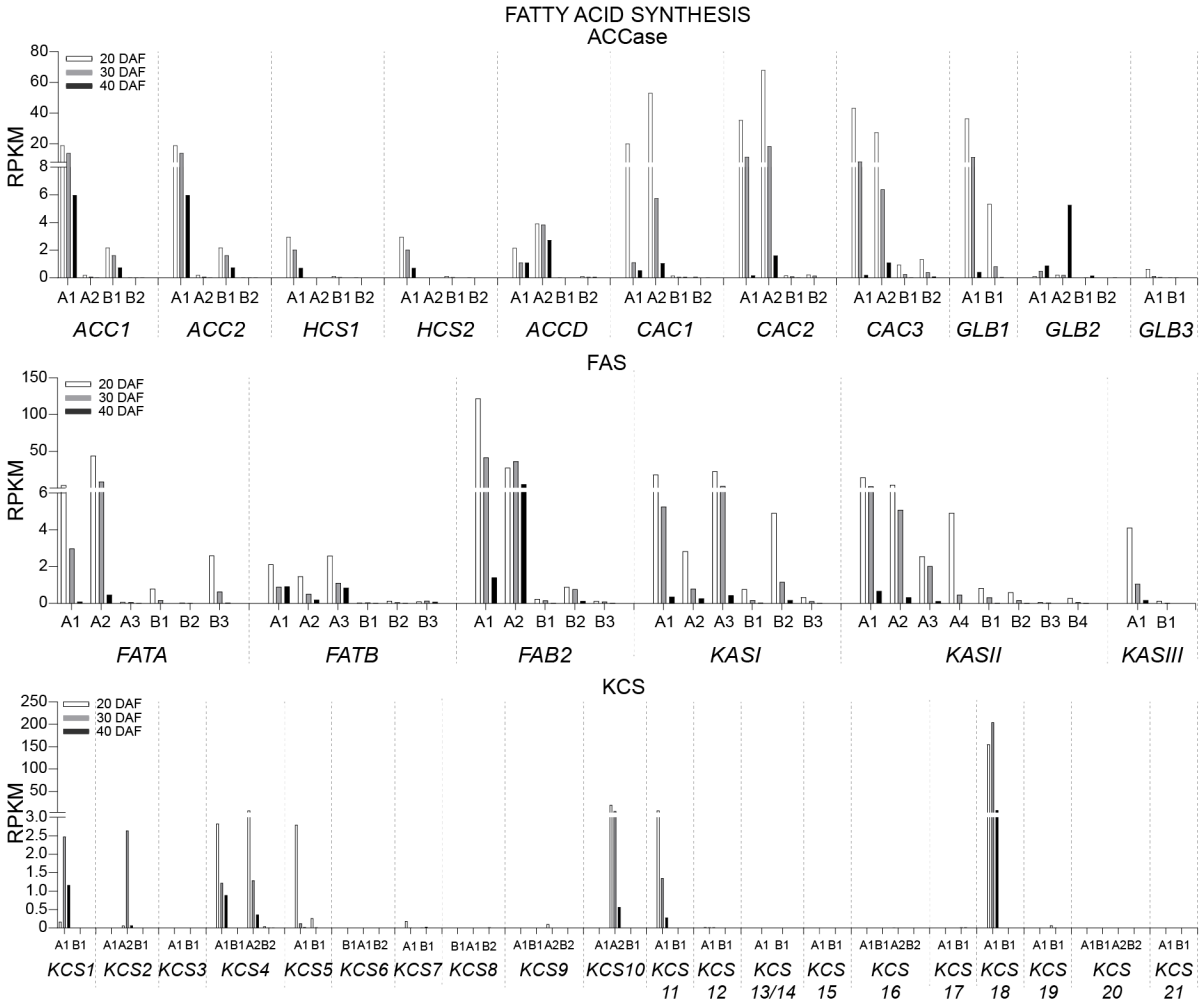
Figure 4.8 Phylogenetic analysis and expression profiling of various genes from at different stages of seed development in *B. juncea*

A, C, E, G, I Phylogenetic relationships of *DGAT*, *PDAT*, *FAD2*, *FAD3*, *FAE1*, *PDCT* genes. Phylogenetic analysis was performed using the coding sequence of genes from *A. thaliana* and the candidate homolog genes identified in *B. juncea* where Br and Bn represent homoeologs from *B. rapa* and *B. nigra* genome. The tree was constructed in the MEGA7 program using the maximum likelihood method. The percentage of replicate trees in which the associated proteins clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, and branch lengths denote the bootstrap value. B, D, F, H, J Expression profile of *DGAT*, *PDAT*, *FAD2*, *FAD3*, *KCS18*, *KCS4*, *KCS9*, *PDCT* genes from seeds at different stages of seed development. Data are represented as reads per kilobase of transcript per million mapped reads.

In addition to the observed differential expression between the *BjuA* and *BjuB* homoeologs, there were differences in expression among the homologs identified within each subgenome *BjuA* and *BjuB*. This redundancy within the subgenome opens the possibility of sub-functionalization and neo-functionalization of duplicated homeologs (Tang and Lyons, 2012). Comparative analysis of transcript expression among *BjuA* and *BjuB* homoeologs identified from different pathways and gene families involved in fatty acid synthesis, acyl editing, TAG biosynthesis, β-oxidation, and seed storage proteins indicate that *BjuA* homoeologs are expressed at a relatively higher level than those of *BjuB* homoeologs (Figure 4.9).

4.3 Discussion

The sequencing and next-generation sequencing (NGS) annotation aids in obtaining quantitative information on gene expression (Rokas and Abbot, 2009) for the genes involved in lipid metabolism. Such resources are available for different plant oil-producing plants, especially those belonging to *Brassica*ceae family including *B. rapa* (Wang et al., 2011) and *B. oleracea* (Cheng et al., 2011). The primary objective of this study was to identify the *B. juncea* homologs for genes encoding key enzymes involved in the metabolic pathways of fatty acid, and TAG biosynthesis and metabolism. Among the gene homoeologs for *BjuA* and *BjuB* identified in developing seeds of *B. juncea*, transcript profiling identified differences in expression of major loci which play an essential role in FA and TAG accumulation in the developing seed.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20 DAF
30 DAF
40 DAF
RPKM
ACC1
ACC2
HCS1
HCS2
ACCD
CAC1
CAC2
CAC3
GLB1
GLB2
GLB3
FAS
FATA
FATB
FAB2
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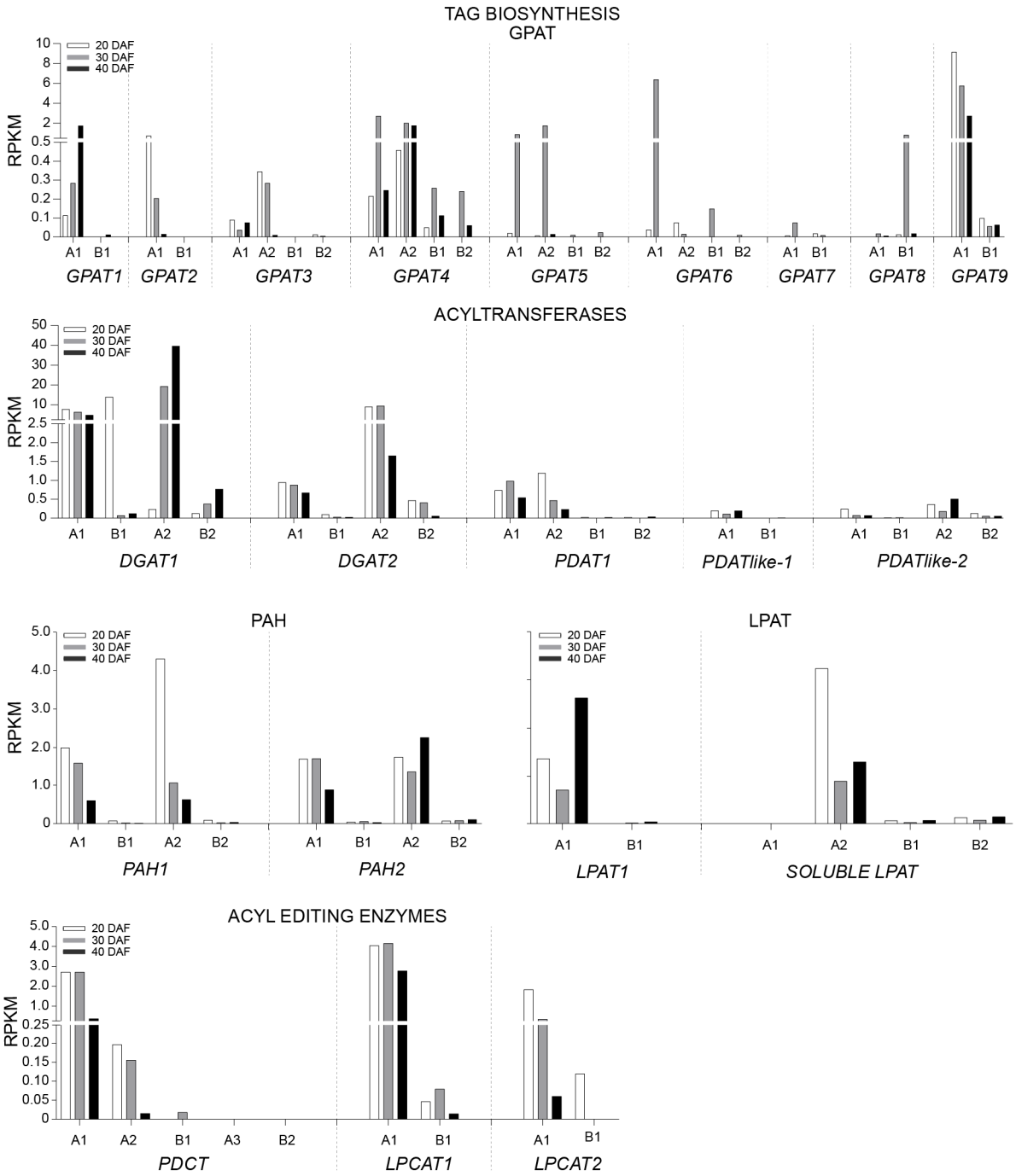
TAG BIOSYNTHESIS
GPAT
20 DAF
30 DAF
40 DAF
RPKM
GPAT1
GPAT2
GPAT3
GPAT4
GPAT5
GPAT6
GPAT7
GPAT8
GPAT9
ACYLTRANSFERASES
DGAT1
DGAT2
PDAT1
PDATlike-1
PDATlike-2
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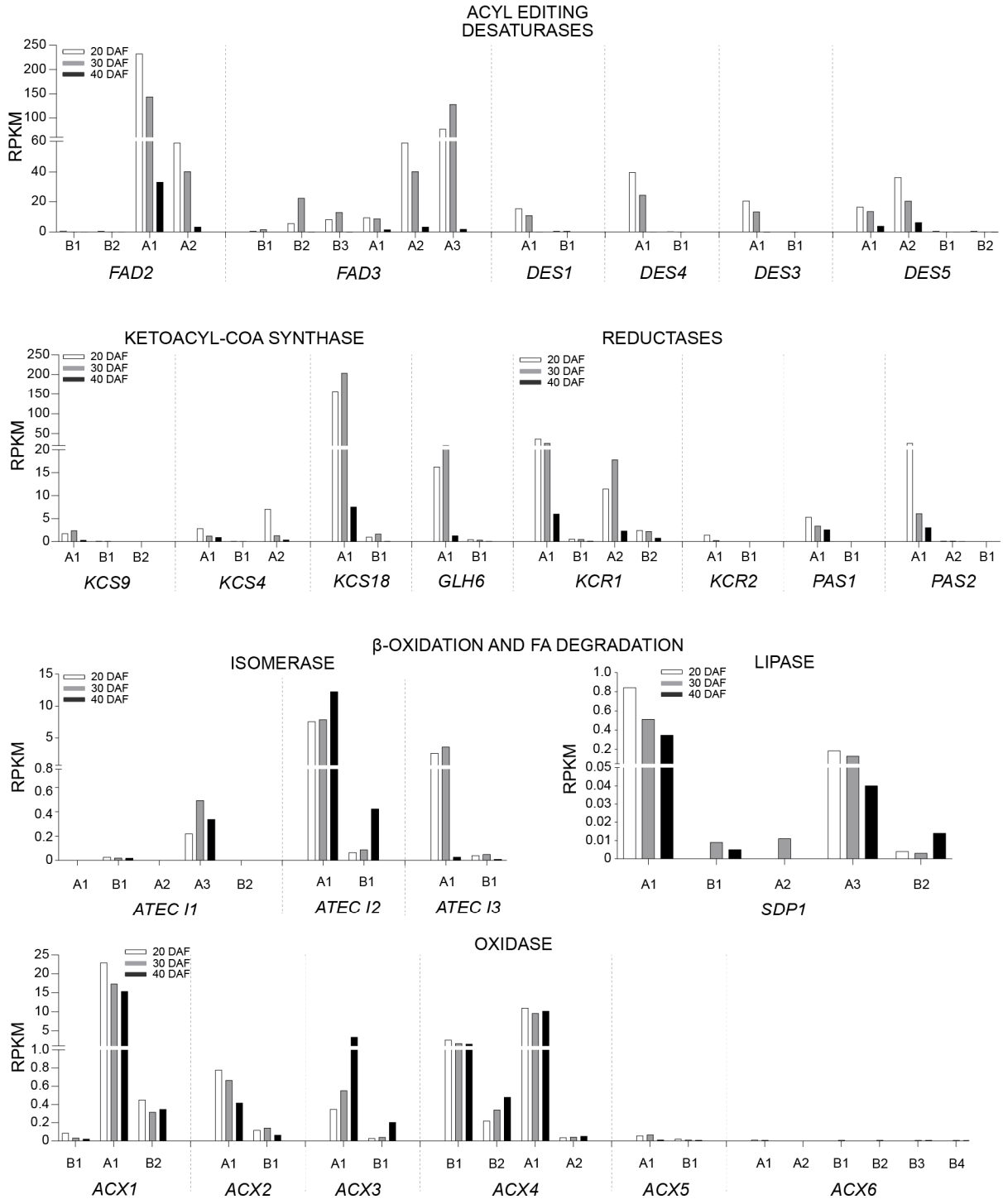
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KETOACYL-COA SYNTHASE
REDUCTASES
β-OXIDATION AND FA DEGRADATION
ISOMERASE
LIPASE
OXIDASE
20 DAF
30 DAF
40 DAF
RPKM
FAD2
FAD3
DES1
DES4
DES3
DES5
KCS9
KCS4
KCS18
GLH6
KCR1
KCR2
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ACX2
ACX3
ACX4
ACX5
ACX6

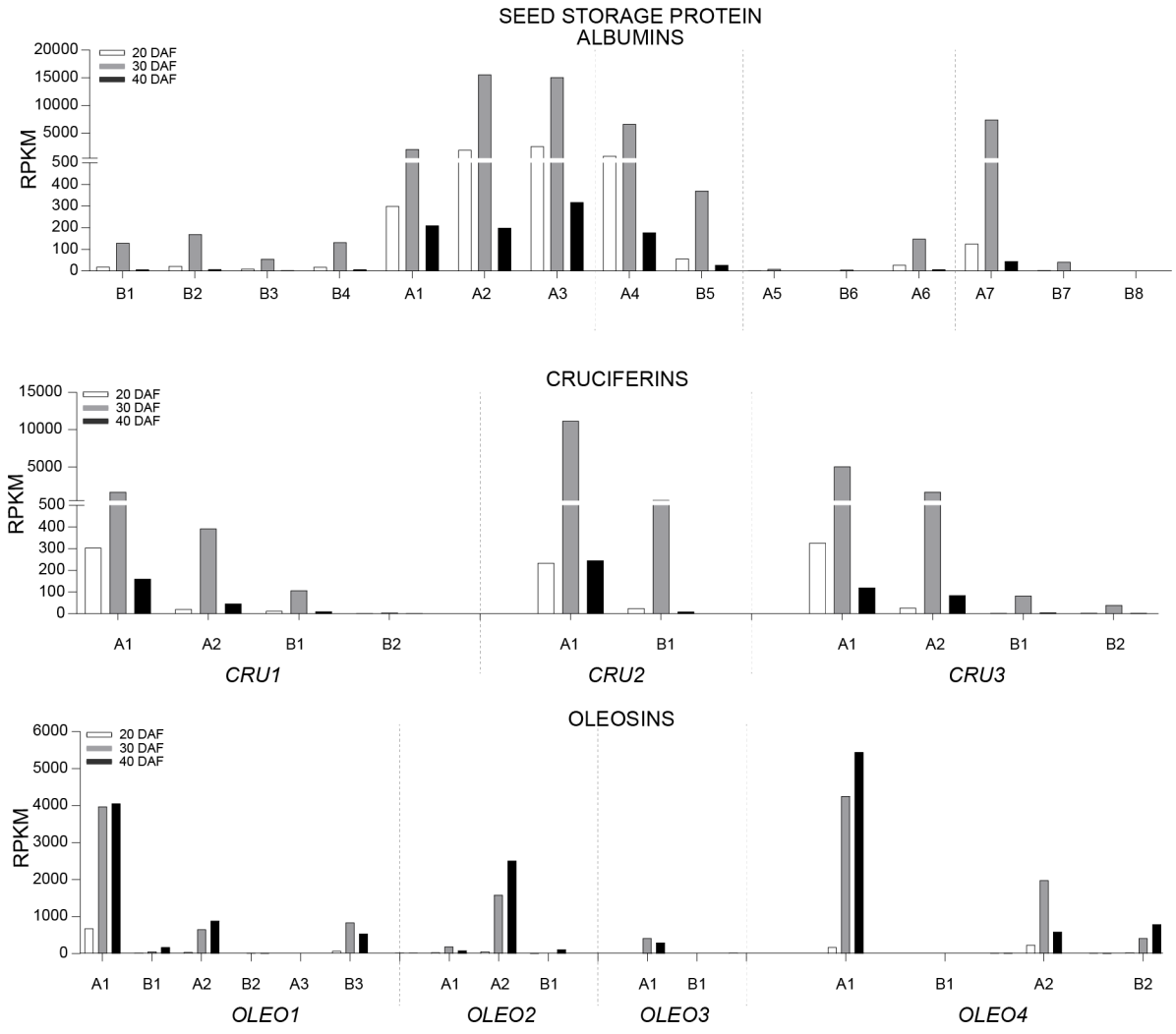

Figure 4.9 Expression profile for gene families involved in lipid metabolism.

Expression profiles of A- ACCase, FAS, KCS, from the fatty acid synthesis pathway, B-Acyltransferases, PAHs, and acyl editing enzymes from TAG biosynthesis pathway, C- desaturases, Ketoacyl CoA synthase, reductases from fatty acid modification pathway, D- isomerase, lipase and oxidase from β oxidation and FA degradation, E- albumins, cruciferins and oleosins from seed storage proteins. Letters A and B on the x axis represents the homoeologs from *BjuA* and *BjuB* subgenomes of *B. juncea*. Numbers next to the letter represents the paralogs within the same subgenome.

4.3.1 Role of desaturases, elongases and acyl editing enzymes in TAG biosynthesis

In the *Brassica* species, seed oil content for *B. juncea*, *B. napus,* and *B. rapa* range between 32.67–39.47%, 37.82–40.56% and 40.35–41.43%, respectively (Rai et al., 2018). In

addition to total seed oil content, there is variation in seed oil composition. Palmitic acid content is in the range of 3.08–3.85, 3.70–5.15, 2.75–3.73% in *B. juncea*, *B. napus* and *B. rapa*, respectively and oleic acid content is in the range of 0.80–48.70, 16.15–37.98 and 6.21–16.15% in *B. juncea*, *B. napus* and *B. rapa*, respectively (Rai et al., 2018). Among the major fatty acids in *Brassica* species, significant variation ($p \leq 0.05$) is observed for linoleic acid content and linolenic acid (Rai et al., 2018). In *B. juncea*, *B. napus* and *B. rapa*, linoleic acid content varied from 11.00-45.30, 18.57-26.93 % and 14.08-18.18%, respectively and linolenic acid content varied from 11.10- 26.72, 9.99- 17.23 and 9.82- 26.66% in *B. juncea*, *B. napus* and *B. rapa*, respectively (Rai et al., 2018).

Fatty acids esterified to *sn*-2 position of PC are the main site of FA modification (Van de Loo et al., 1995; Sperling et al., 1993). In the ER, conversion of oleic to linoleic acid is mediated by FAD2 whereas FAD3 mediates conversion of linoleic to α-linolenic acid (Ohlrogge and Somerville, 1991). Higher 18:1 levels have been obtained in various crops including *B. carinata* and *B. juncea* by RNAi suppressing of the transcript expression for *FAD2* gene (Jadhav et al., 2005; Sivaraman et al., 2004; Liu et al., 2002). Phosphatidylcholine: diacylglycerol cholinephosphotransferase (PDCT) enzyme, encoded by the Arabidopsis *ROD1* gene, catalyzes the interconversion of DAG and PC, thus resulting in the transfer of 18:1 from DAG into PC for desaturation. PDCT also catalyzes the reverse reaction of the transfer of the phosphocholine headgroup from PC to DAG for incorporation of PUFAs into the TAG (Lu et al., 2009).

The erucic acid content is variable in different *Brassica* species and ranges from 0.80-49.40% in *B. juncea* varieties, 10.04- 34.96% in *B. napus* varieties and 43.77- 49.99% in *B. rapa* varieties (Rai et al., 2018). The membrane-bound enzyme complex known as the fatty acid elongation (FAE) complex uses malonyl-CoA as a source of the 2C unit and acyl-CoA as

substrates to form a very long chain fatty acid (VLCFA). Elongation of fatty acids is analogous to de novo fatty acid synthesis in the plastid and involves four enzymatic reactions catalyzed by FAE complex: 3-ketoacyl-CoA synthase (FAE1), 3-ketoacyl-CoA reductase, 3-hydroxyacyl-CoA dehydrase, and enoyl-CoA reductase (Firth and Patrick, 2008; Wu et al., 2008; Blacklock and Jaworski, 2006; Mietkiewska et al., 2004; Roscoe et al., 2001; Rossak et al., 2001; Millar and Kunst, 1997; James et al., 1995). High 22:1 content is not beneficial for edible oil whereas oil rich in 22:1 has diverse applications in plastic, tannery, cosmetic, polyester, and detergent industry (Coonrod et al., 2008). In *B. napus*, using RNAi approach the simultaneous suppression of transcripts for *FAD2* and *FAE1* results in an increase in levels of 18:1 and a corresponding decrease in 22:1 and PUFAs.

4.3.2 *BjuA* homoeologs are expressed at higher levels than *BjuB* homoeologs

Transcript profiling of important genes involved in lipid metabolism provides insights into oil composition and synthesis in developing seeds from *B. juncea*. The transcript expression analysis for *BjuA* and *BjuB* homoeologs indicated increased and predominant expression of *BjuA* gene homoeologs over *BjuB* (Figure 4.9). In future studies, the observed differences in transcript levels among *BjuA* and *BjuB* gene homoeologs for *DGAT1*, *PDAT1*, *FAD2*, *FAD3*, and *PDCT* can be confirmed by qRT-PCR using homoeolog specific primers. Additionally, for enzymes encoded by each identified homoeolog, the substrate specificity and enzyme activity can be characterized by cloning and expression of each homoeolog in a strain of yeast, H1246 which is deficient in TAG synthesis.

The upregulation of *DGAT1* transcript towards later stage of seed development (Figure 4.8B) is consistent with the increase in total lipid content (Figure 4.2). To investigate and

differentiate the contribution of DGAT1 and PDAT1 mediated pathway towards TAG biosynthesis, we analyzed and compared the trends of mol% for the major fatty acids 16:0, 18:1, 18:2, 18:3 and 22:1 from neutral lipid (NL) and polar lipid (PL) fractions across 20, 30 and 40 DAF time points (Figure 4.4). For the NL fraction, the level of 18:1 decreased at 30 DAF and remained unchanged at 40 DAF, whereas for the PL fraction, 18:1 level increased across the time points 20-40 DAF and at 40 DAF time point, the mol % of 18:1 were relatively higher for PL fraction as compared to NL fraction. These findings of lower 18:1 level in NL fraction at 40 DAF suggest that 18:1 acts as a substrate for the synthesis of 22:1 which show increased levels in NL fraction at 40 DAF. Also, lower mol% of 18:2 and 18:3 at 40 DAF in NL compared to PL fraction (Figure 4.4) suggests reduced incorporation of PUFAs into TAG via the FAD2/FAD3 pathway. The observed reduction of 18:2 and 18:3 levels in NL fraction at 40DAF is supported by reduced transcript expression of genes named *FAD2*, *FAD3,* and *PDCT* at 40 DAF (Figure 4.8 F, J Figure 4.9 B, C).

Thioesterases play an important role in the partitioning of de novo–synthesized fatty acids between the prokaryotic and eukaryotic pathways. The substrate specificity of thioesterases determines the chain length and saturation of fatty acids exported from the plastid In Arabidopsis, FATA has a preference for 18:1-ACP, whereas FATB has a preference for saturated and unsaturated acyl-ACP. In our study in *B. juncea*, the expression of *FatA* is relatively higher over *FatB* (Figure 4.9A), which results in the enrichment of 18:1 in the acyl-CoA pool. The expression of *FAD2* and *FAD3* is downregulated consistent with the reduced levels of 18:2 and 18:3 during later stages of seed development. The action of endogenous cytosolic complex fatty acid elongase I (FAE1) which utilizes 18:1-CoA precursor for the synthesis of VLCUFAs, resulting in the minor amount of eicosenoic acid (20:1) and a major

amount of erucic acid (22:1). The transcript expression of *FAE1* is upregulated during later stages of seed development (Figure 4.8H, Figure 4.9A) whereas there is an observed increase in levels of 22:1 at 40DAF (Figure 4.4). Future experiments to test the accumulation of FAE1 protein would confirm the non-linear relationship between mRNA levels and protein levels.

4.3.3 The non-linear relationship between transcript level and protein abundance

The negative correlation between mRNA levels and the corresponding protein levels can be attributed to the fact that under steady-state protein levels, only 40 % of the variation in protein concentration can be explained by the mRNA abundance (Vogel and Marcotte, 2012). Further, mRNA–protein correlations are often weaker during acute stress and developmental changes which involve proteome remodeling, with either mRNA or protein lagging in abundance response (Vogel and Marcotte, 2012). In developing leaves of maize, mRNA profile was quantified using RNA-Seq and protein abundance was measured using high accuracy mass spectrometry (MS) data of tryptic digests (Friso et al., 2011; Majeran et al., 2010). Up to 10-fold variation in protein/mRNA ratio is observed for some of the genes indicating that other factors affect mRNA-protein correlation including codon bias, UTRs, length of mRNA, and degradation of mRNA or protein (Olivares-Hernández et al., 2011; Vogel et al., 2010). To explain the negative correlation between mRNA and protein levels for the FAE1 complex in *B. juncea*, a future study on the regulation of FAE1 complex at post-transcription and post-translation level would be interesting. A similar example of negative correlation between mRNA-protein levels exists in *B. napus* for *BnDGAT1* homologs (Aznar-Moreno et al., 2015) and phytoene synthase family (López-Emparán et al., 2014).

4.4 Material and Methods

4.4.1 Plant materials and growth conditions

Brassica juncea seeds were kindly provided by our collaborator Dr. Iqbal Munir (Khyber Pakhtunkhwa Agricultural University, Pakistan). Seeds were cold stratified for two days at 4˚C before growing on soil mixture (4:2:1 mixture of Metromix Professional Growing Mix: vermiculite: perlite) in the greenhouse under long-day conditions (16 hrs light and 8hrs dark). To isolate seeds at different developmental stages namely 10, 20, 30, 40, 50 DAF and mature seeds, emerging flowers were tagged with the cotton thread at specific time intervals. Seeds were flash frozen in liquid nitrogen and stored at -80^{o}C for further use in RNA and lipid extraction.

4.4.2 Plant transformation

4.4.2.1 Vector and RNAi construction

All plasmids used for the transformation were derived from the binary vector pBinGlyRed3. The Agrobacterium tumefaciens strain GV3101 harboring the binary vector plasmid pBinGlyBar4-EfDAcT was used for the transformation of *Brassica juncea*. To construct this vector, the EfDAcT coding sequence was amplified with the primers 5'-ACCCAATTGATGATGGATGTTCATCAAGAG-'3 and 5'-AGACCTGCAGGTTAAGCGTAATCTGGAACATC-3' which containing *Mfe*I and *Sbf*I recognition sites, respectively. Standard restriction enzyme digests followed by ligation allowed the insertion of the PCR amplified fragment into the *Mfe*I and *Sbf*I sites in the multiple cloning site of pBinGlyBar4. This binary vector contains the glycinin 1 promoter from soybean that

provides seed-specific expression of EfDAcT coding sequence inserted into the multiple cloning site.

The sequence for RNAi suppression contained the two arms of a reverse complement sequence from the *Camelina DGAT1* and *Brassica juncea PDAT1* genes interrupted by the pdk intron from pHANNIBAL (Wesley et al., 2001). This entire hairpin-forming sequence was synthesized by GeneArt and was expressed under the control of the *Brassica napus* oleosin promoter. pBinGlyBar4 contains the *BAR* gene which provides resistance against the herbicide glufosinate (phosphinothricin) allowing for selection of transgenic plants. The completed vector was introduced into *A. tumefaciens* strain GV3101 by electroporation (1.8kV, 200Ω, 50mF) (Figure 4.7) and transformed into *B. juncea* plants.

4.4.3 Lipid extraction and lipid standards

Total lipids were extracted from seeds at different developmental stages using a hexane-isopropanol method (Li et al., 2006) with di15:0 PC and tri 13:0 TAG as internal standards. Total lipids were loaded onto a small silica column and neutral lipids eluted with 5ml 99:1 (v/v) chloroform: methanol. Polar lipids were then recovered with 5ml methanol. These lipid extracts were then analyzed using electro-spray ionization mass spectrometry (ESI-MS) as described previously (Bansal and Durrett, 2016; Li et al., 2014). Seed fatty acid content was determined using direct whole seed transmethylation (Li et al., 2006) using tripentadecanoin as an internal standard. The resulting fatty acid methyl esters were quantified on a Shimadzu GC-2010plus gas chromatograph equipped with a 30 X 0.25mm DB-23 column (Agilent Technologies) as described previously (Aznar Moreno and Durrett, 2017).

4.4.4 RNA isolation and sequencing

Total RNA was extracted from the seeds at different developmental stages using the RNeasy plant mini kit (Qiagen), followed by additional purification with sodium citrate to improve RNA quality (Nybo, 2011). We obtained total RNA yields in the range of 1-3ug for ~50mg seeds. Using an Agilent Bioanalyzer, we confirmed that the RNA Integrity Number (RIN) for all samples was greater than 9. Total RNA (500 ng) was sequenced at the Genomics Core at the University of Kansas Medical Center. The mRNA fraction was enriched with oligo dT capture, sized, reverse transcribed into cDNA and ligated with the appropriate indexed adaptors using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina). Following Agilent Bioanalyzer QC of the library preparation and library quantification using the Roche Lightcycler96 with KAPA SYBR Fast Universal qPCR kit (KAPA Biosystems), the RNA-Seq libraries were adjusted to a 4nM concentration and pooled for multiplexed sequencing. Libraries were denatured and based on qPCR results, diluted to the appropriate concentration, followed by clonal clustering onto the sequencing flow cell using the TruSeq Paired-End (PE) Cluster Kit v3-cBot-HS (Illumina). The clonal clustering procedure was automated using the Illumina cBOT Cluster Station. The clustered flow cell was sequenced on an Illumina HiSeq 2500 Sequencing System using the TruSeq SBS Kit v3-HS (Illumina) to obtain 100bp pair-end reads.

4.4.5 Identification and transcript profiling of *BjuA* and *BjuB* homoeologs

Paired-end reads (2 x 100) obtained from RNA-Seq were trimmed and quality assessed for de novo assembly and mapping using CLC genomics workbench (v 7.5.1). The CDS sequence of genes involved in lipid metabolism from Arabidopsis were selected as bait, and BLASTn algorithm (Altschul et al., 1990) was used against the *Brassica* database (BRAD)

(Wang et al., 2015) for the identification of *BjuA* and *BjuB* homoeologs. For some of the gene families, more than two homologs for *BjuA* and *BjuB* were identified. The CDS sequences of *BjuA* and *BjuB* homologs were aligned with the Arabidopsis gene using MUSCLE (Edgar, 2004), and a phylogenetic tree was generated using Maximum Likelihood method (Tamura and Nei, 1993).

For transcript expression analysis, the sequence of each homolog was used as a reference, and sample reads for each time point were mapped to the reference using mapping parameters using which reads which were 100% identical to the reference sequence were mapped, excluding the mismatch or gap errors. The number of mapped reads was normalized to the gene length, & sample library size and expression of each gene was denoted as RPKM.

4.4.6 Phylogenetic analysis

The phylogenetic analysis was performed using coding sequences (CDS) sequences for the homologs of genes identified in *A. thaliana*, *B. rapa* and *B. nigra* using the MEGA7 software (Kumar et al., 2016). To identify the closest homolog which will be grouped in the clade nearest to *A. thaliana*, the phylogenetic analysis was done using the maximum likelihood method.

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