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Properties and biotechnological applications of acyl-CoA: and phospholipid:diacylglycerol acyltransferases from terrestrial plants and microalgae

-a review dedicated to Professor Randall J. Weselake to celebrate his exceptional career and contributions to the broad field of lipids and AOCS

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Abstract:

Triacylglycerol (TAG) is the major storage lipid in most terrestrial plants and microalgae, and has great nutritional and industrial value. Since the demand for vegetable oil is consistently increasing, numerous studies have been focused on improving TAG content and modifying the fatty acid compositions of plant seed oils. In addition, there is a strong research interest in establishing plant vegetative tissues and microalgae as platforms for lipid production. In higher plants and microalgae, TAG biosynthesis occurs via acyl-CoA-dependent or acyl-CoA-independent pathways. Diacylglycerol acyltransferase (DGAT) catalyzes the last and committed step in the acyl-CoA-dependent biosynthesis of TAG, which appears to represent a bottleneck in oil accumulation in some oilseed species. Membrane-bound and soluble forms of DGAT have been identified with very different amino acid sequences and biochemical properties. Alternatively, TAG can be formed through acyl-CoA-independent pathways via the catalytic action of membrane-bound phospholipid:diacylglycerol acyltransferase (PDAT). As the enzymes catalyzing the terminal steps of TAG formation, DGAT and PDAT play crucial roles in determining the flux of carbon into seed TAG and thus have been considered as the key targets for engineering oil production. Here, we summarize the most recent knowledge on DGAT and PDAT in higher plants and microalgae, with the emphasis on their physiological roles, structural features, and regulation. The development of various metabolic engineering strategies to enhance TAG content and alter fatty acid composition of TAG is also discussed.

Abbreviations

ABI	*ABSCICIC ACID INSENSITVE* transcription factor
ACAT	acyl-CoA:cholesterol acyltransferase
AMPK	AMP-activated protein kinase
cDNA	complementary DNA
CRISPR	clustered regularly interspaced short palindromic repeats
DAG	diacylglycerol
DCR	Defective Cuticle Ridge
DGAT	acyl-CoA:diacylglycerol acyltransferase
EMS	ethyl methanesulfonate
ER	endoplasmic reticulum
FAD	fatty acid desaturase
FAH	fatty acid hydroxylase
G3P	*sn*-glycerol-3-phosphate
GPAT	acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase
IDR	intrinsically disordered region
LCAT	lecithin:cholesterol acyltransferase
LEC	*LEAFY COTYLEDON* transcription factor
LPAAT	acyl-CoA:lysophosphatidic acid acyltransferase
MBOAT	membrane-bound O-acyltransferases
PtdOH	phosphatidic acid
PtdCho	phosphatidylcholine
PDAT	phospholipid:diacyglycerol acyltransferase
RNAi	RNA interference
sn	stereospecific numbering
SnRK1	sucrose non-fermenting1-related kinase1
TAG	triacylglycerol
TILLING	targeting-induced local lesions in genomes
TMD	transmembrane domain
WRI	*WRINKLED1* transcription factor

Introduction

Triacylglycerol (TAG), which is the major component of vegetable oils, consists of three fatty acids esterified to a glycerol backbone. In terrestrial plants, TAG is mainly stored in seeds functioning as an energy reservoir to facilitate germination and early seedling growth. TAG also provides the precursors for membrane biosynthesis and lipid signaling, which are crucial for normal plant growth and development (Fan et al., 2013b; Fan et al., 2014). In microalgae, TAG serves as a source of energy in response to adverse environmental conditions (for review, see Hu et al., 2008). The primary use of seed oils is for edible applications (food and feed). There is, however, a strong interest in using seed oil as renewable feedstock to produce biodiesel, biolubricants and other bioproducts (Biermann et al., 2011).

The global demand for vegetable oils has been steadily growing over the past 50 years and is expected to further increase due to a rising global population and reliance on vegetable-oil-derived chemicals (Chen et al., 2015). Increasing vegetable oil supply is generally accomplished via two approaches: growing more oil crops and increasing oil content in seeds and/or other oil-forming tissues (e.g., mesocarp of palm [*Elaeis* spp.] and avocado [*Persea Americana*]). Direct increase of oil crop planting area, however, is limited by the finite availability of the arable lands (Lu et al., 2011). Recent efforts to meet the rising demands have been focused on increasing oil content of seeds and vegetative tissues by the implementation of metabolic engineering strategies. In addition, considerable research has focused on exploring the potential of oleaginous microalgae to produce TAG, a process that is not expected to utilize arable lands. Since the property of vegetable oils is largely affected by fatty acid composition, research interests have also been directed on the modification of fatty acid composition to increase the nutritional and industrial value of oils.

In general, TAG biosynthesis in terrestrial higher plants and microalgae is similar. Schematically, TAG assembly can be divided into acyl-CoA-dependent and acyl-CoA-independent pathways. Diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) catalyzes the last and committed step in the acyl-CoA-dependent TAG biosynthesis by transferring an acyl group from acyl-CoA to the *sn*-3 position of diacylglycerol (DAG), which has been the target of numerous studies attempting to engineer oil content and fatty acid composition (for review, see Liu et al., 2012). In contrast, phospholipid:diacylglycerol acyltransferase (PDAT; EC 2.3.1.158) catalyzes the acyl-CoA-independent synthesis of TAG using membrane glycerolipids as acyl

donors (Dahlqvist et al., 2000). In this review, we begin by providing background information on storage lipid biosynthesis in higher plants and microalgae. Thereafter, the biochemical and physiological properties of DGAT and PDAT are discussed. This, in turn, is followed by discussion on the metabolic engineering of DGAT and PDAT- catalyzed reactions so as to manipulate oil production in terrestrial plants and microalgae.

Overview of storage lipid biosynthesis in higher plants and microalgae

TAG formation in oleaginous plants and microalgae involves fatty acid biosynthesis and TAG assembly (Fig. 1; for review, see Chen et al., 2015). Depending on the plant species, TAG formation can also involve a complex interplay with membrane metabolism. For example, seed oils enriched in polyunsaturated fatty acids or unusual fatty acids require processes for routing these fatty acids from the site of their synthesis in membranes into TAG.

Higher plants

In developing seeds of oleaginous plants, *de novo* fatty acid biosynthesis and TAG assembly occur in different compartments. Fatty acid biosynthesis operates in the plastid, wherein acetyl-CoAs are converted to fatty acyl chains (for reviews, see Ohlrogge and Jaworski, 1997; Chapman and Ohlrogge, 2012). Acetyl-CoA carboxylase catalyzes the ATP-dependent formation of malonyl-CoA, while the fatty acid synthase complex uses two carbon fragments derived from malonyl-CoA to form an acyl chain while attached to an acyl carrier protein of the fatty acid synthase complex. The main source of acetyl-CoA used for fatty acid synthesis is derived from pyruvate via the catalytic action of the plastidial pyruvate dehydrogenase complex. Pyruvate can be produced from glucose derived from photosynthate through plastidial and cytosolic glycolysis. Specific transporters are required to move some of the cytosolic intermediates of glycolysis into the plastid (for review, see Rawsthorne, 2002). Fatty acyl chains produced in plastids can extend up to 16 or 18 carbons in length, which can further undergo monounsaturation, before being released from the fatty acid synthase complex and transported out of the plastid and converted into acyl-CoA.

TAG assembly occurs in the endoplasmic reticulum (ER) and uses glycerol-3-phosphate (G3P) derived from glycolysis, and acyl-CoA as acyl donor. This process is known as the Kennedy pathway and involves the sequential acylation of the glycerol backbone of G3P at *sn*-1,

2, and 3 positions to yield TAG (Weiss and Kennedy, 1956). Three acyl-CoA-dependent acyltransferases, including *sn*-glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT) and DGAT, participate in this process (for review, see Snyder et al., 2009). GPAT catalyzes the first acylation of G3P to yield lysophosphatidic acid. Lysophosphatidic acid is further acylated by the catalytic action of LPAAT to produce phosphatidic acid (PtdOH), which is then converted to *sn*-1, 2-DAG via the catalytic action of phosphatidic acid phosphatase. DGAT catalyzes the final acylation of the *sn*-3 position of *sn*-1, 2-DAG to form TAG, which is the committed step in acyl-CoA-dependent TAG biosynthesis. TAG can also be synthesized through acyl-CoA-independent pathways via the catalytic action of PDAT, which catalyzes the transfer of an acyl moiety from the *sn*-2 position of phosphatidylcholine (PtdCho) to the *sn*-3 position of *sn*-1, 2-DAG to yield TAG (Dahlqvist et al., 2000; Ståhl et al., 2004). Both DGAT and PDAT play crucial roles in determining the flux of carbon into TAG (Zhang et al., 2009; Harwood et al., 2013; Aznar-Moreno and Durrett, 2017). They also contribute to the routing of modified fatty acids from PtdCho into TAG in some plant species, such as flax (*Linum usitatissimum*), castor (*Ricinus communis*), tung tree (*Vernicia fordii*) and ironweed (*Vernonia galamensis*), which produce relatively high levels of polyunsaturated or unusual fatty acids in their seed oils (Kroon et al., 2006; Shockey et al., 2006; Li et al., 2010a; van Erp et al., 2011; Kim et al., 2011; Pan et al., 2013).

The synthesis of long-chain polyunsaturated or unusual fatty acids generally occurs on PtdCho or in the acyl-CoA pool, where the nascent fatty acids derived from the plastid, mainly saturated [16:0 and 18:0] and monounsaturated [18:1Δ^{9cis}] fatty acids, undergo further modifications, such as elongation and desaturation. The further elongation of the acyl chain is catalyzed by ER-bound fatty acid elongase with acyl-CoA as substrates (Ghanevati and Jaworski, 2001; Rossak et al., 2001). For fatty acid desaturation, fatty acid desaturase (FAD) 2 and FAD3 subsequently introduce double bonds in *cis* configuration at positions Δ-12 and Δ-15 of fatty acids on the *sn*-2 position of PtdCho, respectively (Browse et al., 1993; Vrinten et al., 2005). Other fatty acid modifications, including hydroxylation, epoxidation and conjugation, also utilize PtdCho as the substrate. The contributing enzymes, such as hydroxylase from castor (van de Loo et al., 1995), conjugase from *Momordica charantia* (Cahoon et al., 1999) and epoxygenase from *V. galamensis* (Cahoon and Kinney, 2005), are related to or derived from FAD2.

After being synthesized on PtdCho, the modified fatty acids are moved out of PtdCho and eventually incorporated into TAG via various routes which include acyl-editing (Bates et al., 2007; for reviews, see Chen et al., 2015; Bates, 2016). PDAT is capable of directly moving modified fatty acids from their site of synthesis in PtdCho to TAG. Alternatively, PtdCho-derived fatty acids can also be routed into the acyl-CoA or DAG pool. Fatty acids on PtdCho can enter the acyl-CoA pool via the combined action of phospholipase A_2 and long-chain acyl-CoA synthetase or via the reverse reaction catalyzed by lysophosphatidylcholine acyltransferase. Phospholipase A_2 catalyzes the cleavage of a fatty acid from the *sn*-2 position of PtdCho, the primary site for acyl modification, and the released fatty acid is further ligated to CoA through the catalytic action of long-chain acyl-CoA synthetase, yielding an acyl-CoA. Alternatively, the reverse action of lysophosphatidylcholine acyltransferase generates acyl-CoA and lysophosphatidylcholine from PtdCho and free CoA. In turn, the forward action catalyzed by lysophosphatidylcholine acyltransferase can re-acylate the vacant *sn*-2 position of lysophosphatidylcholine generated by the catalytic action of PLA_2 and/or the reverse reaction of lysophosphatidylcholine acyltransferase. Furthermore, PtdCho-derived fatty acids can also be incorporated into TAG in the form of DAG. *De novo* synthesized DAG can be converted into PtdCho for modification via the catalytic action of CDP-choline:*sn*-1, 2-diacylgycerol cholinephosphotransferase (Slack et al., 1983; Slack et al., 1985). PtdCho can subsequently be converted back to DAG and/or PtdOH via the catalytic action of phospholipase C and/or D, respectively (for reviews, see Chapman and Ohlrogge, 2012; Bates et al., 2013). Alternatively, the PtdCho-DAG conversion could also be achieved via the catalytic action of phosphatidylcholine: diacylglycerol cholinephosphotransferase, which transfers the phosphocholine headgroup of modified-PtdCho to the *de novo* synthesized DAG in the Kennedy pathway (Lu et al., 2009; Wickramarathna et al., 2015). Although the *sn*-2 position of PtdCho is the primary site for acyl-editing, a low amount of acyl-editing flux was also observed to go through the *sn*-1 position (Bates et al., 2007; Bates et al., 2009). The exchange of acyl groups might occur between the *sn*-1 and *sn*-2 positions of PtdCho with involvement of the catalytic action of glycerophosphocholine acyltransferase and lysophosphatidylcholine transacylase (Lager et al., 2015).

In developing seeds of oleaginous plants, TAG accumulates between the outer leaflets of the ER. Eventually, lipid droplets (oil bodies) ranging from 0.2 to 2 microns in diameter pinch

off of the ER and remain surrounded by a monolayer of phospholipid known as a half-unit membrane (for review, see Huang, 1996). In plant seeds, oil-body-membrane associated proteins are embedded on the outside of the lipid droplets (for reviews, see Shimada and Hara-Nishimura, 2010; Pyc et al., 2017; Huang, 2018). The most abundant lipid droplet proteins are oleosins, which appear to prevent oil droplets from coalescing and function in the formation and turnover of lipid droplets (Pyc et al., 2017). In addition, other lipid droplet proteins, such as caleosins and steroleosins, were identified in seeds according to proteomics analyses of isolated lipid droplets (Pyc et al., 2017). The physiological roles of caleosins and steroleosins in seeds, however, are not entirely clear. Some caleosins appeared to be involved in plant stress response (Shimada and Hara-Nishimura, 2010).

Microalgae

TAG biosynthesis in microalgae, in general, is analogous to higher plants involving both acyl-CoA-dependent and acyl-CoA-independent processes (Fig. 1), but has some different features (for reviews, see Hu et al., 2008; Li-Beisson et al., 2015). In the conventional TAG biosynthetic pathways, TAG is generally assembled in the ER from ER-derived DAG and is deposited mainly in lipid droplets in the cytosol. In the halophytic microalga *Dunaliella bardawil*, however, TAG is deposited in plastidial oil droplets-plastoglobuli in addition to cytosolic lipid droplets (Katz et al., 1995). Moreover, the starch-less unicellular green microalga *Chlamydomonas reinhardtii* has a unique plastidial pathway for TAG biosynthesis using DAG derived almost exclusively from the chloroplast (Fan et al., 2011). Plastidial TAG biosynthesis is largely dependent on *de novo* fatty acid biosynthesis, and the resulting TAG is stored in lipid droplets in both the chloroplast and cytosol (Fan et al., 2011). Currently, the chloroplast envelope-based pathway of TAG biosynthesis in microalgae is receiving increasing experimental support (Fan et al., 2011; Goodson et al., 2011; Liu and Benning, 2013; Li et al., 2014; Bagnato et al., 2017); the presence of chloroplast lipid droplets, however, is still debatable. The similar origin and composition of the lipid droplets in cytoplasmic lipid droplets and β-carotene rich plastoglobuli of *D. bardawil* suggested the possibility that β-carotene-rich plastoglobuli are formed in part from hydrolysis of chloroplast membrane lipids and in part from fatty acids or TAG derived from cytoplasmic lipid droplets (Davidi et al., 2014). More recently, it was revealed by microscopy that the chloroplast-associated lipid droplets in *C. reinhardtii* entirely

originated in the cytosol and were distinct from the plastoglobuli in the chloroplast stroma, even though some lipid droplets were associated with the outer envelope of the chloroplast without intervention of the ER (Moriyama et al., 2017). Moreover, some microalgae also possess other pathways of TAG biosynthesis that differ from higher plants. For instance, in the microalga *C. reinhardtii* which lacks PtdCho, the synthesis of TAG involves the participation of the betaine lipid diacylglycerol N,N,N-trimethylhomoserine (for review, see Li-Beisson et al., 2015).

Acyl-CoA-dependent formation of TAG

To date, the majority of studies on the acyl-CoA-dependent formation of TAG have focused on three *DGAT* gene families. The first two gene families, including *DGAT1* and *DGAT2*, encode enzymes embedded in the membrane lipid bilayer, whereas *DGAT3*, the third gene family, encodes a soluble enzyme with DGAT activity (for reviews, see Lung and Weselake, 2006; Liu et al., 2012). DGAT3 is the least investigated of the three DGAT families. In the following several sections, the features of DGAT from each gene family will be discussed along with some discussion of other proteins with DGAT activity.

Membrane-bound DGAT

In 1956, the very first report of DGAT activity was from chicken (*Gallus gallus domesticus*) liver (Weiss and Kennedy, 1956; Weiss et al., 1960), but over four decades passed before the genes were cloned and characterized (Cases et al., 1998). In 1998, the first *DGAT* gene, which is a member of the *DGAT1* family, was isolated from mouse (*Mus musculus*) based on its sequence homology to acyl-CoA: cholesterol acyltransferase (ACAT) 1 (Cases et al., 1998). Shortly thereafter, plant *DGAT1* were identified in the model plant *Arabidopsis thaliana* (hereafter referred to as Arabidopsis) (Hobbs et al., 1999; Routaboul et al., 1999; Zou et al., 1999; Bouvier-Navé et al., 2000) and tobacco (*Nicotiana tabacum*) (Bouvier-Navé et al., 2000). Subsequently, many *DGAT1* have been isolated and characterized from various plant species including (but by no means comprehensive) olive (*Olea europaea*) (Giannoulia et al., 2000), oilseed rape (*Brassica napus*) (Nykiforuk et al., 2002; Greer et al., 2015), castor bean (*R. communis*) (He et al., 2004), burning bush (*Euonymus alatus*) (Milcamps et al., 2005), tung (*V. fordii*) (Shockey et al., 2006), soybean (*Glycine max*) (Wang et al., 2006), garden nasturtium (*Tropaeolum majus*) (Xu et al., 2008), *Echium pitardii* (Manas-Fernandez et al., 2009), flax (*L.*

usitatissimum) (Siloto et al., 2009b; Pan et al., 2013), sesame (*Sesamum indicum*) (Wang et al., 2014), and more recently *Cuphea avigera* var. *pulcherrima* (Iskandarov et al., 2017) and peanut (*Arachis hypogaea*) (Zheng et al., 2017b).

Following the discovery of DGAT1, DGAT2 was found, which essentially has no amino acid sequence similarity with DGAT1 and ACAT1. In 2001, the first *DGAT2* was isolated from the oleaginous fungus *Umbelopsis ramanniana* (formerly *Mortierella ramanniana*) (Lardizabal et al., 2001). In the same study, *DGAT2* homologs were isolated from *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and Arabidopsis, but only *DGAT2* from the former two species encoded active enzymes. Based on the *DGAT2* sequence from *U. ramanniana*, *DGAT2*-related genes were rapidly identified from mammals, such as mouse and human (*Homo sapiens*) (Cases et al., 2001). Although the early attempt to produce active recombinant Arabidopsis DGAT2 (AtDGAT2) was unsuccessful, functional *DGAT2* was isolated and characterized from plants, including tung tree, castor bean, ironweed and flax, on the basis of the putative *AtDGAT2* sequence (Kroon et al., 2006; Shockey et al., 2006; Li et al., 2010a; Pan et al., 2013; Xu et al., 2018).

Physiological roles of DGAT1 and DGAT2

DGAT1 is considered to play a critical role in determining the flux of carbon into seed TAG in some species (Harwood et al., 2013). In oilseed crops such as canola-type *B. napus* and safflower (*Carthamus tinctorius*), the level of DGAT activity was found to be coordinated with oil accumulation during seed development (Tzen et al., 1993; Weselake et al., 1993). Expression analysis of *DGAT1* revealed that this gene is highly expressed in developing embryos in many oilseed crops (Hobbs et al., 1999; Lu et al., 2003) and its expression level is correlated with oil deposition during seed development (Li et al., 2010b). Forward and reverse genetics strategies brought about a more direct piece of evidence that DGAT1 is a major determinant in oil accumulation (Katavic et al., 1995; Zou et al., 1999; Zheng et al., 2008). DGAT1 inactivation resulted in a dramatic decrease in seed oil levels in the Arabidopsis mutant AS11 (Katavic et al., 1995; Zou et al., 1999). Consistently, the activation of DGAT1 [by a phenylalanine insertion in the maize (*Zea mays*) DGAT1] was responsible for the increased embryo oil content in a high-oil maize line (Zheng et al., 2008). Furthermore, DGAT1 appears to play a role in freezing and/or drought stress responses in Arabidopsis, *Boechera stricta* and *B. napus*. The expression of

DGAT1 was found to be highly cold responsive and correlated with the cold tolerance in *B. stricta* lines (Arisz et al., 2018). Consistently, enhanced *DGAT1* expression led to increased freezing tolerance in Arabidopsis (Arisz et al., 2018), whereas Arabidopsis *dgat1* mutant lines were sensitive to freezing (Tan et al., 2018). In addition, over-expression of *DGAT1* during seed development in *B. napus* was shown to decrease the penalty on seed oil content caused by drought (Weselake et al., 2008).

Unlike the substantial contribution of DGAT1 to seed oil accumulation, DGAT2 appears to play a minor role in regulating oil production. The expression of *AtDGAT2* did not restore the TAG synthesizing ability in the *S. cerevisiae* mutant H1246 (Zhang et al., 2009), and the Arabidopsis *dgat2* mutants do not show any changes in TAG accumulation (Zhang et al., 2009). Recently, the functionality of AtDGAT2 in TAG biosynthesis was confirmed in *Nicotiana benthamiana* leaves by transient expression of the encoding cDNA (Zhou et al., 2013), and *S. cerevisiae* yeast by heterologous expression using a codon-optimized version of the cDNA (Aymé et al., 2014). Nevertheless, the physiological role of DGAT2 in Arabidopsis remains to be further explored. Interestingly, by characterizing DGAT2 from different plant species accumulating unusual fatty acids, it was revealed that DGAT2 appears to be important for incorporating unusual fatty acids, such as eleostearic acid (18:3$\Delta^{9cis,11trans,13trans}$) from tung tree (Shockey et al., 2006), ricinoleic acid from castor (Kroon et al., 2006), and vernolic acid (*cis*-12-epoxy-octadeca-*cis*-9-enoic acid) from ironweed (Li et al., 2010a) into storage TAG. Indeed, the expression of *DGAT2* during embryo development was found to be at a higher level than *DGAT1* expression in the developing seeds from plants accumulating unusual or polyunsaturated fatty acids (Kroon et al., 2006; Shockey et al., 2006; Li et al., 2010a; Pan et al., 2013), whereas *DGAT2* transcripts in Arabidopsis and soybean were far below the levels observed for *DGAT1* transcripts (Li et al., 2010b). It appears that TAG production by DGAT1 occurs in a distinct ER subdomain than that of DGAT2, since tung tree DGAT1 and DGAT2 were found to localize to different regions of the ER and they differ in substrate preference (Shockey et al., 2006). The physiological roles of DGAT1 and DGAT2 in microalgae also remain largely to be explored. For example, in most of the microalgal species, one *DGAT1* and one to multiple *DGAT2* genes appear to contribute to the complexity of TAG biosynthesis (Turchetto-Zolet et al., 2011; Chen and Smith, 2012; Gong et al., 2013; Liu and Benning, 2013), and it is still unknown why microalgae need these many redundant copies of *DGAT2*.

In mammals and yeasts, DGAT2 rather than DGAT1 appears to be the dominant enzyme for TAG synthesis. Knocking out of *dgat2* in mouse (*Dgat2*$^{-/-}$) led to lipopenia, abnormal skin and early death (Stone et al., 2004), whereas *dgat1* defective mouse (*Dgat1*$^{-/-}$) was viable and capable of TAG synthesis (Smith et al., 2000). Whilst the *DGAT2* almost ubiquitously exists in all eukaryotes, *DGAT1* is missing in the genome of certain yeasts (*S. cereviseae* and *Candida albicans*) and fungi (*Laccaria bicolor*, *Schizophillum commune* and *Agaricus bisporus*) (Turchetto-Zolet et al., 2011). DGAT2 is likely the primary TAG-synthesizing enzyme in the yeast *Yarrowia lipolytica*, which also has a *DGAT1* gene (Zhang et al., 2012).

Structural and functional features of DGAT

DGAT1 and DGAT2 are integral membrane-bound proteins with multiple transmembrane domains (TMD) (for review, see Liu et al., 2012). Currently, there is no three-dimensional structure available for any DGAT or closely homologous enzymes (Liu et al., 2012; Lopes et al., 2015). The recent insights into DGAT structure-function relationships largely rely on the identification of possible functional motifs and the determination of putative membrane topologies. Very recently, the structure of the hydrophilic N-terminal domain of DGAT1 from *B. napus* (BnaDGAT1) was solved, which resulted in a leap forward in the understanding of the self-regulatory mechanism of this enzyme family (Caldo et al., 2017). Below we discuss the structural and functional features of plant DGAT with relevant reference to the mammalian literature.

DGAT1 is composed of about 500 amino acid residues having a large hydrophilic N-terminal region, followed by 8 to 10 predicted TMD (Fig. 2A; Liu et al., 2012). The N-terminal region of DGAT1 is highly variable and is encoded by the first exon, which is separate from the exons encoding the rest of the polypeptide (Liu et al., 2012; Greer et al., 2015). The N-terminal regions of *B. napus* and mouse DGAT1 enzymes were demonstrated to bind acyl-CoA in a sigmoidal fashion, suggesting positive cooperative binding (Weselake et al., 2006; Siloto et al., 2008). In addition, the N-terminal region of *B. napus* DGAT1 formed dimers and tetramers based on crosslinking experiments (Weselake et al., 2006). Consistently, analysis of mouse and *B. napus* DGAT1 showed that the N-terminal region plays a role in self-oligomerization (McFie et al., 2010; Caldo et al., 2017). Furthermore, the hydrophilic N-terminal region of *B. napus* DGAT1 was shown to constitute the enzyme's regulatory domain, which is not necessary for

catalysis (Caldo et al., 2017). This domain is comprised of two distinct segments, specifically an intrinsically disordered region (IDR) and a folded segment (Fig. 2A). The IDR can form interactions that are important for dimerization and may allow it to partially mediate positive cooperativity. Truncation of this IDR resulted in a more active enzyme form, suggesting the IDR encompasses an autoinhibitory motif. This observation agrees with a previous study on mouse DGAT1, wherein removal of N-terminal fragments led to increased normalized enzyme activity (McFie et al., 2010). The solution NMR structure of the folded segment of the N-terminal region of *B. napus* DGAT1 showed that it is composed of an α-helix near the first predicted TMD (Caldo et al., 2017). Loops and coils connected this helix to the IDR. The loop near the α-helix was shown to contain the allosteric site for acyl-CoA and CoA, which serves as homotropic activator and feedback inhibitor of the enzyme, respectively (Caldo et al., 2017). The small-angle X-ray scattering structure of this domain showed that the monomer has a highly extended structure, exhibiting various heterogeneous conformations. While the domain is predominantly disordered, it appears to exhibit a small gain in secondary structure upon binding to acyl-CoA and CoA. Altogether, these studies demonstrated that the hydrophilic N-terminal domain of *B. napus* DGAT1 comprises a regulatory domain that positively and negatively affects enzyme activity.

The remainder of DGAT1 accounting for more than 75% of the enzyme contains the TMD and the catalytic sites. The TMD is expected to form helical bundles in the membrane, which agrees with circular dichroism profile of purified BnaDGAT1 indicating the predominance of α-helices (Caldo et al., 2017). DGAT1 belongs to a family of enzymes named membrane-bound O-acyltransferases (MBOAT), which were proposed to have highly conserved arginine and histidine residues. Sequence analysis coupled with mutational studies showed a conserved histidine near the C-terminus of mouse DGAT1 represents one of the active site residues (Fig. 2A; McFie et al., 2010). In the same study, mouse DGAT1 was shown to have three TMD, with a cytosolic N-terminus and a C-terminus inside the ER lumen. In contrast, tung tree DGAT1 appeared to have two termini localized in the cytosol, suggesting the presence of even-numbered TMD (Shockey et al., 2006). Furthermore, it was noted that human DGAT1 may have dual topologies as DGAT1 activity was present on both faces of the ER (Wurie et al., 2011). In addition to topological analysis, the membrane-embedded region was also probed for possible substrate binding sites. Two peptide fragments corresponding to the putative binding

sites of bovine (*Bos taurus*) DGAT1 were investigated via synchrotron circular dichroism spectroscopy (Lopes et al., 2014). The first peptide spanning the motif, FYxDWWN, was shown to bind the acyl group of acyl-CoA (Fig. 2A). The second peptide having a candidate DAG binding site (HKWxxRHxYxP), which also exists in protein kinase C and diacylglycerol kinase, interacted with DAG.

DGAT2 is a member of the DGAT2/acyl-CoA:monoacylglycerol acyltransferase family, which also includes acyl-CoA:monoacylglycerol acyltransferases and wax synthases (McFie et al., 2010). It is completely different than DGAT1, being shorter in length and having less TMD (Fig. 2B). The membrane topologies of mouse and *S. cerevisiae* DGAT2 were experimentally determined. Mouse DGAT2 has two TMD with cytosolic N- and C- termini (Stone et al., 2006). On the other hand, *S. cerevisiae* DGAT2 has four TMD, and as in mouse DGAT2, both the N- and C-termini were localized in the cytosol (Liu et al., 2011). The topology of plant DGAT2 has not been determined yet, although preliminary analysis of tung tree DGAT2 showed that both termini are also found in the cytosol (Shockey et al., 2006). The first 30-50 amino acid residues of mouse and yeast DGAT2 were shown to be not essential for catalysis (Stone et al., 2006; Liu et al., 2011). No detailed structural analysis of DGAT2 has been reported although various motifs have been proposed to serve as important binding or active sites. In mouse DGAT2, an FLXLXXXn (n=non polar amino acid) motif is proposed as a binding site for neutral lipid, and substitution of either the first two residues in this motif resulted in decreased DGAT activity (Fig. 2B). Furthermore, the substitution of the second leucine residue in mouse DGAT2 resulted in enzyme inactivation (Stone et al., 2006). As for putative active site residues, a conserved HPHG motif has been implicated to play an important function, since substitution of any residue within this motif either led to lower activity or complete enzyme inactivation (Stone et al., 2006; Liu et al., 2011). It should be noted, however, that the topologies of mouse DGAT2 and *S. cerveisiae* DGAT2 (ScDGAT2) did not agree at certain regions. The HPHG motif, for example, is in the cytosol in mouse DGAT2 but is found within the membrane in ScDGAT2. Other conserved motifs with potentially important function identified in DGAT2 include motifs YFP, RXGFX(K/ R)XAXXXGXX(L/V)VPXXXFG(E/Q) and GGXXE (Liu et al., 2012). An ER retrieval motif responsible for the steady state localization of DGAT2 protein in the ER was identified near the C-terminus of tung tree DGAT2 (Shockey et al., 2006). Even though this ER

retrieval motif is also present in mouse DGAT2, deletion of this region in mouse DGAT2 did not affect the targeting of the mouse acyltransferase into the ER (McFie et al., 2011).

Regulation of DGAT

The activity of enzymes can be regulated at the transcriptional, translational and post-translational levels. Enzymes can differ in their temporal and spatial expression in plants. The expression profile of *DGAT* genes has been determined in different plant species particularly in oil crops. In Arabidopsis, *DGAT1* was expressed in different plant organs such as leaves, roots, flowers, siliques, seeds and seedlings, the last two of which exhibited the highest expression levels (Zou et al., 1999). The high expression of Arabidopsis *DGAT1* (*AtDGAT1*) in developing seeds and pollen correlates with the ability of these organs to accumulate high amounts of TAG (Lu et al., 2003). In addition, *DGAT1* was expressed at lower levels in shoots and roots of seedling, which are sites exhibiting active cell division and growth. *DGAT1* was suggested to be involved in maintaining a balance of DAG and acyl-CoA for the biosynthesis of membrane lipids and recycling of fatty acids to TAG under conditions where catabolic reactions are halted (Lu et al., 2003). Arabidopsis *DGAT2*, however, was expressed at a lower level in seeds compared to other tissues (Li et al., 2010b). Similar to Arabidopsis, the expression level of soybean *DGAT1* was much higher relative to *DGAT2* throughout seed development (Li et al., 2010b). In contrast, oil crops accumulating unusual fatty acids have higher *DGAT2* transcript levels in developing seeds than Arabidopsis and soybean, supporting the possible role of DGAT2 in the accumulation of unusual fatty acids in seed oil (Kroon et al., 2006; Shockey et al., 2006; Li et al., 2010b).

Transcription factors affecting the extent of oil accumulation have been identified including *LEAFY COTYLEDON* genes (*LEC1*, *LEC2*, *LIL* and *FUS3*), *ABSCICIC ACID INSENSITVE* (*ABI*) and *WRINKLED1* (*WRI*) (Santos-Mendoza et al., 2008). These transcription factors have been shown to be involved in the up-regulation of genes in late glycolysis and fatty acid biosynthesis. *ABI* and *WRI* were shown to have a direct effect on *DGAT1* expression (Fig. 3). *ABI4* was found to bind to the *AtDGAT1* promoter and activate transcription under stress conditions, such as nitrogen deficiency (Yang et al., 2011) and increased sucrose content (Wind et al., 2013). Similar to *ABI4*, *ABI5* was also shown to synergistically regulate the expression of *DGAT1* under stress (Kong et al., 2013). In addition, over-expression of *WRI* in *B. napus* increased the expression of *DGAT1* together with *GPAT9* and *LPAAT2*, although the specific

promoter sequence has yet to be identified (Li et al., 2015b). Very recently, the R2R3-type MYB96 transcription factor was shown to regulate TAG biosynthesis by directly activating the expression of *DGAT1* and *PDAT1* (Fig. 3) (Lee et al. 2018). *DGAT1* expression is regulated by MYB96 through binding to the promoter of *ABI4*, whereas MYB96 regulates *PDAT1* expression by directly binding to *PDAT1* promoter (Lee et al. 2018). Transcription factors directly influencing the expression of *DGAT2* in plants, however, have not been identified.

After translating a folded and functional enzyme, its activity can be regulated directly through the binding of allosteric effectors and/or post-translational modifications. DGAT1 has been shown to be modulated by its substrate, acyl-CoA, which binds to an allosteric site at the N-terminus (Weselake et al., 2006; Caldo et al., 2017). The presence of this allosteric site for acyl-CoA agrees with kinetic studies of microsomal and purified plant DGAT1 showing that DGAT1 exhibits positive cooperativity with acyl-CoA (Roesler et al., 2016; Caldo et al., 2017; Xu et al., 2017). Interestingly, CoA was identified as a feedback inhibitor of BnaDGAT1 and was shown to bind to the same allosteric site for acyl-CoA. It is thus possible that the hydrophilic N-terminal domain can act as a sensor of the acyl-CoA:CoA ratio, enabling enzyme activity to adjust to the availability of substrates (Fig. 4). Similarly, acyl-CoA and acyl-ACP were identified as feedback inhibitors of the acetyl-CoA carboxylase, the regulatory enzyme in fatty acid biosynthesis (Davis and Cronan, 2001; Andre et al., 2012). Furthermore, PtdOH was identified as a feedforward activator of plant DGAT1 (Caldo et al., 2018). PtdOH was suggested to aid in relieving possible autoinhibition by interacting with the N-terminal regulatory domain spanning the autoinhibitory motif and convert DGAT1 to a more active state that is also less sensitive to substrate inhibition (Fig. 4). The activity of microsomal DGAT activity has been shown to be activated by proteins such as acyl-CoA binding protein, bovine serum albumin, and human acylation-stimulating protein (Little et al., 1994; Hobbs and Hills, 2000; Weselake et al., 2000; Yurchenko et al., 2014), although specific plant regulatory proteins modulating DGAT1 has yet to be identified. Furthermore, microsomal DGAT activity in Arabidopsis was inhibited by niacin (Hobbs and Hills, 2000); this compound was later shown to non-competitively inhibit mouse DGAT2 (Ganji et al., 2004).

DGAT1 is also regulated through phosphorylation/dephosphorylation (Ghillebert et al., 2011). Initial sequence analysis indicated that mouse DGAT1 contains phosphorylation consensus sequences for tyrosine kinase, protein kinase A as well as protein kinase C (Yen et al.,

2008). Later reports, however, demonstrated that a number of these predicted phosphorylation sites had negligible effect on enzyme activity (Han, 2011; Humphrey et al., 2013). Furthermore, a recent study on mouse DGAT1 identified 24 potential phosphorylation sites and confirmed phosphorylation in several sites through mass spectrometry (Yu et al., 2015). Mutation of three serine phosphorylation sites (S83, S86 and S89) to glutamate to mimic phosphorylation also resulted in enzyme variants with higher activity. Previously, a putative sucrose non-fermenting1-related kinase1 (SnRK1) phosphorylation site was also identified in *T. majus* DGAT1 (Xu et al., 2008). When this residue was mutated to alanine, an increase in enzyme activity was observed. In addition, the over-expression of *DGAT1* with the mutated SnRK1 site translated to higher seed TAG levels in Arabidopsis when compared to an unmodified enzyme. Recent biochemical studies found that purified BnaDGAT1 can be phosphorylated and inactivated by SnRK1, further confirming earlier investigation through mutagenesis (Fig. 4; Caldo et al., 2018). In addition, SnRK1 has also been found to act on the *WRI* transcription factor (Zhai et al., 2017), which subsequently regulates *DGAT* expression. Similar observations were also noted in studies involving the mammalian AMP-activated protein kinase (AMPK), which corresponds to the SnRK1 enzyme in plants. Elevated AMPK activity reduced the expression levels of transcription factors and lipogenesis-related genes (eg., *DGAT1* and *DGAT2*), resulting in decreased TAG accumulation in mouse (Yin et al., 2015). It may be possible that mammalian AMPK can also modulate DGAT1 activity directly as observed in plant systems, since AMPK is a member of a kinase family implicated in transcriptional and post-translational regulation (Ghillebert et al., 2011). Overall, these lines of evidence showed that DGAT1 can be regulated by phosphorylation/dephosphorylation. As for DGAT2, there are no reports discussing the possible regulation of this isoenzyme by phosphorylation. The PhosPhat database indicated that there was no phosphorylated site identified yet in Arabidopsis DGAT2. On the other hand, human DGAT2 was shown to be regulated by ubiquitination via gp78, which is an E3 ligase facilitating ER-associated degradation (Choi et al., 2014).

Soluble DGAT, diacylglycerol acetyltransferases and other enzymes with DGAT activity

The *DGAT3* gene family encodes a soluble enzyme localized in the cytosol. The first *DGAT3* was isolated from peanut (*Arachis hypogea*) through protein purification (Saha et al., 2006). Somewhat later, *DGAT3* was also identified in Arabidopsis (Peng and Weselake, 2011;

Hernández et al., 2012). Another soluble enzyme with DGAT activity was also identified in Arabidopsis and termed Defective Cuticle Ridge (DCR) (Rani et al., 2010). The physiological roles of soluble DGAT in plants, however, remain largely to be explored. It has been reported that Arabidopsis DGAT3 appears to be involved in recycling of linoleic acid (18:2$\Delta^{9cis, 12cis}$) and α-linolenic acid (18:3$\Delta^{9cis, 12cis, 15cis}$) into TAG when TAG breakdown was blocked (Hernández et al., 2012), whereas DCR is likely to be related to the biosynthesis of cutin rather than seed oil (Rani et al., 2010). Unlike the puzzling roles of soluble DGAT in plants, several pieces of strong evidence support the involvement of DGAT3 in TAG biosynthesis in microalgae and diatoms. For instance, the involvement of DGAT3 to TAG biosynthesis in the diatom *Phaeodactylum tricornutum* was confirmed by heterologous expression in *S. cerevisiae* mutant H1246 (Cui et al., 2013). Similarly, a novel DGAT exclusive to green microalgae with moderate similarity to plant DGAT3 was found to participate in the chloroplastidial *de novo* synthesis of TAG (Bagnato et al., 2017). Moreover, many other TAG-biosynthetic biosynthetic enzymes, including GPAT, LPAAT, phosphatidic acid phosphatase and acyl-CoA:monoacylglycerol acyltransferase, have been previously reported to exist in soluble forms (Ichihara et al., 1990; Tumaney et al., 2001; Turnbull et al., 2001; Han et al., 2006; Ghosh et al., 2009). Thus, it is possible that these soluble TAG-biosynthetic enzymes might use different substrate pools and have different physiological roles from the membrane-bound isoforms.

In addition to the aforementioned DGATs, other enzymes with DGAT activity were also identified. One example is the bifunctional wax synthase/DGAT, which predominantly catalyzes the formation of wax esters. This enzyme was first identified in *Acinetobacter* (Kalscheuer et al., 2003) and later characterized in Arabidopsis (Li et al., 2008). Another enzyme with putative DGAT activity is chloroplastic phytyl ester synthase. Two Arabidopsis chloroplastic phytyl ester synthases were characterized and shown to be involved in fatty acid phytyl ester synthesis in chloroplasts (Lippold et al., 2012). A special DGAT (diacylglycerol acetyltransferase) utilizing acetyl-CoA rather than acyl-CoA as acyl donor was also identified in *E. alatus*. This unique DGAT catalyzes the formation of 3-acetyl-1, 2-diacyl-*sn*-glycerol rather than TAG, and the resulting acetyl-TAG has lower viscosity than normal oil (Durrett et al., 2010). The acetyl-CoA utilizing DGAT also belongs to the MBOAT family. Recently, the topology model of diacylglycerol acetyltransferase from *E. alatus* was experimentally determined (Tran et al., 2017). The model shows four TMD with both the N- and C-termini orientated toward the lumen

side of the ER (Fig. 2C). In addition, the MBOAT signature region containing the putative histidine active site is embedded in the third TMD in close proximity with the interface between the membrane and the cytosol, and thus may be readily accessible by the cytosolic acetyl-CoA substrate.

Acyl-CoA-independent formation of TAG

The Kennedy pathway provides a straightforward route towards the formation of TAG using acyl-CoA and G3P. As previously discussed, in many species, however, TAG assembly is intricately associated with membrane metabolism (Fig. 1). PDAT catalyzes the transfer of the acyl moiety at the *sn*-2 position of PtdCho or phosphatidylethanolamine to the *sn*-3 position of *sn*-1, 2-DAG, yielding TAG and *sn*-1 lyso-PtdCho or *sn*-1 lysophosphatidylethanolamine (Dahlqvist et al., 2000; Ståhl et al., 2004).

The identification of PDAT has historically lagged behind that of DGAT probably because of its exclusive presence in plants, algae, and yeast and its absence in mammals (for review, see Chen et al., 2015). In 2000, PDAT activity was first identified in microsomal preparations of the developing seeds from sunflower (*Helianthus annuus*), castor bean (*R. communis*), and *Crepis palaestina* by Stymne and co-workers (Dahlqvist et al., 2000). In the same study, they also isolated the first *PDAT* gene (YNR008w, *LRO1*) from yeast (*S. cerevisiae*). Yeast PDAT has homology with mammalian lecithin:cholesterol acyltransferase (LCAT), which catalyzes the acyl-CoA-independent formation of cholesteryl esters by transferring the acyl group from PtdCho to cholesterol. In addition, *S. cerevisiae* PDAT also displayed low DAG:DAG transacylase activity (Ghosal et al., 2007). In *S. cerevisiae* yeast, PDAT and DGAT2 are the major contributors to TAG biosynthesis and their relative contributions were dependent on the yeast growth stage (Oelkers et al., 2002). PDAT contributed predominantly to yeast TAG accumulation during the exponential growth stage, whereas DGAT2 was involved in the majority of yeast TAG biosynthesis at the stationary growth stage (Oelkers et al., 2002).

Subsequently, two *PDAT* orthologs, *AtPDAT1* (At5g13640) and *AtPDAT2* (At3g44830), with 57% amino acid sequence identify, were identified in Arabidopsis based on sequence homology to yeast *PDAT* (Ståhl et al., 2004). *AtPDAT1* is expressed generally at higher levels in vegetative tissues than in seeds, whereas *AtPDAT2* is highly expressed in seeds (Ståhl et al., 2004; Pan et al., 2015) (For detailed information on expression pattern, refer to AtGenExpress

548 database (http://jsp.weigelworld.org/expviz/expviz.jsp; accessed on 10 July 2018) or Arabidopsis
549 eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; accessed on 10 July 2018). In
550 contrast with the situation in yeast where PDAT is a major determinant of TAG biosynthesis at
551 the exponential growth stage, the contribution of PDAT to TAG biosynthesis in Arabidopsis
552 seeds was unclear. Over-expression of *AtPDAT1* resulted in no effects on the fatty acid and lipid
553 composition, despite the fact that increased PDAT activity was observed in microsomes prepared
554 from *AtPDAT1* Arabidopsis over-expressor lines (Ståhl et al., 2004). In addition, no change in
555 the fatty acid content or composition was observed from the seeds of an Arabidopsis knockout
556 line with a T-DNA insertion in the *AtPDAT1* locus (Mhaske et al., 2005).
557 The contribution of PDAT to Arabidopsis seed TAG accumulation was not realized until
558 *AtPDAT1* was suppressed by RNA interference (RNAi) in a *dgat1* knockout background (Zhang
559 et al., 2009). In a *dgat1* knockout background, RNAi silencing of *AtPDAT1* resulted in up to
560 63% further oil content reduction compared with the *dgat1* control, whereas neither silencing of
561 *AtPDAT2* nor *AtDGAT2* showed further reduction in oil content (Zhang et al., 2009). These
562 results suggested that PDAT1 is a dominant determinant in Arabidopsis seed TAG biosynthesis
563 in the absence of DGAT1 activity. Consistently, *AtPDAT1* expression was found to be highly up-
564 regulated in the seeds of the Arabidopsis *dgat1* mutant, whereas the expression of *AtPDAT2* and
565 *AtDGAT2* was only marginally affected (Xu et al., 2012). Furthermore, the Arabidopsis *pdat1*
566 *dgat1* double mutant displayed abnormal seed and pollen development (Zhang et al., 2009),
567 indicating that PDAT1 and DGAT1 have overlapping functions in TAG biosynthesis in
568 developing seeds and pollen, which are essential for their normal development.
569 Recently, PDAT has been suggested to play a crucial role in mediating TAG biosynthesis
570 in leaves. PDAT functions in diverting fatty acids from membrane lipids to TAG before
571 peroxisomal β-oxidation, thereby maintaining leaf membrane homeostasis in Arabidopsis (Fan et
572 al., 2013a; Fan et al., 2013b; Fan et al., 2014). The relative contribution of PDAT1 and DGAT1
573 to leaf TAG biosynthesis has been examined. Arabidopsis *pdat1* mutant displayed a 57%
574 reduction in TAG content in developing leaves whereas only 31% decrease in TAG level was
575 observed in the *dgat1* mutant (Fan et al., 2013b). In contrast to the 7-fold increase in *PDAT1*
576 over-expressing lines, there was only a marginal increase in leaf TAG levels in *DGAT1* over-
577 expressing Arabidopsis lines (Fan et al., 2013b). These results suggested that PDAT1 may play a
578 more important role in TAG synthesis in young leaves than DGAT1. Pulse-chase radiolabelling

of *pdat1* and *dgat1* mutants using [^{14}C] 12:0, however, showed that the reduction in TAG accumulation was more severe in the *dgat1* mutant rather than the *pdat1* mutant, suggesting that DGAT1 is mainly responsible for the TAG biosynthesis in Arabidopsis leaves (Tjellström et al., 2015). Considering medium chain fatty acids such as 12:0 are rarely incorporated into the *sn*-2 of PtdCho, which is the substrate of PDAT, it is likely that the relative use of PDAT and DGAT in TAG biosynthesis in leaves is dependent on the substrates and acyl flux conditions within the cell (Bates, 2016). Besides Arabidopsis, *PDAT* genes have also been identified and characterized in various plant and microalgal species, including castor (van Erp et al., 2011; Kim et al., 2011), flax (Pan et al., 2013), *Camelina sativa* (Aznar-Moreno and Durrett, 2017; Yuan et al., 2017), green algae *C. reinhardtii* (Yoon et al., 2012) and green algae *Myrmecia incise* (Liu et al., 2016b). It should be noted that PDAT nomenclature in literature lacks consistency. For example, flax PDAT 2 and 4 are homologs of AtPDAT1 whereas flax PDAT 3 and 6 are AtPDAT2 homologs (Pan et al., 2015).

Although a T-DNA insertion in the *AtPDAT1* locus led to no effect on the fatty acid content or composition in Arabidopsis (Mhaske et al., 2005), Aznar-Moreno and Durrett (2017) introduced mutations in genes encoding PDAT1 in *C. sativa* (an AtPDAT1 homolog) using the CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein) system and observed reduced seed oil content and altered fatty acid composition (eg., decreased linoleic acid content) in many transgenic lines, supporting the contribution of PDAT1 in seed oil biosynthesis. Consistently, microRNA mediated down-regulation of *PDAT1* in *C. sativa* led to a decrease in linoleic acid content, whereas over-expression of *PDAT1* had an opposite effect (Marmon et al., 2017). The changes in fatty acid composition in these *PDAT1* over-expression or down-regulation lines, however, were not accompanied with any significant effects on total oil content. In the same study, the relative importance of *C. sativa* PDAT and DGAT in oil accumulation in developing seeds and in different embryo sections was examined. DGAT1 appears to dominate the TAG assembly in cotyledons, while PDAT1 may compensate for TAG accumulation in the absence of DGAT1 by specifically routing linoleic acid from PtdCho into TAG. Unlike PDAT1, PDAT2 homologs from Arabidopsis (AtPDAT2), flax (LuPDAT3 and LuPDAT6) and castor bean (RcPDAT2) did not show an apparent function in TAG biosynthesis (Ståhl et al., 2004; van Erp et al., 2011; Kim et al., 2011; Pan et al., 2013).

In some plants, PDAT is likely to be a key player in directing modified fatty acids from PtdCho into TAG by displaying unique specificity for the acyl group in PtdCho (van Erp et al., 2011; Kim et al., 2011; Pan et al., 2013). PDAT from castor has been shown to preferentially catalyze the incorporation of ricinoleoyl and vernoloyl groups into TAG *in vitro*, while *C. palaestina* PDAT catalyzes the incorporation of vernoloyl groups (Dahlqvist et al., 2000). Consistent with the *in vitro* results, over-expression of castor *PDAT* in Arabidopsis led to large accumulation of hydroxy fatty acids in the seed oil (van Erp et al., 2011; Kim et al., 2011). Similarly, specialized PDAT for selective incorporating α-linolenic acid into TAG was also identified in flax (Pan et al., 2013). These PDATs with unique substrate selectivity, including castor PDAT (RcPDAT1A) and flax PDATs (LuPDAT1 and LuPDAT5), have seed-specific expression pattern and are grouped into a single clade that is different from those of PDAT1 and PDAT2 (Pan et al., 2015).

In oilseed crops accumulating high oleic acid such as *B. napus*, PDAT may be less important than DGAT in affecting seed oil accumulation, since neither the *in vitro* enzyme activity nor the transcripts abundance for PDAT or its encoding gene was higher than that of DGAT (Troncoso-Ponce et al., 2011; Tang et al., 2012). This is also supported by a recent detailed lipidomic analysis of developing *B. napus* seeds, in which the relative contributions of DGAT and PDAT were predicted based on the patterns of their molecular substrates (Woodfield et al., 2018). Furthermore, it has also been recently suggested that PDAT appears to function in stress responses in Arabidopsis (Mueller et al., 2017), *C. sativa* (Yuan et al., 2017) and green algae (Yoon et al., 2012; Liu et al., 2016b). For instance, PDAT1-mediated TAG accumulation was found to increase the heat resistance of Arabidopsis seedlings (Mueller et al., 2017).

Structure-function studies on PDAT have only been limited to *in silico* analysis. Phylogenetic analysis showed that plant PDAT can be grouped into four clades, two of which have one putative TMD while the other two are predicted to be entirely soluble (Pan et al., 2015). The majority of PDAT in the database have the single predicted TMD consisting of a small cytosolic N-terminus and a large C-terminal domain in the ER lumen. The N-terminal region is hydrophilic with arginine clusters similar to those observed in DGAT1 (Liu et al., 2012). The removal of the putative N-terminal TMD in *S. cerevisiae* PDAT did not affect activity (Ghosal et al., 2007). Similar to LCAT-like proteins, PDAT has the conserved features of human LCAT (Peelman et al., 1999). Sequence alignment of PDAT enzymes identified the highly conserved

640 residues constituting a catalytic triad, specifically a histidine residue and an aspartate residue
641 near the C-terminus and an internal serine residue. Other conserved features include a salt bridge
642 between an aspartate residue and an arginine residue and a lid region with a tryptophan residue,
643 which is proposed to bind released fatty acid for efficient acylation. PDAT exhibits homology to
644 human LCAT (26% identity) and phospholipase A_2 (27% identity), the structure of which were
645 recently elucidated (Glukhova et al., 2015; Piper et al., 2015). Using the phospholipase A_2
646 structure, the AtPDAT1 model was determined using PHYRE software with high confidence
647 level (Fig. 2D; Kelley et al., 2015), giving a preliminary glance at the possible orientation of the
648 aforementioned functional motifs. In the model, the components of the catalytic triad are located
649 close to one another, possibly forming contacts through hydrogen bonding. The lid region is
650 composed of loops and a β-sheet on one face of the structure and the salt bridge is in close
651 proximity interacting through electrostatic interaction. About 53% of the polypeptide was
652 modelled using the phospholipase A_2 structure. PDAT1 has a long N-terminal sequence that has
653 been predicted to encompass a TMD that is not included in the model (Fig. 2D).
654 In contrast to DGAT-catalyzed reaction, the equilibrium of which lies far to the right
655 because of the cleavage of a high energy thioester bond of acyl-CoA and the formation of a
656 carbon-oxygen (ester) bond, PDAT-catalyzed TAG formation uses PtdCho as the acyl donor and
657 is considered thermodynamically neutral. Although the reaction mechanisms of PDAT are still a
658 mystery, some insights might be gained from the work on mammalian LCAT, which shares some
659 homology with PDAT from yeast and plants (Dahlqvist et al., 2000; Ståhl et al., 2004; Pan et al.,
660 2013). The recently elucidated structures of human LCAT revealed that the catalytic mechanism
661 of LCAT is similar to that of phospholipase A_2, in which the lid-loop can move aside from a
662 tunnel opening allowing lipids to enter the active site and interact with the catalytic triad (serine
663 residue 181, aspartate residue 345, and histidine residue 377) (Glukhova et al., 2015; Piper et al.,
664 2015). During catalysis, serine residue 181 of the catalytic triad functions as a nucleophile
665 residue by attacking the *sn*-2 acyl-chain of PtdCho and, subsequently, the cleaved acyl-chain is
666 covalently bound to the serine residue to form an acyl-enzyme intermediate before transfer to
667 cholesterol to generate a cholesteryl ester. Unlike the acyl-CoA-dependent acyltransferases
668 requiring an activated acyl-donor, LCAT uses itself as the donor in acyl transfer and thus it is
669 very likely that the acyl-enzyme intermediate has a high chemical potential (Segrest et al., 2015).
670 In addition, recent molecular simulation results suggested that the acyl-LCAT intermediate can

671 facilitate the entry of cholesterol to the active site by decreasing the free-energy cost (Casteleijn
672 et al., 2018).
673
674 **Biotechnological applications of plant DGAT and PDAT**
675 A rising demand for vegetable oil has been witnessed across the globe due to a ballooning
676 human population and increased consumption of plant oil for food, feed, biofuel and industrial
677 applications (for reviews, see Durrett et al., 2008; Weselake et al., 2009). As a consequence,
678 various biotechnological strategies have been used to increase the flow of carbon into TAG in
679 oilseeds, vegetative tissues and microalgae. The importance of DGAT in governing the flux of
680 substrate into TAG was first reported by Katavic and colleagues (1995), wherein an Arabidopsis
681 ethyl methanesulfonate (EMS) mutant with inactivated *DGAT1* exhibited low TAG levels.
682 PDAT is another dominant determinant in plant seed TAG biosynthesis especially in the absence
683 of DGAT1 activity (Zhang et al., 2009). In some plants, PDAT is also characterized as a key
684 player in contributing to directing modified fatty acids from PtdCho into TAG (Dahlqvist et al.,
685 2000). Thus far, *DGAT* and *PDAT cDNAs* have been extensively used as biotechnological tools
686 in many studies aimed at increasing oil content and modifying the fatty acid composition of oils
687 in seeds of higher plants, vegetative tissues and microalgae (Table 1).
688
689 ***Increasing seed oil content***
690 Jako and colleagues (2001) initially demonstrated that the over-expression of native
691 *DGAT1* in Arabidopsis resulted in increased oil accumulation and seed weight. Increased TAG
692 content was also obtained upon over-expression of Arabidopsis and native *DGAT1* in canola-
693 type *B. napus* under either greenhouse or field conditions (Weselake et al., 2008; Taylor et al.,
694 2009). It was further noted that *DGAT1* over-expression improved *B. napus* oil accumulation
695 when compromised under drought condition (Weselake et al., 2008). The over-expression of *T.*
696 *majus DGAT1* in Arabidopsis, high-erucic-acid rapeseed and canola-type *B. napus* also led to
697 enhanced seed oil content (Xu et al., 2008). Since these aforementioned studies, many groups
698 have further used DGAT1 from different species to boost seed content in various crops such as
699 *G. max* (Hatanaka et al., 2016; Roesler et al., 2016), *B. juncea* (Savadi et al., 2015), *Z. mays*
700 (Alameldin et al., 2017), *C. sativa* (Kim et al., 2016) and *Jatropa curcas* (Maravi et al., 2016).
701 Moreover, over-expression of *DGAT1* from microalgae, such as *Chlorella ellipsoidea* and

Nannochloropsis oceanica, also led to increased oil content in Arabidopsis and *B. napus* (Guo et al., 2017; Zienkiewicz et al., 2017). Furthermore, DGAT1 has been used to increase the proportion of unusual fatty acids in seed oil, particularly epoxy fatty acid in *G. max* (co-expressed with an *EPOXYGENASE* gene; Li et al., 2010a) and capric acid in *C. sativa* (in combination with fatty acyl-ACP thioesterase B1 and LPAAT from *Cuphea viscosissima*; Iskandarov et al., 2017). Similar to *DGAT1*, seed-specific over-expression of fungal *DGAT2* resulted in enhanced seed oil content in *G. max* (Lardizabal et al., 2008) and *Z. mays* (Oakes et al., 2011). DGAT2 was also used to increase the proportion of unusual fatty acids in seeds specifically accumulating hydroxy fatty acid in Arabidopsis through co-expression with a cDNA encoding fatty acid hydroxylase 12 (FAH12; Burgal et al., 2008) and epoxy fatty acids in *G. max* through co-expression with a cDNA encoding an epoxygenase (Li et al., 2010a). Over-expression of *DGAT2* alone in Arabidopsis was also shown to increase the percentage of oleic acid in Arabidopsis seed TAG (Zhang et al., 2013; Wang et al., 2016). PDAT has also successfully been applied in engineering transgenic plants with high levels of unusual fatty acids and polyunsaturated fatty acids, such as hydroxy fatty acid and α-linoleic acid, respectively. Over-expression of flax *PDAT* in Arabidopsis led to an increase in the accumulation of α-linoleic acid in its seed oil (Pan et al., 2013). Similarly, co-expression of castor *FAH12* and *PDAT1* in Arabidopsis led to an increase in the accumulation of total hydroxy fatty acid up to 25% (van Erp et al., 2011; Kim et al., 2011). Over-expression of castor *DGAT2* in the above co-expression line increased the hydroxy fatty acid content further to 26.7% (van Erp et al., 2011). As previously suggested by Vanhercke et al. (2013b), the competition between endogenous and transgenically introduced lipid biosynthetic machinery would limit the full potential of the metabolic engineering intervention. In order to reduce the endogenous competition from Arabidopsis *DGAT1*, van Erp et al. (2015) introduced a mutation in *AtDGAT1* in the line expressing castor *FAH12*, *DGAT2* and *PDAT* and the hydroxy fatty acid content was further increased to an average of 31.4%.

Increasing the oil content of vegetative tissue

In addition to increasing seed oil content, it was previously shown that over-expression of *DGAT1* could also boost the oil content of tobacco leaves (Bouvier-Navé et al., 2000). This concept of increasing TAG in vegetative tissues has recently gained traction among researchers

as a viable alternative to meet the ever-growing demand for plant oil. Tobacco (*N. tabacum* and *N. benthamiana*) has served as the most common platform for producing oil in vegetative tissues given its ability to produce high biomass. DGAT1 has been used to boost oil in leaf or/and stem of tobacco through over-expression of *DGAT1* alone (Andrianov et al., 2010; Wu et al., 2013) or in combination with one or more cDNAs encoding proteins/enzymes such as acyl-CoA:monoacylglycerol acyltransferase, WRI, oleosin, cysteine-oleosin and thioesterase (Petrie et al., 2012; Kelly et al., 2013; Vanhercke et al., 2013a; Winichayakul et al., 2013; Vanhercke et al., 2014; Chen et al., 2017; El Tahchy et al., 2017). The latter multi-gene strategies have proven to be more effective in green tissues for enhancing the carbon flux into TAG at multiple metabolic levels, including upregulation of fatty acid biosynthesis ('Push'; e.g., over-expression of *WRI*), enhancing TAG assembly ('Pull'; e.g., over-expression of *DGAT1* and introduction of mouse acyl-CoA:monoacylglycerol acyltransferase) and preventing lipid turnover ('Protect'; e.g., over-expression of *oleosin*) (Vanhercke et al., 2014). The combined over-expression of cDNAs encoding DGAT1, WRI and oleosin, respectively, is a very effective approach for increasing leaf TAG, driving oil accumulation to more than 15% dry weight in *N. tabacum* (Vanhercke et al., 2014). Increased incorporation of medium-chain fatty acids into TAG has also been obtained in tobacco leaves through over-expression of *DGAT1* together with other genes that can increase the flux of medium chain fatty acids in the pathway (Reynolds et al., 2015; Reynolds et al., 2017). Increased oil content in vegetative tissues was also obtained in Arabidopsis (Kelly et al., 2013; Winichayakul et al., 2013), *Saccharum* spp. hybrids (Zale et al., 2016) and *J. curcas* (Maravi et al., 2016) through over-expression of *DGAT1* alone or in combination with other genes. As for DGAT2, over-expression of a *C. reinhardtii DGAT2* in Arabidopsis boosted oil content in leaves (Sanjaya et al., 2013). As mentioned earlier, PDAT1 appears to play a more important role in TAG synthesis in young leaves than DGAT1 (Fan et al., 2013b). The combined over-expression of *PDAT1* and *OLEOSIN* increased leaf TAG accumulation by up to 6.4% and 8.6% of the dry weight in Arabidopsis and the Arabidopsis *trigalactosyldiacylglycerol1-1 mutant*, respectively (Fan et al., 2013b).

Increasing the oil content of microalgae

Microalgae are considered to be one of the most potentially viable sources of storage lipid (TAG) for biofuel production and a future solution to the renewable energy challenge (Hu

et al., 2008; Wijffels and Barbosa, 2010). Whereas mostly green algae and eustigmatophytes are regarded as the best suitable oil producers, oil-rich diatoms such as *P. tricornutum* and several red algal species such as *Porphyridium purpureum* and *Cyanidioschyzon merolae* are also getting considerable research attention (Hu et al., 2008; Liu et al., 2017; Sato et al., 2017; Shuba and Kifle, 2018). Microalgal DGAT and PDAT are potential targets to engineer improved oil-rich biomass accumulation (Goncalves et al., 2016). For instance, genetic engineering of *C. reinhardtii* by expressing a truncated DGAT1 cDNA from *B. napus*, led to increases in the contents of lipids and polyunsaturated fatty acids (Ahmad et al., 2015). Heterologous expression of *DGAT1* from *E. pitardii*, and *DGAT2* from *S. cerevisiae* also resulted in higher TAG accumulation in the marine microalga *Tetraselmis chui* (Úbeda-Mínguez et al., 2017). In *C. reinhardtii*, however, contrary findings were obtained from *CrDGAT2* over-expressors. Deng et al. (2012) reported an increase in lipid content with over-expression of *CrDGAT2-1* or *CrDGAT2-5* in *C. reinhardtii*, whereas La Russa et al. (2012) reported that over-expression of *CrDGAT2* did not increase the lipid content of *C. reinhardtii*. Despite the contrary results in *C. reinhardtii*, over-expression of *DGAT2* in *N. oceanica* increased TAG content up to 2-fold without affecting growth (Li et al., 2016). More recently, increased TAG production with altered fatty acid composition was also observed in the oleaginous microalga *Neochloris oleoabundans* by the over-expression of *DGAT2* (Klaitong et al., 2017). In the diatom *P. tricornutum*, over-expression of *DGAT2* resulted in eicosapentaenoic acid (20:5$\Delta^{5cis, 8cis,11cis, 14cis, 17cis}$)-rich oil and increased neutral lipid accumulation while sustaining similar growth rate in the transgenic microalgae (Niu et al., 2013). Recently, Zulu et al. (2017) used heterologous co-expression of yeast *DGAT2* and a plant *OLEOSIN* as an efficient intervention for enhancing TAG accumulation in *P. tricornutum*. Additionally, it has also been shown that DGAT plays a major role in controlling the photosynthetic carbon flux towards TAG in this diatom (Dinamarca et al., 2017). Interestingly, Xin et al. (2017) investigated the acyl-CoA specificity of different isoforms of DGAT2 in the eustigmatophyte *N. oceanica* (NoDGAT2) for fatty acids with different unsaturation levels. NoDGAT2A preferred substrates with saturated fatty acids, NoDGAT2D preferred substrates with monounsaturated fatty acids, whereas NoDGAT2C exhibited its higher activity toward substrates with polyunsaturated fatty acids. Microalgal transgenic lines were generated with specific saturated fatty acid: monounsaturated fatty acid: polyunsaturated fatty acid proportions in TAG by modulating the ratio of *NoDGAT2A*:*2C*:*2D* transcripts. The authors

have further established a novel strategy to simultaneously improve productivity and quality of oils from microalgae for industrial use.

Increasing DGAT1 activity through protein engineering

Due to the importance of DGAT1 as a molecular tool for increasing oil accumulation, there has been an interest in further increasing the activity of the enzyme through protein engineering. Previous efforts to engineer DGAT enzyme performance, however, have largely relied on the identification of natural variation sites and sequence-based site-directed mutagenesis. For instance, a phenylalanine insertion in maize DGAT1 at position 469, which naturally occurs in a high-oil maize line but not a normal-oil maize line, is responsible for the increased DGAT activity and oil content (Zheng et al., 2008). Similarly, analysis of the protein sequences of eight closely related peanut DGAT2 from a collection of peanut varieties revealed that two out of the six identified amino acid residue substitutions led to increased enzyme activity in DGAT2 variants (Zheng et al., 2017a). Moreover, a previous study on the substitution of a serine residue with an alanine residue in a candidate SnRK1 consensus site in *T. majus* DGAT1 resulted in a variant with higher activity (Xu et al., 2008). Over-expression of the *T. majus DGAT1* variant in Arabidopsis seeds led to higher seed oil content than what could be achieved with over-expression of the cDNA encoding the wild-type enzyme. The potential of protein engineering to boost DGAT1 activity was also demonstrated in mouse DGAT1 wherein the mutation of three serine residues to glutamate residues individually in the N-terminal region also resulted in enzyme variants with increased activity (Yu et al., 2015). The aforementioned switch to glutamates residues was argued to mimic the addition of negatively charged phosphate groups, which was hypothesized to be a stimulatory signal in mouse DGAT1. Moreover, production of a recombinant BnaDGAT1 with an N-terminal poly-His tag in *S. cerevisiae* also resulted in elevated oil accumulation. The added N-terminal tag was shown to minimize the deleterious effect of the N-terminal domain and was able to increase the level of polypeptide production (Greer et al., 2015).

In contrast to the limited target sites identified from natural variation and sequence-based prediction, directed evolution provides a powerful approach for DGAT engineering, especially in the absence of structural information. Pioneering work on this involved the development of a yeast H1246-based high-throughput system for selection of high performance enzyme variants

826 (Siloto et al., 2009a). Many improved BnaDGAT1 variants were generated using the
827 aforementioned method and the two most promising ones were used to increase the oil content of
828 tobacco leaves (Chen et al., 2017). Kinetic analysis indicated that one of the BnaDGAT1 variants
829 exhibited apparent decreased substrate inhibition at concentrations of acyl-CoA beyond 5 μM
830 (Xu et al., 2017). The possible role of the ninth and tenth predicted TMD in enzyme regulation
831 was also identified as a considerable number of beneficial mutations were localized near and
832 within this region (Chen et al., 2017). A similar yeast-based high-throughput system also
833 identified *Corylus americana* and *G. max* DGAT1 variants with improved kinetic properties
834 (Roesler et al., 2016). The over-expression of a cDNA encoding a DGAT1 variant with 14
835 substitutions in soybean resulted in larger increases in seed TAG when compared to seeds
836 resulting from over-expression of the wild-type enzyme. Furthermore, a truncated BnaDGAT1
837 was found to increase TAG accumulation in green microalga (Ahmad et al., 2015).
838
839 **Closing comments**
840 Ever since the isolation of the first plant DGAT in Arabidopsis (Hobbs et al., 1999;
841 Routaboul et al., 1999; Zou et al., 1999; Bouvier-Navé et al., 2000) and the discovery of PDAT
842 activity in plant species (Dahlqvist et al., 2000), our understanding in the terminal steps of plant
843 TAG biosynthesis has grown tremendously. Some of the knowledge has been successfully
844 applied in metabolic engineering of oilseed crops to increase seed oil content and modify the
845 fatty acid composition of seed oil. Meanwhile, the growing interest of using vegetative tissues
846 and microalgae as platforms for industrial oil production has brought them into the spotlight,
847 achieving substantial progress by taking the advantage of the successful applications in oilseed
848 biotechnology. The physiological roles of DGAT and PDAT in regulating plant TAG
849 accumulation underline the potential applicability of over-expression of *DGAT* or *PDAT* in
850 transgenic plants and microalgae for increasing oil content, modifying oil quality and improving
851 plant stress tolerance. It should be noted that the relative contributions of DGAT and PDAT to
852 seed TAG accumulation may vary among species (Ramli et al., 2005; Troncoso-Ponce et al.,
853 2011; Tang et al., 2012; Woodfield et al., 2018), and it is therefore important to choose suitable
854 strategies based on individual plants in manipulating oil production. Thus far, our greatest
855 progress has been in probing the properties and regulation of DGAT1 and exploring the
856 biotechnological uses of DGAT1. In addition to over-expression of cDNA encoding the wild-

type DGAT1, there has been recent successes in the manipulation of oil production using high performance enzyme variants generated via directed evolution (Roesler et al., 2016; Chen et al., 2017). Recent advances in genome editing techniques, such as, CRISPR (Belhaj et al., 2013) and targeting-induced local lesions in genomes (TILLING; Till et al., 2006), open new perspectives on improving enzyme action *in planta*. For instance, the "super DGAT" variants with single amino acid residue substitutions generated in *B. napus* DGAT1 provide valuable candidates for genome editing of *DGAT1* in different species using CRISPR and TILLING (Chen et al., 2017; Xu et al., 2017). The knowledge obtained from directed evolution, in turn, can provide novel and valuable insights into structure-function relationships of DGAT1 (Chen et al., 2017), especially since no detailed three-dimensional structure is available for the entire DGAT1 enzyme. Recently, the structure of the hydrophilic N-terminal domain of BnaDGAT1 was solved and its self-regulatory function was revealed (Caldo et al., 2017). Since DGAT1 from *B. napus* has been successfully purified in an active form (Caldo et al., 2015), first steps are made towards obtaining high-resolution structures. The eventual structural elucidation of DGAT and PDAT will uncover the molecular mechanisms of catalysis and provide for detailed insights into modes of enzyme regulation thus establishing a basis for rational design of acyltransferases for manipulation of oil production.

Conflict of interest

The authors declare that they have no conflicts of interest with the content of this article.

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883 **Table 1.** Metabolic engineering interventions targeting DGAT1, DGAT2 or PDAT to
884 increase/modify oil/triacylglycerol content in higher plants and microalgae.

Gene source	Transgenic crop/organism	In combination with other genes and/or modifications	Observed trait(s)	References
Over-expression of *DGAT1* to increase seed oil content and/or modify oil composition				
Arabidopsis	Arabidopsis		Higher seed oil content and seed weight	(Jako et al., 2001)
Brassica napus, Arabidopsis	*Brassica napus*		Higher seed oil content	(Weselake et al., 2008; Taylor et al., 2009)
Tropaeolum majus	Arabidopsis, *Brassica napus*	Mutated SnRK1 site in DGAT1 (Ser 197 to Ala)	Higher seed oil content	(Xu et al., 2008)
Vernonia galamensis	*Glycine max*	*Stokesia laevis* epoxygenase	Higher epoxy fatty acids	(Li et al., 2012)
Arabidopsis	*Brassica juncea*		Higher seed oil content and seed weight	(Savadi et al., 2015)
Zea mays	*Zea mays*		Higher seed oil content	(Lan et al., 2015)
Corylus americana, *Glycine max*	*Glycine max*, *Saccharomyces cerevisiae*	Engineered DGAT1 variants	Higher seed oil content, lower soluble carbohydrate, higher yeast oil content	(Roesler et al., 2016)
Sapium sebiferum	*Brassica napus*		Higher seed oil content, lower oleic acid and higher linoleic acid	(Peng et al., 2016)
Camelina sativa	*Camelina sativa*		Higher seed oil content	(Kim et al., 2016)
Vernoni galamensis	*Glycine max*		Higher seed oil content	(Hatanaka et al., 2016)
Arabidopsis	*Jatropha curcas*		Higher seed and leaf oil content, higher seed weight	(Maravi et al., 2016)
Sesamum indicum	Arabidopsis, *Glycine max*		Higher seed oil content and seed weight	(Wang et al., 2014)
Cuphea avigera var. pulcherrima	*Camelina sativa*	*FatB1* and *LPAAT* from *Cuphea viscosissima*	Higher capric acid (10:0) in seed oil	(Iskandarov et al., 2017)
Chlorella ellipsoidea	Arabidopsis, *Brassica napus* var. Westar, *Saccharomyces cerevisiae*		Higher seed oil content and seed weight, higher yeast oil content	(Guo et al., 2017)

Over-expression of *DGAT1* to increase leaf/microalgae oil content and/or modify oil composition				
Arabidopsis	*Nicotiana tabacum*		Higher leaf oil content	(Bouvier-Navé et al., 2000)
Arabidopsis	*Nicotiana tabacum*	Arabidopsis *LEC2*	Higher leaf oil content	(Andrianov et al., 2010)
Arabidopsis	*Nicotiana benthamiana*	*Mus musculus MGAT2*	Higher leaf oil content	(Petrie et al., 2012)
Arabidopsis	Arabidopsis, *Saccharomyces cerevisiae*	Cysteine-oleosin (engineered *Sesamum indicum* oleosin containing up to 13 cysteines)	Higher leaf and root oil content; Higher yeast oil content	(Winichayakul et al., 2013)
Arabidopsis	*Nicotiana tabacum*		Higher leaf oil content	(Wu et al., 2013)
Arabidopsis	Arabidopsis *sdp1* (lipase) mutant	Arabidopsis *WRI*	Higher root, stem and leaf oil content	(Kelly et al., 2013)
Arabidopsis	*Nicotiana benthamiana*	Arabidopsis *WRI*	Higher leaf oil content	(Vanhercke et al., 2013a)
Arabidopsis	*Nicotiana tabacum*	Arabidopsis *WRI*, *Sesamum indicum* L oleosin	Higher leaf oil content	(Vanhercke et al., 2014)
Arabidopsis	*Nicotiana benthamiana*	Medium-chain *FATs*, Arabidopsis *WRI*, *Cocos nucifera LPAAT*	Higher medium-chain fatty acid content and Higher leaf oil content	(Reynolds et al., 2015)
Arabidopsis	*Jatropha curcas*		Higher seed and leaf oil content, higher seed weight	(Maravi et al., 2016)
Zea mays	*Saccharum spp.* hybrids	Codon optimized *Z. mays* DGAT1, Arabidopsis *WRI* and Arabidopsis *oleosin* for expression in *Saccharum spp.* hybrids; RNAi mediated down-regulation of ADP-glucose pyrophosphorylase and peroxisomal ABC transporter1	Higher leaf and stem oil content	(Zale et al., 2016)
Arabidopsis	*Zea mays*	*WRI* and *oleosin* from Arabidopsis	Higher leaf TAG content	(Alameldin et al., 2017)
Arabidopsis	*Solanum tuberosum L*	Arabidopsis *WRI*, *Sesamum indicum* L *oleosin*	Higher tuber oil content	(Liu et al., 2016a)

Elaeis guineensis	*Nicotiana benthamiana*	Medium-chain *FAT*s, *GPAT9* and *LPAAT from Cocos nucifera*	Higher medium chain fatty acids in leaf oil and Higher leaf oil content	(Reynolds et al., 2017)
Echium pitardii	*Tetraselmis chui*		Higher oil content in microalgae	(Úbeda-Mínguez et al., 2017)
Arabidopsis	*Nicotiana benthamiana*	Arabidopsis *WRI*, *oleosin*, and *FAT*s	Higher leaf oil content	(El Tahchy et al., 2017)
Brassica napus	*Saccharomyces cerevisiae* *Nicotiana benthamiana*	Single-site variants	Higher leaf oil content and yeast oil content	(Chen et al., 2017)
Over-expression of *DGAT2* to increase seed/leaf/microalgae oil content and/or modify oil composition				
Umbelopsis ramanniana	*Glycine max*		Higher seed oil content	(Lardizabal et al., 2008)
Ricinus communis	Arabidopsis	*FAH12*	Higher hydroxy fatty acids	(Burgal et al., 2008)
Umbelopsis ramanniana	*Zea mays*		Higher seed oil content	(Oakes et al., 2011)
Vernonia galamensis	*Glycine max*	*Stokesia laevis* epoxygenase	Higher epoxy fatty acids	(Li et al., 2012)
Chlamydomonas reinhardtii	*Chlamydomonas reinhardtii*		Higher oil content in microalgae	(Deng et al., 2012)
Thraustochytrium aureum	Arabidopsis *fad3fae1* mutant		Higher oleic acid in seeds	(Zhang et al., 2013)
Chlamydomonas reinhardtii	Arabidopsis		Higher leaf oil content	(Sanjaya et al., 2013)
Phaeodactylum tricornutum	*Phaeodactylum tricornutum*		Higher oil content in microalgae with higher polyunsaturated fatty acid	(Niu et al., 2013)
Brassica napus	*Chlamydomonas reinhardtii*		Higher oil content in microalgae, lower saturated fatty acids content, higher α-linolenic acid content,	(Ahmad et al., 2015)
Sapium sebiferim	Arabidopsis		Higher oleic acid content in seeds	(Wang et al., 2016)
Nannochloropsis oceanica	*Nannochloropsis oceanica*		Higher oil content in microalgae	(Li et al., 2016)
Neochloris oleoabundans	*Neochloris oleoabundans*		Higher oil content in microalgae with altered fatty acid composition	(Klaitong et al., 2017)
Saccharomyces cerevisiae	*Phaeodactylum tricornutum*	Arabidopsis *oleosin*	Higher oil content in microalgae	(Zulu et al., 2017)

Saccharomyces cerevisiae	*Tetraselmis chui*		Higher oil content in microalgae	(Úbeda-Mínguez et al., 2017)
Nannochloropsis oceanica	*Nannochloropsis oceanica*	Modulate the ratio of *DGAT2A*:*2C*:*2D* transcripts by over-expression and knockdown	Modified fatty acid composition in microalgae	(Xin et al., 2017)
Nannochloropsis oceanica (CCMP 1779)	Arabidopsis, *Saccharomyces cerevisiae*		Higher seed and leaf oil content, higher yeast oil content	(Zienkiewicz et al., 2017)
Over-expression of *PDAT* to modify seed/leaf oil content and/or modify oil composition				
Ricinus communis	Arabidopsis	*Ricinus communis FAH12*	Higher hydroxy fatty acid in seed oil	(Kim et al., 2011)
Ricinus communis	Arabidopsis	*FAH12* and *DGAT2* from *Ricinus communis*	Higher hydroxy fatty acid in seed oil	(van Erp et al., 2011)
Ricinus communis	Arabidopsis	*FAH12* and *DGAT2* from *Ricinus communis*; silencing of endogenous *DGAT1*	Higher hydroxy fatty acid in seed oil	(van Erp et al., 2015)
Linum usitatissimum L.	Arabidopsis		Higher α-linolenic acid in seed oil	(Pan et al., 2013)
Arabidopsis	Arabidopsis, Arabidopsis *trigalactosyldiacyl glycerol1-1* mutant		Higher leaf oil content	(Fan et al., 2013b)

Abbreviations: FAH, fatty acid hydroxylase; FAT, fatty acyl-ACP thioesterase; GPAT9, *sn*-glycerol-3-phosphate acyltransferase; LPAAT, lysophosphatidate acyltransferase; LEC, LEAFY COTYLEDON; MGAT, monoacylglycerol acyltransferase; RNAi, RNA interference; SnRK1, sucrose non-fermenting-related protein kinase 1; WRI, WRINKLED1; TAG, triacylglycerol.

Figure Legends

Figure 1. Generalized scheme for triacylglycerol (TAG) biosynthesis in developing seeds of oleaginous higher plants and microalgae. Abbreviation: ACP, acyl carrier protein; ACCase, acetyl-CoA carboxylase; CoA, coenzyme A; CPT, choline phosphotransferase; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; FAD, fatty acid desaturase; FAE, fatty acid elongase; FAS, fatty acid synthase; FAT, acyl-ACP thioesterase; FAX, fatty acid export; FFA, fatty acid; GPAT, *sn*-glycerol-3-phosphate acyltransferase; GPC, glycerophosphocholine; GPCAT, glycerophosphocholine acyltransferase; G3P, *sn*-glycerol 3-phosphate; Hexose P, hexose phosphate; LACS, long-chain acyl-CoA synthase; LPA, lysophosphatidic acid; LPAAT, acyl-CoA:lysophosphatidic acid acyltransferase; LPC, lysophosphatidylcoline; LPCAT, lysophosphatidylcholine acyltransferase; LPCT, lysophosphatidylcholine transacylase; MCAT, malonyl-CoA:ACP acyltransferase; PAP, phosphatidic acid phosphatase; PDAT, phospholipid:diacyglycerol acyltransferase; PDCT, phosphatidylcholine: diacylglycerol cholinephosphotransferase; PDH, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PPP, pentose phosphate pathway; PtdCho, phosphatidylcholine; PtdOH, phosphatidic acid; Pyr, pyruvate; SAD, stearoyl-ACP desaturase; Triose P, triose phosphate. This figure was developed based on information from reviews and articles on lipid biosynthesis (Ohlrogge and Jaworski, 1997; Rawsthorne, 2002; Shearer et al., 2004; Harwood, 2005; Baud and Lepiniec, 2010; Bates et al., 2012; Chapman and Ohlrogge, 2012; Bates et al., 2013; Chen et al., 2015; Li et al., 2015a). Carriers/ transporters on the plastid envelope that are required to move the cytosolic intermediates of glycolysis into the plastid are shown as dark circles.

Figure 2. Current insights into structure and function in triacylglycerol biosynthetic enzymes. A, Structural features of DGAT1 that have been determined using experimental methods. Despite the absence of a crystal structure of DGAT1, some structural features of this enzyme have been determined using a combination of biochemical and biophysical methods. The N-terminal region is found to constitute the regulatory domain, with two distinct segments that influence activity differently. The intrinsically disordered region (IDR) has an autoinhibitory function, while the folded segment has the allosteric binding site (AS) for acyl-CoA/CoA. The transmembrane domains (TMD) have the catalytic sites (catalytic histidine, binding site for acyl

group and diacylglycerol/DAG), SnRK1 phosphorylation site and the endoplasmic reticulum (ER) retrieval motif. This figure was developed based on information from McFie et al. (2010), Liu et al. (2012), Lopes et al. (2014) and Caldo et al. (2017). **B, Experimental topology of *Saccharomyces cerevisiae* DGAT2 based on mutagenesis experiments.** The important motifs are also shown in the topology including the FLXLXXXn motif for binding neutral lipids, HPHG motif with putative active site residues, and the ER retrieval motif. This figure was developed based on information from Shockey et al. (2006), Stone et al. (2006), Liu et al. (2011) and McFie et al. (2011). **C, Experimentally verified topology model of *Euonymus alatus* diacylglycerol acetyltransferase.** The important motifs are shown in the topology including the MBOAT signature region with putative active site residues, and the ER retrieval motif. This figure was developed based on information from Tran et al. (2017). **D, Molecular model of Arabidopsis PDAT using phospholipase A_2 as template and PHYRE2 Protein Fold Recognition Server.** The different functional motifs are shown in the structure including the catalytic triad, salt bridge and a lid region with a tryptophan implicated in binding released fatty acid for efficient acylation.

Figure 3. Possible transcriptional regulation of DGAT1 and PDAT1 in plants. Solid lines represent up-regulation with experimental evidences. Dashed lines represent possible up-regulation. Abbreviation: ABI, ABSCISIC ACID INSENSTIVE; DGAT, acyl-CoA:diacylglycerol acyltransferase; LEC, LEAFY COTYLEDON; MYB96, R2R3-type MYB96 transcription factor; PDAT, phospholipid:diacyglycerol acyltransferase; WRI, WRINKLED. This figure was developed based on information from reviews and research articles on transcriptional regulation of DGAT and PDAT (Baud et al., 2007; Santos-Mendoza et al., 2008; Yang et al., 2011; Wind et al., 2013; Kong et al., 2013; Li et al., 2015b; Lee et al. 2018).

Figure 4. Proposed model for the biochemical regulation of *Brassica napus* DGAT1. BnaDGAT1 has a regulatory N-terminal domain (1-113, NTD, oval) and a membrane-bound domain containing the catalytic site (114-501, MB, rectangle). The N-terminal domain has 2 distinct segments specifically an intrinsically disordered region with autoinhibitory sequences and a folded section with allosteric site for acyl-CoA (FCoA) or CoA. Acyl-CoA and CoA serve as homotropic activator and inhibitor of DGAT1, respectively (Caldo et al., 2017). High CoA

levels inactivate the enzyme whereas the binding of acyl-CoA induces the transition into the moderately active state. SnRK1 can add a phosphoryl group, to further inactivate the enzyme (Caldo et al., 2018). An unknown protein phosphatase may be involved in the dephosphorylation process. An unknown protein phosphatase may be involved in the dephosphorylation process. Lastly, phosphatidic acid (PtdOH) serves as a feedforward activator that can initiate the transition into the more active state possibly by relieving autoinhibition. PtdOH may interact with the N-terminal domain upon activating the enzyme. This figure was adapted from Caldo et al., 2018.

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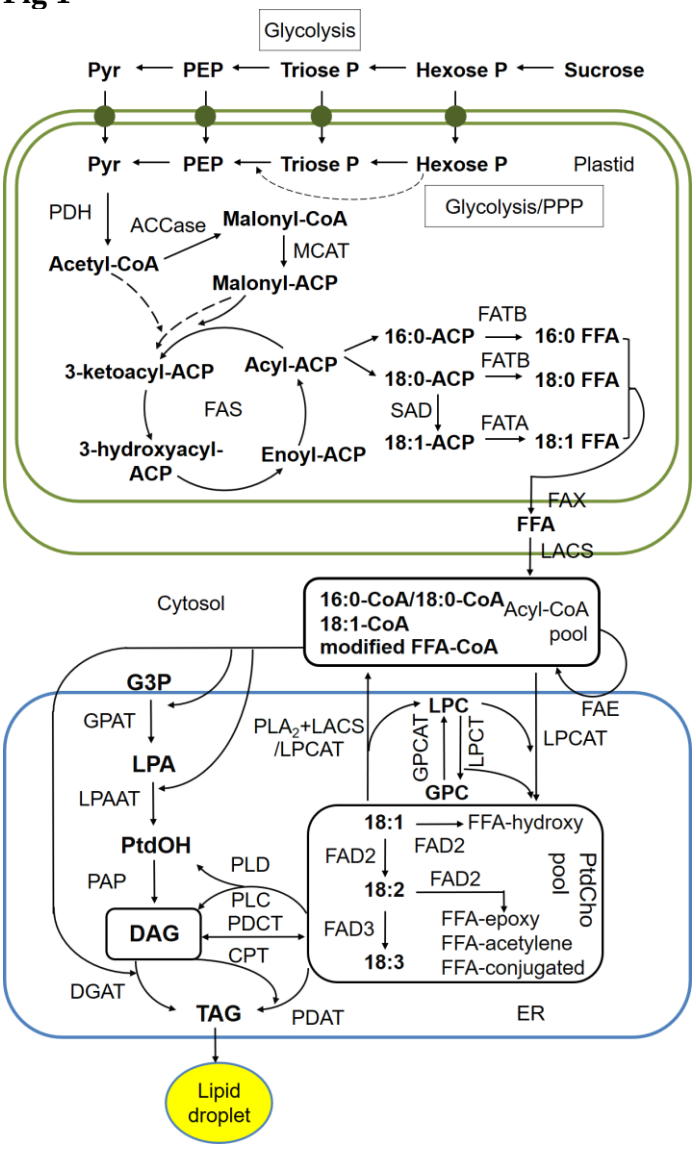
Fig 1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FATB
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16:0 FFA
3-ketoacyl-ACP
Acyl-ACP
18:0-ACP
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3-hydroxyacyl-ACP
Enoyl-ACP
FAX
FFA
LACS
Cytosol
16:0-CoA/18:0-CoA
18:1-CoA
modified FFA-CoA
Acyl-CoA pool
G3P
GPAT
LPA
LPAAT
PtdOH
PAP
PLA2+LACS /LPCAT
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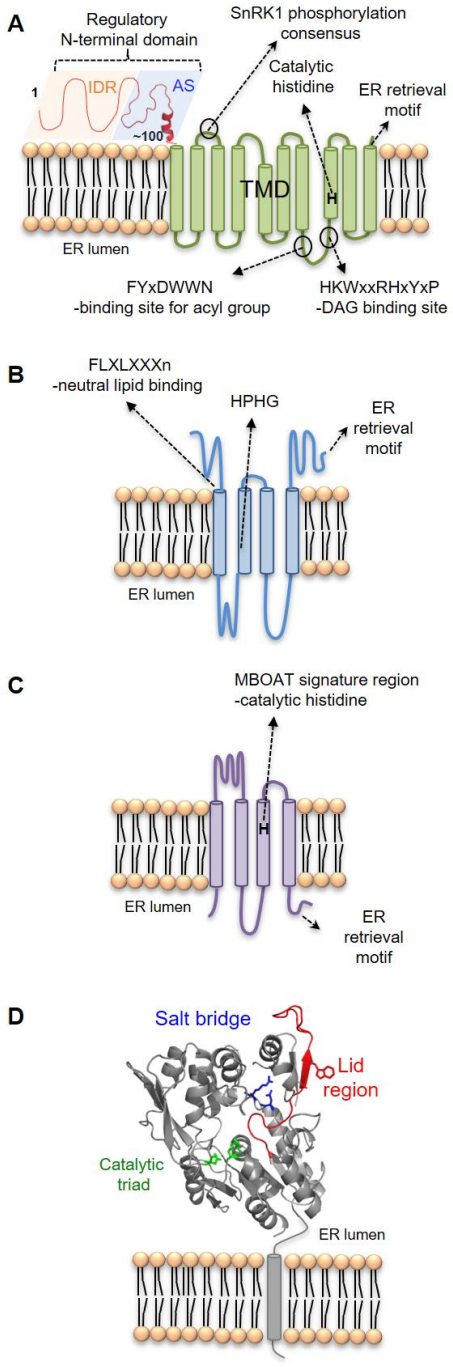
FFA-acetylene
FFA-conjugated
PtdCho pool
Lipid droplet

Fig 2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ER
retrieval
motif
ER lumen
C
MBOAT signature region
-catalytic histidine
H
ER lumen
ER
retrieval
motif
D
Salt bridge
Lid
region
Catalytic
triad
ER lumen

Fig 3

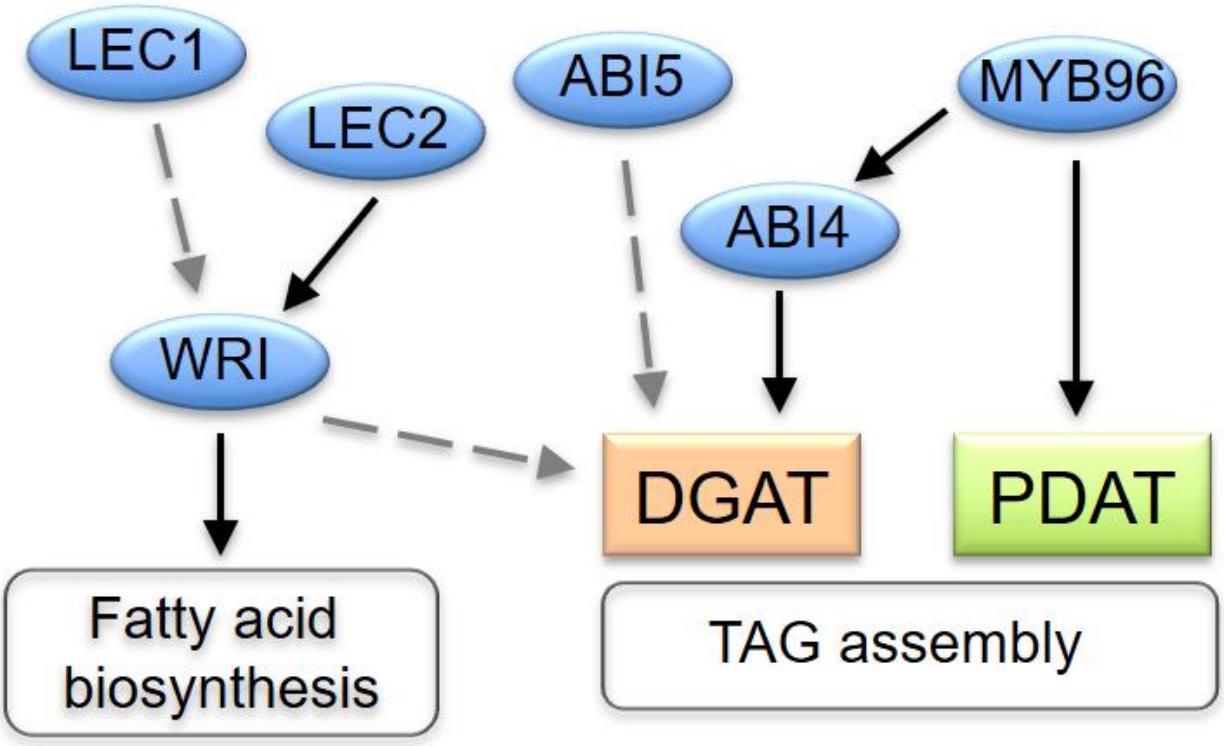
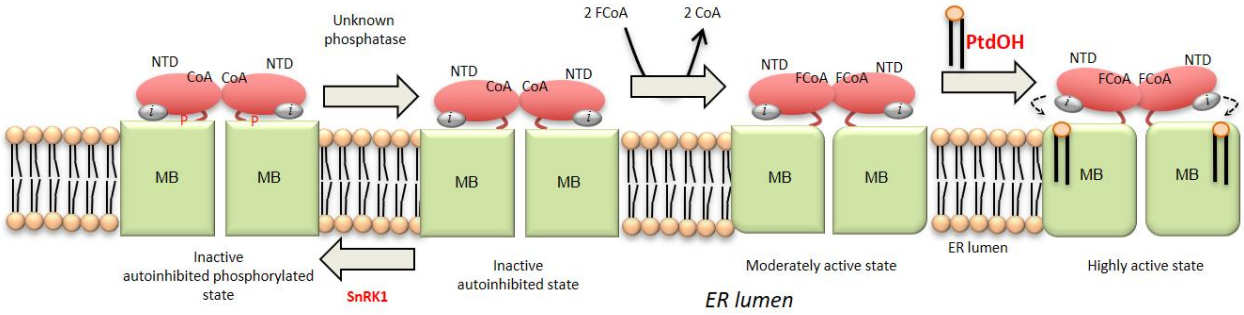

LEC1
LEC2
ABI5
MYB96
ABI4
WRI
DGAT
PDAT
Fatty acid biosynthesis
TAG assembly

Fig 4


Unknown phosphatase
2 FCoA
2 CoA
PtdOH
NTD
CoA
FCoA
MB
Inactive autoinhibited phosphorylated state
SnRK1
Inactive autoinhibited state
Moderately active state
ER lumen
Highly active state
ER lumen