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Review

Acyl-CoA:diacylglycerol acyltransferase: Properties, physiological roles, metabolic engineering and intentional control

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ABSTRACT

Acyl-CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) catalyzes the last reaction in the acyl-CoA-dependent biosynthesis of triacylglycerol (TAG). DGAT activity resides mainly in DGAT1 and DGAT2 in eukaryotes and bifunctional wax ester synthase-diacylglycerol acyltransferase (WSD) in bacteria, which are all

Abbreviations: ABA, abscisic acid; ABI, ABSCISIC ACID INSENSITIVE; ACAT, acyl-CoA:cholesterol acyltransferase; ACBP, acyl-CoA binding protein; ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; ACS, Acetyl-CoA synthetase; acyl-P, acyl-phosphate; AP2/EREBP, APETALA2-ethylene responsive element-binding protein; ARA, arachidonic acid; ARE, acyl-CoA:cholesterol acyltransferase-related enzyme; AUERO1, Aureochrome-1; BCCP, BIOTIN CARBOXYL CARRIER PROTEIN; bHLH, a basic helix-loop-helix TF; BiFC, bimolecular fluorescent complementation; BTA, betaine synthase; bZIP, a basic leucine zipper-domain containing TF; CBL, cocoa butter-like lipid; CCT, CTP:phosphocholine cytidylyltransferase; Core, HCV capsid protein; CPM, 7-diethylaminl-3-(4'-maleimidyl-phenyl)-4-methylcoumarin; CPT, CDP-choline:1,2-diacylglycerol cholinephosphotransferase; CoA, coenzyme A; cryo-EM, cryo-electron microscopy; DAG, 1,2-diacyl-*sn*-glycerol; DAcT, diacylglycerol acetyltransferase; DBTL, design/build/test/learn; DCR, defective in cuticular ridges; DGAT, acyl-CoA:diacylglycerol acyltransferase; DGD, digalactosyldiacylglycerol synthase; DGDG, digalactosyldiacylglycerol; DGTS, diacylglyceryl-3-O-4′-(N,N,N-trimethyl)-homoserine; DHA, docosahexaenoic acid; DHAP, dihydroxyacetone phosphate; Dof-type TF, DNA binding with one finger type TF; DTNB, 5,5'-dithiobis(2-nitro benzoic acid); DW, dry weight; DYRK, dual-specificity tyrosine phosphorylation-regulated kinase; ENR, enoyl-ACP reductase; EPA, eicosapentaenoic acid; ER, endoplasmic reticulum; ESI, electrospray ionization; FA, fatty acid; FAD, fatty acid desaturase; FAE, FATTY ACID ELONGATION; FAEE, fatty acid ethyl ester; FAH, fatty acid hydroxylase; FALDR, fatty aldehyde reductase; FAME, fatty acid methyl ester; FAR, fatty acyl reductase; FatA/B, fatty acid thioesterase A/B; FAX1, fatty acid export 1; FCS, familial chylomicronemia syndrome; FID, flame ionization detection; FFA, free fatty acid; GBSS1, granule-bound starch synthase1; GC, gas chromatography; GPAT, acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase; GPDH, sn-glycerol-3-phosphate dehydrogenase; GRAS, generally recognized as safe; G3P, *sn*-glycerol-3-phosphate; HAD, hydroxyacyl-ACP dehydrase; HCV, hepatitis C virus; HDL, high-density lipoprotein; HTP, high-throughput; *i*, autoinhibitory motif; IDR, intrinsically disordered region; IM, intramuscular; KAR, ketoacyl-ACP reductase; KAS, keto-acyl-CoA synthase; LACS, long-chain acyl-CoA synthetase; LC, liquid chromatography; LD, lipid droplet; LEC, LEAFY COTYLEDON; LPA, lysophosphatidic acid; LPAAT, acyl-CoA:lysophosphatidic acid acyltransferase; LPC, lysophosphatidylcholine; LPCAT, acyl-CoA:lysophosphatidylcholine acyltransferase; LPE, lysophosphatidylethanolamine; LPL, lysophospholipid; MAG, monoacylglycerol; MBOAT, membrane-bound *O*-acyltransferase; MCA, Metabolic Control Analysis; MCMT, malonyl-CoA:ACP malonyltransferase; ME, metabolic engineering; MGAT, acyl-CoA:monoacylglycerol acyltransferase; MGD, monogalactosyldiacylglycerol synthase; MGDG, monogalactosyldiacylglycerol; MS, mass spectrometry; NBD, N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)-methyl]amino; NAFLD, non-alcoholic fatty liver disease; NE, non-esterified; NPL, non-polar lipid; NRP1, pathogenesis-related genes 1; NTD, N-terminal domain; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PC, phosphatidylcholine; PCholine, phosphocholine; PDAT, phospholipid:diacylglycerol acyltransferase; PDCT, phosphatidylcholine: diacylglycerol cholinephosphotransferase; PDH, pyruvate dehydrogenase complex; PE, phosphatidylethanolamine; PEG, polyethylene glycol; PES, phytyl ester synthase; PG, phosphatidylglycerol; PGD1, plastid galactoglycerolipid degradation 1; P_i, inorganic phosphate; PL, phospholipid; PLA_2, phospholipase A_2; PSR1, Pi starvation response 1; PUFA, polyunsaturated fatty acid; PXA1, peroxisomal membrane transporter1; QTL, quantitative trait locus; SAD, stearoyl-ACP desaturase; SCD, stearoyl-CoA desaturase; SDP1, SUGAR-DEPENDENT1; SE, steryl ester; SFR2, SENSITIVE TO FREEZING2; SNP, single nucleotide polymorphism; SnRK1, sucrose non-fermenting-1-related kinase; SPA, scintillation proximity assay; SQD, sulfoquinovosyldiacylglycerol synthase; SQDG, sulfoquinovosyldiacylglycerol; SUC2, sucproton symporter 2; TAG, triacylglycerol; TAR1, tryptophan aminotransferase-related protein 1; TF, transcription factor; TGD, trigalactosyldiacylglycerol; TLC, thin layer chromatography; TMD, transmembrane domain; TORC1, Target of Rapamycin complex 1; WE, wax ester; WIN1, WAX INDUCER1/SHINE1; WRI, WRINKLED; WS, wax ester synthase; WSD, bifunctional wax ester synthase-diacylglycerol acyltransferase; WT, wild type; VLCFA, very-long-chain fatty acid; VLDL, very low-density lipoprotein; 3-D, three-dimensional..

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membrane-bound proteins but exhibit no sequence homology to each other. Recent studies also identified other DGAT enzymes such as the soluble DGAT3 and diacylglycerol acetyltransferase (EaDAcT), as well as enzymes with DGAT activities including defective in cuticular ridges (DCR) and steryl and phytyl ester synthases (PESs). This review comprehensively discusses research advances on DGATs in prokaryotes and eukaryotes with a focus on their biochemical properties, physiological roles, and biotechnological and therapeutic applications. The review begins with a discussion of DGAT assay methods, followed by a systematic discussion of TAG biosynthesis and the properties and physiological role of DGATs. Thereafter, the review discusses the three-dimensional structure and insights into mechanism of action of human DGAT1, and the modeled DGAT1 from *Brassica napus*. The review then examines metabolic engineering strategies involving manipulation of DGAT, followed by a discussion of its therapeutic applications. DGAT in relation to improvement of traits of farmed animals is also discussed along with DGATs in various other eukaryotic organisms.

1. Introduction

Triacylglycerol (TAG), which consists of three fatty acyl chains esterified to a glycerol backbone, represents a highly reduced form of carbon which functions mainly as an energy store in eukaryotes and numerous prokaryotes [1–6]. TAG can also serve a source of fatty acids (FAs) for phospholipid (PL) synthesis [1,6]. In addition, fatty acyl chains can be sequestered in TAG, which is osmotically inert, thereby protecting cells from potentially toxic levels of unesterified FAs and acyl-CoAs [1,6]. Since bacteria and plant-like protists, such as microalgae, can be exposed to unreliable nutritional sources, the ability to form TAG offers them an advantage over competing organisms in an environment where growth substrates are limited [5,7]. An abundant amount of TAG is used to fuel germination and early seedling growth in oleaginous plants [3], whereas in vegetative tissue, TAG occurs in small amounts and appears to act as a temporal reserve for excess unesterified FAs formed during membrane lipid turnover [8–11]. In mammals, including humans, TAG is found throughout the body including adipose, muscle, liver, brain and intestinal tissues, and within circulating lipoproteins [1,4,12]. In prokaryotes and eukaryotes, TAG is sequestered in lipid droplets (LDs) or lipid bodies which range in size from about 0.2 to 100 μm depending on the organism and cell type [7,13–17]. In general, LDs can be envisioned as a core of non-polar lipid (NPL), containing TAG and/or steryl ester, surrounded by a monolayer of PL with various embedded proteins. The TAG produced in seeds and fruits of oil crops is key to feeding a continually increasing global population; indeed, many oil crops are known to produce TAG of high nutritional value [18]. Oleaginous crops are also recognized as valuable sources of TAG for producing biofuel and other useful bioproducts [19–22]. Metabolic engineering (ME) has been used to increase TAG quantity and alter the fatty acyl composition of TAG for both edible and industrial applications using oil crops [23–26] and oleaginous microorganisms [27]. From a medical perspective, understanding TAG metabolism is useful for developing treatments to combat obesity and other metabolic disorders [28].

Formation of TAG is catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) and phospholipid:diacylglycerol acyltransferase (PDAT; EC 2.3.1.22) [29–31]. DGAT catalyzes the acyl-CoA-dependent formation of TAG using 1,2-diacyl-*sn*-glycerol (DAG; refers to *sn*-1,2-DAG unless indicated otherwise) as an acyl acceptor [30,32] whereas PDAT catalyzes the acyl-CoA-independent formation of TAG using nitrogenous PL (e.g., phosphatidylcholine (PC) and phosphatidylethanolamine (PE)) as an acyl donor (Fig. 1) [33–35]. In the latter reaction, the acyl group is transferred to DAG from the *sn*-2 position of the nitrogenous PL. DGAT, however, is ubiquitous among organisms being present in many types of bacteria, plant-like protists, plants and animals. In contrast, the *PDAT* genes originally identified by Stymne and co-workers [33–35] have orthologs in fungi, plant-like protists and plants, which are putative orthologs of human *LECITHIN:CHOLESTEROL ACYL-TRANSFERASE* [29–31,36,37]. It should be noted, however, that PDAT activity was reported in *Streptomyces coelicolor* [38] (Full names of all orgnaisms mentioned in this paper are listed in Supplemental Table S1). Since this prokaryote did not contain a gene(s) with homology to known *PDATs*, it was suggested that microorganisms might contain a gene(s) encoding another class of PDAT.

Although the activity of DGAT was known for several decades [32], it was not until 1998 that a DGAT gene was identified and cloned from mouse (*Mus musculus*) [39]. Three years later, a second DGAT was identified and cloned from the oleaginous soil fungus *Umbelopsis* (formerly *Mortierella*) *ramanniana* [40]. The two gene families were referred to as *DGAT1* and *DGAT2* [40], respectively, and represent integral membrane-bound enzymes which are ubiquitous in eukaryotes [30,40–42]. DGAT1 and DGAT2, however, exhibit no sequence homology to each other. Given the importance of DGAT activity in catalyzing TAG production in eukaryotes, it has been proposed that *DGAT1* and *DGAT2* genes had a functional convergence in eukaryotes [41]. Both DGAT1 and DGAT2 are localized to the endoplasmic reticulum (ER) [28,43], although several studies have shown DGAT2 to also associate with LDs [44–49]. DGAT1s are predicted to have in the range of eight to ten transmembrane domains (TMDs) with fewer of these hydrophobic segments in DGAT2s [29,30,41,50]. Among 55 full-length DGAT1s across species, 8.0% of an average number of 515 amino acid residues are conserved whereas for 54 full-length DGAT2s across species, 4.7% of 344 amino acid residues are conserved [42]. For both DGAT1 and DGAT2 families, most of the conserved amino acid residues are in the C-

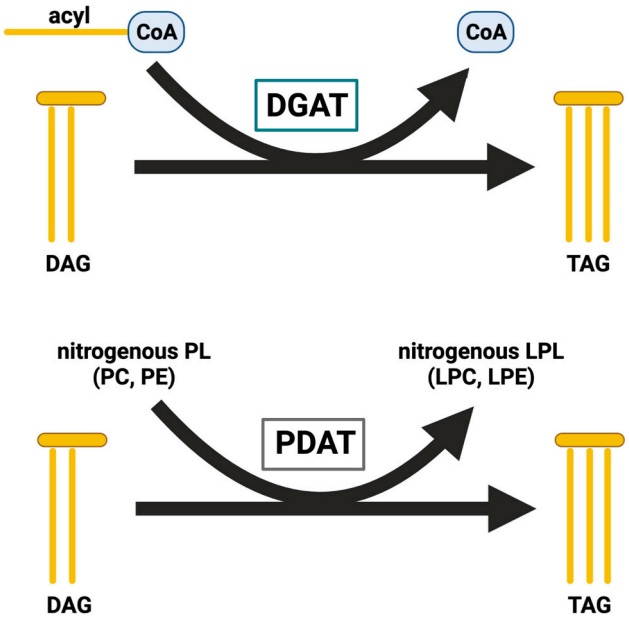

Fig. 1. Acyl-CoA-dependent and acyl-CoA-independent formation of triacylglycerol (TAG) catalyzed by diacylglycerol acyltransferase (DGAT) and phospholipid:diacylglycerol acyltransferase (PDAT), respectively. Nitrogenous phospholipids (PLs) used as acyl donors by PDAT may include phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Other abbreviations: DAG, 1,2-diacyl-*sn*-glycerol; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPL, lysophospholipid.

terminal region of the enzymes [42]. Analysis of two recently published cryo-electron microscopy structures of human (*Homo sapiens*) DGAT1 has shown that there are nine TMDs per DGAT1 protomer [51,52]. The enzyme was also shown to exist as either homodimer or homotetramer. DGAT1 is also known as member of the membrane-bound *O*-acyltransferase (MBOAT) superfamily, of which all members are predicted to have several TMDs [28,53]. MBOATs, which have representatives in all kingdoms of life, are known to catalyze the transfer of fatty acyl chains onto hydroxyl or thiol groups of lipid and protein acceptors. One of the most notable features of MBOATs is an invariant histidine residue, likely to participate in catalysis, which resides in a long hydrophobic segment. In the case of human DGAT1, this corresponds to histidine 415 [28] [51,52]. Amino acid sequences for selected DGAT1 and DGAT2 polypeptides are aligned, respectively, in Fig. 2 and Fig. 3. The invariant histidine in the DGAT1 is indicated with a rectangle Fig. 2. DGAT1s are also characterized by a relatively long and variable hydrophilic N-terminal domain (NTD), on the cytosolic side of the ER, which plays a role in regulation and self-association of DGAT1 protomers [30,54–56]. Many DGAT2 family members feature the consensus amino acid sequence HPHG (corresponding to residues 161-164 in the mouse enzyme) with the second histidine residue being essential for catalysis [28,30]. This highly conserved sequence is indicated with a box in Fig. 3. For DGAT2 from *Arabidopsis thaliana* (hereafter *Arabidopsis*) and *Chromochloris zofingiensis* (formerly known as *Chlorella zofingiensis*), only the two middle amino acid residues (proline and histidine) are conserved in the four-residue motif. The DGAT2 family also includes acyl-CoA: monoacylglycerol acyltransferases (MGATs) 1-3 and wax monoester synthase [28].

A soluble DGAT was highly purified from immature peanut (*Arachis hypogaea*) in 2006 [57]. Amino acid sequence information from this protein was used to generate degenerate primers that were used to clone the first *DGAT3* cDNA. The hydropathy plot for the predicted amino acid sequence of peanut DGAT3 did not reveal any TMDs. Another isoform of peanut DGAT3 was also functionally characterized in 2014 [58]. Genes encoding DGAT3 have been identified in other higher plants (e.g. [59–63]) and plant-like protists [64], but DGAT3s have not received as much attention as DGAT1 and DGAT2. Recent investigations of *Chlamydomonas reinhardtii* and *Arabidopsis* DGAT3 have revealed the presence of a [2Fe-2S] domain in the C-terminal region and an N-terminal putative chloroplast transit peptide [64,65]. Putative DGAT3s have also been identified in other plant species through *in silico* analysis [62,66].

In 2001, Gangar et al. reported the discovery of cytosolic 10 S TAG biosynthetic multi-enzyme complex, containing a DGAT, from the oleaginous yeast, *Rhodotorula glutinis* [67]. The soluble *R. glutinis* DGAT was subsequently purified and cloned by the same research group in 2013 [68].

The recombinant form of another soluble DGAT from *Arabidopsis*, known as defective in cuticular ridges (DCR), was purified and characterized by Rani et al. in 2010 [69]. DCR is encoded by the *At5g23940* gene and various lines of evidence indicated that it might be involved in surface cutin formation. Soluble DGATs have not been identified in animals.

Although most bacteria accumulate polyhydroxyalkanoates as storage compounds [5,70], several genera including *Actinomyces*, *Arthrobacter*, *Dietzia*, *Gordinia*, *Mycobacterium*, *Nocardia*, *Rhodococcus* and *Streptomycetes* produce TAG as a major storage compound [5,71,72]. Members of the genus *Acinetobacter*, however, produce both wax esters (WEs) and TAGs as storage compounds [5,72,73]. The first prokaryotic enzyme with DGAT activity was identified in *Acinetobacter baylyi* (formerly known as *A. calcoaceticus*) sp. ADP1 and was characterized as a bifunctional wax ester synthase-diacylglycerol acyltransferase (WSD, also known as WS/DGAT) [71,73]. This enzyme is not related to the DGAT1 or DGAT2 families. *A. baylyi* sp. ADP1 WSD (AbaWSD, also known as rtfA) was shown to be an amphiphilic protein with one predicted TMD. Subcellular fractionation experiments indicated that the enzyme was associated with cytoplasmic membranes, lipid inclusions and the soluble fraction [71,72]. Genes encoding related WSDs have been identified in *Arabidopsis* [74,75] and other plants [62] (please refer to Section 6.4 for details). Functional characterization of *WSD*-related genes has been conducted with recombinant enzymes from *Petunia hybrida* [76], *Arabidopsis* [74] and plant-like protists such as algae [77–79]. The motif HHXXXDG (corresponding to amino acid residues 133-138 of AbaWSD) is highly conserved in most of the bacterial WSD enzymes [71]. This motif is also present in functionally characterized WSDs from various eukaryotic organisms (e.g., [74,76–78]) and putative WSDs identified via *in silico* analysis [62,66,80].

Analysis of gene structure has shown that *DGAT1* genes from higher plants and animals contain 16-17 exons while *DGAT2* genes contain 8-9 exons [30]. In contrast, *DGAT3* and *WSD* genes from higher plants have been shown to contain 2 exons and 7 exons, respectively [66].

A specialized DGAT from developing seeds of *Euonymus alatus* (Burning Bush) has been shown to catalyze the acetyl-CoA dependent synthesis of TAG with an acetyl moiety at the *sn*-3 position [81,82]. *E. alatus* diacylglycerol acetyltransferase (EaDAcT) is also a member of the MBOAT superfamily, but it lacks the cytosolic hydrophilic NTD associated with regular DGAT1s [82].

Relationships among amino acid sequences of selected polypeptides with DGAT activity from mammals, higher plants, fungi, plant-like protists and bacteria are shown in the dendrogram in Fig. 4. DGAT1 and DGAT2 are two separate groups with high bootstrap values. Although EaDAcT clusters with the DGAT1 family, the enzyme has been shown to be more closely related to the *Simmondsia chinensis* (jojoba) wax ester synthase (WS) and *Arabidopsis* sterol acyltransferase [82]. WSDs, soluble DGAT3s and DCR form another cluster, but these proteins belong to different subdivisions. In addition, WSDs and DGAT3 are more related to each other than to DCR.

It should be noted that there are many examples where DGAT enzymes have been shown to have other activities, which may be physiologically relevant. Conversely, there are also examples of steryl and phytyl ester synthases (PESs) which exhibit some DGAT activity. Mammalian DGAT1 has been shown to exhibit acyl-CoA-dependent MGAT, WS and retinol acyltransferase activities [83]. AbaWSD has also been shown to possess MGAT activity [72]. In contrast, acyl-CoA: cholesterol acyltransferase-related proteins (Are1p and Are2p), which catalyze steryl ester (SE) synthesis in *Saccharomyces cerevisiae*, have also been shown to be capable of catalyzing some TAG formation [84–86]. In addition, PESs, present in chloroplasts from *Arabidopsis*, have been shown to display DGAT activity which was higher than actual PES activity [87]. Microsomal recombinant PES1 or PES2, produced in *S. cerevisiae* deficient in TAG synthesis (strain H1246), catalyzed formation of radiolabeled TAG when microsomes were fed radiolabeled DAG. Both acyl-CoA and acyl-acyl carrier protein (ACP) could serve as acyl-donors for recombinant PES2. In another example, EaDAcT was also shown to catalyze the acylation of fatty alcohols to produce alkyl acetates [88].

S. cerevisiae strain H1246 features a disruption of four genes encoding enzymes contributing to TAG production [85]. It has been used extensively in studies of recombinant DGAT and PDAT. The reader is referred to section 2.7 for a more detailed discussion of yeast strain H1246.

Within the last decade, in depth gene and amino acid sequence comparisons for a plethora of DGATs have been computationally analyzed and studied from an evolutionary perspective [30,41,62,64,66,89–91]. This review builds upon previous reviews about DGATs from various organisms (e.g. [28–31,50,71,92]), and mainly discusses research advances on DGAT within the last decade with a focus on the biochemical properties and physiological roles of DGATs in prokaryotes and eukaryotes, applications involving these acyltransferases in biotechnology and developing treatments for human metabolic disorders. The review begins with coverage of methods used in the assay of DGAT activity. Thereafter, the review systematically discusses TAG biosynthesis and the properties and physiological role of

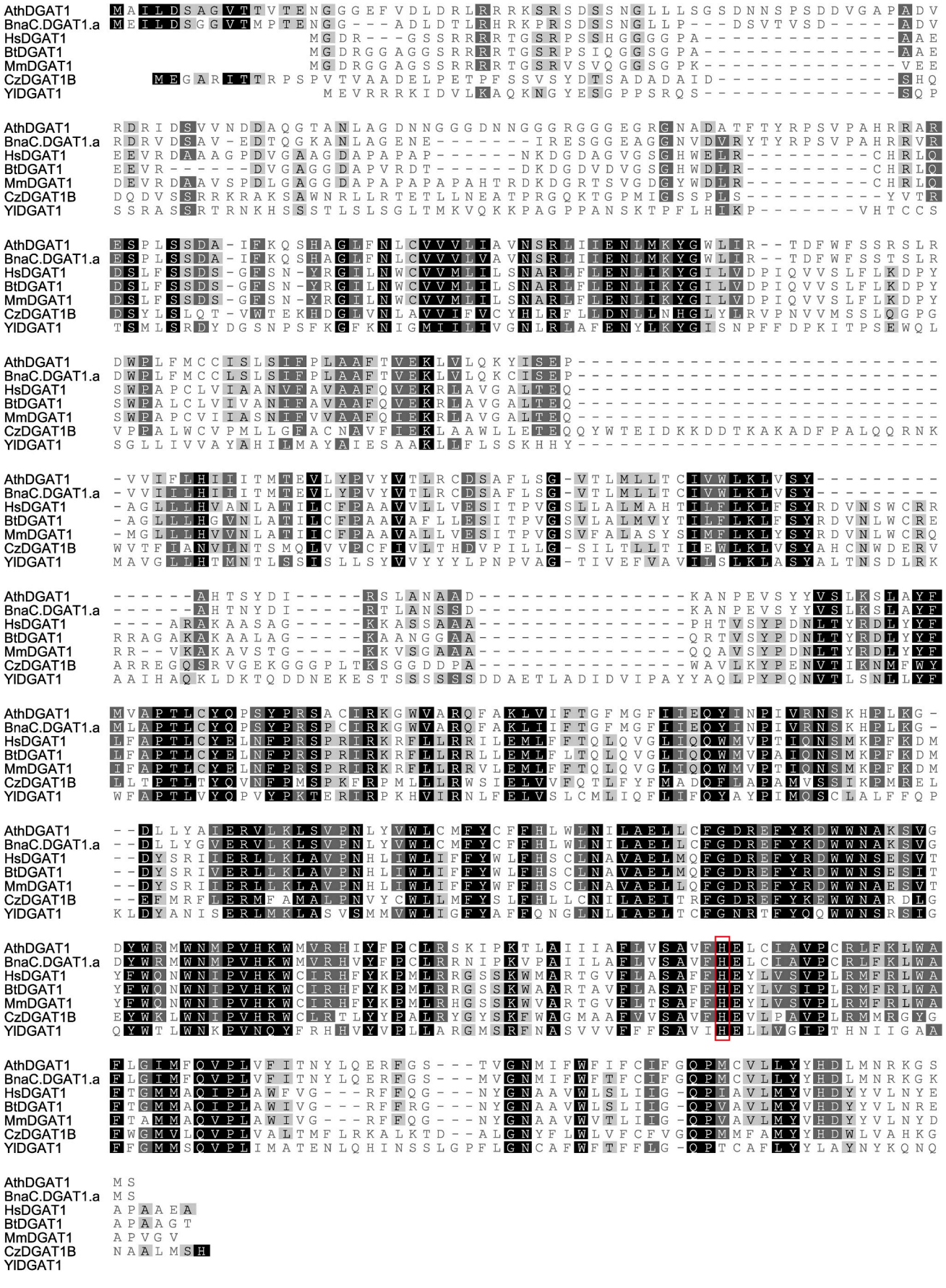

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Fig. 2. Alignment of amino acid sequences of diacylglycerol acyltransferase (DGAT) 1 polypeptides from selected organisms. Similar and conserved amino acid residues are indicated by grey and black backgrounds, respectively. The rectangle indicates the position of the conserved membrane-bound *O*-acyltransferase histidine residue [53] located in a long hydrophobic segment. The organisms and Phytozome/GenBank accession numbers for proteins used are as follows: AthDGAT1, *Arabidopsis thaliana* DGAT1 (NM_127503); Bna.C.DGAT1.a, *Brassica napus* DGAT1 isoform (JN224473); BtDGAT1, *Bos taurus* DGAT1 (NP777118); CzDGAT1B, *Chromochloris zofingiensis* DGAT1B (Cz09g08290.t1); HsDGAT1, *Homo sapiens* DGAT1 (NP03611); MmDGAT1, *Mus musculus* DGAT1 (NP034176); YlDGAT1, *Yarrowia lipolytica* DGAT1 (XP504700). The alignment was conducted with ClusterW.

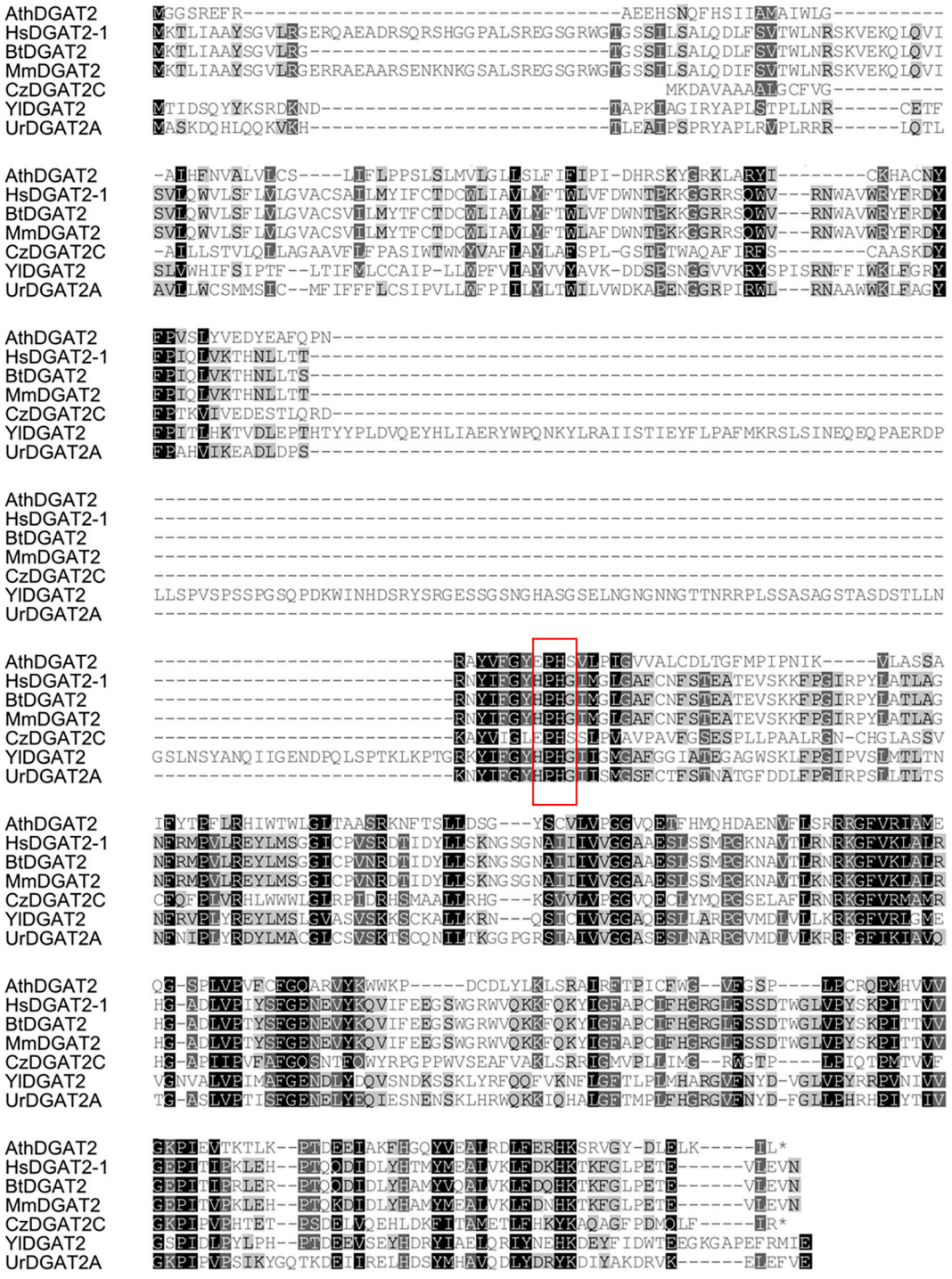

Fig. 3. Alignment of amino acid sequences of diacylglycerol acyltransferase (DGAT) 2 polypeptides from selected organisms. Similar and conserved amino acid residues are indicated by grey and black backgrounds, respectively. The rectangle indicates the position of the conserved HPHG motif [28], which occurs in most of the examples. The organisms and Phytozome/GenBank accession numbers for proteins used are as follows: AthDGAT2, *Arabidopsis thaliana* DGAT2 (NM115011); HsDGAT2-1, *Homo sapiens* DGAT2-1 (NP115953); BtDGAT2, *Bos taurus* DGAT2 (NP_991362); MmDGAT2, *Mus musculus* DGAT2 (NP080660); CzDGAT2C, *Chromochloris zofingiensis* DGAT2C (Cz11g24150.t1); YlDGAT2, *Yarrowia lipolytica* DGAT2 (XP504700); UrDGAT2A, *Umbelopsis ramanniana* DGAT2A (AAK84179). The alignment was conducted with ClusterW.

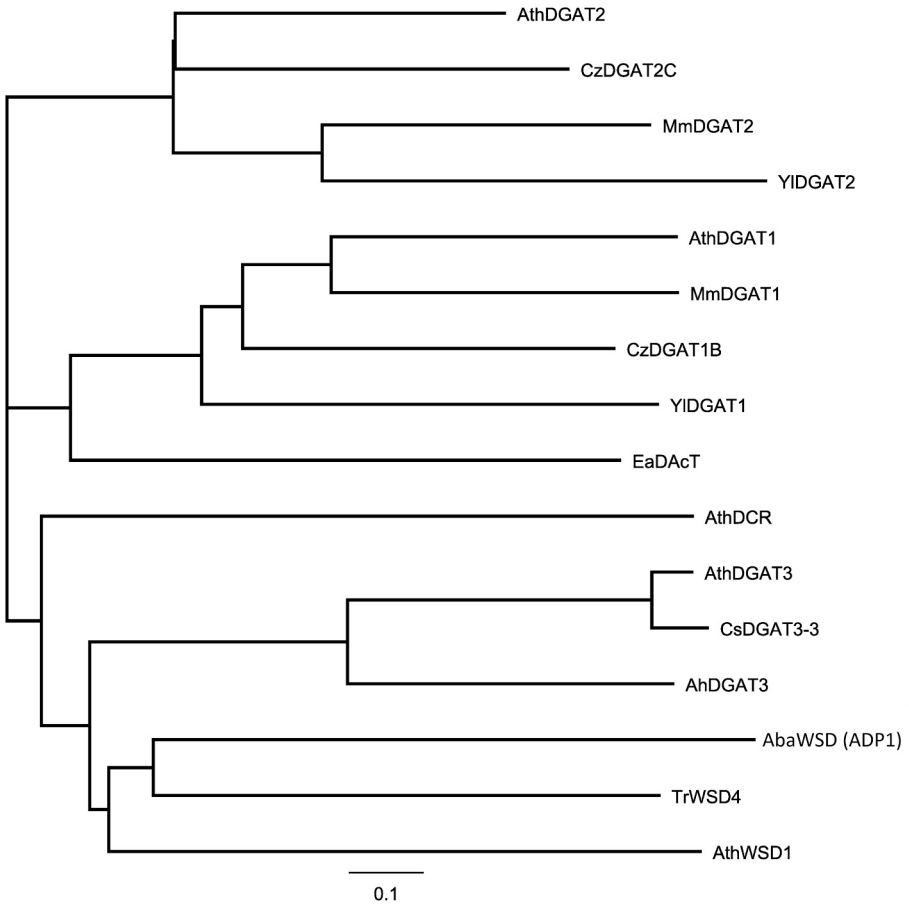

Fig. 4. Dendrogram showing relationships among selected amino acid sequences of diacylglycerol acyltransferase (DGAT) polypeptides from various families. The organisms and GenBank accession numbers for proteins used are as follows: AbaWSD (ADP1), *Acinetobacter baylyi* WSD (AAO17391); AthDGAT2, *Arabidopsis thaliana* DGAT2 (NM115011); AthDGAT3, *Arabidopsis thaliana* DGAT3 (OAP16619); AthDCR, *Arabidopsis thaliana* defective in cuticular ridges (Q9FF86.1); AthDGAT1, *Arabidopsis thaliana* DGAT1 (NM_127503); AthWSD1, *Arabidopsis thaliana* WSD1 (NP_568547); AhDGAT3, *Arachis hypogaea* DGAT3 (ABC41546); CsDGAT3-3, *Camelina sativa* DGAT3-3 (XP_010500353); CzDGAT2C, *Chromochloris zofingiensis* DGAT2C (Cz11g24150.t1); CzDGAT1B, *Chromochloris zofingiensis* DGAT1B (Cz09g08290.t1); EaDAcT, *Euonymus alatus* diacylglycerol acetyltransferase (ADF57327); MmDGAT1, *Mus musculus* DGAT1 (NP034176); MmDGAT2, *Mus musculus* DGAT2 (NP080660); TrWSD4, *Thraustochytrium roseum* WSD4 (ASA49417); YlDGAT1, *Yarrowia lipolytica* DGAT1 (XP504700); YlDGAT2, *Yarrowia lipolytica* DGAT2 (XP504700). WSD, bifunctional wax ester synthase-diacylglycerol acyltransferase. The phylogenetic analysis was conducted with the Neighbor-Joining method using the Geneious Tree Builder module in Geneious Pro 5.3.6.

DGATs in bacteria, fungi, plant-like protists, higher plants and mammals. Both the recent 3-D structure of human DGAT1 [51,52] and the bacterial MBOAT DltB [93] have proven useful in gaining insight into the mechanism of catalysis in MBOAT enzymes. The review moves on to discuss insights into the function of plant and human DGAT1 based on recently available structural information. Following this, a 3-D model for *B. napus* DGAT1 is generated based on the structural information for human DGAT1. In turn, the 3-D model of the plant DGAT1 is further analyzed in the light of information gleaned from variants of the plant enzyme generated through directed evolution [94–96]. Thereafter, the review examines ME strategies involving manipulation of DGAT to alter TAG accumulation and/or FA composition of TAG in bacteria, yeast, plant-like protists and higher plants. The intentional inhibition/down-regulation of DGAT in mammalian systems is then discussed in relation to developing drugs for the treatment of obesity and related metabolic disorders. Thereafter, DGAT in relation to improvement of traits of farmed animals is discussed. Finally, investigations of DGAT in various other organisms are addressed.

2. Assays of DGAT activity

Progress in DGAT research is dependent on suitable enzyme assays, some of which have been adapted to high-throughput (HTP) formats. Recently, a few of these procedures have been compiled and described in detail by Xu et al. (2021) [97]. Various approaches used in the assay of DGAT activity are presented in Table 1. Where appropriate, comments on advantages or disadvantages of the assays are also presented.

2.1. Reaction mixture components and assay optimization

Since DGAT1 and DGAT2 are ER-bound, DGAT assays often involve using microsomes as a source of enzyme. Since DAGs typically contain long chain fatty acyl moieties, rendering them water-insoluble, low concentrations of non-ionic detergent (e.g., Tween-20) in the reaction mixture can aid the dispersion of the acyl acceptors [29,98]. In other cases, a stock solution of DAG was prepared in a water-miscible organic solvent such as ethanol [99–101], acetone [102,103] or DMSO [104]. Investigators have also dispersed DAG by sonication in the presence of PL [105,106]. Possible interference of ethanol in acyltransferase activity, however, should be taken into consideration when using ethanol to prepare a stock solution of DAG. For example, ethanol acylation activity has been reported in microsomes from liver [102,107,108]. Sonication is often used to aid in the dispersion of DAG in the presence of detergent. It should be noted that in many instances, racemic DAG serves equally as well as an acyl acceptor for the DGAT under investigation compared to the regular *sn*-1,2-version of DAG, which is more involved to prepare [109]. Furthermore, bovine serum albumin is often added to DGAT reaction mixtures [3,99,110,111] wherein the protein may prevent the formation of acyl-CoA micelles which could potentially inhibit acyltransferase activity [112]. Bound acyl-CoAs may also be more effective substrates for acyltransferases [113]. In addition, assays of DGAT activity by different researchers have been conducted in absence or presence of $MgCl_2$. For example, assays of DGAT2 activity in *S. cerevisiae* have been conducted in the absence [114] or presence of 8 mM $MgCl_2$ [86]. The concentration of magnesium chloride can have variable effects on DGAT activity [3,115]. For example, mouse DGAT1 was shown to have relatively high activity at 5 mM $MgCl_2$ whereas DGAT2 from the same organism required 20 mM $MgCl_2$ for maximal activity but was inhibited at a higher concentration (100 mM) [116]. Under certain conditions, magnesium ions have also been shown to lead to precipitation of palmitoyl-CoA [117]. Thus, to fully optimize assay conditions for a particular DGAT, the investigator is advised to examine the effects of

Table 1
Assays of diacylglycerol acyltransferase (DGAT) activity.

Method	Comments	References
Radiometric TLC	• Radiolabeled acyl donor or acceptor • Sensitive with low background • Costly with radioisotope safety and disposal issues • Assays using radiolabeled DAG subject to interference by PDAT	[98,99,102,115,118,122–124]
Radiometric SPA	• No lipid extraction or TLC required • HTP format • Requires specialized scintillating beads or microplates • Costly with radioisotope safety and disposal issues	[125,127]
Quantification of TAG based on GC/MS of FAMEs	• Requires lipid extraction and TLC • Requires long reaction times • Possible underestimation of enzyme activity	[101]
LC/MS/MS	• Automated extraction and LC for HTP format • Stable [^{13}C]1,2,3-trioleoyl-*sn*-glycerol used to stabilize the MS signal • Useful for screening for DGAT inhibitors • Expensive complex equipment • Different molecular species of TAG may exhibit different ionization efficiencies	[132,135]
High temperature GC/FID	• Requires lipid extraction and TLC • 1,2-dihexanoyl-*sn*-glycerol used as acyl acceptor • Useful for acyl-CoA specificity and selectivity experiments	[130,136]
GC/MS	• Requires lipid extraction and TLC • 1,2-dihexanoyl-*sn*-glycerol used as acyl acceptor • Increased reaction volume and time may be required • TAG fragmentation ions analyzed	[131]
High temperature GC/MS	• One step lipid extraction with no TLC • 1,2-dioctanoyl-*sn*-glycerol used as acyl acceptor • Gas phase ions of TAG analyzed by selected ion monitoring • Strong correlation with radiometric-TLC assay • Useful for acyl-CoA specificity and selectivity experiments	[137,156]
ESI/MS	• Uses stable isotope of acetyl-CoA • Applied to assay of diacylglycerol acetyltransferase • No TLC required • Three picomoles TAG detected • Good agreement with radiometric-TLC assay	[138]
Fluorescent acyl donor analog	• NBD-palmitoyl-CoA • Requires lipid extraction and TLC • Imaging for TAG quantification • Useful for DAG specificity experiments • Bulky substituent NBD may affect enzyme activity	[103,140]
Fluorescent acyl acceptor analog	• NBD fluorescent label on *sn*-1 or *sn*-2 position of DAG • Requires lipid extraction and TLC • Imaging for TAG quantification • Useful for acyl-CoA specificity experiments • Subject to interference by PDAT • Bulky substituent NBD may affect enzyme activity	[141,142]
Colorimetric	• CoA reacts with DTNB (Ellman's Reagent) • End point or real-time assay • Real time amenable to HTP format • No lipid extraction required • Possible interference by other CoA-generating processes • Inexpensive	[38,78,139]
Fluorescence-based quantification of CoA	• CoA reacts with CPM • Sensitive • End point assay • Amenable to HTP format • Possible interference by other CoA-generating processes • Useful for screening for DGAT inhibitors	[132,149,150]
Fluorescence-based coupled enzyme assay	• Coupling of DGAT-catalyzed reaction to α-ketoglutarate dehydrogenase-catalyzed reaction • Sensitive fluorescence quantification of NADH • Real time assay • HTP format • Possible interference by other CoA-generating processes	[51]
Mammalian cell-based assay	• Radiometric version for quantifying [^{14}C]TAG • LC/MS/MS version for HTP format uses automated extraction	[132,149,174,175]
Yeast cell-based assay	• Fluorescence quantification of NPLs with Nile red • Recombinant DGAT produced in *S. cerevisiae* strain H1246 • Exogenous oleate used for selection of cDNAs encoding functional DGATs • No lipid extraction required • Used for directed evolution of *B. napus* DGAT1	[85,94,95,181,182]
Bacterial cell-based assay	• Fluorescence quantification of NPLs lipids with Nile red • Recombinant DGAT produced in *E. coli* • Used for directed evolution of *T. curvata* WSD	[183]

Abbreviations: CPM, 7-diethylaminl-3-(4'-maleimidyl-phenyl)-4-methylcoumarin; DAG, 1,2-diacyl-*sn*-glycerol; DTNB, 5,5'-dithiobis(2-nitro benzoic acid); ESI, electrospray ionization; FAME, fatty acid methyl ester; FID, flame ionization detection; GC, gas chromatography; HTP, high-throughput; LC, liquid chromatography; MS, mass spectrometry; NBD, N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)-methyl]amino; NPL, non-polar lipid; PDAT, phospholipid:diacylglycerol acyltransferase; SPA, scintillation proximity assay; TAG, triacylglycerol; TLC, thin layer chromatography; WSD, bifunctional wax ester synthase-diacylglycerol acyltransferase.

various reaction mixture additives over a range of concentrations.

2.2. Direct assays based on quantification of triacylglycerol

DGAT activity assays involve either the quantification of TAG or free CoA produced during the reaction. In addition to determination of DGAT activity, these assays can also provide insights into the specificity or selectivity properties of the enzyme [3,29]. For determination of acyl-CoA specificity, separate reactions are set up using different molecular species of acyl-CoA (e.g., palmitoyl-CoA versus oleoyl-CoA) with one molecular species of DAG and the reaction rates are compared to assess relative enzyme performance with each species of acyl-CoA. Conversely, for determination of DAG specificity, different molecular species of DAG are used in combination with one type of acyl-CoA. In the case of substrate selectivity experiments, mixtures (typically equimolar) of different molecular species of acyl-CoA or DAG are presented to the enzyme followed by analysis of the molecular species of TAG produced in the reaction mixture. Some methods for determining DGAT substrate selectivity are discussed after covering the various assay methods. Insights into DGAT activity can also be obtained by using cell-based assays, which will be addressed after discussing the various DGAT assays along with determination of apparent kinetic parameters and potential interference by endogenous DAG.

A well-accepted assay for DGAT activity utilizes a radiolabeled substrate in the form of acyl-CoA with radiolabel on the acyl chain or DAG [98,99,102]. Utilization of DAG with radiolabel on the glycerol backbone prevents problems associated with acyl exchange reactions [118]. Radiometric assays of DGAT activity are sensitive with minimum background signals. Radiolabeled TAG produced can be purified from the reaction mixture using a combination of organic solvent extraction and thin layer chromatography (TLC). Liquid scintillation counting is then used to quantify the amount of radiolabeled TAG produced. If the reaction mixture is relatively small (e.g., 60 μL), it is possible to apply quenched reaction mixture directly to a preparative TLC plate without going through an initial solvent extraction [115]. Direct application of quenched reaction mixtures to TLC plates was previously used in assays of plant triacylglycerol lipase using [carboxyl-^{14}C] 1,2,3-trioleoyl-*sn*-glycerol as a substrate [119]. Moreover, radioactivity has also been analyzed on TLC plates by electronic autoradiography (e.g. [104,120]) and by HPLC coupled with an in-line radioactivity detector (e.g. [120,121]).

Assays of DGAT activity with radiolabeled DAG, using tissue homogenates or microsomes from yeast or plants, are subject to interference by PDAT activity since this enzyme can also utilize DAG in the formation of TAG (Fig. 1) [122–124]. *S. cerevisiae* PDAT was also shown to exhibit some diacylglycerol:diacylglycerol transacylase activity, which results in the transfer of an acyl chain from one molecule of DAG to another molecule of DAG to produce TAG and monoacylglycerol (MAG) [123]. In addition, DGAT assays conducted with homogenates or crude microsomes can contain endogenous DAG and/or endogenous acyl-CoA, the latter of which may arise from acyl exchange with PC [124]. These potential interferences could result in radioisotope dilution and thus affect the determined specific activity of the crude enzyme preparation.

Seethala et al. (2008) have described a homogeneous scintillation proximity assay (SPA) for DGAT activity utilizing ^{3}H-labeled acyl-CoA [125]. In this case, the radiolabeled TAG product binds to polylysine SPA beads resulting in a signal measured using a radiometric imager system or scintillation plate counter. Bovine serum albumin (66 μg/assay) was included in the quenching solution to prevent nonspecific hydrophobic interaction of [^{3}H]acyl-CoA with the polylysine SPA beads. The assay reagents were added to a single well of a multi-well plate, incubated for a specific time, quenched and the resulting signal read in a microplate scintillation counter. This method is particularly suitable for HTP analysis of DGAT activity. A variation of the SPA approach involves the use of scintillating microplates wherein the scintillant is incorporated into the inner surface of the reaction wells [126]. These have been referred to as Flash Plates™. Using this latter approach, Kim et al. (2013) screened 20,000 compounds in a chemical library and identified several inhibitors of recombinant human DGAT2 [127].

Radiolabeled substrates, however, can be costly and there are issues concerning safe usage and proper disposal. In addition, there is a limited range of commercially available radiolabeled FAs. Preparation of DGAT substrates containing desirable acyl chains can also, at times, require further chemical or enzymatic-based synthesis which greatly adds to the underlying work involved just to establish and maintain substrates for DGAT assays. For example, long chain acyl CoAs can be enzymatically synthesized from radiolabeled FA and CoA using a bacterial acyl-CoA synthetase [128]. In another example, DAGs with desirable acyl chains can be synthesized using a combination of chemical and enzymatic-based synthesis [129]. Thus, there has been an impetus to develop DGAT assays which do not utilize radiolabeled substrates.

DGAT assays measuring TAG, which do not use radiolabeled substrates may involve relatively long reaction times, larger volumes of reaction mixtures and/or enriched sources of enzyme such as yeast microsomes containing recombinant DGAT [101,130,131]. Once lipids are extracted from these types of quenched reaction mixtures, TLC is then used to purify the generated TAG. In instances where long chain DAGs (e.g., 1,2-dioleoyl-*sn*-glycerol) are used as acyl acceptors, TLC-purified TAG can be converted to fatty acid methyl esters (FAMEs) which are in turn resolved and quantified using gas chromatography (GC)-mass spectrometry (MS). This approach was used to assay recombinant microalgal DGATs in *S. cerevisiae* strain H1246 [101]. However, if a relatively long reaction time is used without verification with radiolabelled substrates, the time course for TAG formation may be well into the curvilinear portion of the progress curve and thus initial reaction velocity may not be observed. Under these conditions, *in vitro* DGAT activity would be underestimated and substrate selectivity and specificity would possibly be misleading. Therefore, it is important to run a time course for production of TAG to ensure linearity and compare the method with the standard ones with radiolabeled substrates. There have been examples, however, where certain DGAT-catalyzed reactions were shown to exhibit linearity for well over 1 hour [38,132].

Various MS-based approaches have been used in the radiolabel-free quantification and structural analysis of intact TAG produced in DGAT-catalyzed reactions. A few examples of these MS-based assays are presented here. In the last several decades, advances in MS, liquid chromatography (LC) and robotics of liquid-handling have made it possible to analyze low concentrations of relatively small organic compounds in HTP formats [133,134]. MS-based approaches have been particularly useful for screening for small molecular weight inhibitors of human DGATs [132,135]. The mass-resolving capability and high sensitivity of MS-based methods facilitates miniaturization. MS generates gas phase ions from TAG. Relative ion abundances are plotted versus mass-to-charge ratios *(m/z)* providing both quantitative and qualitative information. Thus, MS represents another direct way to examine TAG produced via a DGAT-catalyzed reaction. In many applications, LC has been used to purify TAG extracted from the reaction mixture prior to generation of gas phase ions. Furthermore, the technique of MS/MS provides for two levels of selection resulting in increased selectivity and sensitivity for TAG signal detection. Stable isotopically labeled TAG ([^{13}C]1,2,3-trioleoyl-*sn*-glycerol) was used to stabilize the MS signal [132,135]. An example of a set-up for an LC/MS/MS system consisted of a Thermo Betabasic C4 column, four Agilent binary HPLC pumps and a TSQ Vantage triple quadrupole MS/MS instrument [132]. DGAT-catalyzed reactions (40 μL) were run in deep-well 384 plates followed by plate-based solvent extraction, vortexing and centrifugation. Samples (2 μL) from the top organic phase were injected into the C4 column. The LC/MS/MS approach was shown to offer a considerably greater signal selectivity and sensitivity compared to fluorescence-based assays which indirectly quantify CoA concentration (to be discussed later).

If different molecular species of TAG are analyzed via MS, it would be important to take into consideration their different ionization efficiencies. Thus, the use of appropriate correction factors may be required. This aspect may not be an issue, however, in large scale screening for DGAT inhibitors wherein production of only 1,2,3-trioleyl-*sn*-glycerol is quantified via MS.

Molecular species of TAG have also been resolved intact by GC at higher temperatures than are used for conventional GC of FAMEs. If the DGAT under investigation can utilize DAGs composed of short fatty acyl chains such as 1,2-dihexanoyl-*sn*-glycerol or 1,2-dioctanoyl-*sn*-glycerol, the volatility of the TAG produced by a DGAT-catalyzed reaction is increased [136]. Thus, TLC-purified TAG can be readily resolved by GC at high temperature coupled with analysis of TAG using MS. Bouvier-Navé et al. (2000) used this approach to analyze the activity of recombinant AthDGAT1 in subcellular fractions from transformed yeast [131]. High temperature GC/MS has also been used as a method to quantify BnaDGAT1 (isoform BnaC.DGAT1.a) activity using microsomes prepared from *S. cerevisiae* H1246 expressing *BnaDGAT1* [137]. As previously indicated, this yeast strain is deficient in TAG synthesis. Extracted TAGs produced in the reaction mixture were resolved intact by GC and the gas phase ions generated by MS were analyzed by selected ion monitoring. The use of 1,2-dioctanoyl-*sn*-glycerol as an acyl acceptor increased the volatility of the TAG produced in the reaction mixture. The GC/MS method was highly correlated (R^2=0.9901) with the radiometric method using [1-^{14}C] acyl-CoA as an acyl donor over a wide range of microsomal protein content.

Electrospray ionization (ESI)/MS has been used to assay recombinant EaDAcT, also produced in *S. cerevisiae* strain H1246, without the need for an initial chromatographic separation of TAG [138]. As previously indicated, this enzyme was shown to exhibit enhanced specificity for acetyl-CoA as an acyl donor [81]. The ESI/MS-based assay utilized [1,2-^{13}C] acetyl-CoA to overcome interference caused by high levels of acetyl-TAG found in the microsomes. Endogenous DAG served as the acetyl acceptor. The [^{13}C] acetyl-TAG produced during the enzyme-catalyzed reaction could be effectively monitored by scanning for the neutral loss of the heavier [^{13}C]-labeled acetate group against an endogenous background of normal acetyl-TAG. The ESI/MS-based enzyme assay could detect as little as three picomoles of individual molecular species of acetyl-TAG and resulted in kinetic data for EaDAcT which were in close agreement with the conventional radiometric assay based on the use of [^{14}C] acetyl-CoA as an acyl donor [81].

Matrix-assisted laser desorption/ionization-time-of-flight MS has also been used in the analysis of molecular species of TAG produced by DGAT-catalyzed reactions [139].

Fluorescent substrate analogs have also been used in the direct assay of DGAT activity. One version of this assay approach used the commercially available fluorescent acyl donor [N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)-methyl] amino] (NBD)-palmitoyl-CoA [103]. Membranes from mammalian cells were assayed for DGAT activity by monitoring the production of NBD-TAG. Following extraction of the reaction mixture with organic solvent followed by TLC, newly synthesized NBD-TAG was quantified using an imaging system. NBD has excitation and emission wavelengths of 465 nm and 535 nm, respectively. Recently, this method was used to assay yeast H1246 microsomes containing recombinant forms of soybean (*Glycine max*) DGAT1 [140]. Another direct assay method utilized a fluorescently-labeled DAG [141]. The NBD was bonded to a hexanoyl moiety at the *sn*-2 position of the DAG with a hexadecanoyl moiety at the *sn*-1 position. The assay, however, was subject to possible interference by PDAT since this acyltransferase also uses DAG as a substrate. The assay may also be subject to possible interference by acyl-exchange reactions. Microsomes from rat liver and three species of algae were used as a source of DGAT. A modified version of the method was used in the assay of purified soluble recombinant truncated forms of *Yarrowia lipolytica* DGAT2 fused to maltose binding protein [142]. In this case, NBD was bonded to a decanoyl moiety at the *sn*-1 position of DAG with a decanoyl moiety at the *sn*-2 position. Acyl-CoA specificity studies were also conducted using various acyl donors. Conversely, NBD-palmitoyl-CoA could be used to gain insight into DAG specificity using different molecular species of DAG. Assays with either NBD-acyl-CoA or NBD-DAG avoid problems with the precautions and costs associated with assays using radiolabeled substrates. It is important to note, however, that incorporation of NBD results in DGAT substrates with bulky substituents which may, in some cases, result in altered enzyme activity when compared to the use of natural substrates.

2.3. Indirect assays

DGAT activity has also been assayed indirectly by monitoring the appearance of free CoA using both colorimetric and fluorescence-based methods. It may also be possible to monitor the appearance of CoA directly based on the absorption properties of CoA using spectrophotometry (e.g. [143]). A major advantage of these types of assays is that lipid extraction is not required. DGAT assays based on the appearance of CoA, however, are subject to potential interference by other biochemical reactions producing CoA [132]. Thus, it may not be possible to effectively use these assays until the DGAT under investigation is highly purified or recombinantly produced at relatively high level in a suitable host (e.g., yeast expression system). In the colorimetric assay of DGAT activity, the reagent 5,5'-dithiobis(2-nitro benzoic acid) (DTNB), also known as Ellman's Reagent [144,145], reacts with the sulfhydryl group of CoA producing a colored compound with an absorption maximum of 412 nm [38]. Some researchers have used the DTNB-based approach for end point assays [38,139]. The enzyme reactions were terminated by adding equal volumes of 1% (w/v) trichloroacetic acid to the reaction mixtures followed by centrifugation to pellet precipitated protein. Aliquots of the supernatant were then transferred to the wells of a microtiter plate. DTNB solution was added, and after color development, the absorbances were measured spectrophotometrically. In other instances, DGAT activity as assayed in real time by including DTNB in the reaction mixture. This approach has involved the use of 1 mL cuvettes and a recording spectrophotometer [146] or by use of a microtiter plate system wherein measurements were taken for different wells at regular intervals following initiation of the reactions [78]. The real time version of the assay, however, may not be applicable in situations where DTNB affects the activity of the DGAT being assayed. For example, microsomal recombinant *S. cerevisiae* DGAT2 produced in yeast strain H1246 was inhibited by DTNB [147].

7-Diethylamino-3-(4'-maleimidyl-phenyl)-4-methylcoumarin (CPM) contains a thiol-active maleimide and the fluorescence emission of this agent increases upon reaction with the sulfhydryl group of CoA [148]. HTP end point assays using microtiter plates have been conducted with recombinant microsomal mammalian DGATs produced using a baculovirus expression system involving Sf9 insect cells [132,149]. The use of 1% Triton X-100, a detergent concentration that will solubilize DGAT from the lipid membranes, in the reaction mixture increased the assay window by more than 10-fold for analysis of the activity of recombinant microsomal human DGAT1 and the assay was useful in characterizing inhibitor action [149]. Recently, the fluorescence-based assay was applied to the characterization of recombinant rice (*Oryza sativa*) microsomal DGAT1 produced in the *S. cerevisiae* H1246 [150].

CoA produced in acyltransferase-catalyzed reactions has also been monitored using a coupled enzyme assay. The CoA generated by the acyltransferase reaction was utilized along with α-ketoglutarate and NAD^+ (and the cofactor thiamine pyrophosphate) by α-ketoglutarate dehydrogenase which catalyzed the formation of succinyl-CoA and NADH [151,152]. The production of NADH was monitored in a 96-well format using an excitation of 340 nm and emission of 465 nm. The coupled enzyme assay was recently applied in the kinetic analysis of purified human DGAT1 [51]. The assay allows for the quantification of DGAT activity in real time and is both sensitive and applicable to HTP formats. This indirect assay, however, would be subject to possible

interference by other biochemical reactions generating CoA. In addition, in situations where test compounds, such as inhibitors, are tested for their effects on the DGAT-catalyzed reaction, the investigator would have to ensure that the α-ketoglutarate dehydrogenase-catalyzed reaction is not affected by these substances.

2.4. Determination of substrate selectivity

As mentioned previously, the substrate selectivity of the DGAT under investigation can be assessed by presenting the enzyme with two or more molecular species of either acyl donor or acyl acceptor. Examples of some approaches to determine DGAT substrate selectivity are discussed here. In one approach for determining acyl-CoA selectivity [98], a equimolar mixture of [1-^{14}C]acyl-CoAs was incubated with microsomal DGAT from various developing seeds of oleaginous plants followed by extraction with organic solvent and purification of radiolabeled TAG by TLC. The radiolabeled TAG from the silica gel was then converted to non-esterified FAs which in turn were resolved by reverse phase TLC. The distribution of radiolabel between/among the resolved FAs, based on liquid scintillation counting, provided insight into the acyl-CoA selectivity of the enzyme. In a similar approach to determine acyl-CoA selectivity, Wiberg et al. (1994) fed microsomes from developing seeds of sunflower (*Helianthus annuus*), *Cuphea procumbens* or castor bean (*Ricinus communis*) with combinations of two molecular species of [^{14}C]acyl-CoA (two combinations of the following: 10:0, 18:1Δ^{9cis} and 12-OH 18:1Δ^{9cis}) [109]. Each reaction mixture contained one molecular species of unlabeled DAG. TLC-purified radiolabeled TAGs generated in the DGAT-catalyzed reactions were converted to FAMEs and analyzed using radio-GC to determine the distribution of FAs at the *sn*-3 position. A more recent study for determining acyl-CoA selectivity of different isoforms of BnaDGAT1 involved incubating recombinant microsomal DGAT (produced in *S. cerevisiae* H1246) with a mixture of [1-^{14}C]palmitoyl-CoA and [1-^{3}H]oleoyl-CoA [153]. The proportions of radiolabeled palmitoyl and oleoyl in isolated TAG is then determined by quantifying the radioactivity from ^{14}C and ^{3}H, respectively, using a two-channel external standard method. In an early study, using particulate fractions from developing seeds of a high erucic acid variety of *B. napus* [154] and DGAT assays using non-radiolabeled acyl-CoA, the percent distribution of various acyl groups at the *sn*-3 position of TAG generated in the reaction mixture was quantified using the Brockerhoff method [155] for assessing the positional distribution of acyl moieties on the glycerol backbone of TAG.

As previously indicated, if the DGAT under investigation can utilize DAGs composed of hexanoyl or octanoyl acyl chains, then the more volatile TAG produced by the DGAT-catalyzed reaction can be resolved using GC (Table 1) [131,136,137]. In an early investigation of the acyl-CoA selectivity properties of DGAT in particulate fractions from developing safflower (*Carthamus tinctorius*) seeds, Ichihara et al. (1988) used 1,2-dihexanoyl-*sn*-glycerol as an acyl acceptor with different combinations of palmitoyl-CoA and stearoyl-CoA, or palmitoyl-CoA and oleoyl-CoA [136]. Following organic solvent extraction and purification of TAG by TLC, the different molecular species of TAG were resolved and quantified using GC-flame ionization detection. More recently, Greer et al. (2014, 2016) used an enriched source of recombinant microsomal BnaDGAT1 in acyl-CoA selectivity experiments with 1,2-dioctanoyl-*sn*-glycerol as an acyl acceptor. Following a simple extraction (without the use of TLC), molecular species of TAG were resolved by GC and then analyzed and quantified by MS using selected ion monitoring [137,156].

Examples of experiments involving substrate selectivity assays of DGAT with different molecular species of DAG are less common than examples of experiments to determine acyl-CoA selectivity. In addition to performing acyl-CoA selectivity experiments with microsomes from developing seeds of sunflower, *C. procumbens* and castor bean, Wiberg et al. (1994) also examined DAG selectivity [109]. Microsomes were presented with combinations of two molecular species of unlabeled DAG and one molecular species of [^{14}C]acyl-CoA (10:0, 18:1 Δ^{9cis} or 12-OH18:1Δ^{9cis}). Different molecular species of radiolabeled TAG were purified by TLC; in some experiments, two TLC runs with two different solvent systems were required to fully resolve the different TAGs. The radioactivity in sections of silica gel containing TAG was measured using a liquid scintillation analyzer. The combined information from acyl-CoA and DAG selectivity experiments allowed the investigators to conclude that *C. procumbens* microsomal DGAT selectively utilized acyl-CoA and DAG species with 10:0 whereas developing castor bean microsomal DGAT selectively utilized substrates with 12-OH 18:1Δ^{9cis}. In a more recent example, Yu et al. (2006) examined the DAG selectivity of microsomes from developing seeds of *Vernonia galamensis*, *Stokesia leavis* and soybean [157]. *V. galamensis* and *S. leavis* are known accumulators of seed TAG enriched in vernolic acid (12,13-epoxy-9-octadecenoic acid). Among the various experiments performed, [1-^{14}C]oleoyl-CoA or [1-^{14}C]vernoloyl-CoA were incubated with a combination of 1,2-dioleoyl-*sn*-glycerol, 1-palmitoyl-2-vernoloyl-*sn*-glycerol and 1,2-divernoloyl-*sn*-glycerol. Following the enzyme reaction, lipids were extracted with organic solvent, dried under a stream of N_2 and then redissolved in ethyl acetate and passed through an NH_2 column to remove non-esterified FAs. The effluent was dried under a stream of N_2 and subjected to TLC on a silica gel 60-Å plate using the solvent mixture hexane/methyl tertiary butyl ether/acetic acid (75:25:1, v/v/v). Resolved radiolabeled molecular species of TAG were visualized with a phosphorimager and subsequently scraped off the TLC plate and quantified using a liquid scintillation analyzer. Experiments with [1-^{14}C]vernoloyl-CoA and microsomes from *V. galamensis* or *S. leavis* yielded a similar distribution of label among the three acylated molecular species of DAG with the highest incorporation occurring when 1,2-divernoloyl-*sn*-glycerol was the acyl acceptor. This finding supported the enhanced vernolic acid content observed for the seed TAG of *V. galamensis* or *S. leavis*. The increased polarity of molecular species of TAG containing vernoloyl moieties decreased their mobility during TLC, thus making it possible to resolve the various TAG species.

Investigations with recombinant DGATs from higher plants have shown that the molecular species of acyl-CoA substrate and DAG substrate can exhibit a combinatorial effect on enzyme activity [120]. In addition, the use of short chain DAG substrates, such as 1,2-dihexanoyl-*sn*-glycerol, may not be indicative of the *in planta* specificity that is observed with more natural DAG substrates comprised of long acyl chains [104,120], Artificial short chain DAG substrates, however, are attractive due to their increased solubility in comparison to long chain DAGs such as 1,2-dioleoyl-*sn*-glycerol. Combinatorial substrate effects on higher plant DGAT activity and the use of artificial DAG substrates are discussed in more detail in section 6.2.2.3.

2.5. Determination of kinetic parameters

Although numerous studies have determined the kinetic parameters in the characterization of various DGATs, it is important to note that the investigator is dealing with a complex assay mixture involving dispersed acyl acceptor and production of insoluble TAG. The situation becomes further complicated when the DGAT is introduced into the reaction mixture in a particulate form such as microsomes. The kinetics of an acyl-CoA-dependent acyltransferase, such as DGAT, may also be affected by the detergent properties of acyl-CoAs if acyl donor concentrations reach or exceed the critical micelle concentration of the thioester. At its critical micelle concentration, the acyl-CoA will self-associate to form micelles and the monomer will no longer be the only form of thioester in solution [158,159]. In some studies, relatively high concentrations of acyl-CoA have been shown to inhibit acyltransferase activity [39,160,161], but this was not always the case [51,99,139,149,150]. In addition, as previously discussed, the insoluble nature of long chain DAGs requires that the acyl acceptor be dispersed in non-ionic detergent or dissolved in a water-miscible organic solvent, such as ethanol, prior to being introduced into the reaction mixture. Thus, the term “bulk concentration” is sometimes used to describe changes in the apparent

concentration of DAG in plots of initial reaction velocity versus acyl acceptor concentration [115]. Given the above complexities, it is appropriate to refer to "apparent" V_{max}, K_m and $S_{0.5}$ (substrate concentration at 50% of maximum velocity; for an allosteric situation).

2.6. Interference by endogenous diacylglycerol

Membrane fractions used in DGAT assays often contain endogenous DAG which can potentially interfere with the assay, especially when studying DAG specificity and/or selectivity [3,29,131,162–164]. In addition, as indicated previously, if using radiolabeled DAG, the presence of unlabeled endogenous DAG can result in dilution of the radiolabeled acyl acceptor, thus reducing the specific activity of the enzyme preparation. When using a specific quantity of certain molecular species of exogenous DAG for the assay of microsomal DGAT containing endogenous DAG, one cannot simply correct for the enzyme activity by subtracting the enzyme activity in the absence of exogenous DAG from the activity in the presence of exogenous DAG. In assay situations involving endogenous DAG, the overall DGAT activity is likely supported by a complex mixture of different molecular species of DAG. In the case of DGAT assays using microsomes from developing embryos of maize (*Zea mays*), the level of [1-^{14}C]oleoyl incorporation, from [1-^{14}C] oleoyl-CoA into TAG was essentially the same in the absence or presence of 0.3mM 1,2-dipalmitoyl-*sn*-glycerol [143]. Thus, DGAT from maize embryo microsomes prefers utilizing endogenous DAG. Given these complications, some investigators have conducted assays of microsomal DGAT which only rely on endogenous DAG as an acyl acceptor [138,165].

An earlier investigation by Slack et al. (1985) demonstrated that assays of microsomal CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase (CPT) activity from developing cotyledons of safflower benefited from the introduction of radiolabeled DAG dissolved in benzene to freeze-dried microsomes [166]. CPT catalyzes the transfer of a phosphocholine moiety from CDP-choline to DAG resulting in the formation of PC and cytidine monophosphate [167]. After the removal of benzene using a stream of N_2 gas, the dried microsomes were resuspended in water at 0°C and then used in assays with unlabeled CDP-choline [166]. Improved dependence of microsomal DGAT activity on exogenous DAG has also been achieved by freeze-drying microsomes prior to the addition of exogenous DAG in the presence of benzene. Following the removal of benzene by evaporation, the redried microsomes are sonicated on ice in presence of assay buffer prior to initiating the reaction. Either radiolabeled DAG or acyl-CoA can be used. In the case of microsomes from maize embryos, PC or Tween 20 was introduced into the reaction mixture along with exogenous DAG [143]. Examples of this DGAT assay procedure, with slight variations in the protocols, have been reported for microsomes from developing seeds [118,143,168], bovine muscle and adipose tissue [169–171] and *S. cerevisiae* H1246 producing a recombinant yeast DGAT2 [172]. Endogenous DAG has also been removed from yeast microsomes, producing recombinant EaDAcT, by treatment with cold acetone [173].

2.7. Cell-based assays

Cell-free *in vitro* assays of DGAT activity typically involve the use of relatively high substrate concentrations and conditions wherein the initial reaction velocity is observed. In contrast, cell-based assays of DGAT activity are affected by substrate availability and various cellular processes. Thus, cell-based assays provide an indication of DGAT performance under more physiological conditions (Table 1). In medical research, cell-based systems have been particularly useful for probing the effects of small molecular weight DGAT inhibitors [132,149,174,175]. Some of these methods have involved pretreating cells with DGAT activity with inhibitor followed by feeding radiolabeled oleic acid (18:1) and then checking for the level of [^{14}C]TAG produced [149,175]. In an example study, Cao et al. (2011) examined inhibitor effects in HEK293A mammalian cells over-expressing the cDNA encoding human DGAT1 [149]. Cells were preincubated with small molecular weight DGAT1 inhibitors at various concentrations for 1 hour followed by incubation with 10 μM [^{14}C]oleic acid (50 Ci/mmol), with 0.1% FA-free bovine serum albumin, for 4 hours. Cellular lipids were then extracted, and TAG and PL isolated using TLC. The lipid classes were quantified based on phosphorimaging signals from the radiolabeled lipids. Other mammalian cell-based assays of DGAT action involved inhibitor treatment and feeding of the stable isotope of oleic acid [^{13}C] [132,174]. Lipids were extracted and TAG was purified and quantified using LC/MS/MS in a HTP format.

One particularly attractive and useful cell-based assay involves the use of *S. cerevisiae*, strain H1246, wherein four genes encoding enzymes contributing to TAG production were disrupted [85]. The genes *DGA1*, *LRO1*, *ARE1* and *ARE2* encode the proteins Dga1, Lro1p, Are1p and Are2p, respectively (Fig. 6). The nomenclature used here is based on rules put forward by the yeast community [176]. Dga1 and Lro1p represent enzymes with DGAT and PDAT activity, respectively. Are1p and Are2p primarily catalyze the synthesis of SEs. Since the yeast strain was still viable with no apparent growth defects under standard conditions, it was concluded that storage lipid biosynthesis was non-essential in *S. cerevisiae*. Thus, without interference from endogenous TAG biosynthesis, *S. cerevisiae* H1246 represents an excellent host system for producing recombinant DGATs from various sources (e.g., [94,177–180]). The strain has also been used as a host for producing recombinant PDATs (e.g., [36,180]). A HTP procedure for quantifying intracellular NPLs produced by the action of recombinant DGAT in *S. cerevisiae* H1246 has been developed which is based on the fluorescence of the NPL-staining agent Nile red (9-diethylamino-5H-benzo[α] phenoxazine-5-one) [94,181,182]. The predominant NPLs of eukaryotic cells are TAG and SE [48]. In some examples of applications, Nile red has been used to quantify the NPL content of bacteria [183], fungi [184], microalgae [185,186], *B. napus* microspore-derived cell suspension cultures [187] and mammalian oocytes [188]. Recombinant castor bean DGAT1, along with various truncations of the enzyme, were produced in the yeast strain H1246 followed by quantification of NPLs using the Nile red assay and *in vitro* assays of microsomal DGAT activity [94]. There was a strong positive correlation between endogenous NPL content based on fluorescence analysis and microsomal DGAT activity. Exogenous application of oleate or other unsaturated FAs has been shown to inhibit the growth of *S. cerevisiae* strains devoid of TAG biosynthesis [48,94,181,189–191]. When a functional recombinant DGAT was introduced into *S. cerevisiae* H1246, however, the action of the enzyme rescued the yeast strain from the toxic effects of oleate and restored growth. Both oleate toxicity and the HTP Nile red assay formed the basis of a directed evolution approach for enhancing the performance of BnaDGAT1 [95,181,182]. *BnaDGAT1* cDNA was subjected to error-prone PCR and the resulting variants were expressed in yeast strain H1246. Addition of 1 mM oleate to the growth medium facilitated the identification of functional DGAT variants while excluding non-functional enzyme variants which could not rescue the yeast from the toxic effects of oleate. Yeast expressing the remaining functional variants were then subjected to screening for NPL production using the Nile red assay. Putative high-performance DGAT variants identified via the directed evolution approach with Nile red staining were confirmed via quantification of extracted TAG [95] and microsomal DGAT assays [96]. *S. cerevisiae* features a mechanistic connection between the import and activation of exogenous FAs [192]. Thus, exogenous FAs can be fed to *S. cerevisiae* H1246 expressing a functional recombinant DGAT to provide insight into the acyl selectivity properties of the enzyme under cellular conditions [193].

Escherichia coli has been used extensively as a model system to investigate bacterial lipid synthesis [194]. Although *E. coli* does not naturally produce TAG, the microorganism has been engineered to produce this lipid through the introduction of recombinant DGAT from another source [57,69,73,183,195]. Experiments to gain insight into

recombinant DGAT activity under conditions in the *E. coli* intracellular environment involved feeding [1-^{14}C]acetate followed by lipid extraction, and purification and analysis of radiolabeled TAG [57,69]. HTP screening of *E. coli* cells for NPL production using Nile red has recently been applied in the directed evolution of a WSD from *Thermomonospora curvata* [183]. Bacteria with WSD variants resulting in a substantially increased Nile red response were also analyzed for TAG content. The variant with amino acid substitution P35L resulted in 2.5 times more TAG than the wild type (WT).

3. Bacterial DGATs

3.1. Triacylglycerols and wax esters in bacteria

In most storage lipid-producing bacteria, the bifunctional WSD catalyzes the last step in both TAG and WE biosynthesis. Therefore, TAG and WE will be briefly discussed in this section prior to a discussion of DGAT in bacteria. Many prokaryotes can accumulate storage lipids, with only a few exceptions, which generally exist in nutrient rich habitats such as lactobacilli and methanogens (for a recent review, see [196]). Some actinomycetes and streptomycetes can accumulate 10-80% of their cellular dry weight (DW) as TAG, while some species in the Gram-negative genera *Acinetobacter* and *Alcanivorax* can produce minor amounts of TAGs [196–204]. A variety of *Acinetobacter* spp. can synthesize large amounts of WE as the predominant storage lipid, and some species in *Moraxella*, *Micrococcus*, *Alcanivorax* and other genera have the capacity to accumulate certain levels of WEs [196,198,200,203,205–211]. A few oleaginous species such as *Rhodococcus opacus* PD630 have been used as model oleaginous organisms for NPL metabolism studies and industrial strains for TAG production [5].

The accumulations of storage lipids in bacteria are substantially influenced by cultivation conditions and are generally promoted under stresses such as nitrogen deficiency and adverse environmental conditions [17,212]. Bacterial TAG and WE consist of mainly long-chain saturated and monoenoic FAs such as palmitic and oleic acids, and their compositions can vary largely depending on culture medium and conditions [213–216]. Many heterotrophic bacterial species can produce unusual TAGs and WEs when grown in the presence of different hydrocarbons as carbon sources [73,217,218]. In addition, although cyanobacteria (also called blue-green algae) are a phylum of gram-negative bacteria that typically do not accumulate TAG, several species such as *Nosto commune* and *Synechocystis* sp. PCC 6803 can synthesize TAG [219–221]. Recently, slr2103 from *Synechocystis* sp. PCC 6803 has been reported to have DGAT activity and to be involve in TAG and phytyl ester biosynthesis, and interestingly slr2103 is not a WSD ortholog [221]. To simplify the discussion, the term bacteria will refer to species other than cyanobacteria, whereas cyanobacteria will be discussed separately at the end of each subsection.

Storage acyl lipids have important physiological functions in bacteria. Acyl moieties in these biomolecules are in their most reductive form and their oxidation can provide more energy than other storage compounds [197]. As the major storage compounds for energy and carbon in some bacterial species, TAGs and WEs may play important roles for the prokaryote's survival under adverse environments [222]. Storage lipids may also have other important physiological functions in bacterial cells, such as contributing to the biosynthesis and regulation of membrane lipid composition, detoxifying FAs from plasma membrane and phospholipid biosynthesis, and serving as a source of intermediates for secondary metabolism [223,224]. In addition, TAG may have special functions in the long-term survival and drug resistance of *Mycobacterium tuberculosis*, which causes tuberculosis and has infected approximately one quarter of the world's population [225].

Bacterial TAGs and WEs also have potential applications as renewable feedstocks to produce biofuel, oleochemicals, cosmetics, nutritional or pharmaceutical products, and other bioproducts [220,226]. Some oleaginous bacteria are promising candidates for the commercial production of TAGs and WEs on a large scale. These species can be cultured in fermenters under controlled conditions with minimal influence of environmental factors. Thus, there is great potential to convert agriculture waste, forest by-products and other industrial wastes to lipids, which can be engineered with biotechnology without concerns of social barriers related to genetic modification.

In recent years, the rising interests of academia and industry in bacterial storage lipids have promoted the research on the biochemical and molecular basics of storage lipid biosynthesis. In the following subsections, TAG and WE biosynthesis in bacteria is briefly described, followed by discussion of the biochemical and physiological properties of the enzymes possessing DGAT and/or WS activities. The biotechnological application of the enzymes will be discussed in Section 9.1.

3.2. Overview of triacylglycerol and wax ester biosynthesis in bacteria

In general, TAG formation in lipid-accumulating bacteria involves *de novo* FA biosynthesis and storage lipid assembly [12,194,227,228]. *De novo* FA biosynthesis starts with the ATP-dependent formation of malonyl-CoA catalyzed by acetyl-CoA carboxylase (ACCase), which consists of multiple subunits including biotin carboxylase, biotin carboxyl carrier protein and carboxyltransferase in most prokaryotes [229]. The fatty acyl chain is then synthesized by the fatty acid synthase multienzymatic complex that uses malonyl-ACP (from malonyl-CoA) as a two-carbon donor [194]. FA chains can be extended up to 16 or 18 carbons in length while attached to the acyl carrier protein (ACP) subunits of the fatty acid synthase complex. Fatty acids are released in a reaction catalyzed by thioesterases (also known as fatty acid thioesterase and named TesA in *E. coli*) and the unesterified FAs can be converted to fatty acyl-CoAs by acyl-CoA synthetase (also named FadD in *E. coli*). Both acyl-ACP and fatty acyl-CoA can be used in the assembly of storage lipids in prokaryotes.

A generalized scheme for the assembly of TAG and WE in prokaryotes is outlined in Fig. 5. TAG biosynthesis in bacteria is mainly carried out via the Kennedy pathway, which involves the sequential acylation of the glycerol backbone of *sn*-glycerol-3-phosphate (G3P) [230]. The first step is catalyzed by acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase (GPAT; also named PlsB in *E. coli*) to produce lysophosphatidic acid (LPA; also known as acylglycerol-3-phosphate) from G3P. In *r*-proteobacteria, this enzyme is localized to the cytoplasmic membrane and can use both acyl-CoAs and acyl-ACPs as acyl donors [230,231], In bacterial species not belonging to the *r*-proteobacteria class, this reaction is catalyzed by PlsY (23 kDa), which is a unique class of membrane-bound GPAT enzymes that uses acyl-phosphate (acyl-P) as an acyl donor [232]. Acyl-P is produced from acyl-ACP with the catalysis of a soluble phosphate acyl-ACP acyltransferase PlsX [232–235].

LPA is then converted to phosphatidic acid (PA) by the catalytic action of acyl-CoA:lysophosphatidic acid acyltransferase (LPAAT; also known as PlsC). The first bacterial LPAAT was identified in *E. coli* [236,237], followed by the characterization of homologous enzymes in other bacterial species. *E. coli* LPAAT can use both acyl-ACP and acyl-CoA as substrates, while those from gram-positive bacteria such as *Bacillus subtilis* can only use acyl-ACP as an acyl donor [232,237,238]. *E. coli* has two LPAATs enzyme, and Multiple LPAATs with overlapping or distinct functions have been identified in some bacterial species such as *Neisseria meningitides* [239], *Pseudomonas fluorescens* [240] and *Shewanella livingstonensis* [241].

GPAT and LPAAT share the highly conserved catalytic motif HxxxxD [242]. The crystal structure of *Thermotoga maritima* LPAAT reveals that this enzyme has an unusual hydrophobic/aromatic N-terminal two-helix motif linked to an acyltransferase αβ-domain that contains HxxxxD, which anchors the protein to one leaflet of the membrane. This allows the active site to use soluble acyl-donors to acylate lysophospholipids within the membrane bilayer [243].

PA can subsequently be dephosphorylated to produce DAG by a reaction catalyzed by phosphatidate phosphatase (PAP; also named PgpB

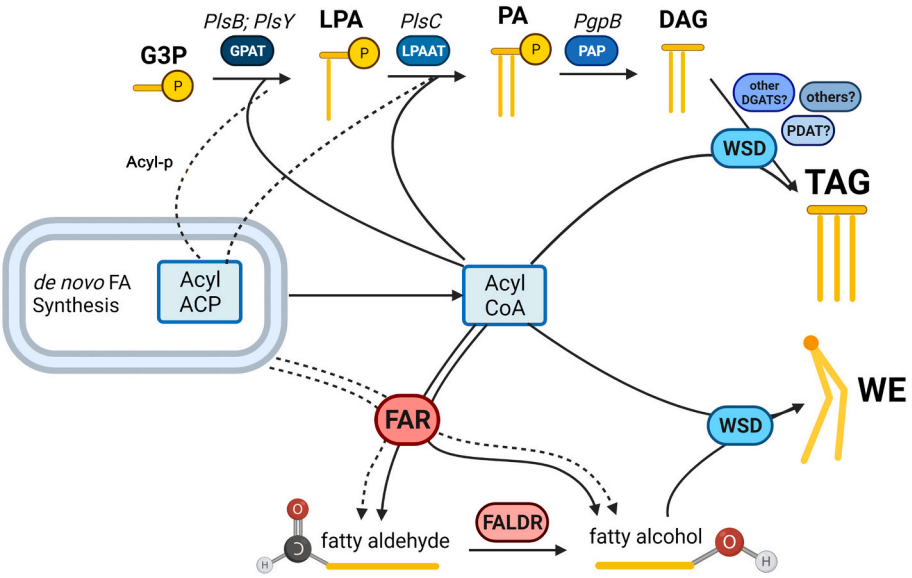

Fig. 5. Generalized scheme for triacylglycerol (TAG) and wax ester biosynthesis in bacteria. Abbreviation: ACP, acyl carrier protein; Acyl-P, Acyl-phosphate; CoA, coenzyme A; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; FA, fatty acid; FALDR, fatty aldehyde reductase; FAR, fatty acyl reductase; GPAT (also known as PlsB/PlsY), acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase; G3P, *sn*-glycerol-3-phosphate; LPA, lysophosphatidic acid; LPAAT (also known as PlsC), acyl-CoA:lysophosphatidic acid acyltransferase; PA, phosphatidic acid; PAP (Also known as PgpB), phosphatidic acid phosphatase; PDAT, phospholipid:diacyglycerol acyltransferase; TAG, triacylglycerol; WE, wax ester; WSD, bifunctional wax ester synthase-diacylglycerol acyltransferase. Dotted lines represent possible pathways.

in *E. coli*) [244]. Two types of PAP enzymes (Mg^{2+}-dependent (PAP1) and Mg^{2+}-independent (PAP2)) have been identified in eukaryotes (for review, see [245]). However, only PAP2 has been identified in prokaryotes [246–249]. The first bacterial PAP2 enzyme was identified from *E. coli*. This 28-kDa membrane-associated enzyme uses PA and phosphatidylglycerol phosphate as substrates to generate DAG and phosphatidylglycerol, respectively [246,250]. The recent structural studies reporting its apo and lipid-bound forms substantially expanded our understanding of the mechanisms of substrate binding and catalysis by PAP2 [251–254]. PAP2s have also been characterized in other bacterial species, such as *B. subtilis* [255], *S. coelicolor* [248], and *Mycobacterium smegmatis* [249]. In addition to the biosynthesis of DAG for storage lipids, PA also serves as the precursor for membrane phospholipids. PA can also be generated by the phosphorylation of DAG in a reaction catalyzed by DAG kinase (dgk in *E. coli*) [12,194,227,228]. Therefore, the production of storage lipids and phospholipids can be competitive under cellular regulation in terms of carbon flow.

The last step in the acyl-CoA-dependent biosynthesis of TAG is mainly catalyzed by WSD in bacteria. Unlike the existence of DGAT1, DGAT2 and other enzymes with DGAT activities in plants and mammals, WSD appears to be the most common DGAT type catalyzing TAG synthesis in bacteria. Bacterial WSD (AbaWSD, also known as AtfA) was first identified and characterized from the gram-negative bacterium *A. baylyi* ADP1 (formerly known as *A. calcoaceticus* ADP1), which can accumulate WEs and TAGs intracellularly during cultivation under growth-limiting conditions [73]. WSDs have been subsequently identified from various bacterial species [38,256–261].

Some representative WSDs that have been functionally characterized are listed in Table 2. Nevertheless, when the *AbaWSD*-like homologous genes were disrupted in *Streptomyces* species, the strains could still synthesize TAG and the crude cell extracts showed considerable acyl-CoA-dependent DGAT activities [38,262], indicating the existence of unknown DGATs. In addition, other bacterial acyltransferases may also have DGAT activity, such as the *M. tuberculosis* mycolyltransferase Ag85A [139].

The bifunctional WSDs also catalyze the biosynthesis of WEs from acyl-CoA and long-chain fatty alcohol in bacteria [73]. Long-chain fatty alcohols can be synthesized from acyl-CoA or acyl-ACP, either in one-step catalyzed by a fatty acyl reductase (FAR), or in two steps catalyzed by FAR and then fatty aldehyde reductase (FALDR) with fatty aldehyde as the intermediate compound (Fig. 5) [228,263–269]. In addition, some bacterial WSDs have been reported to have only WS activity [257,270].

Although the acyl-CoA independent formation of TAG by a reaction catalyzed by PDAT has been reported in animals, plants, microalgae and yeast, this type of enzyme has not been identified in bacteria yet. Nevertheless, phospholipid: diacylglycerol acyltransferase activities have been detected in bacteria [38]. When *S. coelicolor* cell extract was incubated with radiolabeled DAG and PL substrates (especially PE) in an *in vitro* assay, TAG was produced. This PDAT activity was not affected by the disruption of the AbaWSD-homologous genes [38]. Further studies are needed to explore the occurrence of acyl-CoA-independent synthesis of TAG and identify the enzymes in bacteria.

Bacterial TAG and WE are generally stored in LDs, which are similar to LDs in eukaryotes. Our knowledge regarding the biogenesis and structural and functional dynamics of LDs, however, is rather limited [196,271,272]. The formation of LDs has been observed and analyzed in *A. baylyi* ADP1 and *R. opacus* PD630 [17]. Some LD-associated proteins have been identified from *Rhodococcus jostii* RHA1, *R. opacus* PD630, and *M. tuberculosis* [273–276]. For example, a putative heparin-binding hemagglutinin homolog protein tadA (Triacylglycerol Accumulation Deficient) was reported to localize to LDs in *R. opacus PD630*. When *tadA* was disrupted, TAG levels were reduced by 60-70% compared to the WT [274]. In *M. tuberculosis*, the phage shock protein ortholog Rv2744c localized to the surface of LDs and regulated their number and size [276,277]. Omics studies have identified many bacterial proteins associated with LDs, and about 200 of these proteins have been previously shown to associate with LDs and have known roles in lipid metabolism [273,275]. Further analysis of these LD-associated proteins using biochemical and molecular approaches would expand our knowledge of LD formation in bacteria.

Cyanobacteria have high photosynthetic yields and can convert up to 10% of solar energy to biomass, which is much higher than eukaryotic microalgae and conventional energy crops such as sugarcane (*Saccharum officinarum*) or corn (*Z. mays*), and thus are considered as attractive platforms for the production of fatty acids and their derivatives [278,279]. Genomic analysis has revealed that genes related to FA biosynthesis are highly conserved between cyanobacteria and *E. coli* [220]. The first condensation reaction catalyzed by β-keto-acyl-ACP synthase III (KAS III, also known as fabH in *E. coli*) seems to be the sole rate-limiting step of the FA synthase in *Synechococcus* sp. PCC 7002 [280]. Moreover, the crystal structures of several enzymes in *de novo* FA synthesis of *Synechocystis* sp. have been reported at the resolution range of 1.54 to 2.42 Å [281–283]. The first cyanobacterial DGAT has been recently identified in *Synechocystis* sp. PCC 6803 [221]. This enzyme encoded by *Slr2103* has both DGAT and PES activities and does not

Table 2
Some characterized bifunctional wax ester synthase-diacylglycerol acyltransferases (WSDs) and diacylglycerol acyltransferases (DGATs) in bacteria

Strain	NCBI Reference Sequence	Protein names in this review	Protein names used in publications	Protein ID used in publications	Reference
Acinetobacter baylyi ADP1	*WP_004922247.1*	*AbaWSD*	AtfA; Aba-WS/DGAT; Ac1; AbWSD1	ACIAD0832	[294]
Marinobacter hydrocarbonoclasticus DSM 8798 (*M. nauticus ATCC 49840*)	ABO21020.1	MhyWSD1	WS1	ABO21020	[257]
	ABO21021.1	MhyWSD2	WS2	ABO21021	[257,297]
Marinobacter aqualeolei VT8	WP_011783747.1	MaqWSD1	Ma1; Ma-WS/DGAT1	Maqu_168	[268,296]
	WP_011786509.1	MaqWSD2	Ma2; Ma-WS/DGAT2	Maqu_3067	[268]
	ABM20482.1	MaqWSD5	MaWSD5		[270]
Alcanivorax borkumensis SK2	WP_011590012.1	AboWSD1	AtfA1	ABO_2742	[261]
	WP_011589085.1	AboWSD2	AtfA2	ABO_1804	[261]
Streptomyces coelicolor (Strain ATCC BAA-471 / A3(2) / M145)	WP_011027469.1	ScoWSD1		SCO0958	[38]
Streptomyces avermitilis MA-4680	WP_010988651.1	SavWSD		SAV7256	[260]
Streptomyces sp G25	WP_067270919.1	SspWSD1	Atf-G25	STSP_03080	[262]
Rhodococcus opacus PD630	EHI42943	RopWSD1	Atf1	OPAG_07257	[286]
	EHI41112	RopWSD2	Atf2	OPAG_00138	[259]
Rhodococcus jostii RHA1	WP_011594556.1	RjoWSD6	Rh1; Atf6		[146,287]
	WP_011597548.1	RjoWSD8	Atf8		[146]
Mycobacterium tuberculosis H37Rv	CCP45940.1	MtuWSD1	Tgs1	Rv3130c	[258,311,312]
	CCP46561.1	MtuWSD2	Tgs2	Rv3734c	[258]
	CCP46053.1	MtuWSD3	Tgs3	Rv3234c	[258]
	CCP45897.1	MtuWSD4	Tgs4	Rv3088	[258]
	CCP46633.1	MtuAg85A	Mycolyltransferase Ag85A	Rv3804c	[139]
	CCP46192.1	Rv3371		Rv3371	[284]
Mycobacterium bovis BCG Pasteur 1173P2	WP_003899932.1	MboWSD1	Tgs1	BCG3153c	[285]
	WP_003420440.1	MboWSD2	Tgs2	BCG3794c	[285]
Mycobacterium abscessus	CAM63627.1	MabWSD1	Tgs1	MAB_3551c	[313]
	CAM64613.1	MabWSD4	Tgs4	MAB_4544c	[313]
Thermomonospora curvata DSM 43183	ACY99349.1	TcWSD	tDGAT	ACY99349	[288]
Synechocystis sp. PCC6803	BAA17260.1	slr2013		slr2013	[221]

belong to the groups of AbaWSD, DGAT1, DGAT2, or PDAT [221]. In addition, another recent study reported that TAG in this strain might be mainly produced by unknown acyltransferases and not by Slr2103 [219]. A thorough characterization of enzymes involved in lipid metabolism in cyanobacteria would substantially expand our understanding of TAG biosynthesis in this microorganism.

3.3. Properties and physiological roles of bacterial DGATs

3.3.1. Identification of bacterial bifunctional wax synthase-diacylglycerol acyltransferases and their nomenclature

As mentioned previously, the first WSD, AbaWSD, was identified from *A. baylyi* ADP1 [73], which led to the functional characterization of WSDs in several other bacterial species including *Marinobacter hydrocarbonoclasticus* DSM 8798 [257], *Marinobacter aqualeolei* VT8 [268], *Alcanivorax borkumensis* SK2 [261], *S. coelicolor* A3(2) [38], *Streptomyces avermitilis* MA-4680 [260], *Streptomyces sp. G25* [262], *M. tuberculosis* H37Rv [258,284], *Mycobacterium bovis* BCG [285], *R. opacus* PD630 [259,286], *R. jostii* RHA1 [287], *Psychrobacter cryohalolentis* K5, [287] and *T. curvata* [288] (Table 2). Two enzymes with no similarity to AbaWSD, mycolyltransferase Ag85A of *M. tuberculosis* and Slr2013 of *Synechocystis* sp. PCC 6803, also have DGAT activity [139,221].

Although almost all bacterial WSDs are related to AbaWSD from *A. baylyi* ADP1, the enzymes were not named consistently. For example, AtfA, WS1, MA1, Atf-G25, Atf1, Tgs1, and tDGAT are all homologs of AbaWSD (Table 2), but the names are not indicative of their function. The existing nomenclatures are complicated and confusing. In this review, a uniform nomenclature (WSD + number) for bacterial WSDs is used. Other names used in the literature and their corresponding protein accession numbers are presented in Table 2. We have attempted to keep the numbers consistent with those in the literature. For example, *A. baylyi* ADP1 only has one WSD, so AbaWSD is not be given a number. *R. jostii* RHA1 has 14 AbaWSD homologs. Since Atf6 has been used to describe one of these forms in previous publications [146,289], here it is named RjoWSD6. In addition, three letters for strain names are used because two letters are not enough to distinguish some species such as *M. aqualeolei* (Maq) and *Mycobacterium abscessus* (Mab).

3.3.2. WSDs from Acinetobacter baylyi and Marinobacter spp.

The most in-depth biochemical information available for a bacterial WSD is from studies of WSDs from three gram-negative bacteria strains, *A. baylyi* ADP1, *M. aqualeolei* VT8 and *M. hydrocarbonoclasticus* DSM 8798 [5,71–73,257,268,287,290–297]. Therefore, WSDs from these three strains are discussed together in this subsection. AbaWSD has been functionally expressed in *E. coli*, *Pseudomonas citronellolis* [73], *S. cerevisiae* and other microorganisms for biochemical and biotechnological studies [178,298,299]. AbaWSD is an amphiphilic protein consisting of 458 amino acid residues with a theoretical molecular mass of 51.8 kDa and pI of 9.05 [73]. In *A. baylyi* ADP1, AbaWSD has been shown to be predominantly associated with the cytoplasmic membrane and LDs, with some presence in the cytosol [72]. The recombinant AbaWSD in *E. coli*, with molecular mass of 53 kDa, has been purified to apparent homogeneity [72]. This enzyme exhibits a typical high WS activity with classical Michaelis-Menten kinetics and 10-folder lower DGAT activity, which does not conform to Michaelis-Menten or cooperative enzyme kinetics [72]. Although the highest activities of AbaWSD were achieved with C16-CoA and C14-C18 fatty alcohols, this enzyme utilized a broad range of acyl donors such as saturated or unsaturated acyl-CoAs with various acyl chain lengths. AbaWSD also had a broad range of acyl acceptors that included linear alcohols from C2 to C30, branched, cyclic and aromatic alcohols, MAGs and DAGs [72,294,295]. However, AbaWSD cannot use polar substrates such as sugars, amino acids or organic acids [290].

M. hydrocarbonoclasticus is a gram-negative aerobic marine bacterium with the capacity to synthesize isoprenoid WEs [300]. It should be noted that *M. hydrocarbonoclasticus* and *M. aqualeolei* are actually the same species, with the former being the earlier synonym used [301]. WSDs from the *M. hydrocarbonoclasticus* DSM 8798 and *M. aqualeolei* VT8 strains have been reported to have some different properties and therefore are discussed here with different names (Table 2). Four putative WSD genes have been identified in *M. hydrocarbonoclasticus* DSM 8798 but only MhyWSD1 and MhyWSD2 exhibit WS activity [257]. *In*

vitro assays with purified recombinant proteins from *E. coli* revealed that MhyWSD1 had both WS and DGAT activities [257]. MhyWSD2 exhibited high WS activity but had no detectable DGAT activity in *in vitro* assays [257]. Similar to AbaWSD, MhyWSD1 and MhyWSD2 both have broad substrate specificities and can utilize various acyl-CoAs and acyl alcohols, as well as phytol and farnesol [257,297,298]. When *MhyWSD2* was expressed in *S. cerevisiae* deficient in TAG synthesis (strain H1246), the yeast could not produce any TAG [297]. The recombinant protein displayed high WS activity but no DGAT activity in direct *in vitro* assays using radiolabeled substrates. In addition, MhyWSD2 used a broad range of substrates, including substrates not naturally present in *M. hydrocarbonoclasticus* such as ricinoleoyl-CoA and ricinoleoyl alcohol, indicating the potential value of the enzyme in biotechnology [297]. In contrast to MhyWSD1 and MhyWSD2, MhyWSD3 had neither WS nor DGAT activities, and *MhyWSD4* was a pseudogene [257].

Three functional WSDs were characterized from *M. aqualeolei* VT8 [267,270,287,291,292,302]. MaqWSD1 and MaqWSD2 differ from MhyWSD1 and MhyWSD2 by only two amino acid residues (194E/D and 321E/G) and one amino acid residue (395 D/G), respectively. Both enzymes can use various substrates, but MaqWSD2 had significant DGAT activity when assayed using an indirect coupled *in vitro* assay [287,291,302,303]. This finding was surprising since MhyWSD2 had no DGAT activity [297] and differs from MaqWSD2 by only one amino acid residue. Further experimentation is required to shed light on this aspect. In addition, the 3-D structure of MaqWSD1 has been recently reported, which is the first one for this class of enzymes [296]. MaqWSD3 and MaqWSD4 are similar to MhyWSD3 and MhyWSD4, respectively. MaqWSD5 (also referred to as MaWSD5) consists of 452 amino acid residues and shares about 19% sequence identity with AbaWSD [270]. MaqWSD5 has WS activity but no DGAT activity, and its expression in the model plant *Arabidopsis* resulted in the accumulation of 20:1Δ^{11} (hereafter 20:1)/18:1 and 20:1/20:1 WEs, indicating its potential industrial value [270].

3.3.3. Identification and characterization of other bacterial WSDs

A. borkumensis is a gram-negative marine bacterium which can accumulate a large amount of TAG [261]. Two WSDs have been identified in *A. borkumensis* SK2 which share 49% and 40% amino acid sequence identity to AbaWSD, respectively [261]. *In vitro* enzyme assays showed that AboWSD1 (also referred to as AtfA1) exhibits a high level of WS activity with lower DGAT activity, whereas AboWSD2 (also referred to as AtfA2) has high WS activity with negligible DGAT activity. Directed gene insertional mutagenesis in *A. borkumensis* SK2 revealed that AboWSD1, but not AboWSD2, is the main contributor to TAG and WE biosynthesis *in vivo*. However, a double knockout mutant still produced substantial amounts of TAG. Since *AboWSD1* and *AboWSD2* are the only two *AbaWSD*-like genes in the sequenced genome of *A. borkumensis*, the strain likely has an alternative non-WSD-dependent TAG biosynthesis pathway [261,304].

Streptomyces are gram-positive bacteria with a complex secondary metabolism with some strains accumulating TAG in the stationary phase [305]. DGAT activity has been reported for *S. coelicolor*, *S. avermitilis* and *Streptomyces lividans* [38,260,305]. *S. coelicolor* M145 has three putative AbaWSD homologs Sco0958 (ScoWSD1), Sco1280 and Sco0123 [38]. Deletion and over-expression of *ScoWSD1* (sco0958) in *S. coelicolor* M145 led to 70% decrease and 38% increase in TAG content, respectively, and the recombinant protein showed strong *in vitro* DGAT activity. In contrast, neither Sco1280 deletion or Sco0123 knockout led to detectable decreases in TAG content, and none of the recombinant proteins exhibited DGAT activity [38]. Therefore, ScoWSD1 is the main contributor to TAG biosynthesis in *S. coelicolor* [38]. A triple knockout *S. coelicolor* mutant, however, still had DGAT activity, indicating the existence of uncharacterized acyl-CoA-dependent pathway in the strain [38].

Searching the genome of *S. avermitilis* MA-4680 for deduced amino acid sequences for homologs of WSD, DGAT1 and DGAT2 resulted in one putative WSD-like protein (sav7256; SavWSD) [260]. The recombinant SavWSD exhibited high WS activity and low DGAT activity [260]. In contrast, crude extracts of *S. avermitilis* cells grown under conditions which promote storage lipid accumulation had very low WS activity and very high DGAT activity, which correlated with higher levels of TAG accumulation [260]. Since no other putative homologs of WSD, DGAT1, or DGAT2 could be deduced from the genome of *S. avermitilis* MA-4680, the low DGAT activity of recombinant SavWSD protein but high DGAT activity of the crude cell extracts suggested the possible presence of unknown DGAT isoforms [260], and/or perhaps missing activator(s) in the recombinant protein that is present in the cell extract. In addition, the high WS activity of SavWSD, but with no WE accumulation in the bacterium, indicated that *S. avermitilis* lacked the ability to reduce acyl-CoA to fatty alcohols and therefore could not form WEs. The lack of WE biosynthesis ability is consistent with the fact that *S. avermitilis* has no homolog of *A. baylyi* acyl-CoA-reductase in the genome [260].

Recently, an oleaginous strain *Streptomyces* sp. G25, isolated from desert soil, was shown to contain 41% TAG on a DW basis when glucose was used as a carbon source [204]. A single WSD-encoding gene was identified from the draft genome sequence of the microorganism, and *in vitro* and *in vivo* analyses indicated the enzyme was an active WSD [262]. Although the draft genome sequence only had one WSD-encoding gene, the knockout mutant could still accumulate lower but substantial amounts of TAG, indicating the existence of other enzymes catalyzing the biosynthesis of TAG [262].

Some species in the genus *Rhodococcus* can accumulate very high levels of TAG and therefore could have promising industrial applications [306]. For instance, *R. opacus* PD630 and *R. jostii* RHA1 have been shown to accumulate 76% and 57% of biomass as TAG, respectively, when gluconate is the carbon source [201,289]. *R. opacus* PD630 has 17 AbaWSD homologous and two of them have been functionally characterized [259,286]. The disruption of *RopWSD1* (also knowns as *Atf1*) and *RopWSD2* (also known as *Atf2*) in *R. opacus* PD630 decreased TAG by 50% and 30%, respectively [259,286]. *In vitro* assays revealed that the recombinant RopWSD1 purified from *E. coli* exhibited high WS activity (4.65 pmol/mg/min) and very low DGAT activity (0.37 pmol/mg/min), whereas the recombinant RopWSD2 had high WS activity (4.02 pmol/mg/min) and high DGAT activity (7.19 pmol/mg/min) [286]. Nevertheless, the expression of *MhyWSD1* with the knockout of DAG kinase (*dgkA*) in *E. coli* led to TAG production, while over-expression of either *RopWSD1* or *RopWSD2* in the *E. coli dgkA* mutant did not produce any TAG [307]. Similarly, co-expression of *RopPAP* with either *AbaWSD* or *R. jostii WSD8* (also refer to as *aft8*) in *E. coli* produced TAG, whereas co-expression of *RopPAP* with either RopWSD1 or RopWSD2 did not result in TAG accumulation [308]. The results indicated that RopWSD1 or PopWSD2 might not have strong *in vivo* DGAT activity. It will be interesting to determine if the other putative RopWSDs, especially RopWSD8, contribute to TAG production.

In *R. jostii* RHA1, 14 putative genes encoding WSD-like proteins have been identified [289] and the expression of the encoding genes under different nitrogen conditions was analyzed [146]. *RjoWSD8* was the most highly expressed during nitrogen limitation in the medium and deletion of the gene led to a 70% decrease in TAG content, indicating this WSD has a major role in TAG biosynthesis during this stress [146]. The DGAT activity of RjoWSD8 was confirmed by enzymatic assay [146]. In addition, the purified recombinant RjoWSD6 (also named as atf6 or Rh1) exhibited WS activities and could use a broad range of substrates [287].

Many species in the genus *Mycobacterium* can cause serious diseases in mammals. Some species, such as *M. tuberculosis*, *M. bovis* and *M. abscessus*, can accumulate large amount of TAG in the peripheral deposits associated with the cell envelope and LDs, which may serve as energy reservoir and thus ensure survival of the pathogen during dormancy and reactivation [225,258,309,310]. The most well-studied strain, *M. tuberculosis* H37Rv, has 15 *AbaWSD*-like genes [258]. Recombinant MtuWSD1-4 proteins exhibited high DGAT activities with

oleoyl-CoA and dioleoyl-*sn*-glycerol as substrates [258]. MtuWSD1 was the major contributor to TAG synthesis in *M. tuberculosis* H37Rv, *in vivo* [311]. Furthermore, disruption of *MtuWSD1* resulted in decreased antibiotic tolerance of *M. tuberculosis* H37Rv, which could be restored by complementation with *MtuWSD1* [312]. Although recombinant Rv3371 (one of the 15 AbaWSD-like proteins) showed very low DGAT activity, *in vitro* [258], the *Rv3371* deletion *M. tuberculosis* H37Rv mutant had altered sensitivity to anti-mycobacterial drugs [284]. In addition to WSDs, *M. tuberculosis* mycolytransferase Ag85A also exhibited DGAT activity with serine 126 as the catalytic residue. The over-expression of Ag85A in the non-pathogenic strain *M. smegmatis* Mc^2155 resulted in the formation of a massive LDs, modified lipid composition and a thickened cell wall [139].

In the *M. bovis* BCG Pasteur strain (ATCC 35734), two functional WSDs, MboWSD1 and MboWSD2, were reported to be associated with LDs [285]. The TAG content in both of the single knockout mutant strains was moderately diminished, which could be fully restored by the complementation of the same genes [285].

WSDs were also identified in *M. abscessus*, a non-tuberculous mycobacteria that is receiving greater attention as a serious infectious agent [313]. *M. abscessus* has seven putative *WSD*-encoding genes. The deletion of *MabWSD1* resulted in a nearly 80% reduction in TAG production but the deletion of *MabWSD2* did not lead to decrease of TAG content. When the seven putative *WSDs* were overexpressed in the *MabWSD1*-knockout mutant, MabWSD1 and, to a lesser extent, MabWSD4 could substantially recover cellular TAG content. Similarly, over-expression of *MabWSD1* in WT strain led to notably higher TAG accumulation than the control, followed by *MabWSD4*. These *in vivo* and *in vitro* assays indicated that among the seven putative WSDs, MabWSD1 and MabWSD4 had major and minor functions in supporting TAG biosynthesis in *M. abscessus*, respectively [313].

3.3.4. Conserved motifs and active sites of bacterial WSDs

Bacterial WSDs share relatively low overall amino acid sequence similarity within species and with their eukaryotic homologues. Most WSDs, however, have the highly conserved essential motif HHxxxDG [71,290,296,303]. This essential motif is also conserved in several other acyltransferase classes, including non-ribosomal peptide synthetases and polyketide-associated proteins [314–317]. During catalysis, the second histidine residue of bacterial WSD likely acts as the general base to abstract a proton from the hydroxyl group of the acyl receptor (e.g., fatty alcohol or DAG) and generate a nucleophilic alkoxide [290,296]. A tetrahedral intermediate is then produced during the attack of the alkoxide onto the carbonyl carbon of the acyl-CoA thioester (the acyl donor), and the subsequent collapse of the tetrahedral intermediate produces WE or TAG and releases free CoA [5,296]. Direct mutagenesis and structural modeling have expanded our understanding of the conserved HHxxxDG and the substrate preference of WSD. In AbaWSD, mutation of H132 or H133 to a leucine residue decreased WS activity from 46.42 pmol/mg/min to 0.50 pmol/mg/min and 0.11 pmol/mg/min, respectively. Simultaneous substitution of both histidine residues to leucine residues led to the complete loss of activity [290]. These results indicated both histidine residues are important for enzyme activity, and that H133 may have a more important role than H132 [290].

The functions of amino acid residues outside the conserved HHxxxDG motif have also been explored [183,291–293,303,313]. Random mutagenesis of AbaWSD identified 17 amino acid residues outside of the HHxxxDG motif that were important for catalysis [293]. Some of the residues, such as E15, W67, A126, S374 and G378, are highly conserved in other WSDs [293]. The influence of G355 in AbaWSD (A360 in MaqWSD1) on substrate specificity and enzyme activity has been analyzed [291,292]. Barney et al. (2013) reported that AbaWSD-G355I has higher specific activity toward n-dodecanol than wild-type AbaWSD [291]. In contrast, Röttig and Steinbüchel (2016) did not observe this improvement in AbaWSD-G355I, but found this mutant had a positive impact on its affinity and catalytic efficiency, and the *E. coli* producing recombinant AbaWSD-G355I produced a significantly higher content of fatty acid ethyl esters (FAEEs) than that hosting the WT AbaWSD [292].

Based on structural modeling, amino acid residue substitutions have been carried out on several residues from MaqWSD2 and MabWSD1, which led to decreased WSD activities [303,313]. In MaqWSD2, P118A, L119A, N270A, D271A or R305A, as well as H140A, H141A or D145A in the HHxxxDG domain, led to significantly reduced WS and DGAT activities; all these amino acid residues are highly conserved in WSDs [303]. In MabWSD1, H144A or Q145A in the HHxxxDG domain also resulted in lower enzymatic activity than the WT enzyme [313]. Interestingly, although the second histidine residue is key in catalysis, MabWSD1 has a glutamine residue at this site, which also had a substantial role in enzymatic activity [313]. In addition, directed evolution has been used to screen WSD from *T. curvata* WSD variants with enhanced performance in TAG and/or WE production [183]. Characterization of these variants would also expand our understanding of the enzymatic properties of WSDs.

The recent crystallization and structural determination of MaqWSD1, as well as the associated elucidation of the reaction mechanism has greatly enhanced our understanding of the catalytic mechanism of WSDs [296]. The protein structure, bioinformatics and mutational analyses indicated that the second histidine residue in the HHxxxDG motif indeed serves as the key residue involved in catalysis, while the first histidine residue has an important structural role. The aspartate residue also has a structural role and its replacement with an alanine residue led to significant loss of activity [296]. Structural information and biochemical data will provide the basis to guide protein engineering of bacterial WSDs toward the production of desired lipid-related products.

3.3.5. Identification and characterization of DGATs from cyanobacteria

The model unicellular freshwater cyanobacteria strain *Synechocystis* sp. PCC6803 can accumulate low but detectable amounts of TAGs and WEs (FA phytyl esters) under abiotic stress conditions [219,221]. Protein BLAST searches with the C-terminal acyltransferase domain of *Arabidopsis* PES2 led to the discovery of slr2103, which is not closely related to the acyltransferase domain of DGAT1 or bacterial WSD [221], but is similar to a conserved domain (Pfam PF03982) found in eukaryotic DGAT2s [219]. Slr2103 is composed of 294 amino acid residues with a molecular mass of approximately 32 kDa [221].

When slr2013 was recombinantly produced in *E. coli*, the strain could synthesize phytyl ester and TAG with the feeding of phytol and 1,2-dioctanoyl-*sn*-glycerol, respectively [221]. The recombinant enzyme exhibited low PES activity (1.9 pmol min^{-1} mg^{-1} protein) and high DGAT activity (~300 pmol min^{-1} mg^{-1} protein) in *in vitro* assays with phytol and 1,2-dioctanoyl-*sn*-glycerol as acyl receptors, respectively, with 16:0-CoA as the acyl donor [221,318]. The results might not lead to a conclusion if slr2103 prefers phytol or DAG as preferred acyl receptor for two reasons. Firstly, phytol is poorly dissolved in the *in vitro* reaction mixture. Secondly, slr2103 exhibited lower activity with 1,2-dipalmitoyl-*sn*-glycerol as the acyl receptor compared to 1,2-dioctanoyl-*sn*-glycerol [221]. Additionally, slr2103 had little preference for 16:0-ACP, unesterified 16:0 or monogalactosyldiacylglycerol (MGDG)-bound fatty acyl chains as acyl donors in the *in vitro* assays [221].

In vivo functions of slr2103 have also been investigated [219,221]. Slr2103 deletion in *Synechocystis* sp. PCC6803 has been shown to lead to significantly reduced phytyl ester content under dark and salt stress with phytol feeding, salt and dark stress, and normal growth conditions, respectively, indicating the major role of slr2103 in phytyl ester synthesis [221]. TLC analysis of NPLs revealed that Synechocystis had a lipid that co-migrated with the TAG standard. This lipid was absent in the *Δslr2103* deletion mutant [221]. Subsequent analysis using quadrupole-time-of-flight MS confirmed the significant difference in the TAG content of the WT and mutant strains (~ 5 vs ~0.3 nmol/OD750, respectively). The deletion of slr2103 also led to ~50% reduction in LD

numbers [221]. The results indicated that TAG production in *Synechocystis* was dependent on slr2103 [221]. Another study also reported a lipid that co-migrated with the TAG standard when the NPLs from WT *Synechocystis* were separated on TLC. This lipid was not present in the Δslr2103 mutant. Interestingly, this lipid migrated slightly slower than the TAG standard [219]. Subsequent LC-MS-MS analysis showed that only 10% of the compounds in this TLC band region was TAG, and the TAG content was quite similar to that on the silica gel of the corresponding position in the Δ2103 sample [219]. The authors concluded that the trace amount of TAG in *Synechocystis* sp. was not produced by slr2103 but instead by other acyltransferases [219]. Further studies of TAG biosynthesis in cyanobacteria would be of benefit in developing ME strategies for altering storage lipid biosynthesis in these fast-growing prokaryotic organisms.

4. Fungal DGATs

4.1. Overview of non-polar lipid metabolism in Saccharomyces cerevisiae

Lipid metabolism has been extensively studied in *S. cerevisiae* (often referred to as Baker's yeast or budding yeast) [319] and these studies has benefited from an enormous wealth of genetic resources available for this organism [320,321]. A plethora of investigations with this organism has also contributed to our understanding of lipid metabolism in mammals. Indeed, *S. cerevisiae* represents a useful model system to investigate the acyl-CoA-dependent formation of TAG as key pharmacological target in the development of therapeutic interventions to combat obesity and other related metabolic disorders [86,319].

Unlike many other yeasts, *S. cerevisiae* is only capable of producing saturated and monounsaturated FAs, mostly consisting of C16 and C18 FAs [322]. Although *S. cerevisiae* produces FAs via *de novo* synthesis, growth of the organism can be supported by uptake of exogenous FAs [322,323]. About 80% of the saturated FAs are desaturated via the catalytic action of an ER-resident Δ9 desaturase which utilizes saturated acyl-CoA as substrate [324]. A discussion of *de novo* FA biosynthesis and FA desaturation in *S. cerevisiae*, however, is beyond the scope of this review. Excellent reviews have been published describing these processes (e.g., [322,324])

TAG and SE accumulation during the growth of *S. cerevisiae* is affected by nutritional and environmental conditions [325] and the intracellular content of these NPLs is the result of a balance between synthesis and catabolism [48]. An overview of pathways involved in the biosynthesis and degradation of TAGs and SEs is depicted in Fig. 6. Proteins catalyzing these biochemical reactions are denoted according to the nomenclature developed by the yeast community [176].

The glycolytic intermediates G3P and dihydroxyacetone phosphate (DHAP) are used in the formation of LPA. Gat1p and Gat2p are acyltransferases which can catalyze the acylation of either G3P or DHAP [326]. An NADPH-dependent reductase (Ayr1p) catalyzes the conversion of 1-acyl-DHAP to LPA [327]. In turn, enzymes with LPAAT activity (Slc1p, Slc4p and Loa1p) catalyze the acyl-CoA-dependent acylation of LPA to produce PA [6,328]. Pah1p with PAP activity catalyzes the dephosphorylation of PA to generate DAG and DAG kinase (Dgk1p) catalyzes the phosphorylation of DAG to produce PA [48,319]. PAP is considered a key enzyme for the regulation of lipid synthesis in both yeast and mammals [329].

Dga1p and Lro1p represent enzymes with DGAT and PDAT activity, respectively. Lro1p contributes more to TAG formation during the exponential phase of growth whereas Dga1p contributes more to TAG biosynthesis during the early stationary phase [86,325]. Lysophospholipid (LPL) generated through PDAT action can be re-acylated to form PL via the catalytic action of acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT) which is also known as Ale1p [330–335]. Ale1p is an integral membrane protein of the ER and is enriched in mitochondria-associated membranes [330,334]. Although Are1p and Are2p primarily catalyze the acyl-CoA-dependent biosynthesis of SEs, these enzymes also catalyze a low level of TAG formation [85,86,336,337]. Acyl-CoAs used as substrates for TAG and SE biosynthesis can be derived from the cytosolic *de novo* biosynthesis of FAs [322,324] or through the import and activation of exogenous FAs by the action of long-chain acyl-CoA synthetases (LACS) ([192]).

TAG in LDs is progressively degraded to non-esterified FAs and glycerol by a cascade of hydrolytic reactions catalyzed by various lipases and SE hydrolases catalyze the degradation of SEs [6,48,319]. Lipolysis of TAG is most active during the exponential growth phase of yeast cells and is linked to the cell cycle and environmental conditions [338,339].

Growth of *S. cerevisiae* cells in oleate has been shown to selectively stimulate TAG formation at the expense of SE and the TAG produced was more enriched in oleate compared to cells grown in glucose-containing medium [323]. Switching from glucose to oleate was shown to increase the amount of PL in extracts and LDs but the PL composition was not dramatically affected.

4.2. Properties and physiological role of fungal DGATs

4.2.1. DGAT1 and DGAT2

As mentioned previously, the first DGAT2 was cloned from *U. ramanniana*, [40], an oleaginous soil fungus. The DGAT2 was solubilized from LD membranes and further purified using chromatography. In the final heparin chromatography step, two polypeptides (36 and 36.5 kDa) were identified in the column fractions that possessed high levels of DGAT activity. These polypeptides were partially sequenced and degenerate primers were then used to obtain full-length cDNAs which were expressed in insect cells. In each case, the insect cell microsomes exhibited high levels of DGAT activity. The two isoforms of DGAT2 shared 54% identity with each other and were designated DGAT2A (see Fig. 3) and DGAT2B. The investigators also showed that unidentified orthologues of *U. ramanniana* DGAT2 were present in other fungi and other eukaryotes. Although *U. ramanniana* primarily produced TAG containing C18 acyl moieties, enzyme assays with C6-C10 DAG as acyl acceptors indicated that DGAT2A, more so than DGAT2B, exhibited enhanced specificity for substrates containing medium chain fatty acids (12:0-CoA) relative to substrates containing long acyl chains (18:1-CoA). In later research, *U. ramanniana* DGAT2A was used to increase seed oil content in soybean [340]. As a part of this study, the recombinant enzyme was purified to >95% purity from the mature seed of transgenic soybean plants.

The cloning of *U. ramanniana* DGAT2 quickly led to the cloning of *S. cerevisiae* DGAT2 (Dga1) which was encoded by the *DGA1* gene [44,86]. Fungal DGAT2 has also been functionally characterized in numerous other fungal organisms including the fission yeast *Schizosaccharomyces pombe* [341,342], *Mortierella alpina* [343–345], *Claviceps purpurea* [346]; *Y. lipolytica* [347–350], the methylotrophic yeast *Pichia pastoris* [351], *Rhodosporidium diobovatum* [172], *Rhodosporidiobolus fluvialis* DMKU-RK253 [352], *Mucor circinelloides* [353] and the fungal pathogen *Botrytis cinerea* [354].

Some fungi, however, have been shown to also produce DGAT1 (encoded by the *DGA2* gene). The fact that *DGA1* and *DGA2* encode DGAT2 (Dga1p) and DGAT1 (Dga2p), respectively, can be a source of confusion! Type-1 fungal DGATs have been functionally characterized in *Y. lipolytica* [348–350], *C. purpurea* [355] and *M. circinelloides* [353]. *Y. lipolytica*, *M. circinelloides*, and some of the yeasts producing DGAT2, are oleaginous fungi that produce intracellular lipids at greater than 20% of their DW [356,357].

Given the importance of *S. cerevisiae* as a model system and an industrial yeast, numerous investigations have focused on the characterization of TAG biosynthesis in this organism. *S. cerevisiae DGAT2* (*DGA1*) was first functionally expressed in insect cells [40] and then later in *S. cerevisiae* mutant strains devoid of TAG synthesis [44,86]. ScDGAT2 is predicted to code for a polypeptide consisting of 418 amino acid residues which shares about 36% identity with *U. ramanniana* DGAT2A and DGAT2B [40,86]. ScDGAT2 (Dga1p) and ScPDAT (Lro1p) are major

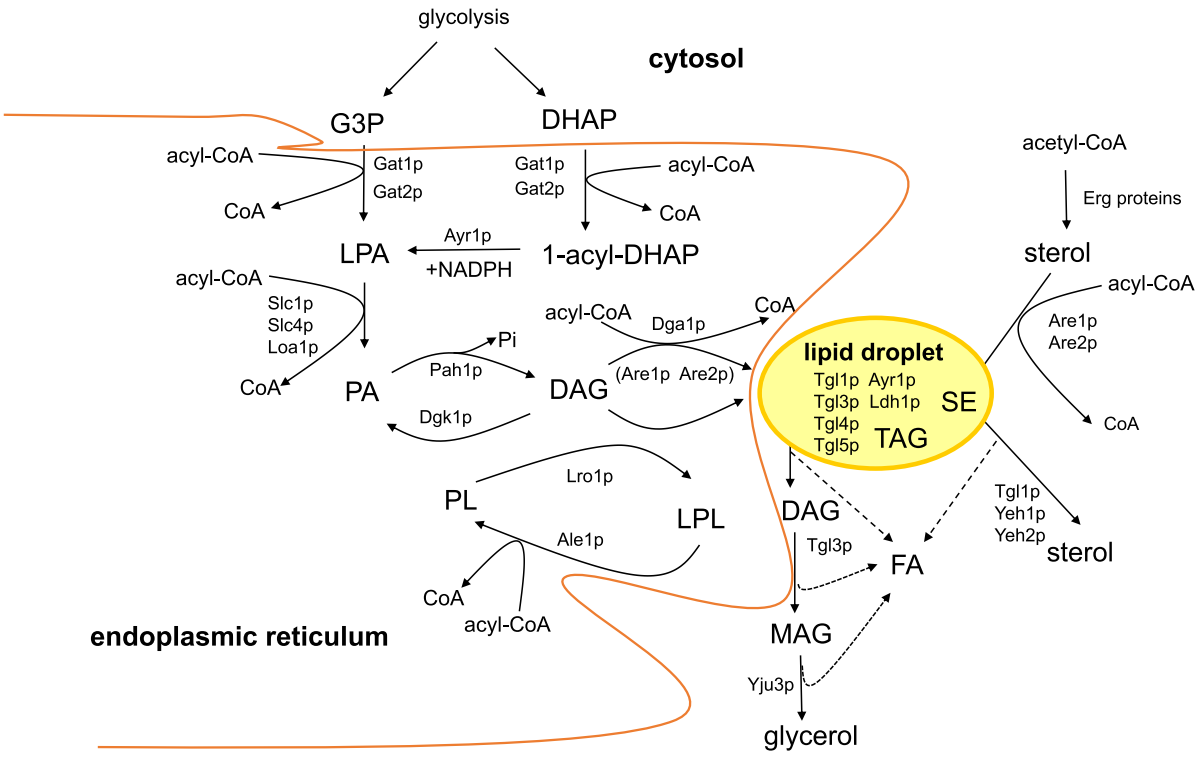

Fig. 6. Overview of the biosynthesis and degradation of triacylglycerol (TAG) and steryl ester (SE) in *Saccharomyces cerevisiae*. *Abbreviations:* DAG, diacylglycerol; DHAP, dihydroxyacetone phosphate; FA, fatty acid; G3P, *sn*-glycerol-3-phosphate; LPA, lysophosphatidic acid; LPL, lysophospholipid; MAG, monoacylglycerol; PA, phosphatidic acid; PL, phospholipid. Adapted from [6,48,319,334,791]. Tgl1, Tgl3, Tgl4, Tgl5, Ayr1, and Ldh1 catalyze the mobilization of TAG, whereas Tgl1, Yeh1, and Yeh2 function as SE lipases. The enzymes involved in catalyzing the various reactions are presented according to the nomenclature of the yeast community [176].

contributors to LD formation but ScDGAT2 is present in both the LDs and ER while PDAT is not present in LDs [44,86,358]. In *S. cerevisiae*, LDs have been shown to be functionally linked to the ER membrane [45,49]. The continuity between LDs and the ER allows DGAT2 to move in both directions in an energy and temperature independent process [45]. During the early stationary phase, DGAT2 moves from the ER to the LDs and is responsible for their growth [49]. When *S. cerevisiae* was transferred to fresh medium, LD degradation was shown to be facilitated by the ER protein Ice2p which has a cytosolic domain for interacting with LDs. Ice2p was required for effective channeling of DAG from LDs to membrane synthetic processes in the ER. Ice2p also caused DGAT2 to return to the ER thereby providing a model of how yeast cells switch from production of TAG to degradation of the non-polar storage lipid.

ScDGAT2 contains seven cysteine residues [40]. In studies with recombinant microsomal ScDGAT2 produced in yeast strain H1246, it has been shown that none of the cysteine residues are directly involved in catalysis or formation of disulfide linkages [147]. Cysteine 314, however, appears to be close to the possible active site of the enzyme or the residue is somehow involved in correct folding of the polypeptide. In a follow-up study, site directed mutagenesis in conjunction with chemical modification and *in silico* analysis was used to gain insight into the function of ScDGAT2 and determine the enzyme's topology in microsomal vesicles derived from fragmented ER [359]. ScDGAT2 was shown to contain four TMDs with the N-terminal and C-terminal segments localized to the cytosolic side of the ER. The first TMD is followed by a relatively large luminal loop which contains the conserved motif, ^{129}YFP^{131}, and a distinctive hydrophilic segment. Histidine 195, which was shown to be essential for enzyme activity, is in the conserved motif, ^{193}HPHG^{196}, in the second TMD. Since ScDGAT2 has been shown to move between the ER and LDs [45,49], it would be interesting to also determine the topology of the enzyme in LDs.

Full length and N-terminally-truncated forms of recombinant ScDGAT2 have been highly purified from solubilized protein fractions prepared from LDs [360]. A C-terminal FLAG™ tag was used to facilitate anti-FLAG™ agarose chromatography. FLAG™ is an eight amino acid peptide designed for applications in immunoaffinity chromatography [361]. Purified truncated ScDGAT2 was immobilized to a BIACORE sensor chip that had been coupled to the anti-FLAG™ antibody and was used to study possible interactions of the truncated enzyme with molecules in the soluble phase using surface plasmon resonance [362]. Binding of oleoyl-CoA to the ScDGAT2-chip was specific because butyryl-CoA, which was not utilized by the enzyme as an acyl donor, did not induce any signals via surface plasmon resonance. The modified immobilized enzyme was also shown to interact with small molecular weight inhibitors of DGAT activity. It was suggested that the technique may be useful for probing structure/function relationships of DGAT and screening for DGAT inhibitors.

Hydroxy FAs, such as ricinoleic acid (12-OH 18:1Δ^{9cis}), have industrial uses in the preparation of environmentally friendly bioproducts [22,346,363]. *C. purpurea*, a fungal pathogen affecting cereal crops, produces high levels of acylglycerols containing ricinoleic acid in its sclerotia [364]. The cloning and functional characterization of *C. purpurea* DGAT2 has been described [346]. The enzyme was recombinantly produced in the yeast strain H1246. *In vitro* assays with microsomes indicated that CpDGAT2 exhibited an enhanced specificity for ricinoleoyl-CoA as an acyl donor in comparison to thioesters containing 18:1Δ^{9}, 18:2$\Delta^{9,12}$ (hereafter18:2) or α-18:3$\Delta^{9,12,15}$ (hereafter 18:3) acyl chains. In addition, co-expression of *C. purpurea FATTY ACID HYDROXYLASE* (*FAH)12* with *CpDGAT2* resulted in enhanced production of ricinoleic acid in strain H1246 when compared to co-expression of *CpFAH12* with *ScDGAT2* (*ScDGA1*). Thus, the investigators provided both *in vitro* and *in vivo* evidence in support of the enhanced preference of CpDGAT2 for substrates containing ricinoleic acid. In a later study, the investigators also cloned CpDGAT1 and functionally expressed the encoding cDNA in strain H1246 [355]. The activity of CpDGAT1 was much higher than that of CpDGAT2.

DGAT2 (*DGA1*) from the oleaginous fungus *R. diobovatum* has also been functionally expressed in the yeast strain H1246 [172]. In this case, the acyl-CoA specificity observed in *in vitro* assays was 18:2 > 18:1 > 16:0 > 18:0 which agreed with *R. diobovatum* TAG being enriched in 18:2. The acyl acceptor, 1,2-dioleoyl–*sn*-glycerol, was introduced to freeze-dried microsomes in benzene followed by 30 seconds of sonication and then evaporation of benzene under a stream of nitrogen gas.

M. alpina is an oleaginous fungus which accumulates polyunsaturated fatty acids (PUFAs) [343]. When comparing cultures grown in the absence or presence of different concentrations of NaCl, a concentration of 2% NaCl resulted in increased TAG accumulation [343]. The increase in TAG content was accompanied by an increase in the expression of *DGAT2*. The investigators suggested that the increase in TAG content may be related to NaCl-stimulated *DGAT2* expression. Insight into the substrate specificity and selectivity properties of codon-optimized MaDGAT2 has come from FA supplementation experiments with the yeast strain H1246 expressing the encoding cDNA [344]. Various 18-20C PUFAs were fed to the yeast separately and in various combinations. Without PUFA supplementation, the TAG fraction contained endogenous saturated and monounsaturated FAs, which were characteristic of the usual FA composition of *S. cerevisiae* TAG. n-6 (ω-6) PUFAs tested included 18:2, γ-linolenic acid (18:3$\Delta^{6,9,12}$), dihomo-γ-linolenic acid (20:3$\Delta^{8,11,14}$) and arachidonic acid (ARA; 20:4$\Delta^{5,8,11,14}$). n-3 (ω-3) PUFAs included α-linolenic acid (18:3$\Delta^{9,12,15}$), eicosapentaenoic acid (EPA; 20:5$\Delta^{5,8,11,14,17}$) and docosahexaenoic acid (DHA; 22:6$\Delta^{4,7,10,13,16,19}$). For single PUFA treatments, n-6 PUFAs were more abundant in TAG than n-3 PUFAs, except for EPA which was also highly abundant in TAG. Similar results were found using mixtures of different PUFAs, showing that EPA was preferred over α-linolenic acid and DHA. The enhanced preference of MaDGAT2 for EPA agreed with the lipid phenotype of *M. alpina*.

For certain phytopathogenic fungi, DGAT may play a role in pathogenesis [354,365]. In gray mold disease, which is caused by *B. cinerea*, disruption of the *BcDGAT2* gene has been shown to lead to defective spore production with no sclerotia formation due to less TAG accumulation [354]. Thus, BcDGAT2 was required for effective penetration of the host and virulence.

M. circinelloides is a filamentous oleaginous fungus which produces storage lipid enriched in γ-linolenic acid. Putative DGAT genes in this organism were shown to include *DGAT1A*, *DGAT1B*, *DGAT2A* and *DGAT2B* [353]. Only McDGAT2B was shown to restore TAG biosynthesis in yeast strain H1246. In addition, *DGAT2B* gene expression correlated with TAG biosynthesis in *M. circinelloides*. Insights into the substrate preference of McDGAT2B were based on feeding exogenous unesterified FAs to H1246 expressing *McDGAT2B*. The enzyme preferred saturated FAs and monounsaturated FAs and exhibited a preference for γ-linolenic acid.

Unlike *S. cerevisiae*, the oleaginous yeast, *Y. lipolytica*, has been shown to produce TAG via the collective action of DGAT1 (Dga2p), DGAT2 (Dga1p) and PDAT (Lro1p) which are encoded, respectively, by *DGA2*, *DGA1* and *LRO1* [347–349]. Although each of the TAG synthases contributed substantially to TAG accumulation, DGAT2 was shown to be the greatest contributor [348,349]. In addition, *DGA1* was expressed later than *DGA2* in the yeast growth stages [348]. Although *Y. lipolytica* DGAT1, encoded by *DGA2*, is homologous to the *ARE* genes of *S. cerevisiae,* the enzyme exhibited little or no sterol acyltransferase activity [348,349]. Unlike *S. cerevisiae*, *Y. lipolytica* only contains one *ARE* gene and its gene product, Are1p, did not exhibit DGAT activity [348]. Using fusions of YlDGAT1 or YlDGAT2 to green fluorescent protein, microscopic observation indicated that YlDGAT1 was associated with a structure resembling the ER whereas YlDGAT2 was localized to the surface of LDs [350]. Proteomic analysis of purified LDs from *Y. lipolytica* has also revealed the presence of DGAT2 [366]. *DGA2* overexpression has been shown to result in large LDs whereas *DGA1* overexpression has been shown to result in smaller and more abundant LDs [350]. Interestingly, the *Y. lipolytica* triple mutant Δ*dga1*Δ*dga2*Δ*lro1*, unlike *S. cerevisiae* H1246, did not exhibit oleate sensitivity [349]. This has also been shown to be the case for the triple mutant Δ*dga1*Δ*lro1*Δ*Are2* of the methyltrophic yeast, *P. pastoris* [351]. *P. pastoris* has only one SE synthase (Are1p).

In studies aimed at increasing lipid accumulation in *Y. lipolytica*, it was noted that effective lipid accumulation was dependent on leucine-mediated signaling [367]. More recently, a multifactorial study of a control strain of *Y. lipolytica* and a strain over-expressing *DGA1*, under either nitrogen limitation or carbon limitation has been conducted [368]. *DGA1* over-expression with nitrogen limitation resulted in increased lipid accumulation with down-regulation of several amino acid biosynthetic pathways, including the leucine pathway. With nitrogen limitation, the regulator Target of Rapamycin complex 1 (TORC1) kinase was repressed and lipid accumulation was induced. The highest lipid accumulation was observed when TORC1 activity was modulated by leucine concentration. The interaction of *DGA1* over-expression and nitrogen limitation revealed this mode of regulation. TORC1 kinase plays a central role in a complex signal transduction network which allows cells to adjust growth and metabolism in response to environmental changes [369,370].

Two soluble recombinant truncated forms of YlDGAT2 have been produced in *E. coli* as fusions with maltose-binding protein and highly purified as active forms [142]. Fusion with maltose-binding protein rendered the recombinant enzymes soluble and facilitated affinity chromatography using amylose resin. The investigators were not able to produce the full-length recombinant enzyme. YlDGAT2Δ19 lacked the N-terminal hydrophilic segment while YlDGAT2Δ85 lacked both the N-terminal hydrophilic segment and the N-terminal TMD. Although, YlDGAT2Δ19 was more active than YlDGAT2Δ85, both recombinant enzyme forms exhibited enhanced specificity for saturated acyl-CoAs. YlDGAT2Δ85, however, exhibited a preference for long-chain saturated acyl-CoA. Although the N-terminal TMD appeared to influence acyl donor specificity, it was not essential for TAG biosynthesis. In contrast, deletion of the first N-terminal TMD in ScDGAT2 has been shown to result in complete loss of enzyme activity [359]. It should be noted, however, that recombinant ScDGAT2 was produced in yeast strain H1246 and was assayed using microsomes whereas the soluble truncated forms of YlDGAT2 were produced in *E. coli*.

4.2.2. Naturally soluble yeast DGAT

A cytosolic 10 S TAG biosynthetic multienzyme complex has been isolated and characterized from the oleaginous yeast *R. glutinis* [67,371]. The soluble complex contained LPAAT, PAP, DGAT and ACP which were held together by protein-protein interactions. The complex incorporated non-esterified FAs, or acyl moieties from acyl-CoA, into TAG. A cDNA encoding the DGAT component of the complex was cloned and expressed in yeast strain H1246 [68]. The recombinant RgDGAT, with a poly-histidine tag at the C-terminal end to facilitate immobilized nickel ion chromatography, was purified to near homogeneity and further characterized. A Kyte-Doolittle hydropathy plot of the polypeptide did not reveal any putative TMDs. The polypeptide contained the modified acyltransferase motif, ^{432}SXXXD^{437} and multiple sequence analysis indicated that RgDGAT belonged to the dehydrogenase enzyme superfamily. *In vitro* acyl-CoA specificity studies with the purified recombinant enzyme, with *sn*-dioleoyl-1,2-glycerol as the acyl acceptor, indicated the following acyl preference: 18:1 > 16:0 > 18:0. The acyl-CoA specificity data were in line with the fact that *R. glutinis* TAG was enriched in 18:1 (47%) and 16:0 (37%). Another cDNA, encoding a N-terminally truncated version of the enzyme (described as the C-terminal domain), was expressed in *E. coli* and also purified. The acyltransferase motif was also present in the truncated enzyme and the activity of the full-length enzyme was 1.3-fold greater than that of the truncated RgDGAT, indicating the function of N-terminus in its activity.

5. Plant-like protist DGATs

5.1. Overview of triacylglycerol synthesis in algae

In general, acyl lipid biosynthesis, including that of TAG, in algae is similar to that in higher plants. There may be certain modifications, however, which are species specific (see e.g. [31]). Earlier work was summarized by Guschina and Harwood [372] and has been subsequently covered in recent reviews [373–375]. There has been a particular focus on *C. reinhardtii* [376] or on diatom [377,378] lipid biosynthesis. Marine protists have also been summarized recently [379]. Thraustochytrids used to be considered as algae but they lack plastids, do not photosynthesise and rely exclusively on heterotrophic nutrition. Nor are they true fungi although they can be considered fungi-like. Useful examples for lipid synthesis are *Schizochytrium* spp. [380].

5.1.1. Fatty acid biosynthesis

Given that the study of storage lipid biosynthesis in plant-like protists is relatively new compared to that of many other organisms, it is worthwhile devoting some discussion to FA biosynthesis. For acyl lipids, their biosynthesis can be said to begin with FA formation (Fig. 7). Since the rate of FA synthesis is often dependent on the amount of carbon precursors, attention has been paid to this aspect [375]. Various sources and the use of ACCase have been reviewed [381]. Relevant enzymes include pyruvate dehydrogenase, acetyl-CoA synthase and ATP:citrate lyase. Particular attention has been paid to *C. reinhardtii* and the diatom, *Phaeodactylum tricornutum*. In addition, experiments have been conducted to highlight sources of reducing equivalents and ATP for fatty acid biosynthesis [375].

The pathway and organisation of *de novo* FA formation in algae is mostly derived from knowledge of the individual enzymes and regulatory mechanisms in higher plants [375]. The first algal studies were in *C. reinhardtii* [382,383] and these have been expanded to include diatoms, the eustigmatophyte *Nannochloropsis* spp. and red algae [375]. This research indicates that FA biosynthesis in different algae is similar in Chlorophyta such as *C. reinhardtii* and to higher plants [376,384,385]. The first committed step is catalyzed by ACCase. There are homomeric and heteromeric forms of ACCase with the former having a role in the supply of malonyl-CoA for FA elongation in the cytosol. ACCase plays an important role in control of carbon flux to FAs in plants [386] and its regulation is discussed in Li-Beisson et al. (2019) [375].

Malonyl-ACP is generated by the catalytic action of malonyl-CoA: ACP malonyltranferase; over-expression of the encoding gene has been shown to increase TAG accumulation in *P. tricornutum* [387]. Malonyl-ACP is used by KAS enzymes. KAS III catalyzes the initial condensation while catalysis by KAS I continues the 2C addition cycle up to 16:0. The final condensation to 18:0 is catalyzed by KAS II. For each addition of 2C, ketoacyl-ACP reductase, hydroxyacyl-ACP dehydrase and enoyl-ACP reductase are needed [375]. Once synthesis of the 16:0- and 18:0-ACPs has occurred, the latter can be desaturated to 18:1 and the acyl groups are either retained in the plastid or hydrolyzed by the catalytic action fatty acyl-ACP thioesterases to release non-esterified FAs. For metabolism in the plastid, the FAs are esterified to G3P and then converted to plastid membrane lipids. Such molecules typically contain 18:1 at *sn*-1 position and palmitate at the sn-2 position i.e. 18/16C distribution initially. This signifies plastid metabolism or the so-called 'prokaryotic' pathway. If fatty acyl-ACP thioesterase activity takes place, the FAs are linked to CoA by the catalytic action of LACS in the outer plastid envelope. Such acyl-CoAs enter the cytosolic pool for use in complex glycerolipid formation in the ER.

5.1.2. Glycerolipid formation

The main glycerolipids in algae are the phosphoglycerides, the glycosylglycerides, various betaine lipids and TAGs. All are produced by the Kennedy pathway —see [32,388] for early publications. The Kennedy pathway, which is an acyl-CoA-dependent process, is usually taken to be the four enzyme steps leading from G3P to TAG (although Kennedy's main contribution was to identify the use of cytidine derivatives from phosphoglyceride formation (see [389]). Here, the focus is on the production of TAG, which is largely confined to the ER [378,390].

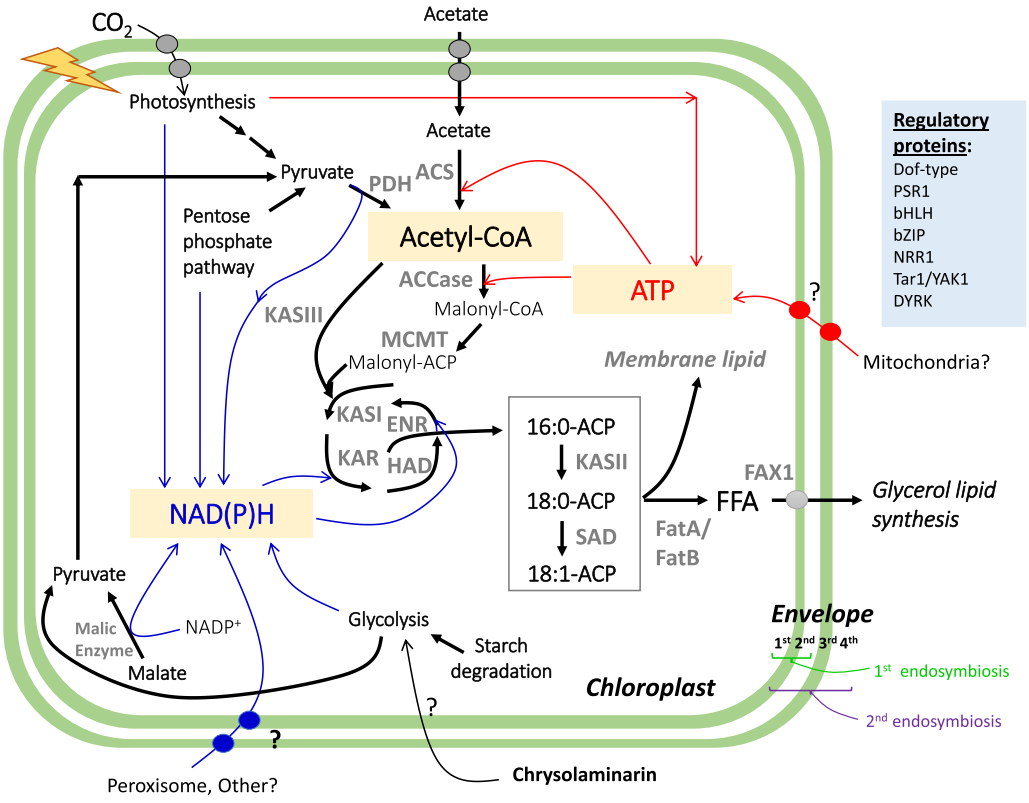

Fig. 7. *De novo* fatty acid synthesis in microalgae – carbon and energy sources. Abbreviations: ACCase, acetyl-CoA carboxylase; ACP, acyl-CoA binding protein; ACS, Acetyl-CoA synthetase; bHLH, a basic helix-loop-helix TF; bZIP, a basic leucine zipper-domain containing TF; Dof-type TF, DNA binding with one finger type transcription factor; DYRK, dual-specificity tyrosine phosphorylation-regulated kinase; ENR, enoyl-ACP reductase; FatA/B, fatty acid thioesterase A/B; FAX1, fatty acid export 1; FFA, free (non-esterified) fatty acid; HAD, hydroxyacyl-ACP dehydrase; KAR, ketoacyl-ACP reductase; KAS, keto-acyl-CoA synthase; MCMT, malonyl-CoA:ACP malonyltransferase; NRP1, pathogenesis-related genes 1; PDH, pyruvate dehydrogenase complex; PSR1, Pi starvation response 1; SAD, stearoyl-ACP desaturase; TAR1, tryptophan aminotransferase-related protein 1. Diagram and Figure legend were reproduced from Li-Beisson et al. (2019) [375] with permission and slight modification.

In the Kennedy pathway, fatty acyl moieties are transferred from the acyl-CoA pool (where the acyl-CoAs may be bound to acyl-CoA binding proteins (ACBPs)) [391,392] first of all to G3P (Fig. 8) [375]. As in bacteria and yeast, this reaction is catalyzed by GPAT. Candidate genes have been listed in several algae [375] and some characterisation carried out for *P. tricornutum* [393], *Thalassiosira pseudonana* [394] and *Lobosphaera incisa* [395].

Also, as in bacteria and yeast, LPAAT catalyzes the esterification of LPA at its *sn*-2 position to yield PA. Two candidate enzymes have been noted in *C. reinhardtii*. CrLPAAT1 is chloroplast-located [396] while CrLPAAT2 is an ER isoform [397]. Both enzymes prefer 16C FAs (e.g., palmitate) and are functionally implicated in TAG accumulation. Genome-wide analysis of *LPAAT* genes has been carried out in algae [398] and, in *Nannochloropsis*, two isoforms of the enzyme have been identified. As in *C. reinhardtii*, the two isoforms have a different subcellular location but are both needed for TAG biosynthesis [399]. In *P. tricornutum*, a potentially important role for LPAAT (called AGPAT, acylglycerolphosphate acyltransferase) has been discussed [400].

The product of LPAAT action, PA, is at a branch-point in the Kennedy pathway where it can be metabolised to CDP-DAG, the precursor of anionic lipids, such phosphatidylglycerol or phosphatidylinositol. For TAG formation, also as in bacteria and yeast, PA is dephosphorylated by the catalytic action of PAP to produce DAG. Eleven putative phosphatases were identified in *C. reinhardtii* [376] and two in *P. tricornutum* [378]. CrPAP2 was found to be important for the induction of TAG accumulation under nitrogen stress [401].

The DAG intermediate is also a branch-point in the Kennedy pathway. In *C. reinhardtii*, the plastid-located DAG is a precursor for glycosylglyceride biosynthesis while the ER-located DAG is used to produce diacylglyceryl-3-O-4′-(N,N,N-trimethyl)-homoserine (DGTS) or zwitterionic phosphoglycerides (PE in *C. reinhardtii* but PC in other algae). Glycosylglycerides include MGDG, digalactosyl diacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (see [375] for details and references). For TAG biosynthesis, also as in yeast, the *sn*-3 position of DAG is acylated from the acyl-CoA pool by the catalytic action of DGAT or by transfer of an acyl chain from a phosphoglyceride via the catalytic action of PDAT. The relative contributions of DGAT versus PDAT to TAG formation is still a matter of discussion [402]. Several PDAT genes have been described in algae [378,403–405] and some characteristics noted [375]. Evolutionary and biochemical analysis of PDATs indicated that PDAT underwent divergent evolution in algae, where positive selection might serve as a major driving force [36].

DGAT1 and DGAT2 are the two main types of DGAT in algae. In general, algae have a single *DGAT1* gene (three picoplankton (*Micromonas pusilla, O. tauri, Ostreococcus lucimarinus*) had no detectable *DGAT1*) but multiple copies of *DGAT2* genes. The confusion in nomenclature of *DGAT2* genes was referred to in Li-Beisson et al. (2019) [375], but most authors nowadays seem to use descriptors such as *DGAT2A* and *DGAT2B*. In *C. reinhardtii*, DGTT1, 2, 3, and 4 refer to DGAT2B, E, D, A, respectively. However, it should be noted that the same letters after DGAT2 in different algal species might not mean the enzymes are the closest homologs (e.g. CrDGAT2A is closer to CzDGAT2C but far from CzDGAT2A in phylogenetic analysis [406]). In the first wide-ranging look at DGATs in algae, Chen and Smith [92] made a careful search of existing algal genomes including verifications with EST-sequence information where possible. They considered the absence of DGAT1 in the three picoalgae (noted above) could have been due to gaps in the genomic sequence or because their DGAT1s may be so divergent that sequence similarity searches could not pick them up [92]. Dendograms of the DGATs were constructed and showed that those from green algae clustered with higher plant enzymes. In contrast, all the other algal sequences were distinct from existing eukaryotic ones. One clade contained the three diatoms together with the brown alga, *Ectocarpus silicosus*. The DGAT1s from *Cyanidioschyzon merolae* and *Emiliania huxleyi* were separate from all the other sequences [92].

In general, algae have multiple DGAT2s. In their survey, Chen and Smith (2012) found a single DGAT2 in *C. merolae* and in *M. pusilla* but none for *E. huxleyi* [92]. Other algae all contained multiple DGAT2s (5 in *C. reinhardtii*, 4 in *Coccomyxa*, 5 in *Volvox carteri,* 3 in *O. lucimarinus*, 4 in *Ostreococcus tauri*, 5 in *P. tricornutum*, 3 in *F. cylindrus*, 2 in *T. pseudonana*, 6 in *Ectocarpus siliculosus*) [92].

Although DGAT1 and DGAT2 are the main forms of DGAT found in higher plants and algae, a soluble form has been detected in a few higher plants [57,61,69], as summarized in section 1. A similar enzyme has been reported in green algae and heterologous expression of the *CrDGAT3* caused accumulation of TAG in yeast under certain incubation conditions [64]. This protein appears to be mainly localized to the chloroplast and the expression of the encoding gene is activated by light, in correspondence with TAG accumulation [407]. In addition, a *DGAT3* gene was detected in *P. tricornutum* and characterised further following heterologous expression in yeast [408,409].

In addition to DGAT1, DGAT2 and DGAT3, WSDs have also been reported in algal species such as *Euglena gracilis* [77], *P. tricornutum* [79] and *C. zofingiensis* [80]. *E. gracilis* WSDs do not exhibit DGAT activity *in*

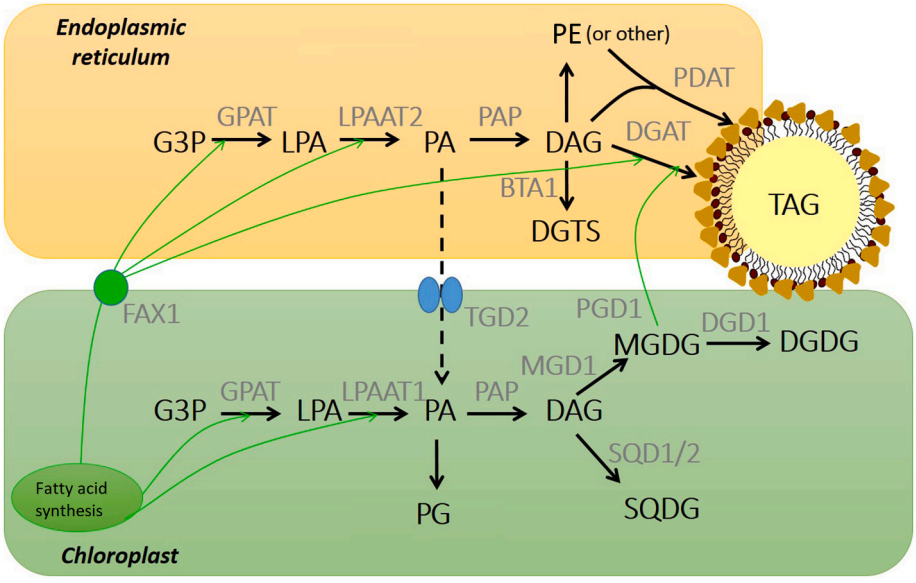

Fig. 8. Glycerolipid synthesis in *Chlamydomonas reinhardtii*. Abbreviations: BTA, betaine synthase; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DGD, digalactosyldiacylglycerol synthase; DGDG, digalactosyldiacylglycerol; DGTS, diacylglyceryl-3-O-4′-(N,N,N-trimethyl)-homoserine; FAX1, fatty acid export 1; G3P, *sn*-glycerol-3-phosphate; GPAT, acyl CoA:glycerol-3-phosphate acyltransferase; LPA, lysophosphatidic acid; LPAAT, acyl-CoA:lysophosphatidic acid acyltransferase; MGD, monogalactosyldiacylglycerol synthase; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PDAT, phospholipid:diacylglycerol acyltransferase; PGD1, plastid galactoglycerolipid degradation 1; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SQD, sulfoquinovosyldiacylglycerol synthase; SQDG, sulfoquinovosyldiacylglycerol; TGD, trigalactosyldiacylglycerol; TAG, triacylglycerol. Diagram and Figure legend were reproduced from Li-Beisson et al. (2019) [375] with permission and slight modification.

vivo [77], but *in vitro* and *in vivo* assays showed that the *P. tricornutum* WSD could function as either a WS or a DGAT [79]. Over-expression studies showed that the DGAT activity was dominant while the WS activity was condition-dependent and lower. In *WSD* over-expressing *P. tricornutum*, increased levels of TAG were found and this was nitrogen stress independent. Phylogenetic analysis showed that putative *WS/DGAT* genes were present broadly in microalgae [79,80]. The evolution of *WSD* genes has been driven primarily by purifying selection and partially by positive selection, where positive selection may have contributed to functional divergence [80]. Although *C. zofingiensis* can accumulate TAG but not WE, this alga has a WSD with high WS activity but low DGAT activity [80].

Further details of the various algal *DGAT* genes and properties of the gene products are presented in the next section.

5.2. Properties and physiological roles of DGATs in algae

As described in section 5.1., DGAT1 and DGAT2 are the most widely described and characterized forms of DGAT in algae. Current research suggests that they are also the most important functionally. Similar to their homologs in higher plants, DGAT1 and DGAT2 which are generally membrane-bound do not share any significant amino acid sequence similarities. For oil crops, DGAT1 seems to dominate in plants producing TAGs with common FAs while DGAT2 tends to be important for oils with unusual FAs [410], but this is not always the case (to be discussed in section 6.2.). DGAT1s are larger than DGAT2s and both have TMDs, though these can be very variable in algae [92].

5.2.1. DGAT1

As expected, the first studies of DGATs in algae were those that identified putative enzymes from their sequence analogies to those from other organisms. Thus, DGAT1 was predicted for *C. reinhardtii* [403] and for *P. tricornutum* [411]. The latter was characterized biochemically by heterologous expression of the gene in yeast showing a preference for 16:0-CoA and 18:0-CoA. Its mRNA was processed by alternate splicing and resulted in two cDNA products (designated *PtDGAT1short* and *PtDGAT1long*, respectively) and the *PtDGAT1short* encoded a 565 amino acid residue protein that was homologous to several higher plant DGAT1 proteins and 55% identical to a putative DGAT1 protein in the diatom *T. pseudonana* [411]. PtDGAT1 is likely localized to the ER adjacent to the chloroplast and has a significant contribution to TAG biosynthesis [409]. For the putative gene in *C. reinhardtii*, up-regulation was noted under various nutrient-stress conditions (including -nitrogen, -sulfur, -zinc and low Fe) [403].

In their bioinformatic search, Chen and Smith (2012) found putative DGAT1 protein sequences in several green algae, the red alga *C. merolae*, the diatom *Fragilariopsis cylindrus*, a haptophyte *E. huxleyi* and a brown alga, *E. siliculosus* [92]. All but three picoplankton (*M. pusilla, O. tauri, O. lucimarinus*) were found to contain putative DGAT1 sequences. There were two suggestions as to why no sequences were detected (see section 5.1) - gaps in the available gene sequences or significantly divergent sequences in these organisms [92].

A *DGAT1* gene from *Myrmecia incisa* was predicted to encode a protein of 745 amino acids (more than twice the size for two *DGAT2* genes in *M. incisa* [412]. Multiple sequence alignment indicated a pleckstrin homology domain. Heterologous expression in a yeast mutant devoid in TAG biosynthesis confirmed its biochemical ability to restore TAG accumulation and LD formation. This freshwater microalga is now named *L. incisa* (also called *Parietochlorus incisa*) and is interesting because it accumulates ARA (up to 60% of total FAs) in its TAG under nitrogen-starvation conditions. A *DGAT1* gene was identified and shown to be increased by nitrogen-stress in high light. The gene restored TAG formation in a yeast mutant devoid in TAG biosynthesis and favoured saturated over monounsaturated fatty acyl-CoAs. It could also use a variety of n-3 and n-6 PUFAs including ARA. Structure/function analysis of *LiDGAT1* revealed that the pleckstrin homology domain was important (but not essential) for TAG formation in the heterologous system. Pleckstrin homology domains were found in a number of other DGAT1s from algae including a number of chlorophytes, in the multicellular *Klebsormidium flaccidum* and in two diatoms (*P. tricornutum, T. pseudonana*) but seemed absent from red algae [413]. Further analysis of *L. incisa* revealed the presence of five putative DGAT isoforms including one DGAT1, three DGAT2s and one DGAT3. Feeding yeast transformants with FAs confirmed that LiDGAT1 had a broad substrate specificity. It localized to the ER in close proximity to developing LDs [414].

DGAT1s have also been characterized in several *Chlorella* species. The green microalga *Chlorella vulgaris* can be induced (e.g., by nitrogen-limitation) to accumulate large amounts (40% DW) of TAG. Under such conditions, expression of a *DGAT1* gene (*CvuDGAT1*) was induced [415]. The *C. vulgaris* enzyme (460 amino acid residues) has been shown to share motifs characteristic of other plant or algal DGATs including distinct substrate binding sites and a putative active site, as shown in the predicted tertiary structure [415]. Unlike the *C. reinhardtii DGAT1*, that from *C. vulgaris* was constitutively expressed and, thus, may explain the alga's high oil levels of around 40% DW. A second *Chlorella*, *C. zofingiensis* [416], has now been studied for DGAT enzymes [417]. This species is recognized as an industrially-important alga because it can provide good yields of both TAG and the high-value carotenoid, astaxanthin [418]. Its algal genome was predicted to contain eleven putative DGAT-encoding genes [416]. Later, ten DGATs were verified, including two DGAT1s, CzDGAT1A and CzDGAT1B [417]. Both DGAT1 isoforms showed TAG synthesis activity on heterologous expression in yeast, but CzDGAT1A was more active, and expression of the gene was induced by nitrogen-depletion. In addition, its heterologous expression in *Nannochloropsis oceanica* approximately doubled TAG accumulation suggesting its use for industrial oil production [417,419]. In *Chlorella ellipsoidea*, a putative *DGAT1* was identified, designated *CeDGAT1* with the cDNA encoding a protein of 713 amino acid residues [420]. Expression levels were increased under nitrogen-starvation, leading to TAG accumulation. Activity was confirmed by heterologous expression in yeast and over-expression in *A. thaliana* and *B. napus* enhanced seed oil by up to 37% [420].

N. oceanica is of interest as an industrially important oleaginous alga. It shows fast growth, an available genome sequence, established genetic tools and has a high TAG content (around 23% DW). In one *N. oceanica* strain (IMT1) [421], two of the thirteen *DGAT* genes were designated *DGAT1A* and *DGAT1B*. Nitrogen depletion increased expression of *DGAT1A*. Moreover, *in vivo* and *in vitro* studies showed that NoDGAT1A but not NoDGAT1B had acyltransferase activity. Knockdown of *NoDGAT1A* caused about a 25% decrease in TAG levels while over-expression caused around 39% increase upon nitrogen- depletion. In addition, its over-expression increased TAG (~2.4-fold) under nitrogen-replete conditions without compromising growth [422].

Another green microalga, *Haematococcus pluvialis*, is known for its ability to accumulate LDs with high concentrations of mono- or di-esters of astaxanthin. DGATs have an important role in the accumulation of astaxanthin and one or some of them may catalyze the biosynthesis of astaxanthin acyl esters [423], and a following up study confirmed the hypothesis that HpDGAT1 has xanthophyll acyltransferase activity [424]. It would be interesting to test if other DGATs, especially the WSD, also have xanthophyll acyltransferase activity, though the *C. zofingiensis* WSD did not show this activity in an *in vitro* assay [80]. The same alga accumulates TAG and has been proposed as a new source of biodiesel [425]. One homologue of *DGAT1* and four *DGAT2s* were identified in the transcriptome database [426]. The HpDGAT1 contained a pleckstrin homology domain and nine TMDs. Heterologous expression in a TAG-deficient yeast confirmed its ability to synthesize TAG with a broad acyl-CoA substrate specificity including PUFAs such as n-3 or n-6 linolenic acids and ARA [427].

Although DGATs have been characterized from a growing number of algal species, many studies are focused on elucidating their role and application in TAG accumulation. Studies in other eukaryotes indicated

that the structure/function of DGATs are essential for our understanding of these enzymes (refer to sections 4, 6 and 7 for details). Nevertheless, such research in algal DGATs is still in its infancy. Xu et al. (2020) recently investigated the evolutionary and structural features of green algal DGAT1s with *in silico* analysis [419]. Although DGAT1s from green microalgae have similar structural features to their homologs in higher plants and mammals, such as the variable hydrophilic NTD and highly conserved C-terminal hydrophobic region forming multiple TMDs, the NTDs of algal DGAT1s are much more varied in length (approximately 20 - 326 amino acids) than that of plant DGAT1s (approximately 110 amino acid residues) and animal DGAT1s (approximately 94 amino acid residues) [419]. Secondary structure analysis indicated the NTD of algal DGAT1s has much less propensity to be disordered and possibly has less conserved allosteric sites than the majority of plant and animal DGAT1s, where the NTD is disordered with an autoinhibitory motif but the small, folded portion contains a conserved allosteric site [54,419]. The unique features of green algal DGAT1s were also supported by enzymatic analysis of the truncated *C. zofingiensis* DGAT1B [419]. The NTD of *C. zofingiensis* DGAT1B has 107 amino acid residues, which is similar to that of plant and animal DGAT1s. Although the first 80 amino acid residues in the NTD of *B. napus* DGAT1 (BnaDGAT1) down-regulated enzyme activity and its removal resulted in higher DGAT activity [54], the removal of the equivalent region to the autoinhibitory motif (80 amino acid residues) in *C. zofingiensis* DGAT1B diminished the enzyme activity by 10-fold [419]. Moreover, the NTD of *C. zofingiensis* DGAT1B is likely not involved in mediating positive cooperativity, but is important for maintaining high enzyme activity [419].

5.2.2. DGAT2

As mentioned previously, in contrast to the common existence of a single DGAT1 enzyme, algae usually contain multiple isoforms of DGAT2s. In the paper by Chen and Smith [92], of the green algae *C. reinhardtii* contained 5 (see [403,428,429]), *Coccomyxa* C-169 had 4, *V. carteri* had 5, *M. pusilla* had 3, *O. lucimarinus* had 3 and *O. tauri* had 4 [92]. Similarly for diatoms (P. *tricornutum*, 5; *F. cylindrus*, 3; *T. pseudonana*, 2) and the brown alga *E. siliculosus*, (6) there were multiple copies [92,411,430].

For the model green alga, *C. reinhardtii*, Miller et al. (2010) carried out homology searches and identified five putative *DGAT2* genes (called *DGTT1-5*, also known as *DGAT2A-E*) [429] and there were four further reports in 2012 [403]. The expression of three *CrDGAT2s* genes was induced by nitrogen-starvation and the encoded enzymes were likely to have a role in TAG accumulation [403]. One of these was functionally characterized as a DGAT2 and was named DGTT1 (DGAT2B). La Russa et al. (2012) examined three *DGAT2* candidate genes and constructed over-expressing strains, but over-expression did not result in increased TAG levels [431]. In contrast, Deng et al. (2012) used RNAi silencing of the five *CrDGAT2s* and found that *CrDGAT2-1* and *CrDGAT2-5* were important, as deduced by such experiments [432]. Heterologous over-expression of these two *DGAT2s* in *C. reinhardtii* in a TAG-deficient yeast confirmed their conclusions [432]. Msanne et al. (2012) again used nitrogen-stress and noted that *DGAT2B*, in particular, was increased in expression [428]. There was little change in expression of the other *DGAT2s* and *DGAT1* expression was unchanged. Further details of the role of the *DGAT2* gene family in *C. reinhardtii* showed that *DGAT2B*, *E*, and *D*, but not *DGAT2A*, complemented the TAG-deficient phenotype in a yeast mutant. Complementation with *DGAT2E* was particularly effective, resulting in yeast yielding TAG levels which were around 20-times those of WT yeast. Some differences were noted in the FA compositions of the yeast storage lipids for the four genes upon heterologous expression [433]. Further work on three DGAT2 isoforms also revealed differences in their substrate specificities [434]. In *in vitro* enzyme assays, CrDGAT2B preferred polyunsaturated acyl-CoAs and CrDGAT2E preferred monounsaturated acyl-CoAs while CrDGAT2D preferred 16C-CoAs. The DGAT2 isoforms also differed in their DAG substrate preferences. *In vivo* knockdown of the three *DGAT2* genes gave 20-35% decreases in TAG content and a change in TAG FA composition that agreed with the results from *in vitro* enzyme assays [434].

Another green microalga *C. zofingiensis* also has several putative *DGAT2* genes [406]. Of the seven gene sequences, *CzDGAT2C* was plant-like, *CzDGAT2F* and *CzDGAT2G* were animal/fungus-like and the others were algal-like, which was quite similar to the diverse structures of *DGAT2s* from other green microalgae. The putative functional motifs of the deduced amino acid sequences of CzDGAT2s and CrDGAT2s were analyzed and compared with their homologs in other eukaryotes *in silico*. These algal DGAT2s do not have the conserved putative lipid binding FVLF motif that is found in ScDGAT2 (FLXLXXXn in mouse DGAT2) [359,406,435]. CzDGAT2s and CrDGAT2s in the plant-like and animal/fungus-like groups, but not the ones in the algal-like group, have the highly conserve YFP motif, an essential one for DGAT2 activity in animal and higher plant DGAT2s [359,406]. Interestingly, both CzDGAT2s and CrDGAT2s have the longest conserved motif of plant and animal DGAT2s corresponding to RXGFX(K/R)XAXXXGXX(L/V) VPXXXFG(E/Q) [406]. It would be interesting to investigate the functions of the motifs with mutagenesis and enzymatic activity analysis in the future. When the putative genes were expressed in a TAG-deficient yeast without codon-optimization, only CzDGAT2C (the closest homolog of AthDGAT2 and CrDGAT2A) was able to restore TAG formation. The performance of algal DGAT2s in yeast, however, may be influenced by other factors such as codon usage. Therefore, this does not mean other CzDGAT2s have no functions in lipid biosynthesis in *C. zofingiensis*. *In vitro* enzyme assays confirmed CzDGAT2C as showing typical DGAT activity. Moreover, CzDGAT2C has a short hydrophilic tail and two TMDs in the N-terminal region and a large C-terminal hydrophilic fragment with one putative TMD. Truncation mutagenesis results indicated that similar to ScDGAT2 [359], both termini are important and sensitive to modifications [406]. In addition, yeast two-hybrid assays suggested DGAT2 activity was likely mediated through homodimer/heterodimer formation with the different isoforms [406].

Another green alga that has been renamed is *L. incisa* (previously *Myrmecia* and then *Parietochloris*). Three putative *DGAT* genes were identified, one *DGAT1* and two *DGAT2s* (*LiDGAT2A* and *LiDGAT2B*) [412]. The latter two genes encoded proteins of 351 and 355 amino acid residues, respectively. Either recombinant protein was able to restore TAG synthesis in a yeast mutant; they also contained a HPHG sequence, typical of DGAT2 enzymes. Neither isoform changed the typical FA composition of yeast TAG and the *LiDGAT2A* transcript was regulated by nitrogen-starvation [412]. A new DGAT2 (LiDGAT2C) was reported by the same laboratory. It could restore TAG synthesis in a yeast mutant. Its acyl-CoA preference was compared to the other *L. incisa* DGAT2s. DGAT2A was the most effective at utilizing ARA-CoA (the major FA in *L. incisa*) while DGAT2C had no activity with this acyl-CoA, in *in vitro* assays with high concentrations of non-radiolabelled substrates [436]. The DGAT family in *L. incisa* was examined further by Zienkiewicz et al. (2018) [414]. The investigators confirmed the existence of one DGAT1, three DGAT2s and a single isoform of DGAT3. All the DGAT2 isoforms localised to the ER when expressed in yeast.

In *H. pluvialis*, four putative *DGAT2* genes were identified by querying a transcriptome shotgun assembly sequence database [437] with *AthDGAT2* and *CrDGAT2s* sequences [438]. Recombinant HpDGAT2D could substantially recover TAG synthesis in the TAG-deficient *S. cerevisiae* H1246 mutant, and its DGAT activity was further confirmed using *in vitro* assays [438]. In another study, four putative *DGAT2s* were also identified from *H. pluvialis* based on genomic analysis and all showed DGAT activity when recombinantly produced in yeast, except for HpDGAT2E (also known as DGTT2), which turned out to be a LPAAT [427]. In addition, the DGAT2s in *H. pluvialis* have less activity than the HpDGAT1 in producing TAG [427]. This was similar to results obtained for other algae such as *C. zofingiensis* [417]. *Haematococcus lacustris*, a related species that also accumulates astaxanthin simultaneous with TAG, contains five putative *DGAT2* genes [439]. Recombinant HlDGAT2A, 2B, 2D or 2E could restore TAG synthesis in

yeast and all, except HlDGAT2B, worked well with monounsaturated or polyunsaturated acyl-CoAs. *HlDGAT2B* was heterologously expressed in *Arabidopsis* and *C. reinhardtii* and significantly increased TAG and PUFA contents in both cases [439].

Nannochloropsis spp. microalgae in the class Eustigmatophyceae have attracted interest as industrial feedstocks for the production of significant amounts of EPA. Remarkably, despite its small genome size, *Nannochloropsis* has a large pan-genome of >38,000 genes. Eleven *DGAT2* genes were found in all five oleaginous species that were examined. It was considered that they had originated from three ancient genomes including a secondary endosymbiosis host and green and red algae [421]. A particular *DGAT2* was identified and overexpressed in *N. oceanica*, increasing TAG biosynthesis and NPL content (by ~ 70%). The FA profile of the *N. oceanica* hosting this *DGAT2* was changed with saturated and PUFAs, increasing at the expense of monounsaturated fatty acids. Growth rates were unchanged [440]. A particular species of *N. oceanica* (CCMP1779) was studied later and found to contain putative genes for one DGAT1 and twelve DGAT2s [441]. Six of the latter, *NoDGTT1-NoDGTT6* were expressed in a heterologous yeast system; *NoDGTT5* was chosen for further work because it caused accumulation of the highest TAG levels. Over-expression of *NoDGTT5* resulted in TAG levels equivalent to those caused by nitrogen-starvation but with reduced growth rates [441]. Different *N. oceanica* DGAT2s were examined in terms of their substrate specificities and potential for producing designer oils [442]. FA-feeding experiments and *in vitro* assay with non-radiolabeled acyl-CoA substrates indicated that NoDGAT2A preferred saturated FAs (especially 16:0). No DGAT2D was active with monounsaturated FAs while NoDGAT2C showed the highest activity with PUFAs (especially 20:5). By modulating the ratio of *DGAT2* transcripts, *N. oceanica* strains were developed that were suitable for nutritional supplementation or biofuel production [442]. In contrast to Zienkiewicz et al. (2017) [441], Xin et al. (2019) considered that there were eleven full-length DGAT2s [443]. Of these, TAG synthesising activity was only detected in TAG-deficient yeast for three DGAT2s, which included NoDGAT2A, NoDGAT2C and NoDGAT2D. The eight inactive DGAT2s may have lacked proper substrates in the yeast system. In further work, two DGAT2s were found to be PUFA-preferring enzymes (by feeding yeast with separate potential substrates). NoDGAT2J used linoleic acid while NoDGAT2K was active with EPA. By modulating transcript abundance of the *DGAT2s*, strains were developed where the proportions of linoleic acid and EPA in TAG could be varied ~19-fold and ~35-fold, respectively. This provided strains of good potential in the health food or biofuel sectors [443]. Finally, *N. oceanica* (CCMP1779) was studied during its transition from quiescence to autotrophy in response to nitrogen availability [444]. The reorganisation of cell membranes together with TAG accumulation was accompanied by up-regulation of six of the *DGATs* during nitrogen deprivation but a strong decrease in their expression occurred following reintroduction of nitrogen. This contrasted with the single *DGAT1* gene which did not change. Other genes involved in FA recycling or LDs were also studied [444].

Diatoms are the major phytoplankton species [378] and contribute to 20% of global carbon fixation [377]. As in other algae, diatoms usually contain a single *DGAT1* gene but multiple *DGAT2* genes [92,378]. Most studies in diatoms have concentrated on *P. tricornutum* because of its obvious commercial utility. Five putative isoforms of DGAT2 were observed [92] and the TAG synthase activity of PtDGAT2B was confirmed [409,445]. In contrast, to the much larger DGAT1, which had at least eight TMDs, the DGAT2 isoforms (PtDGAT2A, B, C and D) contained two, four, one and one putative TMDs, respectively. Expression of *PtDGAT2B* led to increased proportions of unsaturated FAs in yeast TAG [445]. Although the recombinant production of PtDGAT2A could not recover TAG biosynthesis in the TAG-deficient *S. cerevisiae* quadruple mutant strain (H1246) [409,445], its over-expression in *P. tricornutum* was reported to lead to increased LDs and TAG accumulation [446].

In *O. tauri*, predicted to have up to four isoforms of DGAT2, but no DGAT1 [92], a *DGAT2* gene was identified and characterized [430]. The latter publication predicted three *DGAT2* homologs and confirmed that no homologue for either *DGAT1* or *DGAT3* was present in the *O. tauri* genome. Either OtDGAT2A or OtDGAT2B restored TAG synthesis in TAG-deficient yeast. OtDGAT2B showed a broad substrate selectivity including polyunsaturated acyl-CoAs [430]. A DGAT2 was also studied in *T. pseudonana* where the enzyme shifted its location from the chloroplast to the ER when growth changed from exponential to the stationary phase. Over-expression of the enzyme caused increases in TAG and a change in FA composition but did not affect growth [447]. In addition, Chen and Smith [92] reported two putative DGAT2 proteins in the diatom *T. pseudonana* and three in *F. cylindrus*.

6. Higher plant DGATs

6.1. Overview of triacylglycerol biosynthesis in developing seeds of oleaginous plants

A vast amount of the research on TAG biosynthesis in higher plants has focused on developing seeds of oleaginous plants. As in bacteria, fungi, plant-like protists and mammals, glycerolipid biosynthesis in developing seeds involves the Kennedy pathway which allows production of the glycerolipids present in membranes and storage TAGs. Many of the FAs used in glycerolipid biosynthesis are produced through *de novo* FA biosynthesis in the plastid. For a detailed discussion of *de novo* FA biosynthesis in higher plants, the reader is encouraged to consult other reviews dealing more specifically with this process [448,449]. A generalized scheme for the Kennedy pathway of the ER and its relationship with membrane lipid metabolism in developing seeds of oleaginous higher plants producing seed oil containing PUFAs is outlined in Fig. 9. In oleaginous plants, the Kennedy pathway involves four steps in the production of TAG, and the intermediates, PA and DAG, are involved in the biosynthesis of anionic and zwitterionic phospholipids, respectively [9,448]. The three acyltransferase-catalyzed reactions are dependent on acyl-CoA as the acyl donor.

The first two acyltransferase-catalyzed reactions, involving GPAT and LPAAT, were originally reported by Kornberg and Pricer [450], but it was Eugene Kennedy who added further details and, critically, revealed the key role of CDP-derivatives in complex lipid biosynthesis [32]. G3P is produced by reduction of DHAP which is an intermediate in glycolysis. Increased supply of G3P and/or increased GPAT activity have been suggested to raise TAG accumulation in oil crops [451,452]. Isoform GPAT9 has been identified as the acyltransferase catalyzing the first reaction in the Kennedy pathway in developing seeds of *Arabidopsis* [453,454].

LPAAT is the second enzyme in the pathway and tends to have high activity compared to the other three acyltransferases [455]. Nevertheless, heterologous expression of a gene encoding either a yeast *sn*-2 acyltransferase or *Tropaeolum majus* LPAAT has been shown raise TAG levels in *B. napus* despite the low intrinsic flux control coefficient for this reaction [456,457].

The product of the LPAAT-catalyzed reaction is PA which is at a first branch-point in the Kennedy pathway leading to production of anionic phospholipids. PA can also be dephosphorylated by the catalytic action of PAP to yield DAG. Multiple forms of PAP have been reported in plants [458–461]. Once DAG has been formed, it can be used for production of zwitterionic phospholipids, glycosylglycerides or TAG.

As in bacteria, fungi and plant-like protists, the acyl-CoA-dependent acylation of DAG is catalyzed by DGAT (Fig. 1. and Fig. 9.). Several isoforms are found in plants [62]. As indicated in the introduction, these include DGAT1, DGAT2, naturally soluble DGATs (including DGAT3), dual function WSD and the specialized DAcT. In higher plants, as a generalization, DGAT1 appears to be used for catalyzing the synthesis of common molecular species of TAG whereas DGAT2 appears to be involved in catalyzing TAG containing less common FAs (such as

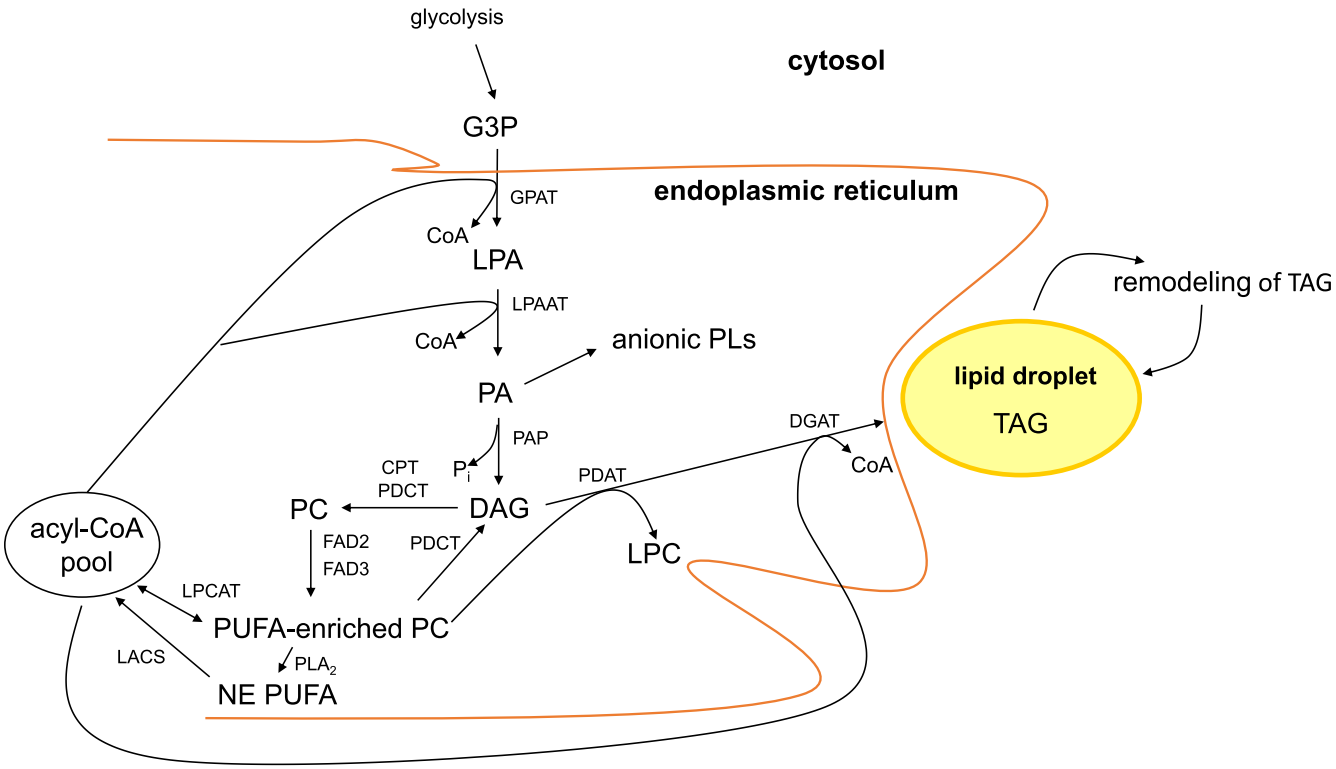

Fig. 9. Generalized overview of the biosynthesis of triacylglycerol (TAG) enriched in polyunsaturated fatty acids (PUFAs) in developing seeds of oleaginous plants. The Kennedy pathway is shown in relation to some possible acyl-trafficking reactions along with phospholipid:diacylglycerol acyltransferase (PDAT) action. Possible specialized pools of 1, 2-diacyl-*sn*-glycerol (DAG), including DAG synthesized *de novo* in the Kennedy pathway and phosphatidylcholine (PC)-modified DAG [589], are not specifically depicted. Phosphatidylethanolamine can also serve as an acyl donor for the PDAT-catalyzed reaction. Other abbreviations: CPT, CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase; FA, fatty acid; FAD, fatty acid desaturase; GPAT, acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase; G3P, *sn*-glycerol-3-phosphate; LACS, long-chain acyl-CoA synthetase; LPA, lysophosphatidic acid; LPAAT, acyl-CoA:lysophosphatidic acid acyltransferase; LPC, lysophosphatidylcholine; LPCAT, acyl-CoA:lysophosphatidylcholine acyltransferase; NE, non-esterified; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PDCT, phosphatidylcholine:diacylglycerol cholinephosphotransferase; P_i, inorganic phosphate; PL, phospholipid; PLA_2, phospholipase A_2. The Figure is based on information from [9,448,465,471–473].

hydroxy FAs discussed in [462]). There are, however, exceptions where the plant DGAT2 under investigation has been shown to catalyze the production of TAG enriched in more common FAs such as α-linolenic acid (e.g., [193]).

An alternative way of producing TAG from DAG is by the reaction catalyzed by PDAT (Fig. 1 and Fig. 9). The enzyme was first reported in yeast as well as several plants [34] and later the enzyme from *Arabidopsis* was cloned and functionally characterized [35]. DGAT and PDAT have been shown to have overlapping functions in *Arabidopsis* [463] but with DGAT more important for TAG formation [464]. Nevertheless, the relative contribution of these acyltransferases for overall TAG formation has been the subject of considerable research [465]. The current data suggests that PDAT is used more in oleaginous plants with highly unsaturated or unusual FAs [18,124,180,466–468]. Evolutionary analysis suggests that PDATs are present in all higher plants, lycophytes, plant-like protists and fungi [36,37].

In developing seeds of oleaginous plants which produce TAG containing PUFAs or unusual FAs, such as ricinoleic acid, biosynthesis of these FAs occurs at the level of PC. DAG produced in the Kennedy pathway can be converted to PC via the catalytic action of CPT [448] or phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) (Fig. 9) [469]. In the latter reaction, the phosphocholine group of PC is transferred to DAG generated in the Kennedy pathway and the DAG resulting from PC (after removal of phosphocholine) is available for incorporation into TAG via the DGAT-catalyzed reaction. In an example of PUFA formation, FAD2 and FAD3, sequentially catalyze the formation of *sn*-2-18:2-PC and *sn*-3-18:3-PC, respectively [448]. Formation of *sn*-2-ricinoleoyl-PC involves the catalytic action of a FAH which results in hydroxylation of the twelfth carbon from the carboxyl end of the oleoyl moiety [470]. Depending on the species, various enzymes, including PDAT, PDCT, LPCAT, phospholipases (e.g., phospholipase A_2 [PLA_2]) and LACS, are involved an acyl trafficking processes which result in PUFAs and/or unusual FAs being incorporated into storage TAG [465,471,472]. These processes are outlined in relation to the Kennedy pathway in Fig. 9.

A recent study has shown that TAG may not represent a metabolic endpoint during seed development in oleaginous higher plants, but may in fact undergo remodeling [473]. Evidence for TAG remodeling was demonstrated through *in vivo* isotopic tracing experiments using developing embryos of *Physaria fendleri* obtained 30 days after pollination. The study has implications for developing novel approaches in the ME of TAG which could potentially be applied after initial synthesis of the storage lipid.

6.2. *DGAT1 and DGAT2*

6.2.1. *Functional expression in microorganisms*

cDNAs encoding DGATs from numerous species of higher plants have been functionally expressed in microorganisms, with most of the heterologous expression studies conducting using the *S. cerevisiae* strain H1246. Most of these studies were based on the expression of cDNAs representing transcripts in oil-forming tissues of oleaginous plants. These species have included members of the *Brassicaceae* [63,65,69,74,104,120,131,153,177,474–478], castor (*R. communis*) [94,479–481], cocoa (*Theobroma cacao*) [482,483], coconut (*Cocos nucifera*) endosperm [484], *Cuphea* sp. [485], oil-rich tubers of *Cyperus*

esculentus [486], *Echium pitardii* [487], flax (*Linum usitatissimum*) [180], garden nasturtium (*T. majus*) embryos [179], *Jatropha curcas* [488], *Idesia polycarpa* [489], *Litchi chinensis* [490], *Macadamia tetraphylla* [491], maize (*Z. mays*) embryos [492], oil palm (*Elaeis guineensis*) kernel [493], oil palm mesocarp [494,495], peanut [57,58,496,497], rice (*O. sativa*) [150], chia (*Salvia hispanica*) [498], shea (*Vitellaria paradoxa*) fruit kernels [499], *Sorghum* (*Sorghum bicolor*) [500], soybean [140,165,501–503], sunflower (*H. annuus*) [504], *Tetraena mongolica* [505], tung tree (*Vernicia fordii*) [43,506–508], *V. galamensis* [509], *Xanthoceras sorbifolia* [510], and acetyl-TAG-producing species [81,82,511].

In some cases, the functional expression of plant *DGAT2s* in yeast has been shown to result in poor production of recombinant enzyme unless the encoding genes are codon-optimized [177,193]. This was first demonstrated for a plant *DGAT2* cDNA through the codon-optimization of the gene encoding *Arabidopsis* DGAT2 [177]. The cDNA based on the natural gene could, however, be transiently expressed producing a functional recombinant *Arabidopsis* DGAT2 in *N. benthamiana* leaves [512].

6.2.2. Biochemical properties

6.2.2.1. Early studies and sources of DGAT for characterization. AthDGAT1 was first identified by four different groups, in reports published in 1999 and 2000, shortly after the cloning of mouse *DGAT1* [131,474,513,514]. In addition, a truncated cDNA encoding a *B. napus* DGAT1 was cloned by Nykiforuk et al. in 1999 [515]. Later the same year, a full-length cDNA encoding a *B. napus* DGAT1 was also cloned [516]. Interestingly, each cDNA was functionally expressed in *P. pastoris* [475]. It is now known that the first *BnaDGAT1* cloned corresponded to *BnaA.DGAT1.b* [476], which was named according to nomenclature suggested for *Brassica* species [517].

Characterization studies on DGAT1 and DGAT2 from higher plants have benefited from the production of specific recombinant enzyme forms in expression systems such as yeast devoid of TAG biosynthesis. In depth characterization, however, requires the availability of highly purified and active enzyme, or at least microsomal fractions from the host organism which are enriched in active recombinant enzyme. Earlier studies on the solubilization of membrane-bound DGATs from higher plants and their partial purification likely involved mixtures of different isoenzymes [110,143,518,519]. Tung tree DGATs, with maltose-binding protein and a poly-histidine affinity tags, have been recombinantly produced in *E. coli* [520,521]. In the case of DGAT1, the recombinant protein was mostly targeted to membranes and insoluble fractions [521]. Multiple proteins copurified with the DGAT1 fusion protein from the soluble fraction. Recombinant DGAT was also solubilized from the insoluble fraction and partially purified, although there was extensive precipitation following immobilized metal ion affinity chromatography. Tung tree DGAT2 was highly purified but in an inactive form [520]. The investigators suggested that the loss in enzyme activity may have been due to certain post-translational events not occurring or improper folding associated with the production of a eukaryotic recombinant protein in a prokaryotic expression system.

In addition to *BnaA.DGAT1.b*, *B. napus* has been shown to contain three additional highly homologous gene forms (homeologous forms) of *DGAT1* [153,156,476]. The A and C genomes have been shown to each contribute two gene forms. Recombinant isoform BnaC.DGAT1.a was the first membrane-bound DGAT1 to be highly purified in an active form [522]. The final purification step, which involved size-exclusion chromatography, suggested that the majority of enzyme eluted as a dimer with some apparent tetramer. Cross-linking studies also indicated that BnaC.DGAT1.a could form a dimer and this capability was associated with the first 80 amino acid residues of the enzyme [54]. Structural studies, described in detail below, confirmed this dimeric form.

6.2.2.2. Biochemical regulation of DGAT1. Despite not being purified, recombinant plant DGAT1 in yeast microsomes has been useful in gaining insight into the biochemical properties of the enzyme. The activity of microsomal recombinant DGAT1 from the American hazelnut shrub (*Corylus americana*) has been shown to exhibit a sigmoidal response to increasing acyl-CoA concentration [165]. In addition, several variants of the enzyme, generated using a DNA shuffling approach, were also shown to exhibit sigmoidal kinetics. The same study also reported sigmoidal kinetics for DGAT1-2 from maize which was previously shown to be encoded by a *DGAT1* allele (ASK allele) associated with a maize high-oil line [492]. The sigmoidal kinetics were considered metabolically meaningful in that when cellular acyl-CoA concentrations were low, less of the thioesters would be used to support DGAT action and more would be available to support membrane synthesis. Once acyl-CoA concentrations increased, more of the acyl-CoAs would be directed to storage TAG accumulation because of the increased activity of the DGAT1 induced by higher concentrations of acyl-CoA.

The most in depth information on structure and function for a membrane-bound higher plant DGAT has been achieved using BnaC.DGAT1.a [54,522–524]. These investigations involved the use of both full length and truncated forms of the recombinant enzyme. Some of the experiments used yeast microsomes enriched in full-length enzyme or N-terminally-truncated enzyme whereas other experiments used purified full-length enzyme or various purified N-terminal segments of the enzyme. When using microsomal fractions for activity analysis, it was important to normalize enzyme production based on the use of a tag which was immunochemically recognized [54,96]. Thus, DGAT1 polypeptide production level could be quantified through image analysis of DGAT bands in Western blots of SDS-PAGE gels.

A model describing the biochemical regulation of BnaC.DGAT1.a is shown in Fig. 10. Much like the hazelnut enzyme [165], BnaC.DGAT1.a has been shown to exhibit a sigmoidal response to increasing concentrations of acyl-CoA [54,96,523] suggesting that it is an allosteric enzyme showing positive cooperativity. The results are consistent with the predicted BnaDGAT1 structure (Please refer to Fig. 17 for details), in which the second segment of the soluble NTD (residues 61-113) of one subunit interacts with the membrane domain of the other subunit. The binding of acyl-CoA in the allosteric site of the NTD may initiate structural changes within the same subunit and this change may be directly transmitted to the other subunit by the soluble domain. Furthermore, intermolecular communication between subunits can also be facilitated by the first transmembrane helix of one subunit, which forms multiple interactions with the helices of the other subunit. Interestingly, an earlier study using a recombinant N-terminal fragment of BnaA.DGAT1.b with poly-histidine tag (13,278 Da) indicated that the fragment could bind radiolabeled acyl-CoA through positive cooperativity [111]. The fragment bound erucoyl ($22{:}1\Delta^{13}$ hereafter 22:1-CoA) with greater affinity than 18:1-CoA. Previously, digestion of microsomes from microspore-derived cell suspension cultures of *B. napus* with Proteinase K, followed by Western blot analysis, failed to reveal immunoreactivity with antibodies raised against the segment of BnaA.DGAT1.b corresponding to amino acid residues 21-35. This observation suggested that the hydrophilic NTD of the enzyme was localized to the cytosolic side of the ER [475]. Although the NTD of BnaC.DGAT1.a did not exhibit DGAT activity, it was proposed to contain an intrinsically disordered region (IDR) that had an autoinhibition function and a dimerization interface [54,524]. A folded segment was also present towards the C-terminal end of the NTD which represented an allosteric non-catalytic site for binding of acyl-CoA and/or CoA. These interactions have been shown to occur at micromolar affinity [111,524]. With increasing acyl-CoA concentration, the non-catalytic site was shown to facilitate homotropic allosteric activation and, under limiting acyl-CoA conditions, CoA was shown to act as a non-competitive feedback inhibitor through the same folded segment of the NTD [54]. Thus, the hydrophilic NTD acts as a sensor of the cytosolic ratio of acyl-CoA: CoA, adjusting the activity of the enzyme

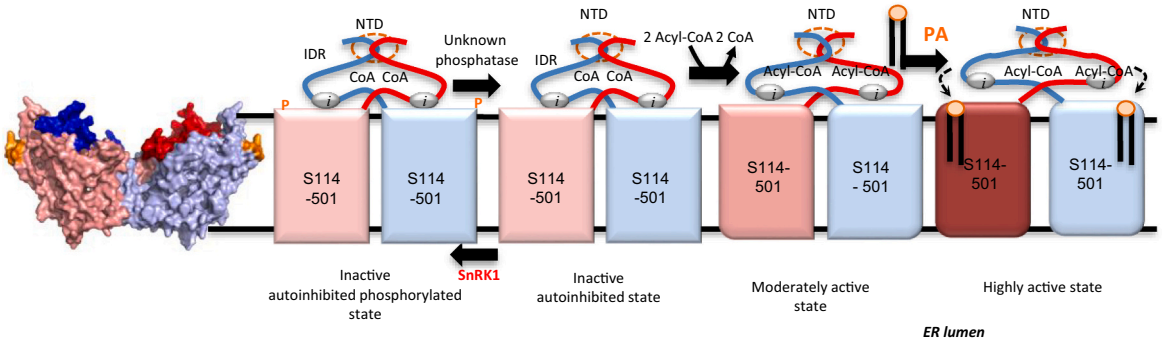

Fig. 10. Model depicting biochemical regulation of *Brassica napus* diacylglycerol acyltransferase 1 (DGAT1). BnaDGAT1 is composed of a regulatory N-terminal domain (NTD; blue) and a membrane-bound domain with catalytic site (S114-501; red). The NTD has an intrinsically disordered region (IDR) with autoinhibitory motif (*i*) and a folded section with allosteric site for CoA/acyl-CoA. Acyl-CoA or CoA can interact with the hydrophilic NTD of BnaDGAT1 and allosterically modulate catalysis within S114-501 which lies beyond the NTD [54]. High acyl-CoA levels promote the moderately active state whereas high CoA levels promote the more inactive state. Phosphatidic acid (PA) acts as a feedforward activator of BnaDGAT1, possibly by interacting with and bringing about a conformational change in region S114-501 which displaces the proposed autoinhibitory motif (*i*). BnaDGAT1 is also a substrate of sucrose non-fermenting-1-related kinase (SnRK1), which catalyzes the phosphorylation of the enzyme thereby resulting in a less active form of the enzyme. An unidentified protein phosphatase may be involved in catalyzing dephosphorylation of BnaDGAT1, thus resulting in activation of the enzyme. Soluble domains of each subunit are shown in darker shade and the SnRK1 site in orange. The Figure is revised from Caldo et al. (2017, 2018) [54,523] and the protein structure at the left side is in consistent with Fig. 17. The figure is reproduced with the permission from John Wiley & Sons and Society for Experimental Biology.

accordingly. Interestingly, increasing CoA concentration has been shown to displace radiolabeled acyl-CoA in the earlier binding study with the recombinant NTD of BnaA.DGAT1.b [111].

BnaC.DGAT1.a has also been shown to be activated by PA through its specific interaction with the NTD [523]. The interaction of PA with BnaC.DGAT1.a is shown in the model in Fig. 10. The impetus for investigating the potential effect of PA was based on a previous study which demonstrated that PA activates DGAT activity in microsomes from microspore-derived cell suspension cultures of *B. napus* [115]. In the presence of PA, the acyl-CoA saturation plot became more hyperbolic and desensitized BnaC.DGAT1.a to inhibition by CoA [523]. PA may have also relieved possible autoinhibition of the enzyme, thus facilitating the transition of the enzyme to a more active state. In addition, PA might have a biophysical effect on membrane curvature or fluidity thereby contributing to activation of the recombinant enzyme. PA is known to be a second messenger in signaling processes underlying plant growth and development [525] and the anionic PL has also been shown to activate CTP:phosphocholine cytidylyltransferase 1 (CCT1) in *Arabidopsis* via binding to a C-terminal amphipathic lipid binding domain [458]. CCT is a key enzyme in the nucleotide pathway for PC biosynthesis catalyzing the conversion of phosphocholine to CDP-choline and the importance of CCT in plants was firstly demonstrated in peas [526] where control mechanisms were also studied [527]. In the Kennedy pathway, LPAAT is responsible for catalyzing the production of PA (see Fig. 9). Interestingly, heterologous constitutive expression of a cDNA encoding a yeast *sn*-2 acyltransferase has been shown to increase seed oil content in *Arabidopsis* and *B. napus* [456], and soybean through seed-specific expression [528]. Seed-specific expression of *T. majus LPAAT* during seed development in *B. napus* has also shown to increase seed oil content [457]. Furthermore, expression of a *B. napus LPAAT* in *Arabidopsis* has been shown to increase seed oil content [529]. Since the DGAT-catalyzed reaction in developing seeds of *B. napus* exhibits strong flux control in the Kennedy pathway [455,530], it is possible that the seed oil content associated with increased LPAAT activity may be due to PA-mediated activation of DGAT1. The split-ubiquitin based membrane yeast two-hybrid assay has been used to demonstrate that *B. napus* LPAAT2 physically interacts with BnaC.DGAT1.a$_{81\text{-}501}$ under *in vivo* conditions [523]. Weak but clear interaction between *Arabidopsis* LPAAT2 and DGAT1 has also been observed [453]. Therefore, the PA product of the LPAAT-catalyzed reaction may be more effectively channeled to the PA binding site on BnaC.DGAT1.a. Interestingly, the effect of PA was specific for DGAT1 since recombinant BnaDGAT2 was not subject to activation by PA [523]. Co-activation of DGAT1 and CCT1 by PA is intriguing to consider in terms of the regulation of LD formation. As LDs pinch off from the ER, PL is also removed from the membrane. Thus, activation of CCT1 may allow PC biosynthesis to keep pace with increased TAG formation due to increased DGAT1 activity (Fig. 11). Despite this generalization, it is important to note that LD formation is a complex process involving several interacting proteins [531].

Plant DGAT1 may also be modulated by sucrose non-fermenting-1-related kinase (SnRK1) (Fig. 10) which belongs to a sub-family of serine/threonine kinases regulating metabolism in response to energy status [523]. In plants, SnRK1 serves as a sensor in metabolism affecting growth, development, and the stress response [532]. A targeting motif for SnRK1 was first identified in AthDGAT1 [474]. *T. majus* DGAT1 has been shown to undergo activation when site-directed mutagenesis was used to convert the putative phosphorylation target of SnRK1, serine residue 197, to an alanine residue [179]. In a more recent study, BnaC.DGAT1.a was shown to be partially inactivated following phosphorylation of the enzyme catalyzed by SnRK1 [523]. Thus, SnRK1-mediated covalent regulation of DGAT1 to reduce TAG accumulation links the control of the enzyme to broader aspects of cellular metabolism including energy status and sugar signaling [523,533]. In the model depicted in Fig. 10, the predicted SnRK1 site (orange) is located on the cytosolic side of the membrane, and phosphorylation catalyzed by SnRK1 contributes to preserving the inactive state of BnaC.DGAT1.a. An unknown phosphatase would be required to catalyze the removal of phosphate so as to up-regulate the BnaC.DGAT1.a to a more active form [523].

Recent studies on recombinantly produced DGAT1 from forage *Sorghum* and rice (*O. sativa*) have also suggested a regulatory role for the NTD [150,500]. In addition, amino acid residue substitutions of numerous putative phosphorylation sites in the NTD of rice DGAT1-1, via site directed mutagenesis, have been shown to result in lower enzyme activity, suggesting that phosphorylation of the NTD may play a further role in regulation of the enzyme [150]. IDRs are well-recognized components of the NTDs of plant DGAT1s [54,150,500,524]. Interestingly, the first 16 amino acid residues of soybean DGAT2A has also been shown to be an IDR which may have an autoinhibitory function much like the IDR of plant DGAT1 [503]. Indeed, the N-terminal truncation, GmDGAT2A$_{20\text{-}337}$, led to an increase in TAG accumulation when recombinantly produced in H1246 yeast when compared to the effect of producing the full-length recombinant enzyme.

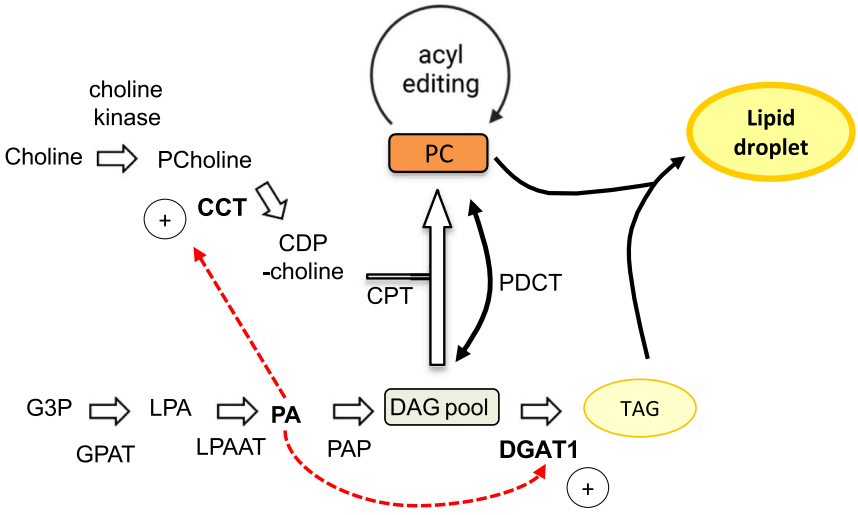

Fig. 11. Co-activation of diacylglycerol acyltransferase 1 (DGAT1) and CTP:phosphocholine cytidylyltransferase (CCT) 1 by phosphatidic acid (PA) in the possible control of lipid droplet body formation during seed development in oleaginous higher plants. PA results in the concomitant activation of phosphatidylcholine (PC) and triacylglycerol (TAG) biosynthesis via feedforward activation of CCT1 and DGAT1, respectively. PC is required for the phospholipid monolayer to package TAG into oil bodies. 1, 2-diacyl-*sn*-glycerol (DAG) generated *de novo* via the Kennedy pathway can also be converted to PC to facilitate formation of polyunsaturated fatty acids (PUFAs) and acyl-editing as a means of generating DAG and acyl-CoA enriched in PUFAs. Other abbreviations: CPT, CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase; G3P, *sn*-glycerol-3-phosphate; GPAT, acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase; LPA, lysophosphatidic acid; LPAAT, acyl-CoA: lysophosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; PCholine, phosphocholine; PDCT, phosphatidylcholine:diacylglycerol cholinephosphotransferase. The Figure was generated based on information from previous publications [458,471,472,654]. The image was reproduced with permission from John Wiley & Sons and Society for Experimental Biology.

It has been known for a long time, in developing seeds of oleaginous plants, that the forward and reverse reactions of LPCAT are responsible for acyl exchange between PC and the acyl-CoA pool [534]. This aspect is outlined in Fig. 9. In developing seeds of oleaginous plants producing TAGs enriched in PUFAs, biochemical coupling of the LPCAT-catalyzed reverse reaction to the DGAT-catalyzed forward reaction may represent a process of routing PUFAs synthesized at the level of PC into TAG. The common intermediate in this coupling process is acyl-CoA. Both *in vivo* and *in vitro* evidence in support of this process has been demonstrated using recombinant flax lipid biosynthetic enzymes and a quintuple mutant of *S. cerevisiae* devoid of TAG synthesis also displaying extremely low LPCAT activity [535]. For the *in vivo* study, flax *FAD2-1* and *FAD3B* genes were introduced into the quintuple mutant, expressing flax *LPCAT1* and/or flax *DGAT1-1*, to generate PC enriched in 18:3. PUFA-enriched TAG was only produced when both *LPCAT1* and *DGAT1-1* were co-expressed. Thus, the endothermic LPCAT reverse reaction could be pulled in the direction of acyl-CoA and LPC via coupling to the DGAT1-1-forward reaction, which is strongly exothermic. Furthermore, the specificity of LPCATs has been shown to differ in the forward and reverse reactions [536]. In the study with flax recombinant enzymes, both *in vivo* and *in vitro* evidence demonstrated that PUFA-containing substrates were preferred in the routing of PUFA from PC into TAG [535]. Therefore, the coupling process involving LPCAT1 and DGAT1-1 was apparently further aided by enhanced selectivity for PUFA-containing substrates. In a more recent study, recombinant flax LPCAT2, which is highly homologous to LPCAT1, was shown to physically interact with recombinant flax DGAT1-1 under *in vivo* conditions in both yeast and *Nicotiana benthamiana* leaves [537]. *Arabidopsis* DGAT1 and LPCAT2 also showed physical interaction [453]. Thus, the coupling process may even be further aided by substrate channeling between the participating enzymes. The study also showed that various other transferases involved in TAG biosynthesis in developing flax seed could interact, suggesting that enrichment of TAG with 18:3 was facilitated by a transferase interactome. Feeding studies with exogenous FAs have also shown that recombinant flax DGAT2-3 (produced using a codon-optimized cDNA) is even more selective for 18:3-containing substrates than flax DGAT1-1 [193]. A similar study has shown that recombinant chia DGAT2-1 is also highly selective for substrates containing ALA [498]. Both flax and chia seed contain TAG that is highly enriched in 18:3 [538,539].

The regulation of DGAT activity during seed development in oleaginous higher plants can also be viewed in terms of the contribution of the DGAT-catalyzed reaction to overall process involving FA biosynthesis and TAG assembly. This aspect is addressed in section 9.4.1 which includes the application of Metabolic Control Analysis (MCA) during seed development in *B. napus* and how this type of analysis can provide useful information to guide ME strategies aimed at increasing seed oil content.

6.2.2.3. Substrate specificity and selectivity. Many of the functional expression studies on higher plant *DGATs*, previously referred to, have involved feeding exogenous FA(s) to a TAG-deficient yeast mutant producing the recombinant DGAT under investigation. Some of these studies have shown enhanced preference for the incorporation of FAs into TAG that are known to be naturally produced in the plant from which the DGAT under investigation was sourced (e.g., [193,487,491,498,500]). It should be noted, however, that these exogenous FAs need to be incorporated into acyl-CoA and DAG for utilization by the recombinant DGAT under investigation. Therefore, more accurate information on the specificity and selectivity properties of the enzyme can be obtained using *in vitro* assays involving the direct substrates. From a physiological perspective, the substrate specificity/selectivity determinations based on *in vitro* enzyme assays, however, may not reflect the substrate selectivity under *in vivo* conditions [402]; furthermore, developing plants are exposed to ambient temperatures and various other environmental conditions.

In vitro DGAT assays with microsomal fractions from developing seeds and other oleaginous tissues/cells have been useful in gaining insight into the specificity and/or selectivity properties of the overall DGAT activity in these membranes [3,29]. Some specific examples include studies with microsomes from developing microspore-derived embryos of *B. napus* [162], *Cuphea* seeds [98], flax seeds [540], *V. galamensis*, *Stokesia laevis* and soybean seeds [157], and *Crambe abyssinica* seeds [168]. Since it is now known that the overall DGAT activity of a developing seed is probably due to a mixture of DGAT isoforms (along with PDAT if radiolabeled DAG is used as an acyl acceptor), more recent studies of substrate preferences have been conducted with specific recombinant forms of DGAT.

Earlier research on the specificity/selectivity of specific recombinant isoforms of plant DGAT focused on acyltransferases which might be useful in enhancing the level of industrially useful unusual FAs in transgenic plants. In this regard, as mentioned previously, plant DGAT2 has emerged as a category of membrane-bound DGATs with increased preference for substrates containing unusual FAs. Thus, plant DGAT2s may be more important than DGAT1s in routing unusual FAs formed at

the level of PC into TAG.

Tung tree oil is enriched in α-eleostearic acid (18:3$\Delta^{9cis,11trans,13trans}$), which is a conjugated FA imparting industrially useful drying properties to the oil [541]. Therefore, there has been an interest in engineering high-yielding oilseed crops to produce this FA [43], Functional expression experiments with tung tree *DGAT1* and *DGAT2* have shown that recombinant DGAT2 exhibits an enhanced propensity for catalyzing the formation of trieleostearin [43].

Castor oil, as mentioned previously, is enriched in ricinoleic acid, and thus represents another industrially useful oil [22,363]. The presence of noxious proteins in the seed meal left over from oil extraction has been a major factor in suppressing the wide-spread cultivation of the crop [363] and, therefore, there has been an interest in producing oils enriched in ricinoleic acid in high-yielding oilseed crops [470,481]. Recombinant castor bean DGAT2 has been shown to be very effective in utilizing substrates containing ricinoleoyl moieties [480,481]. It should be noted, however, that castor bean DGAT1 has been shown to exhibit an increased specificity for catalyzing the transfer of [^{14}C]18:1 from [^{14}C]18:1-CoA to 1,2-diricinoleoyl-*sn*-glycerol than to 1,2-dioleoyl-*sn*-glycerol or 1,2-dipalmitoyl-*sn*-glycerol [479]. In addition, castor bean DGAT1 was more active with 1,2-diricinoleoyl-*sn*-glycerol than *AthDGAT1* at DAG bulk concentrations below 1 mM [542].

Vernolic acid (*cis*-12-epoxyoctadeca-*cis*-9-enoic acid), which is enriched in oil from *V. galamensis* seeds, is industrially important for epoxy coatings [543]; thus, there has been an interest in ME of oil crops to produce seed oils enriched in this epoxy FA [544]. Recombinant *V. galamensis* DGAT2 was more effective then recombinant VgDGAT1 in catalyzing the production of TAG containing vernoloyl moieties [509,545,546].

Although 18:3 is not considered an unusual FA, the routing of this PUFA from its site of synthesis in PC to TAG may also involve DGATs with enhanced selectivity for substrates containing 18:3. As mentioned previously, flax DGAT1 or DGAT2 have been shown to exhibit enhanced specificity for 18:3-CoA, with DGAT2 exhibiting a substantially higher specificity for this acyl-CoA [193,535].

In-depth studies on the substrate preferences of specific forms of recombinant plant DGATs have been conducted with various species in the *Brassicaceae*. The acyl-CoA specificity properties of *AthDGAT1* and *AthDGAT2* have been investigated using microsomes prepared from *N. benthamiana* leaves transiently expressing the cDNA of each enzyme type under the control of a 35S promoter [512]. In general, AthDGAT2 activity was higher than AthDGAT1 activity. 18:1, 18:2 and 18:3-CoA were all effective acyl donors for AthDGAT2. AthDGAT1 displayed the highest specificity for 18:1-CoA followed by 18:2-CoA, and the enzyme had essentially no activity with 18:3-CoA. It should be noted, however, that 1,2-dihexanoyl-*sn*-[^{14}C]glycerol was used as an artificial acyl acceptor. Different substrate specificity data might be obtained, however, using more conventional long-chain DAGs [120]. Transient expression of *AtDGAT2* also led to significant increases ($P < 0.001$) in the 18:2 and 18:3 content of leaf TAG [512].

Globally, *Brassica* oilseed species represent the third largest source of vegetable oil, with low erucic acid/low glucosinolate varieties of *B. napus* (known as canola in North America) being the most widely grown [24,547]. *B. napus* is an allotetraploid species containing two (A and C) of three ancestral *Brassica* genomes [548]. Phylogenetic analysis of the amino acid sequences encoded by four transcriptionally active *DGAT1* genes from *B. napus* has suggested that the gene forms separated over time into two clades (I and II) with the A and C genomes each having a member of clade I and II [156,476]. *In vitro* enzyme assays using microsomal forms (produced in H1246 yeast) of the four recombinant BnaDGAT1s indicated that each isoform could effectively utilize a range of acyl-CoA substrates which represented the FAs found in the seed oil of the *B. napus* double haploid line used to provide the sequence information [156]. 1,2-dioleoyl-*sn*-glycerol was used as the acyl acceptor. In each case, 18:3-CoA was the most effective acyl donor. The clade II acyltransferases (BnaA.DGAT1.b and BnaC.DGAT1.b), however, displayed increased specificity for 18:2-CoA over the clade I enzymes (BnaA.DGAT1.a and BnaC.DGAT1.a). Based on differences in amino acid residues in three motifs in clade I versus clade II BnaDGAT1s, the investigators suggested that it may be useful to use a site directed mutagenesis approach to potentially change the acyl-CoA substrate specificity of a clade II enzyme to that of a clade I enzyme (or vice versa). Highly purified recombinant BnaC.DGAT1.a (a member of clade I), in a solubilized form, has been shown to display similar acyl-CoA specificity properties to the recombinant enzyme in yeast microsomes [522]. Each of the four recombinant BnaDGAT1s were shown to exhibit enhanced specificity for 16:0-CoA over 18:1-CoA when using 1,2-dioleoyl-*sn*-glycerol as the acyl acceptor [153,156]. Somewhat different results were obtained using 1-palmitoyl-2-oleoyl-*sn*-glycerol as the acyl acceptor [153], indicating that the nature of the acyl acceptor can affect acyl-CoA specificity. Nonetheless, each isoform still used16:0-CoA more effectively than 18:1-CoA. During the peak of TAG biosynthesis in developing seeds of *B. napus*, the ratio of 18:1-CoA to 16:0-CoA, however, was shown to be 3:1 [153]. Acyl-CoA selectivity assays performed using a 3:1 ratio of 18:1-CoA to 16:0-CoA in the reaction mixture, resulted in the four isoforms of BnaDGAT1 incorporating 18:1 in amounts two- to four-fold higher than 16:0 [153]. In another selectivity experiment with isoform BnaC.DGAT1.a, in this case using an equimolar mixture of 16:0-CoA and 18:1-CoA, the ratio of enzyme activity supported by 16:0-CoA to 18:1-CoA was 2.6 [137]. When the enzyme was assayed separately with each acyl-CoA, the ratio was 1.7. In these experiments, however, the artificial acyl acceptor 1,2-dioctanoyl-*sn*-glycerol was used.

Earlier assays of DGAT activity, using long chain DAG as an acyl acceptor, in membrane fractions from developing seeds or microspore-derived embryos of high-erucic acid versus low-erucic acid varieties of *B. napus* showed similar substrate specificities for either oleoyl- or erucoyl-CoA [98,162]. In the case of the microspore-derived embryos, similar activity was observed up to 5 μM acyl-CoA, and thereafter, higher activities were seen with erucoyl-CoA [162]. These early results suggested that *DGAT* genes were unaffected by the breeding process used to produce low-erucic acid varieties of *B. napus*. Indeed, the low erucic acid trait in *B. napus* has been linked to the *FATTY ACID ELONGATION (FAE) 1* gene [549–551] and mutations in the gene have been associated with a loss of elongation activity [552,553]. More specifically, *FAE1* encodes a β-ketoacyl-CoA synthase that catalyzes the first condensation reaction in the extra-plastidial elongation pathway for conversion of 18:1-CoA to 22:1-CoA [168,551,554]. Another earlier study examining the substrate preferences of microsomal acyltransferase activities, however, has shown that DGAT activity from a very high erucic acid variety of *B. napus* (54% erucic acid content) exhibits a much higher specificity for erucoyl-CoA over oleoyl-CoA [154]. Thus, depending on the cultivar, changes in the acyl-CoA selectivity of DGAT may have also occurred during the breeding process.

Recently, assays of microsomal DGAT activity have been conducted during seed development in *B. napus* using an edible low-erucic acid cultivar (MONOLIT) versus a nonedible high-erucic acid cultivar (MAPLUS) [477]. Enzyme assays were conducted with 18:3-CoA or 22:1-CoA as acyl donors using radiolabeled 1,2-dihexanoyl-*sn*-[^{14}C] glycerol as an artificial acyl acceptor. DGAT activity of the microsomes from each cultivar effectively used 18:3-CoA at all stages of seed development examined. When 22:1-CoA was used as the acyl donor, however, substantial microsomal DGAT activity was only seen with the high-erucic acid cultivar (MAPLUS). The results of this experiment suggested that BnaDGAT isoforms with different acyl-CoA specificities were operative during seed development in the two cultivars. Various isoforms of BnaDGAT1 or BnaDGAT2 were then recombinantly produced in H1246 yeast followed by acyl-CoA substrate specificity experiments with microsomal fractions. cDNAs encoding the BnaDGAT2 isoforms were codon optimized for expression in *S. cerevisiae*. The BnaDGAT1 isoforms showed similar and broad acyl-CoA substrate specificities, but with relatively poor utilization of 22:1-CoA. The broad acyl-CoA specificities observed for the BnaDGAT1s was in general

agreement with a previous study on the acyl-CoA specificity of four isoforms of BnaDGAT1, although 22:1-CoA was not used here [156]. In this previous study, however, relatively high acyl-CoA specificities were reported when using 18:2- and 18:3-CoA. These differences may have been attributable to the enzyme variants being investigated [477]. In contrast, the recombinant BnaDGAT2 isoforms tested displayed narrow acyl-CoA substrate specificities with 18:3-CoA being the most effective acyl donor in all cases [477]. Two of the BnaDGAT2 isoforms also exhibited high activity with 22:1-CoA and were referred to as 22:1-CoA-accepting [477,478]. The isoforms that did not utilize 22:1-CoA effectively were referred to as 18:3-specific [477,478]. It was thus hypothesized that either one, or both, of the 22:1-CoA-accepting isoforms were more active in MAPLUS during seed development thereby accounting for enhanced utilization of 22:1-CoA [477]. The specificity study on BnaDGAT2 isoforms in high erucic acid MAPLUS suggests that DGAT specificities have also been affected (via mutations in *DGAT2* genes) during the selection process to develop high erucic acid cultivars with high seed oil content [477]. In a follow-up study, a range of chimeric enzymes produced from 18:3-specific BnaDGAT2 and 22:1-CoA-accepting BnaDGAT2 led to the identification of a polypeptide region, containing two predicted transmembrane helices, which was linked to the differences in acyl-CoA substrate specificity [478]. Replacement of the equivalent region in *Arabidopsis* DGAT2 with the segment from 22:1-CoA-accepting BnaDGAT2 resulted in a modified *Arabidopsis* DGAT2 with enhanced specificity for 22:1-CoA.

Crambe (*C. abyssinica*), another member of the Brassicaceae, also produces seed oil enriched in erucic acid [555]. The specific activity of DGAT for 22:1-CoA, in microsomes from developing seeds of *Crambe*, has been shown to double at 19 days after flowering (DAF) compared to earlier stages [168]. These results suggested that a DGAT isoform(s) with increased specificity for 22:1-CoA was up-regulated at the beginning of rapid erucic acid and oil accumulation. Recently, four forms of each *DGAT1* and *DGAT2* were isolated from cDNA prepared from developing *Crambe* seeds [120]. The different isoforms of *DGAT1* and codon-optimized isoforms of *DGAT2* were expressed in H1246 yeast. Assays of microsomal DGAT activity indicated that all forms of recombinant DGAT1 and DGAT2 could utilize a range of acyl-donors along with 22:1-CoA when using the artificial acyl acceptor 1,2-dihexanoyl-*sn*-[14C]glycerol. When 1,2-dierucoyl-*sn*-glycerol was used as an acyl acceptor (using various radiolabeled acyl-CoAs), with a representative isoform (DGAT1 D) of DGAT1, 22:1-CoA was used at substantially lower rate then for 1,2-dioleoyl-*sn*-glycerol. The various isoforms of DGAT2, however, were not capable of catalyzing the acylation of 1,2-dierucoyl-*sn*-glycerol. The results further suggested the DGAT activity levels and acyl-CoA substrate specificities are affected by the acyl composition of the DAG acyl acceptor, and that the use of artificial acyl acceptors, such as 1,2-dihexanoyl-*sn*-glycerol, may not reflect the *in planta* situation.

High-erucic acid pennycress (*Thlaspi arvense*) is also a member of the Brassicaceae. A recent study of the heterologous functional expression of pennycress *FAE1* in *Arabidopsis* backgrounds Col-0 (accumulating < 2.5% erucic acid in the seed oil) or an *fae1-1* mutant (deficient in very-long chain fatty acids) suggested that erucic acid was incorporated into TAG via DGAT1 [556].

Garden nasturtium (*T. majus*) is a member of the Tropaelaceae family and also produces seed oil containing very high levels of erucic acid (60-80%) [557]. The substrate specificity properties of recombinant garden nasturtium DGAT1 have also been examined [179]. Enzyme assays were conducted with lysates of yeast strain H1246, which was used to produce the recombinant enzyme. [^{14}C]Labeled palmitoyl, oleoyl, eicosenoyl (20:1Δ^{11}) or erucoyl-CoA were used in combination with either 1,2-dioleoyl-*sn*-glycerol or 1,2-dierucoyl-*sn*-glycerol as acyl acceptors. For either acyl acceptor, the acyl-CoA specificity was 22:1>20:1>18:1>16:0-CoA. Oleoyl-containing DAG was a somewhat better acceptor than erucoyl-containing DAG.

It thus appears, depending on the plant species under investigation, that DGAT1 and/or DGAT2 isoforms may contribute to the incorporation of erucic acid into TAG. Although erucic acid is considered an unusual FA, its production is based on the extra-plastidial elongation of acyl-CoA rather than synthesis at the level of PC as is the case for α-eleostearic, ricinoleic or vernolic acid.

Camelina sativa, another member of the Brassicaceae family, has been emerging as important industrial crop which can be grown under many different environmental conditions and on marginal land with low input requirements [558,559]. The plant is also easily transformed [559,560] and the seed is about ten times larger than *Arabidopsis* making *C. sativa* more amenable to biochemical studies than that of the model oilseed plant [104,561]. A recent study of recombinantly produced DGAT1 and DGAT2 from *C. sativa* has shown major differences in preferences of the two enzyme forms for long chain acyl acceptors [104]. DGAT1 preferred acyl acceptors consisting of saturated and monounsaturated fatty acyl chains whereas DGAT2 exhibited high specificity for acyl acceptors containing polyunsaturated fatty acyl chains. In these experiments, 18:1-, 18:2- and 18:3-CoA were used as acyl donors. Interestingly, the combination of substrates that resulted in the highest DGAT2 activity, but lowest DGAT1 activity, corresponded to the molecular species of TAG which were shown to increase in transgenic *C. sativa* where *DGAT1* expression was reduced [561]. DGAT assays using the artificial acyl acceptor, 1,2-dihexanoyl-*sn*-[^{14}C]glycerol, showed considerably different results in terms of acyl-CoA preferences. For example, DGAT2 exhibited 25 times lower activity with 18:3-CoA when using 1,2-dioleoyl-*sn*-glycerol instead of 1,2-dihexanoyl-*sn*-glycerol as an acyl acceptor. Thus, the investigators indicated that the use of artificial acyl acceptors might not necessarily reflect the *in planta* DGAT selectivity with natural long chain acyl acceptors. The investigators further suggested that DGAT specificity studies based on the use of fluorescent substrate analogues may also not be reflective of the physiological situation and that caution should be exercised in the interpreting results.

In terms of characterization of soybean DGAT, a combination of 18:1-CoA and 1,2-dioleoyl-*sn*-glycerol has been reported to be an effective substrate combination for the assay of recombinant soybean microsomal DGAT1A or DGAT1B [501]. Acyl donors or acyl acceptors, containing the unusual FA, vernolic acid, were not effective substrates indicating that soybean DGAT1 isoforms would not be useful in the ME of soybean to produce vernoloyl-enriched TAG. Recently, recombinant microsomal soybean DGAT2A has been shown to utilize 16:0-, 18:0-, 18:1- or 18:2-CoA, but the activity was highest with 18:2-CoA as an acyl donor [503].

It is also possible that the hydrophilic NTD of plant DGAT1 contributes to the specificity/selectivity properties of this category of DGAT. Acyl-CoA binding experiments using the recombinant NTD of BnaA. DGAT1.b have shown that oleoyl-CoA-binding exhibited a dissociation constant of 17 μM whereas binding of erucoyl-CoA was stronger with a dissociation constant of 4 μM [111]. DGAT1 sequences from various plant species are highly identical except for the region of the hydrophilic NTD before the acyl-CoA binding motif (see Fig. 2.; [485,493]). This observation has prompted researchers to suggest that differences in substrate preference between DGAT1s of different species may be linked to amino acid sequence differences in their NTDs [485,493]. Generating recombinant chimeras involving the exchange of NTDs between DGAT1s of different species or the generation of amino acid residue substitutions in the NTD may shed light on this aspect.

6.2.3. High-performance DGATs

Single amino acid residue substitutions or insertions can have a substantial effect on the activity and specificity properties of a DGAT. In an early example, the non-conservative substitution of a lysine residue by alanine at position 232 (K232A) in bovine DGAT1 has been associated with a large change in milk fat content, wherein the lysine-encoding allele was associated with high milk fat content [562,563]. It was suggested that lysine residue 232, together with nearby conserved lysine residues might be critical for the interaction of the CoA moiety with DGAT1 [563]. In the *DGAT1* allele encoding DGAT1 with an

alanine residue at position 232, binding of acyl-CoA would be negatively affected thereby resulting in lower milk fat content.

An early demonstration of an amino acid residue substitution bringing about a change in activity for a plant DGAT1 was for maize DGAT1-2 [492]. A high-oil quantitative trait locus (QTL) (*qHO6*) represents a *DGAT1-2* allele encoding a high-performance form of DGAT1-2. A phenylalanine residue inserted at position 469 resulted in an enzyme which was associated with increased seed oil content and increased 18:1 content. Ectopic expression of the high oil DGAT1-2 allele increased seed oil content and 18:1 content by up to 41% and 107%, respectively. Position 469 is in the last putative TMD. Deletion of F469 in the putative TMD would shift the side chain positions of all residues from F469 to the end of the helix (positions 469 to 482) which could alter the hydrophobicity of the helix and influence overall protein structure and function.

The regulation of plant DGAT1 by SnRK1-catalyzed phosphorylation has already been discussed in section 6.2.2 [179,523]. Site directed mutagenesis of cDNA encoding *T. majus* DGAT1 is another early example of a single amino acid substitution having a large effect on DGAT1 activity [179]. In this case, substitution of serine 197 with an alanine residue resulted in a 38%-80% increase in the activity of the recombinant *T. majus* DGAT1 produced in H1246 yeast. The alanine residue at position 197 no longer provided a substrate for SnRK1 and thus the modified enzyme could not be down-regulated by SnRK1-catalyzed phosphorylation.

As described in section 2.7, cell-based assays have proven useful for assaying DGAT activity. A cDNA encoding a functional DGAT can rescue the H1246 yeast strain from the lipotoxic effects of 18:1 and other unesterified FAs [94]. The amount of NPL produced in H1246 yeast, transformed with a cDNA encoding a functional DGAT, can then be quantified using Nile red fluorescence in a procedure that is amenable to a 96-well format for analyzing the effects of the functional expression of many mutated *DGAT* cDNAs [94,181]. When combined with error-prone PCR to introduce mutations into a *DGAT1* cDNA, this has resulted in a powerful system for the directed evolution of DGAT1 [95,181]. A cartoon outlining this procedure is shown in Fig. 12. For single amino acid residue substitutions resulting in modified DGAT1 activity, the effect of swapping this residue with each of the other 19 amino acid residues can be conducted using site-saturation mutagenesis (Siloto and Weselake 2012; [95]). Site-saturation mutagenesis has been conducted at position 447 of BnaC.DGAT1.a [95]. Variants generated by site-saturation mutagenesis were recombinantly produced in H1246 yeast and the NPL content of the cells was assessed by Nile red fluorescence. The recombinant enzyme form with an isoleucine (I) at position 447 was used as the reference activity. The highest levels of NPL produced were associated with variants F447, L447 and V447. Since position 447 is buried in a putative TMD, changing the hydrophobic isoleucine residue to another hydrophobic residue (phenylalanine, leucine or valine) had a positive effect on enzyme activity.

Selected BnaC.DGAT1.a variants generated by directed evolution were recombinantly produced in H1246 yeast and microsomes obtained from the various yeast transformants were used to assess both enzyme activity and polypeptide abundance [96]. Activity enhancements in the variants were generally associated with an increase in the apparent maximum velocity. Depending on the amino acid residue substitution (s), different mechanisms came into play which included increased enzyme activity, increased polypeptide accumulation and possibly reduced substrate inhibition. A kinetic model was developed which took both sigmoidicity and substrate inhibition into consideration. Indeed, concentrations of acyl-CoA > 5 μM have been shown to inhibit recombinant WT BnaC.DGAT1.a activity [54,96]. In variant L441P, where a leucine residue was replaced with a proline residue, the enzyme was not affected by acyl-CoA concentrations up to 25 μM. Essentially, this variant was de-sensitized to the potential inhibitory effects of higher acyl-CoA concentrations. Thus, variant L441P may prove useful in ME strategies aimed at maximizing DGAT activity under cellular conditions where acyl-CoA levels have increased. A few of the high-performance BnaC.DGAT1.a variants were used as templates to install the

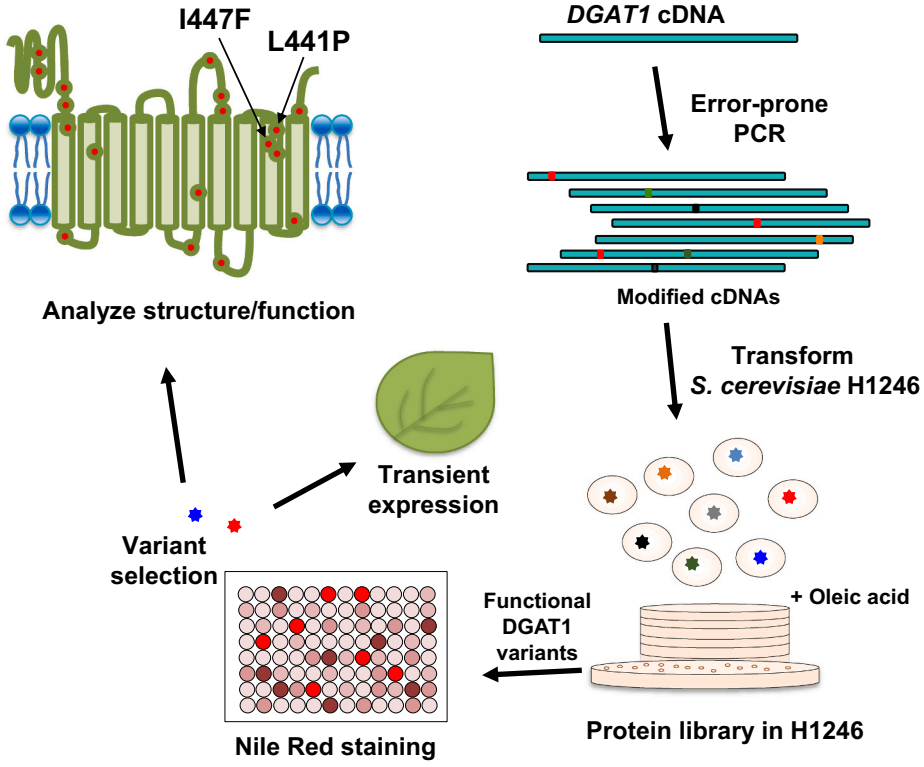

Fig. 12. Cartoon depicting mutagenesis and screening procedure used to identify and test diacylglycerol acyltransferases 1s (DGAT1s) with increased enzyme activity [1096]. The image was reproduced with permission from John Wiley & Sons and Society for Experimental Biology.

equivalent amino acid residue substitutions into *Camelina* DGAT1B [96]. The resulting *Camelina* DGAT1B variants exhibited increased performance.

DNA shuffling of the American hazelnut shrub *DGAT1* has been shown to result in several enzyme variants with increased performance [165]. In turn, a high-performance soybean DGAT1 variant with 14 amino acid residue substitutions was generated, based on the most promising American hazelnut shrub DGAT1 variant.

In another recent study, peanut DGAT2a was mutagenized to generate the site-specific variants D3V, N6D, A9V, A26P, T37M and S118P [496]. When recombinantly produced in yeast strain H1246, all six variants retained DGAT activity and produced LDs *in vivo*. Variants N6D and A26P, however, resulted in a significant increase in enzyme activity relative to recombinant WT DGAT2a and their introduction also resulted in increased total cellular FA content. In addition, variant N6D increased the content of palmitoleic acid while A26P enhanced palmitic acid content.

Very recently, Hatanaka et al (2022) compared DGAT1s from seven plants and found that in yeast expression culture, DGAT1s from *Vernonia*, sunflower, *Jatropha*, and sesame significantly increased TAG content (>10-fold) more than those from *Arabidopsis*, castor bean, and soybean [564]. Their further analysis identified three conserved amino acid residues in the former four DGAT1s but not in the latter three DGAT1s, and they proved that the three amino acid residues could be key factors underlying the high performance DGAT1s generated by site-directed mutagenesis.

Directed evolution of plant DGAT1s has resulted in variants which have proven useful in increasing plant oil content [95,165]. Genome editing is a promising tool for generating *in planta* amino acid residue substitutions to activate endogenous DGAT1 isoforms in oil crops as means of increasing seed oil content [565]. DGAT1 variants have also provided insights into structure/function in the absence of a 3-D structure [95,96,165]. Amino acid residue substitutions leading to enhanced BnaC.DGAT1.a activity along with various putative functional motifs in this isoform will be discussed in section 8.2 where the plant enzyme is modeled based on the 3-D structure available for human DGAT1 [51,52].

6.2.4. Role of DGATs in non-seed and non-mesocarp tissue

Most knowledge on the properties and physiological roles of DGATs in higher plants are based on studies of these acyltransferases from seed and mesocarp tissues of oleaginous plants. Typically, vegetative tissues contain relatively low levels of TAG, but they may have important physiological roles [8,566–569]. A previous survey of the leaves of 13 plants has shown levels of TAG up to 5 mg per g fresh weight [8]. In a specific example, 15-day-old seedlings of *Arabidopsis* were shown to contain 0.04% TAG on a DW basis [570]. In leaves, especially during senescence, it has been suggested that TAG acts as a buffer for storing fatty acyl chains when they are in excess [567,571]. During leaf senescence in *Arabidopsis*, DGAT1 has been implicated in having role in sequestering FAs, liberated from the degradation of galactolipids, into TAG which ends up in the plastiglobuli [572,573]. It was further suggested that the accumulation of leaf plastidial TAG was transient and that the storage lipid was eventually converted to phloem-mobile sucrose during senescence [572]. Given that a plethora of studies have localized DGAT1 to the ER, the plastidial localization of DGAT1 observed by Kaup et al. in 2002 [572] during senescence is worthy of further investigation. Similarly, during nitrogen deprivation in *Arabidopsis*, phytyl esters may represent a transient sink for phytol and FAs released from chlorophyll and galactolipids, respectively [87]. As indicated previously, PES1 and PES2 catalyze the formation of these phytyl esters. Once the nutrient stress is removed, the phytyl esters can be reutilized for production of chlorophyll and galactolipids. It also possible, that the DGAT activities of PES1 and PES2 may contribute to the production of transient TAG, although there is no clear evidence for the presence of long-chain acyl-CoAs in the interior of the chloroplast [87]. In terms of DGAT activity, PES1 or PES2, however, utilized various acyl donors including acyl-CoA, acyl-ACP or MGDG. FAs released from the fatty acid synthase complex are exported to plastid envelope where LACS catalyzes the formation of acyl-CoA [448,471]. LACS9, which catalyzes the synthesis of acyl-CoA at the chloroplast envelope membrane, has been shown to require the first 20 amino acid residues for plastidial localization in rice in a process that is not dependent on an N-terminal transit peptide [574]. In another previous study, using naturally senescing leaves of *Arabidopsis*, experiments with a double mutant in FA degradation and *dgat1* also implicated DGAT1 in the partitioning of FAs into TAG [566]. The relative contributions of DGAT1, DGAT2 and PDAT activities to accumulation of TAG in vegetative tissues appears to be tissue- and species specific [25]. Radiolabeling experiments with [^{14}C]lauric acid (12:0) applied to young leaves of WT *Arabidopsis*, and *dgat1* or *pdat1* mutants of *Arabidopsis*, have shown that the *dgat1* mutant displayed 76% reduction in TAG biosynthesis suggesting that DGAT1 has a major role in TAG biosynthesis in leaves [575]. Other studies with *Arabidopsis* and mutants of *Arabidopsis* have indicated that DGAT1 plays an important role in vegetative tissue during stress [576–579]. In contrast, soybean *DGAT2D* expression has been shown to be up-regulated by abiotic stresses [502]. In other studies, PDAT has been shown to play a critical role in catalyzing TAG formation in *Arabidopsis* [10,580,581] and *Camelina* [582] vegetative tissues.

It is important to note, however, that there some examples of plants which produce substantial amounts of TAG in tissues other than seed or mesocarp. For example, yellow nutsedge (*C. esculentus*) is grass-like perennial, which has been shown to accumulate about 25% oil and 25% (DW) starch in tubers and may thus represent a model system to study oil formation in non-seed tissue [583,584]. In another example, “oil firewood” (*T. mongolica*) has been shown to have stem tissue with about 10% TAG (DW basis) occurring in the phloem [585]. Recent studies have indicated that DGAT2 is probably a major contributor to TAG biosynthesis in *C. esculentus* tubers [486,586]. In addition, two *DGAT1* genes from *T. mongolica* have been functionally expressed in yeast strain H1246, soybean hairy roots and *T. mongolica* calli [505].

6.2.5. Localization and interaction with other lipid biosynthetic enzymes

In tung tree, *DGAT1* has been shown to be expressed in different organs whereas *DGAT2* is highly expressed in developing seeds at the start of oil biosynthesis [43]. Tung tree DGAT1 and DGAT2 have also be shown to be localized in different subdomains of the ER [43]. Both enzymes contained a similar C-terminal ER retrieval motif, but this motif alone was not sufficient for assisting in the localization of the two enzyme forms to the different subdomains of the ER. Further investigation indicated that the N and C termini of tung tree DGAT1 and DGAT2 are on the cytosolic side of the ER.

There is also evidence for *Arabidopsis* DGAT1 and DGAT2 associating with LDs. An immunogold technique has been used to demonstrate that *Arabidopsis* DGAT2 associates with LDs associated with microtubules [47]. The investigators used rabbit antibodies raised against human DGAT2 which cross-reacted with a 44kDa protein in a Western blot of proteins extracted from 5-day-old *Arabidopsis* seedlings. *Arabidopsis DGAT1* and *DGAT2* have also been codon-optimized for expression in *S. cerevisiae* [177]. Although *AthDGAT1* could be expressed in yeast without codon-optimization, the procedure improved production of the recombinant enzyme. When expressed in H1246 yeast, codon optimized *AthDGAT1* or *AthDGAT2* led to the production of LDs and both isoenzymes were associated with LDs. Interestingly, expression of *AthDGAT1* also increased the levels of the triterpene, squalene, in LDs.

DGATs from higher plants have also been shown to interact with other lipid biosynthetic enzymes [523,537,587–589]. Tung tree GPAT8 and GPAT9 have been shown to both co-localize with tung tree DGAT2 in the same ER subdomain [587]. Using a split-ubiquitin based membrane yeast two-hybrid assay, GPAT8 was further shown to interact with itself, GPAT9 and DGAT2, but not with DGAT1. The first predicted TMD of GPAT8 was required for interaction with DGAT2 and for its DGAT2-

dependent co-localization to ER subdomains. As previously indicated in section 6.2.2, BnaC.DGAT1.a was shown to interact with *B. napus* LPAAT2, an interaction which may serve to facilitate channeling of PA produced by the LPAAT-catalyzed reaction to the PA-binding site on BnaC.DGAT1.a leading to activation of the DGAT [523]. Biomolecular fluorescent complementation (BiFC) analysis using *Arabidopsis* protoplasts has been used to demonstrate the physical interaction of AthDGAT1 and AthPDAT1 [588]. The investigators suggested that the two enzymes, which use different acyl donors, may be coordinated in catalyzing the production of TAG. Both the split-ubiquitin based membrane yeast two-hybrid assay and BiFC assay have also been used to demonstrate physical interactions of flax DGAT1 and DGAT2 with other transferases involved in lipid biosynthesis [537]. Yeast two-hybrid assays involved the use of a yeast system whereas BiFC analysis used the lower epidermis of transformed *N. benthamiana* leaves. DGAT1 and DGAT2 each interacted with transferases in the acyl-editing pathways, and physical interactions among the acyl-editing enzymes were also identified. It was proposed that these physical interactions constituted an interactome that facilitated the channeling of PUFA from PC to TAG during seed maturation. In a very recent study, analysis of physical interactions among recombinant transferases, involving DGAT1 and DGAT2, was combined with metabolic flux analysis in the *Arabidopsis* AS11 mutant to develop a multidimensional model for seed TAG biosynthesis which involved specific enzyme isoforms utilizing spatially separate DAG pools operating in distinct subdomains of the ER [589]. The role of PDAT, and various recombinant plant DGAT2s, in the utilization of different DAG pools were probed in a system without competing endogenous DGAT1 activity. Yeast two-hybrid analysis revealed the nature of possible metabolons involved in TAG biosynthesis. Among the findings, AthDGAT1 was shown to utilize a distinct rapidly produced PC-derived pool from that of AthPDAT1, or various plant DGAT2s, which acted on a more slowly turned over bulk-PC-derived DAG pool. The two processes likely involved separate TAG-producing metabolons or interactomes.

6.2.6. Gene expression in various tissues

Numerous valuable studies have been conducted on the analysis of *DGAT* gene expression and expression of other genes encoding lipid biosynthetic enzymes and associated proteins in higher plants (e.g., [11,59,62,66,90,410,590–595]). *DGAT* gene expression is generally increased in tissues that accumulate TAG and specific gene forms of *DGAT* have been implicated in various tissues and species. Early studies with *Arabidopsis* have shown that *DGAT1* is expressed in various tissues including roots, leaves, flowers, developing siliques, germinating seeds and young seedlings [474,596,597]. Analysis by Western blotting has also shown that DGAT1 protein in various tissues with the greatest abundance in developing seeds [597]. In allotetraploid *B. napus* and allohexaploid *Camelina*, which are other members of the Brassicaceae, *DGAT1* mRNA has been shown to be highest in developing embryos, flower petals and developing buds, with much lower levels of transcript in leaf and stem tissue [513,598]. Other examples of *DGAT1* being expressed in various plant organs include *Echium* [487] and sesame [599]. Differences in the relative expression levels of four closely related gene forms of *B. napus DGAT1* have been observed [153]. *B. napus* lines with high seed oil content, however, have been shown to exhibit effective expression of all the closely related gene forms *DGAT1* [594]. Differences have also been observed in expression levels of peanut *DGAT1-1* and DGAT1-2 in various tissues [58]. *DGAT1-1* showed high expression in flowers and seeds whereas *DGAT1-2* was expressed highest in roots, seeds and cotyledons.

Analyses of gene expression in oleaginous plants producing unusual FAs, such as eleostearic acid and ricinoleic acid, have shown high expression of *DGAT2* during seed development [43,61,480]. These results have provided support for the role of DGAT2 in catalyzing the biosynthesis of TAGs enriched in unusual FAs. This, however, is not always the case. For example, it has been shown that peanut *DGAT2* mRNA is abundant in root, stem, leaves, flowers and during seed development thus suggesting both 'seed-specific' and housekeeping roles for the enzyme [496].

In other cases, expression of both *DGAT1* and *DGAT2* appeared to be important in supporting oil biosynthesis. In olive mesocarp, *DGAT1* and *DGAT2* have been shown to share an overlapping expression pattern with a strong up-regulation of *DGAT2* expression and a decrease in *DGAT1* expression with later stages of mesocarp growth [600]. *DGAT1* and *DGAT2* expression have also been shown to be high in developing flax seed [180] and *Jatropha* [601].

Tetraploid soybean contains several putative *DGAT* genes [66,502]. Several gene expression studies with soybean have generally suggested that DGAT1 isoforms are more important in supporting seed oil biosynthesis than DGAT2 isoforms [140,410,501,503]. Increases in the expression of *DGAT2* gene forms during soybean seed development, however, have also been reported [66,502]. Constitutive expression of soybean *DGAT1A* or *DGAT2D* in *Arabidopsis* Col-0 resulted in some transgenic lines exhibiting significant increases in seed oil content [502]. Heterologous expression of soybean *DGAT1A* also resulted in significant increases in the 18:3 and 20:1 content of TAG whereas soybean *DGAT2D* expression resulted in significant increases in the 18:2 and 18:3 content of TAG. The investigators emphasized that increased *DGAT2* expression in higher plants was not always associated with production of TAG containing unusual FAs. Interestingly, an insertion/deletion mutation in the soybean *DGAT2B* gene in OAC Wallace has been significantly associated with decreased seed oil content across three environments [602].

The expression of *DGAT1* and *DGAT2* gene forms has been shown to occur in both vegetative and reproductive growth stages in maize [90]. High expression of *DGAT1.1* was observed in developing embryos (16-24 days after pollination) whereas high expression of *DGAT1.2* occurred throughout most embryo and seed development stages. In contrast, *DGAT2.1* was expressed highest at later endosperm and seed developmental stages.

DGAT1-1 from oil palm has been shown to be expressed at the onset of lauric acid accumulation in the kernel [591] and the recombinant enzyme produced in H1246 yeast exhibited a preference for medium-chain FAs [493]. Other studies have shown enhanced expression of both oil palm *DGAT1-3* and *DGAT2-2* in developing mesocarp tissue [494,495].

6.2.7. Regulation of gene expression

In addition to affecting genes involved in plant development, transcription factors (TFs) are known to affect genes encoding enzymes involved in carbon metabolism and lipid accumulation [23,603]. For example, the *Arabidopsis* mutant *wrinkled* (*wri*) *1* has been shown to exhibit an 80% decrease in seed oil content [604]. Genes associated with plastidial glycolysis and FA biosynthesis have shown to be among the targets of WRI1 [605]. *WRI1* was later shown to encode an APETALA2-ethylene responsive element-binding protein (AP2/EREBP) and constitutive expression of *WRI1* was shown to increase seed oil content in *Arabidopsis* [606]. Later it was shown that the *WRI1* gene is a target of the master regulator, LEAFY COTYLEDON (LEC) 2, and is thus required for the regulatory effect of LEC2 on FA biosynthesis [607]. TFs acting at the level of TAG bio-assembly have been identified more recently (see below).

Nitrogen deprivation has been shown to lead to the induction of *DGAT1* expression in *Arabidopsis* seedlings [576]. ABSCISIC ACID INSENSITIVE (ABI) 4, which is a key TF in the abscisic acid (ABA) signaling pathway, was shown to interact with CE1-like elements (CACCG) in the *Arabidopsis* DGAT1 promoter [576]. In addition, *DGAT1* expression and TAG accumulation were decreased in the *abi4* mutant. Thus, ABA signaling is involved in the accumulation of TAG in vegetative tissue. ABI5 has also been implicated in the regulation of *DGAT1* expression during nitrogen deficiency in *Arabidopsis* seedlings [576]. A follow-up study demonstrated a synergistic effect of ABI4 and ABI5 in

regulating *Arabidopsis DGAT1* expression in tobacco transient assays [577].

FUSCA3 is part of a network of TFs that affect embryo development and oil accumulation [23,59,603,608,609]. FUSCA3 is a B3 domain-containing TF that interacts with the RY *cis*-motif (CATGCATG) in the promoters of many seed-specific genes [608–610]. Over-expression of *Arabidopsis FUSCA3* has been shown to activate TAG biosynthesis in *Arabidopsis* seedlings and tobacco BY2 cells, which are both non-seed systems [609]. Over-expression of *FUSCA3*, driven by an estradiol-inducible promoter, resulted in a 50-fold increase in the TAG content of young *Arabidopsis* seedlings to 6% of the DW. Growth in 1.5% sucrose intensified FUSCA3-enhanced TAG biosynthesis in the seedlings. Over-expression of *FUSCA3* in tobacco BY2 cells also increased TAG accumulation. Co-expression of *FUSCA3* and *AthDGAT1* had an additive effect resulting in a TAG level of 4% of the DW. Gene expression analysis indicated that a higher proportion of genes associated with TAG biosynthesis were affected than those involved in other lipid biosynthetic processes. *DGAT1* was up regulated about three-fold by *FUSCA3* expression under sucrose conditions. Interestingly, *ABI4* and *ABI5* increased by about 23-fold and 11-fold, respectively, under sucrose conditions. Thus, the effect of *FUSCA3* expression on promoting TAG accumulation may involve the effects of ABI4 and ABI5 on TAG biosynthesis via activation of *DGAT1* expression. In addition, analysis of the expression of other genes encoding TFs suggested that FUSCA3-mediated enhancement of TAG biosynthesis was independent of LEC1, LEC2, WRI3 and WRI4.

The CCCH zinc finger TFs have also been shown to activate the expression of DGAT1 and several other genes, including other TFs in soybean and *B. napus* [611,612]. In soybean, *DGAT1* expression was activated by GmZF351 [611]. In a more recent study, GmZF392 was also shown to activate the expression of lipid biosynthetic genes [613]. In addition, GmZF392 was shown to physically interact with GmZF351 to cooperate in activating the expression of downstream genes. A nuclear factor, GmNFYA, was further shown to act as an upstream activator of both GmZF351 and GmZF392. Electrophoretic mobility shift assays have been used to demonstrate that the *B. napus* zinc finger TF, BnaZFP1, interacts with the promoter region of a gene encoding one of the isoforms of BnaDGAT1 [612].

In investigations with *Arabidopsis*, the R2R3-type MYB96 TF has been shown to regulate seed oil accumulation via activation of DGAT1 and PDAT1 expression in a process which is independent of WRI1-mediated FA biosynthesis [614]. MYB96 was shown to specifically interact with the *PDAT1* promoter. Activation of *DGAT1* expression was due, however, to an increase in the abundance of ABI4. MYB96 was shown to interact with the promoter of ABI4 thereby leading to increased expression. As indicated in section 6.2.5, AthDGAT1 has been shown to physically interact with AthPDAT1 to possibly enhance the efficiency of TAG bio-assembly [588]. This represents an interesting example of a possible combination of genetic and biochemical regulation of TAG bio-assembly.

In another recent study, the WAX INDUCER1/SHINE1 (WIN1) TF, which is a member of the AP2/EREBP family, has been shown to have dual function in promoting wax accumulation and seed oil biosynthesis in *B. napus* [615]. Over-expression of *BnaWIN1* resulted in the up-regulation of many genes involved in *de novo* FA biosynthesis, wax accumulation and seed oil assembly. The promoters of *GPAT9* and *DGAT2* were among the targets of BnaWIN1.

A complex network of TFs regulating plant lipid biosynthesis is emerging [616]. Transcriptome analyses of oil-forming tissues have provided clues regarding possible TFs involved in regulating TAG biosynthesis in developing seeds and leaf tissue (e.g., [59,592,593]). In a comparative study using two *B. napus* near-isogenic lines, differing greatly in seed oil content, *ABI4*, *ABI5* and *FUSCA3* were shown to be among the *TF* genes up-regulated along with increases in the expression of *DGAT1* and *DGAT2* [593].

There is also some evidence that alternative splicing may be involved in the regulation of DGAT1 activity in peanut [497]. Seven different splicing variants of *AhDGAT1* were identified (*AhDGAT1.1-AhDGAT1.7*). Some of the splicing variants displayed organ-specific expression patterns. When evaluated for functional expression in H1246 yeast, the five longest variants resulted in the production of recombinant DGATs with high activity which complemented the FA-lethality phenotype in this strain.

6.2.8. DGAT1 and DGAT2 in relation to plant development and various stresses

DGAT1 and PDAT1 have been shown to have overlapping roles in *Arabidopsis* seed oil formation along with roles that are essential for normal pollen and seed development [463,617]. The *dgat1* mutants of *Arabidopsis* have been shown to exhibit delayed seed development and post-germinative growth [514,618] and mutant seedlings have been shown to exhibit altered carbohydrate metabolism [596]. In contrast, seed-specific *DGAT1* over-expression in *Arabidopsis* has been shown to lead to an increase in seed weight [619], and transcriptional and hormonal changes that are not restricted to TAG biosynthesis [620]. TAG biosynthesis is known to occur during germination and early seedling growth (e.g., [597,621,622]). As indicated in section 4.1, anabolic and catabolic processes of TAG have been shown to operative in parallel in *S. cerevisiae* [325] and the homeostasis between TAG formation and degradation appears to also operative in *Arabidopsis* seedlings, especially under low nitrogen conditions [576]. TAG biosynthesis is also active to varying extents in more mature and senescing vegetative tissues (see section 6.2.4). Seed-specific anti-sense suppression of *DGAT1* gene expression in *B. napus* has also been shown to lead to severe developmental abnormalities along with reduced seed yield and germination rates [623]. In a more recent study with *Arabidopsis*, WRI1 and DGAT1 have been implicated in the regulation of tocochromanol metabolism, thus revealing a more complex physiological role for DGAT1 [624].

Several environmental stresses are known to increase TAG accumulation in vegetative tissues [87,505,576–579,596,625–630]. Early studies with *Arabidopsis dgat1* mutants have shown that during germination and early seedling growth, there is an increased sensitivity to osmotic stress, ABA, salt treatment and cold treatment [596]. Previously, it has also been shown that increasing sucrose concentration from 2 to 22% (w/v) results in increased TAG accumulation in microspore-derived cell suspension cultures of *B. napus* [631]. Increasing sucrose concentration, however, was also associated with progressively decreased fresh weight of cells. In the same study, growth of the cell suspension in 6% and 14% sucrose also resulted in increased total DGAT activity per fresh weight and *OLEOSIN* gene expression. Further studies by the same group showed that culturing of the *B. napus* cell suspension at increased sucrose concentrations resulted in increased *DGAT1* (*BnaA.DGAT1.b*) expression and DGAT1 polypeptide [475,632,633]. The response to increased sucrose concentration, however, is difficult to interpret since alterations in sucrose concentration can affect carbon/nitrogen ratio, osmotic pressure, induction of sucrose transporters and possible events related to sucrose-signaling [533,578]. In this regard, it is interesting to note that *FUSCA3*-induced enhancement of TAG accumulation increased in *Arabidopsis* seedlings grown on medium containing 1.5% sucrose [609].

Seed-specific over-expression of *Arabidopsis DGAT1* in *B. napus* has been shown to reduce the penalty on seed oil content caused by drought suggesting that there may be environmental influences on the regulation of DGAT activity [530]. The increased 'pull' of carbon towards TAG biosynthesis due to *DGAT1* over-expression may have offset the decrease in seed oil content seen in drought conditions [530,578]. Given that the *Arabidopsis dgat1* mutant showed increased sensitivity to osmotic stress [596] suggests that *DGAT1* may have a role in the response to this type of stress [578].

As discussed in the previous section (6.2.6), the TF, ABI4, has been shown to activate *DGAT1* expression in *Arabidopsis* seedlings during nitrogen deficiency [576]. Further investigation by the same group

revealed that *DGAT1* expression in *Arabidopsis* seedlings was significantly induced by ABA, jasmonic acid, salicylic acid, high salt or increased osmotic pressure [577]. The growth regulator, ABA, has a critical role in the regulation of seed maturation and mediating responses to abiotic stresses [634], whereas jasmonic acid and salicyclic acid are activated by biotic factors [635]. The transcript encoding the TF, ABI5, was shown to substantially increase in *Arabidopsis* seedlings subjected to various stresses [577]. In contrast, *DGAT1* expression in the *Arabidopsis* mutant *abi5* was only induced by small extent by the stressors. When *ABI5* was over-expressed in *Arabidopsis*, both TAG accumulation and *DGAT1* expression were induced. The relationship between ABI4 and ABI5 action during stress was probed by comparing *DGAT1* expression in the *Arabidopsis* mutants *abi4*, *abi5* and *abi4abi5*. *DGAT1* expression levels were similar in *abi4* and *abi4abi5* but lower in *abi5*. In a tobacco leaf transient assay, *ABI4* expression activated *DGAT1* expression by about 14-fold. *DGAT1* expression was increased 30-fold, however, when tobacco leaf was co-transfected with *ABI4* and *ABI5*. The various lines of evidence indicated a synergistic effect of ABI4 and ABI5 in controlling TAG accumulation in stressed *Arabidopsis* seedlings. The investigators suggested that ABI5 somehow influences the ABI4-*DGAT1* promoter complex.

As previously mentioned, *T. mongolica* has been shown to accumulate substantial amounts of TAG in stem tissues [585]. *DGAT1a* and *DGAT1b* expression *T. mongolica* plantlets have been shown to be substantially induced by growth in media containing 800 mM NaCl, 20% (w/v) polyethylene glycol (PEG)-2000 or 15% (w/v) sucrose [505]. The stress-induced elevation in *DGAT1a* and *DGAT1b* expression was concomitant with substantial increases in TAG content. Time-dependent increases in *DGAT1* and *DGAT2* expression were similar for growth of plantlets in 20% PEG 2000 or 15% sucrose.

The effect of cold stress (4°C), 200 mM NaCl treatment, 20% PEG-6000 treatment (to mimic drought stress) or 100 μM ABA on expression of two genes encoding two DGAT1 isoforms has been examined in leaf and root tissue of peanut [58]. *DGAT1-1* expression increased in all tissues except for cold-, salt- and ABA-treated leaves. *DGAT1-2* expression was substantially increased for all stress treatments except for cold-stressed leaves.

The effect of environmental stresses and hormones on *DGAT1* and *DGAT2* expression in soybean have been examined in both developing seeds and seedlings [502]. Expression of *DGAT2D* gene was up regulated by abiotic and biotic stresses. During seed development, *DGAT2D* expression increased 12 hours after cold treatment (4°C) and then markedly dropped. In contrast, expression of the *DGAT1A* gene was down-regulated during seed development by cold treatment. With the application of heat stress (42°C) during seed development, the expression levels of *DGAT1A* and *DGAT2D* increased at 12 hours of treatment and then decreased. In leaves, expression of *DGAT1A* and *DGAT2D* decreased after insect biting or treatment with methyl jasmonate (50 μM), with a more pronounced decrease for *DGAT2D*, suggesting a possible connection between jasmonic acid-signaling and DGAT2D action. Treatment of seedlings with 100 μm ABA led to increases in *DGAT1A* and *DGAT2D* expression over time, with the most significant increases associated with *DGAT2D* expression. Interestingly, the *DGAT2D* promoter contained several jasmonic acid- and ABA-responsive *cis*-elements with fewer of these *cis*-elements in *DGAT1A*.

The effect of cold (4°C), salt (200 mM NaCl), alkali (100 mM $NaHCO_3$) or drought treatment (20% PEG-6000) on expression of *DGAT1* and *DGAT2* gene forms has been examined in maize leaves and roots [90]. Cold treatment significantly induced the expression levels of *DGAT1.1*, *DGAT1.2*, *DGAT2.1* and *DGAT2.2* in leaves with a more delayed increase in expression in roots. Treatment with salt resulted in induction of gene expression for the four gene forms after 12 hours, with quicker responses occurring in roots. With alkali treatment, *DGAT1* and *DGAT2* expression was initially down-regulated in leaves followed by a gradual up-regulation. In contrast, expression of the four gene forms was significantly up-regulated in roots, especially at early stages of alkali treatment (6-12 hours). Drought stress resulted in a similar increased in gene expression among the four gene forms with a quicker and stronger response occurring in roots. The investigators suggested that the DGATs might have different roles in response to abiotic stresses in different parts of the maize seedling.

Cold stresses include chilling and freezing [636]. Two recent studies have linked increased DGAT1 production to increased freezing tolerance in higher plants [579,629]. *Arabidopsis dgat1* null mutants have been shown to be more sensitive to chilling and freezing stresses than the WT plant [629]. Exposure to freezing resulted in decreased TAG content in the *dgat1* mutants with increased levels of DAG and PA. In contrast, knockdown mutants of three gene forms encoding diacylglycerol kinase exhibited enhanced tolerance to freezing. Collectively, the results suggested that modulation of the DAG to TAG conversion catalyzed by DGAT1 and DAG to PA conversion catalyzed by diacylglycerol kinase played a role in the plant's response to cold stress. In the same year, another study provided complementary evidence to support the role of DGAT1 in contributing to increased freezing tolerance [579]. QTL analysis of *Boechera stricta*, a relative of *Arabidopsis* adapted to the Rocky Mountain environment, identified a single QTL containing the *DGAT1* gene whose expression was strongly responsive to cold. Freezing tolerant plants exhibited increased *DGAT1* expression during acclimation to cold in comparison to more cold sensitive plants. In addition, TAG accumulation increased in response to subsequent freezing. Oligogalactolipids were also increased in freezing-tolerant plants. Production of the oligogalactolipids is due to the action of SENSITIVE TO FREEZING 2 (SFR2), a previously identified component of freezing tolerance in *Arabidopsis* [637]. SFR2 catalyzes the transfer of galactosyl groups from MGDG to other galactolipids to produce oligogalactolipids, which may act to stabilize chloroplast membranes and DAG [638,639]. Constitutive over-expression of *Arabidopsis DGAT1* in *Arabidopsis* Col-0 led to increased freezing tolerance [579]. The investigators proposed that DGAT1 imparts freezing tolerance in plants by supporting SFR2-mediated adjustment of the composition of chloroplast membranes.

PDAT, of course, also catalyzes the formation of TAG in leaves. Expression of various *PDAT* gene forms have been shown to be altered in response to various abiotic stresses applied to *Camelina* seedlings [582]. PDAT activity has been shown to contribute to TAG accumulation in response to thermal stress [626]. The TF MYB96 has also been shown to mediate ABA-dependent TAG accumulation in *Arabidopsis* seedlings under drought conditions in a process involving up-regulation of *DGAT1* and *PDAT1* expression [627]. Thus, environmental effects on TAG accumulation in vegetative tissues of higher plants appear to involve DGAT1, DGAT2 and PDAT action. This is all further complicated through effects of abiotic stress on *DGAT3* expression (to be discussed in the next section). Regulation of the provision of lipid substrates for membrane-desaturation under temperature stress has been linked to metabolic adjustments in different intracellular glycerolipid pathways [640]. Various forms of DGAT and PDAT may operate with other lipid metabolic enzymes to result in membrane adjustments in this context.

6.3. Naturally soluble DGATs

As indicated in section 1, a soluble DGAT, which eventually was categorized as a DGAT3 [30,31], was initially highly purified and cloned from developing peanut cotyledons [57]. The isolated gene exhibited about 13% identity with the bacterial *WSD* and likely orthologs were identified in *Arabidopsis*, rice, soybean and many other plant species [57,58]. In addition, the peanut *DGAT3* mRNA was only detected in early stages of seed development (8-24 DAF). In the same study, recombinant peanut DGAT3 with poly-histidine tag was produced in *E. coli* and purified using immobilized metal ion affinity chromatography. Both the highly purified enzyme and recombinant enzyme exhibited enhanced specificity for 18:1-CoA over 18:0- or 16:0-CoA. *E. coli* expressing peanut *DGAT3* produced both TAG and WEs.

In more recent research, two additional isoforms of peanut DGAT3

have been identified [58]. The original peanut DGAT3 isolated by Saha et al. in 2006 [57] was named AhDGAT3-1 with the two additional isoforms named AhDGAT3-2 and AhDGAT3-3 [58]. All three isoforms lack a TMD and ER retention sequence, which confirms their cytosolic nature [57,58]. Analysis of the deduced amino acid sequences of AhDGAT3-1 and AhDGAT3-3 have revealed some putative functional motifs [57,58]. In AhDGAT3-1, an invariant proline residue is found at position 34 between a putative phosphopantetheine attachment site and a thiolase acyl enzyme intermediate signature [57]; in AhDGAT3-3, the invariant proline residue, however, is located at position 33 [58]. AhDGAT3-1 and AhDGAT3-3 also have putative tyrosine kinase phosphorylation sites at R^{183}KAETMIY^{190} and P^{177}KAETMIY^{184}, respectively [57,58]. *AhDGAT1-1*, *AhDGAT1-2* and *AhDGAT3-3* have also been functionally expressed in yeast strain H1246 [58]. In terms of the FA composition of the transformed yeast, there was a decrease in 16:1 and 18:0 accompanied by an increase in 16:0, 18:1 and 18:2. Unlike *AhDGAT3-1* mRNA [57], the high levels of *AhDGAT3-3* mRNA occurred in flowers and leaves [58]. During seed development, relatively high levels of *AhDGAT3-3* mRNA were observed at 10, 20 and 50 days after pollination [58].

Through bioinformatic analysis of a public transcriptome data set for *Arabidopsis* [641], it has been shown that *DGAT3* (At1g48300) exhibits a similar expression pattern with *DGAT1* during seed development, although the highest expression occurred during late seed maturation [59]. In another study, Hernández et al. (2012) have shown that *Arabidopsis DGAT3* may have a role in modulating the acyl-CoA pool size and composition in response to requirements of membrane biosynthesis in young seedlings [60]. Active recycling of 18:2 and 18:3 into TAG occurred when seed oil degradation was blocked. In the same study, *Arabidopsis* Δ75DGAT3 fused to green fluorescent protein was shown to localize to the cytosol when the gene was transiently expressed in *N. benthamiana* leaves. Thus, this study also suggested a physiological role for DGAT3 in vegetative tissue. More recently, however, *Arabidopsis* DGAT3 has been shown to be an iron-sulfur protein with a [2Fe-2S] cluster with an N-terminal putative chloroplast transit peptide [65]. In this more recent study, it was suggested that the exclusive cytosolic localization of recombinant *Arabidopsis* DGAT3 observed in tobacco leaves by Hernández et al. (2012) [60] may have been attributable to the absence of the chloroplast targeting signal in the truncated DGAT3 (Δ75DGAT3). Recombinant poly-histidine-tagged full-length *Arabidopsis* DGAT3 purified by immobilized metal ion affinity chromatography has been shown to result in an active enzyme preparation; however, removal of either 46 or 75 N-terminal residues resulted in inactivation of the enzyme [65]. Removal of the first 46 N-terminal residues from AthDGAT3 essentially produced an enzyme which was devoid of the putative chloroplast transit peptide. Interestingly, unesterified FAs have been shown to accumulate during a time course analysis of DGAT activity for either purified recombinant peanut DGAT3 [57] or *Arabidopsis* DGAT3 [65] suggesting that these enzymes possess acyl-CoA hydrolase activity in addition to DGAT activity. Thus, Aymé et al. (2018) [65] have speculated that the increase in TAG accumulation observed by Hernández et al. (2012) [60] may have been a consequence of the acyl-CoA hydrolase activity of Δ75AthDGAT3 together with involvement of other enzyme activities. The observations that *AthDGAT3* is over-expressed in leaves and roots during iron deficiency [642] and down-regulated in flowers of a mutant accumulating iron [643] has suggested that the physiological function of AthDGAT3 may be linked to iron availability [65]. It would be useful to conduct chloroplast import experiments with full-length AthDGAT3. Since AthΔ46DGAT3 was found to be inactive, removal of the N-terminal putative transit peptide might result in an inactive DGAT3 released into the stroma. Thus, if chloroplast uptake of processed DGAT3 occurs, the inactive truncated enzyme may serve another function which perhaps involves the [2Fe-2S] cluster.

The *DGAT3* gene has also been cloned from tung tree [61]. Phylogenetic analysis showed that tung DGAT3 was closely related to castor DGAT3. There was complete conservation of 11 amino acid residues among 27 DGAT3s from 19 species of plants with 10 of the residues occurring within the last 100 residues from the carboxy terminus. The data suggested that the catalytic regions of DGAT3s are likely located in the carboxyl termini, as was also found for DGAT1s and DGAT2s [42]. Gene expression experiments indicated that tung tree *DGAT3* was expressed in leaves, flowers and at early stages of seed development, prior to active oil biosynthesis [61]. In contrast, *DGAT2* expression was well coordinated with seed oil biosynthesis. Thus, it was suggested that DGAT3 plays a role in TAG metabolism in non-seed tissues.

Recently, two DGAT3s from oil palm (*E. guineensis*) were compared to several orthologs from other species [62]. The various DGAT3 proteins had predicted lengths ranging from 340 to 360 amino acid residues. EgDGAT3-1 and EgDGAT3-2 exhibited, respectively, 36% and 39% identity with *Arabidopsis* DGAT3.

Interestingly, *DGAT3A* has been shown to be more highly expressed than *DGAT1*, *DGAT2* or *WSD* genes during soybean seed development [66]. The same study also showed relatively high expression of *DGAT3* in soybean leaves. *DGAT3* has also been shown to be highly expressed in developing maize embryos and vegetative tissues [90].

Recently, *DGAT3* expression has been analyzed in *Camelina* [63]. Three gene forms exhibited divergent expression in various tissues with *DGAT3-1*, *DGAT3-2* and *DGAT3-3* highly expressed in roots, flowers and young seedlings, and developing seeds, respectively. Given the apparent importance of DGAT3-3 in seed oil accumulation, *CsDGAT3-3* was functionally expressed in H1246 yeast. The TAG content of yeast could be increased by providing various exogenous unsaturated FAs in growth medium. Eicosenoic acid ($20{:}1\Delta^{11}_{?}$ hereafter 20:1) was particularly effective in promoting both increased TAG accumulation and enrichment of TAG with this FA. *In vitro* DGAT assays using recombinant CsDGAT3-3 produced in *E. coli* indicated an acyl-CoA specificity of 20:1 > 18:3 > 18:2. Transient expression of CsDGAT3-3 in *N. benthamiana* leaves resulted in a two-fold increase in leaf oil content along with increases in 18:2, 18:3 and 20:1 content of 29.5%, 41.2% and 225.4%, respectively. Seed-specific expression of *CsDGAT3-3* in developing tobacco seed resulted in seed oil content increasing from 35.3-36.1% in the control to 45.8% in CsDGAT3-3-lines. Unsaturated FA content (18:2, 18:3 and 20:1) was also enhanced, with the largest increase associated with 20:1. A *CsDGAT3-3/green fluorescent protein* fusion protein was introduced into the inner epidermis of onion bulb leaves by particle bombardment. Fluorescence microscopy indicated a cytosolic localization.

Expression of upland cotton *DGAT3* (*GhDGAT3*) has been shown to be higher than that of *GhDGAT1* or *GhDGAT2* during seed development [644]. A more recent study on DGATs in upland cotton has shown that *GhDGAT3* is highly expressed in the ovule and during fiber development [645]. When *GhDGAT3D* was heterologously expressed in *Arabidopsis* using a 35S promoter, there was a significant increased in seed oil content accompanied by decreases in 18:2 and 18:3, and an increase in 18:1 [645].

The effect of abiotic stress on *DGAT3* expression has been studied in peanut and maize [58,90]. In peanut leaves and roots, cold treatment enhanced *DGAT 3-3* at 3 hours and 12 hours, respectively, and was then followed by a decrease in transcript level [58]. Treatment with 200 mM NaCl resulted in *DGAT3-3* transcripts decreasing rapidly from 1 to 72 hours in seedling roots, but there was a 7-fold increase in the transcript in leaves after 3 hours. Treatment with 100 μM ABA increased *DGAT3-3* in peanut leaves about 3-fold after 6 hours. Cold treatment has also been shown to induce maize *DGAT3* expression in leaves and roots [90]. Treatment with 200 mM NaCl increased maize *DGAT3* expression in leaves but expression was repressed in roots. Treatment with 150 mM $NaHCO_3$ resulted in enhanced maize *DGAT3* expression at early stages of leaf development. Simulation of drought stress with 20% PEG 6000 led to repression of maize *DGAT3* expression in both leaves and roots. Together with the previously described data on *DGAT1*, *DGAT2* and *PDAT* expression in response to environmental stress (see section 6.2.7),

it appears that the various TAG-biosynthetic enzymes exhibit differential responses.

DCR (At5g23940) from *Arabidopsis* is another soluble enzyme which has also been shown to exhibit DGAT activity [69]. DCR is a member of the BAHD acyltransferases which feature the HXXXD consensus motif [646–648]. The BAHD acyltransferase family was named based on the first four characterized members of the family which include benzylalcohol *O*-acetyltransferase, anthocyanin *O*-hydroxycinnamoyltransferase, anthranilate N-hydroxycinnamoyl/benzoyltransferase and deacetylvindoline 4-*O*-acetyltransferase [646]. *Arabidopsis DCR* has been cloned and functionally expressed as a TAG-producer in both *E. coli* and H1246 yeast [69]. Within the amino acid sequence, the BAHD acyltransferase motif and a VXXGF lipid-binding motif were located, respectively, at the N-terminal end and in the middle of the sequence. The purified recombinant enzyme, produced in *E. coli*, exhibited increased specificity for oleoyl-CoA compared to palmitoyl- or stearoyl-CoA when assayed with 1,2,-dioleoyl-*sn*-glycerol as an acyl acceptor. Feeding of H1246 cells, producing recombinant DCR, with 16-hydroxyhexadecanoic acid resulted in incorporation of the hydroxy FA into TAG. The investigators suggested that DCR may play a role in sequestering hydroxy FAs into TAG and preventing them from entering membranes during cutin biosynthesis. Yeats and Rose (2013) [649], however, have suggested that a role for cytoplasmic TAG intermediates in cutin biosynthesis was not consistent with the known steps within the pathway. They further suggested that more research was required to determine the native substrate of DCR. In an examination of genes encoding TAG biosynthetic enzymes from flax, DCR1, an ortholog of *Arabidopsis* DCR, did not exhibit DGAT activity [180]. Flax *DCR1* could not be functionally expressed in H1246 and was incapable of complementing the reduced TAG phenotype of the *AS11* Arabidopsis mutant.

6.4. Bifunctional wax synthase-diacylglycerol acyltransferases

Early research on the functional characterization of higher plant *WSDs* has been conducted with petunia and *Arabidopsis* [74,76]. The petunia ortholog of the bacterial *AbaWSD* was shown to be mainly expressed in petals [76]. The enzyme was predicted to contain two TMDs and was associated with membranes. Recombinant petunia WSD1 failed to restore TAG biosynthesis in a yeast mutant deficient in TAG biosynthesis, but isoamyl esters of FAs were detected.

Arabidopsis contains 11 WSD orthologs of AbaWSD, based on their sequence similarity [76], and *Arabidopsis WSD1* has been heterologously expressed in *E. coli* for characterization [74]. The crude extract exhibited about 10-fold greater WS activity than DGAT activity. 1-[^{14}C]16:0-CoA was used as an acyl donor with either octadecanol or 1,2-dipalmitoyl-*sn*-glycerol as an acyl acceptor, respectively, to determine WS and DGAT activity. Heterologous expression of *Arabidopsis WSD1* in H1246 yeast resulted in the production of WEs when the growth medium contained palmitic acid and a long-chain alcohol. Octadecanol (C18), tetracosanol (C24) and octacosanol (C28) were all effective acyl acceptors. No TAG formation, however, was detected in the transformed H1246 yeast. *Arabidopsis WSD1* was highly expressed in stem, leaves and flower bud. The enzyme was localized to the ER. WSD1 was proposed to be the enzyme responsible for catalyzing the biosynthesis of WEs in *Arabidopsis* shoots.

In soybean and *Arabidopsis*, *in silico* and real-time quantitative polymerase chain reaction analyses have shown that *WSD* transcripts are restricted to fewer tissue types than for *DGAT3* transcripts [66]. The highest expression of *WSD* in soybean occurred in leaf tissue.

In a more recent study, *Arabidopsis WSD1* has been shown to be positively regulated by binding of WRI4 to the gene's promoter [650]. *WRI4* encodes an AP2/EREBP TF. WRI4 also interacted with promoters of other genes encoding enzymes involved in WE biosynthesis. *WRI4* was mainly expressed in stem tissue, was up regulated by salt stress and was involved in activating cuticular wax biosynthesis.

WSD1, *WSD6* and *WSD7* have been shown to be induced during drought, salt stress or ABA treatment in *Arabidopsis* [75]. The *wsd1* mutants exhibited reduced WE content in leaves and stems while WE content was unchanged in the mutants *wsd6*, *wsd7* or *wsd6wsd7*. Enzyme assays with recombinant proteins produced in insect cells indicated that WSD6 and WSD7 had WS activity but exhibited a different substrate specificity than WSD1. WSD6 and WSD7 were localized to the ER and Golgi apparatus.

WSD-1 has been shown to be expressed in association with increased oil accumulation in ripening mesocarp but the transcript was only detected at a low level in the oil palm kernel [495]. Recombinant production of oil palm WSD-1 in H1246 yeast failed to produce TAG. Introduction of exogenous alcohols, however, resulted in WE production with recombinant WSD-1.

In silico analysis of *DGAT* genes in oil palm has revealed two *WSD* genes [62]. The study also involved comparisons with 12 other oleaginous plant species. Twelve WSD sequences for soybean were shown to cluster with oil palm WSD-2. Out of 97 higher plant WSD sequences, 52 were predicted to be cytosolic with no TMDs. Thirty-one of the plant WSDs had one predicted TMD and 14 had two predicted TMDs. Actual subcellular localization experiments conducted thus far, however, have indicated that higher plant WSDs are associated with membranes. Expression analysis of *DGAT* and *WSD* genes has indicated that *WSD* is the most diversified gene family with all plants having putative *WSD* genes [66]. The proposed active-site motif HHXXXDG is present in the N-terminal region of all putative WSD identified thus far [62,66]. In general, it appears that WSDs seem to function more as WSs than DGATs in higher plants. In addition, some important plant WS enzymes such as jojoba WS have predominant WS activity but very weak DGAT activity [651–653], and thus are not discussed here.

6.5. Diacylglycerol acetyltransferase

Endosperm and embryo tissue from *E. alatus* are enriched in 1,2-diacyl-3-acetyl-*sn*-glycerol, which has a lower viscosity than conventional long-chain TAGs [81]. The aril tissue outside of the seed, however, produces conventional long-chain TAGs. Comparative deep transcription profiling of endosperm and aril tissue led to the discovery of EaDAcT, which is also a member of the MBOAT family [81]. The recombinant enzyme has been shown to catalyze the synthesis of acetyl-TAG in both yeast and *Arabidopsis*, with levels of acetyl-TAG reaching 40 mol% of the total TAG in the seed oil from the transgenic *Arabidopsis* line. Although EaDAcT could not utilize long-chain acyl-CoAs, the enzyme exhibited activity with acyl-CoAs less than 8 carbons and could acetylate a wide range of DAGs including short-chain DAGs [81,88]. EaDAcT was also shown to exhibit increased selectivity for DAGs containing more double bonds over DAGs with fewer double bonds [88]. The recombinant enzyme, however, was also shown to utilize DAGs with medium-chain FAs. As indicated previously in section 1, the enzyme also catalyzed the acetylation of fatty alcohols [88]. Recently, it was experimentally demonstrated that EaDAcT has four TMDs with the N-terminus and C-terminus both residing in the lumen of the ER [82]. There is a large cytosolic loop between the first and second TMD. The MBOAT signature region is embedded in the third TMD near the junction between the membrane and cytosol. Of the 9 cysteine residues, residues 187 and 293 were most important for enzyme activity. Site-directed mutagenesis showed that serine 253, histidine 257, aspartate 258 and valine 263 are necessary for EaDAcT activity. Further mutagenesis experiments suggested that multiple amino acid residues are responsible for the unique acyl donor specificity of the enzyme. In addition to the genus *Euonymus*, DAcTs have also been identified in the genera *Celastrus* and *Adonis* [82].

7. Mammalian DGATs

7.1. Overview of triacylglycerol biosynthesis in mammals

As in other organisms discussed, in mammalian tissues, the synthesis of TAG also occurs mainly via the Kennedy (G3P) pathway (Fig. 13) [12,28,388,654,655]. This pathway is present in all mammalian cell types [28,656]. The first reaction catalyzed by GPAT is considered to have a substantial effect on the flow of carbon into TAG within the Kennedy pathway [1]. DAG for TAG synthesis can also be generated by the MGAT pathway, which is also shown in Fig. 13. This alternative pathway has historically been restricted to the small intestine where it has an important role in dietary TAG absorption [657,658]. This will be discussed in more detail in Section 7.2.3.

7.1.1. Introduction to mammalian DGATs

Two mammalian DGAT genes have been identified that encode the enzymes designated as DGAT1 and DGAT2 [39,116]. These proteins do not share any sequence similarity at the protein or DNA levels. As indicated in section 1, DGAT1 belongs to the MBOAT family of enzymes that includes ACAT1 and 2, which catalyze cholesteryl ester biosynthesis [659]. DGAT2 belongs to a different gene family that includes several MGAT isoforms (MGAT1–3) and WS [660–665]. Although DGAT1 and DGAT2 catalyze the same biochemical reaction, they appear to be structurally very different and have broad substrate specificities, both utilizing the same long-chain fatty acid substrates [116]. Interestingly, both *DGAT1* and *DGAT2* are ubiquitously expressed with the highest levels of expression in tissues that are active in TAG synthesis, such as white adipose tissue, small intestine and liver [39,116].

7.1.2. DGAT1

Mammalian DGAT1 is an integral membrane protein present in the ER that was initially identified based on its homology to ACAT1 [39]. Algorithms used to identify hydrophobic regions of proteins predicted that DGAT1 had 6–12 TMDs. However, experiments using protease protection assays demonstrated that DGAT1 had only three TMDs, with its N-terminus exposed to the cytosol and its C-terminus in the ER lumen [55]. Unexpectedly, most of the protein was present in the ER lumen. This topology model placed a potential active site histidine residue in DGAT1 in the ER lumen. This histidine residue is conserved in ACAT1 and ACAT2 and was found to be essential for ACAT activity [666,667]. Similarity, this histidine residue was also required for the full catalytic activity of DGAT1 [55].

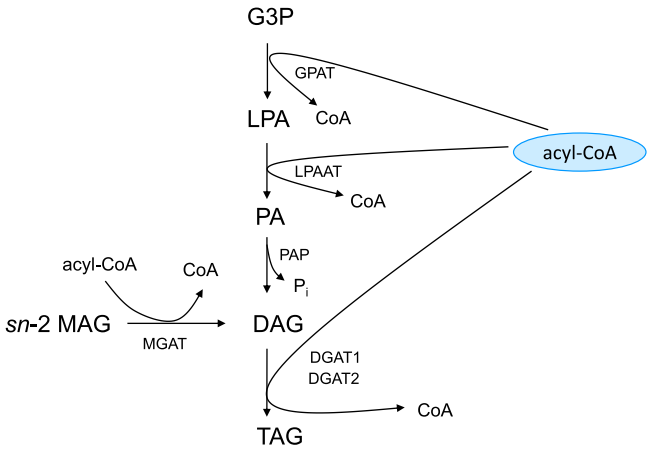

Fig. 13. Mammalian triacylglycerol (TAG) biosynthesis involves both the Kennedy pathway and acyl-CoA:monoacylglycerol acyltransferase (MGAT) pathway. The MGAT pathway is operative in the intestine. Other abbreviations: DAG, 1,2-diacyl-*sn*-glycerol; DGAT, diacylglycerol acyltransferase; G3P, *sn*-glycerol-3-phosohate; GPAT, acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase; LPA, lysophosphatidic acid; LPAAT, acyl-CoA:lysophosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; *sn*-2 MAG, 2-monoacyl-*sn*-glycerol.

Recent structural studies using cryo-electron microscopy have shown that purified human DGAT1 has nine TMDs, which is different from the previous DGAT1 topology model, and functions as a dimer [51,52]. A more detailed description of the structural features of DGAT1 will be discussed in section 8.

It has also been proposed that DGAT1 has a dual topology in the ER membrane [668]. Using *Dgat1* knockout mice and selective DGAT1 inhibitors, it has been suggested that DGAT1 has approximately equal activities on both sides of the ER membrane. How DGAT1 might insert into the ER membrane such that its active site has two different orientations remains to be determined. DGAT1 activity in the ER lumen would suggest that DGAT1 produces TAG utilized for very low-density lipoprotein (VLDL) production in the liver while the cytosolic facing activity generates TAG that is stored in cytosolic LDs. However, pharmacological inhibition of DGAT1 in primary hepatocytes and in mice had no effect on TAG secretion indicating that neither the luminal nor cytosolic DGAT1 activities are necessary for VLDL assembly [669,670].

7.1.3. DGAT2

DGAT2 is also an integral membrane protein that resides in the ER where TAG synthesis occurs [435,671,672]. Detailed mapping of the membrane topology of DGAT2 has demonstrated that it has two TMDs that are connected by a short loop of ~5–10 amino acid residues [46]. The bulk of the protein, including both its N- and C-termini, are exposed to the cytosol.

Using both immunofluorescence microscopy and subcellular fractionation methods, DGAT2 has also been found to be associated with LDs. The localization of DGAT2 to LDs appears to be dependent upon the addition of exogenously added FAs, such as oleate, to the cell culture medium which stimulates TAG synthesis. The presence of DGAT2 on LDs is thought to allow for their efficient expansion via localized TAG synthesis. It remains unclear where the DAG for TAG biosynthesis on LDs would come from.

The LD localization of DGAT2 raises an interesting question as to how an integral membrane protein that spans the ER lipid bilayer membrane interacts with the PL monolayer of LDs. Membrane topology studies demonstrated that DGAT2 has two TMDs near its N-terminus that are separated by ~5–10 amino acid residues. It has been suggested that the TMDs form a hairpin structure that does not completely span the ER lipid bilayer. Instead, DGAT2 is embedded in the ER membrane laterally which would facilitate its incorporation into the PL monolayer of the LD surface. However, experiments using a cysteine accessibility assay demonstrated that the loop between the TMDs of DGAT2 does extend through the ER lipid bilayer into the ER lumen [46]. Deletion mutagenesis and experiments using fluorescent reporters demonstrated that, while the first TMD of DGAT2 functioned as an ER targeting signal, neither of the TMDs had any apparent role in LD targeting. Available evidence suggested that even after cells are oleate-loaded, DGAT2 remains in the ER and is tethered to LDs via a domain in its C-terminal region that is predicted to be an amphipathic α-helix. Indeed, imaging DGAT2 in adipocytes by immunogold electron microscopy showed that DGAT2 was present in distinct ER structures in close proximity to the LD membrane [672]. Immunofluorescence microscopy demonstrated that the staining of DGAT2 in the ER and LDs were not segregated [673]. Instead, DGAT2 staining of LDs was continuous with the ER which was wrapped around LDs. Lastly, DGAT2 has not yet been detected in any mammalian LD proteomes from cells or tissues [674]. Taken together, these data suggest that DGAT2 is a resident ER protein that can interact with LDs to promote their expansion.

7.1.4. Regulation of DGAT1 and DGAT2

DGAT1 and *DGAT2* expression can both be regulated transcriptionally. In the liver and adipose tissue, *DGAT1* gene expression is increased

by fasting and decreased by refeeding. The counterintuitive increase in expression during fasting is to protect against lipotoxic stress brought on by increased non-esterified FA levels [675–677]. In contrast, *DGAT2* expression in the liver does not change under either of these conditions. In adipose tissue, *DGAT2* expression increases during feeding to promote TAG storage, but decreases during fasting.

Both DGAT1 and DGAT2 also appear to be regulated post-translationally but the exact mechanisms remain poorly understood. Mice lacking DGAT2 only in adipose tissue have normal TAG levels in this tissue along with increased DGAT1 activity [678]. There was no corresponding increase in *DGAT1* mRNA or DGAT1 protein levels, which suggested that DGAT1 is regulated post-translationally. Several reports have suggested that DGAT activity can be regulated by reversible phosphorylation [28]. Both DGAT1 and DGAT2 have several potential phosphorylation sites [28]. Global phosphor-proteomic studies of cells and tissues have identified multiple residues in DGAT1 that are phosphorylated (in mouse DGAT1: Y327, T15, S17, S20, S40 and S67) but their roles in modulating DGAT1 function were not determined [679–685]. A more recent proteomic study identified many of the same phosphorylated amino acid residues in mouse DGAT1 that was expressed in CKC12 cells [686]. Mutagenesis of these and other potential phosphorylation sites revealed that only S83, S86 and S89 of mouse DGAT1 might be important for DGAT1 activity. However, the authors of this study reported that they could not demonstrate that any of these three serine residues were phosphorylated using MS. It is possible that the cell culture conditions were such that DGAT1 was dephosphorylated at the time of isolation. Alternatively, these three serine residues may be important for DGAT1 activity but in a phosphorylation-independent manner.

To date, there are no reports directly demonstrating that DGAT2 is phosphorylated. However, DGAT2 is a relatively unstable protein (T½ of ~30 minutes) that is rapidly degraded in an ubiquitin-dependent manner by the proteasome [687,688]. In addition to changes in gene expression, the rapid turnover of DGAT2 could serve to modulate its activity. For example, in adipocytes, DGAT2 is inactivated during lipolysis to promote FA release and not storage as TAG in LDs [676]. It has been speculated that the rapid degradation of DGAT2 could facilitate this process. However, treating cells with lipogenic agents that promote TAG storage did not stabilize DGAT2 [688].

7.2. Physiological roles of DGATs in mammals

7.2.1. Adipose tissue

White adipose tissue has the highest DGAT activity of mammalian tissues and consequently the highest concentration of TAG [116,689]. Furthermore, both *DGAT1* and *DGAT2* are highly expressed in this tissue relative to most others [39,116]. This raises the question of whether it is DGAT1 or DGAT2 that is the predominant contributor to TAG synthesis in adipose tissue. Using quantitative gene expression analysis, *DGAT2* was found to be expressed at much higher levels than *DGAT1* in 3T3-L1 mouse adipocytes [690]. This was corroborated by large-scale human gene expression analyses where *DGAT2* mRNA levels were found to be higher than that for *DGAT1* in adipose tissue [691,692].

Because of the near complete absence of TAG in the global *Dgat2* knockout mice compared to a ~50% reduction of TAG in global *Dgat1* knockout mice, DGAT2 was presumed to be the major contributor to TAG storage in adipose tissue [689,693,694]. However, because mice lacking DGAT2 died shortly after birth, definitive comparisons to adult *Dgat1* knockout mice could not be made [694].

Subsequent studies in mice in which either *Dgat1* or *Dgat2* were individually disrupted only in adipose tissue showed that both enzymes contributed significantly to TAG synthesis in this tissue [678]. Surprisingly, there were normal amounts of adipose tissue in mice without DGAT2 in this tissue when fed either chow or high fat diets indicating that DGAT1 is compensating for the absence of DGAT2. Chow fed mice lacking only DGAT1 in adipose tissue had only a modest reduction in adipose tissue content. These observations are consistent with those observed in single *Dgat1* and *Dgat2* knockout adipocytes generated from mouse embryonic fibroblasts [695]. Adipocytes with only one of the DGAT enzymes had normal rates of TAG synthesis and storage. This suggested that DGAT1 and DGAT2 do not have completely distinct functions and are not essential for TAG storage in adipose tissue as they can compensate for each other.

Unlike global *Dgat1* knockout mice, which were resistant to diet-induced obesity and had improved glucose metabolism, mice lacking DGAT1 only in adipose tissue were only partially resistant to diet-induced obesity and were glucose intolerant [678,696]. Thus, it is apparent that the loss of DGAT1 in other tissues also contributes to the healthier metabolic phenotype observed in global Dgat1 knockout mice.

DGAT1 also has a protective role in preventing FA-induced toxicity in adipocytes during lipolysis where a large proportion of FAs released from LDs are re-esterified back to TAG [676]. A reason for this futile cycle was unknown. Although counterintuitive, DGAT1 becomes activated during lipolysis which promotes the resynthesis of TAG from released FAs. The net effect of this cycling between TAG breakdown and its resynthesis keeps intracellular concentrations of FAs low enough to prevent lipotoxicity. In the absence of DGAT1 in adipose tissue, the accumulation of unesterified FAs promotes ER stress leading to insulin resistance and impaired glucose metabolism.

Of particular consequence was that mice lacking DGAT2 only in adipose tissue did not die after birth, suggesting that the lethality observed in the global *Dgat2* knockout arose mainly from skin abnormalities [678,694]. This was confirmed as mice with *Dgat2* disrupted only in the skin died shortly after birth. These mice had an apparent skin defect leading to a defective skin permeability barrier causing them to become rapidly dehydrated. In the global *Dgat2* knockout, there was a decrease in skin acylceramide content which would contribute to a defective skin permeability barrier. It was originally proposed that DGAT2 produced a pool of TAG in the skin enriched in the essential FA, 18:2, which would then be utilized by other enzymes to produce acylceramide, a key component of the skin permeability barrier [697]. However, a more recent study has shown that, in addition to TAG synthesis, DGAT2 can also directly catalyze the formation of acylceramide, utilizing ceramide and a fatty acyl CoA as substrates [698]. Thus, DGAT2 may directly contribute to the maintenance of the skin permeability barrier by synthesizing this essential lipid.

Regardless of their individual contributions, it appears that DGAT1 and DGAT2 account for all TAG synthesized in adipocytes. TAG was almost completely absent in adipocytes lacking both enzymes [695]. Interestingly, although these cells lack TAG, they were considered fully differentiated adipocytes as determined by increased expression of multiple markers of adipocyte differentiation. Thus, although TAG is a hallmark of adipocytes, TAG does not appear to be required for adipogenesis.

DGAT1 and *DGAT2* are expressed at higher levels in brown adipose tissue, compared to white adipose tissue, suggesting that the encoded enzymes might produce TAG whose FAs contribute to thermogenesis [699]. When DGAT2 was inhibited in both mouse primary brown adipocytes or IMBAT-1 cells (brown adipocyte-derived cell line), there was a reduction in the amount of *de novo* synthesized FAs incorporated into TAG that were utilized for FA oxidation and thermogenesis [699]. Inhibition of DGAT1 had no effect on this process.

In vivo studies in mice with both *Dgat1* and *Dgat2* disrupted in only brown adipose tissue demonstrated that these DGAT enzymes are not essential for thermoregulation [700]. Despite the absence of TAG in brown adipose tissue, the double *Dgat* knockout mice were able to thermoregulate even in a chronically cold environment. The core body temperature of these mice appeared to be maintained through adaptive changes in gene expression and by utilizing circulating FAs and glucose and glycogen stored in brown adipose tissue. Although DGAT2, more so than DGAT1, can contribute substrates for thermogenesis, neither of these enzymes are essential for this process.

7.2.2. Liver

7.2.2.1. Lipoprotein metabolism. The liver has a prominent role in TAG metabolism, specifically in the assembly of VLDL and its secretion into the circulation [701]. The contribution of DGAT1 and DGAT2 to the synthesis of TAG incorporated into VLDL has primarily been studied in cell cultures and animal models over-expressing *DGATs*. An early model proposed that a latent, luminal facing DGAT activity (possibly DGAT1) contributed to the formation of VLDL, while the overt, cytosolic-facing DGAT activity (possibly DGAT2) might synthesize TAG stored in cytosolic LDs [106].

Studies in primary mouse and human hepatocytes suggested that DGAT2, more so than DGAT1, generated a pool of TAG utilized for VLDL assembly which was contrary to previous models [28,669]. Pharmacological inhibition of DGAT1 demonstrated that DGAT1 preferentially utilizes fatty acyl-CoA substrates derived from exogenous FAs. FAs released from hydrolysis of these TAGs were utilized for oxidation and not incorporated into VLDL [669,670]. In contrast, inhibition of DGAT2 in primary mouse hepatocytes led to decreased TAG secretion but did not affect the amount of apoB secreted. Instead, the secreted lipoprotein particles were less-lipidated. The use of dual radiolabeling experiments demonstrated that DGAT2 has a prominent role in VLDL secretion through the re-esterification of FAs released from a pre-existing TAG pool. Furthermore, DGAT2 was proposed to be to be involved in the maturation of nascent VLDL.

To address the specific roles of DGAT1 and DGAT2 in liver TAG metabolism, animal models lacking these enzymes specifically in this tissue have now been generated. Mice in which DGAT1 has been disrupted only in the liver had normal hepatic TAG levels when fed a chow diet [702,703]. However, when challenged with a high fat diet or fasted, there was a marked reduction in liver TAG which was protective against hepatic steatosis. Similarly, inhibition of DGAT2 in mouse liver also reduced hepatic TAG levels with protection from hepatic steatosis [677,704–706].

Unlike the studies performed with primary hepatocytes where inhibition of DGAT1 had no effect on TAG secretion, genetic disruption of *Dgat1* in mouse liver resulted in decreased plasma TAG after an overnight fast [702,703]. Similar results were obtained by pharmacological inhibition of DGAT1 [174]. The amount of ApoB100 secreted was unaffected by Dgat1 deficiency [703]. However, the VLDL secreted were less lipidated than in WT mice and had decreased TAG. This suggested that DGAT1 is required for the complete lipidation of nascent VLDL particles.

Using rodent models, DGAT2 has also been implicated in hepatic TAG metabolism. Both intracellular TAG and VLDL secretion were reduced when DGAT2 function was disrupted specifically in the liver [677] or inhibited with antisense oligonucleotides, siRNA or pharmacologically [670,705,706]. The reduction in hepatic TAG was accompanied with decreased lipogenesis and increased FA oxidation. ApoB secretion was not evaluated in these *in vivo* studies. However, there was no change in apoB secretion from either human or mouse primary hepatocytes when DGAT2 was inhibited which suggested that DGAT2 can also participate in the maturation of VLDL [669]. Unexpectedly, high-density lipoprotein (HDL) cholesterol was reduced in several of these models [677,705,706]. Inhibition of DGAT2 in the liver reduced apoA1 secretion, decreasing the number of HDL particles secreted. The precise mechanism for this is not clear.

Interestingly, unlike in rodent models, inhibition of DGAT2 in non-human primates did not reduce VLDL secretion from the liver and had very little effect on hepatic TAG production [670]. The reason for this appears to be that *DGAT2* is expressed at much greater levels than *DGAT1* in rodent liver. In contrast, in humans and non-human primates, *DGAT1* and *DGAT2* expression are more equalized. Thus, DGAT2 may have a greater role in hepatic TAG metabolism in rodents than compared to human and non-human primates.

Using different radiolabeled precursors (i.e., oleate, glycerol, acetate) in conjunction with selective inhibition of DGAT1 or DGAT2, it is apparent that DGAT1 preferentially utilizes exogenous unesterified FAs for TAG synthesis, while DGAT2 prefers to utilize endogenously synthesized FAs [670,702,707]. It is also apparent that both DGAT1 and DGAT2 are capable of contributing to the production of TAG that can be utilized for VLDL production, in *in vivo* models. It has been proposed that DGAT2 acts upstream of DGAT1 where it synthesizes TAG from *de novo* synthesized FAs [708]. Some of this TAG is secreted while some is stored in cytosolic LDs. DGAT1, functioning downstream from DGAT2, would primarily function in the lipolysis/re-esterification of stored TAG utilizing already existing or exogenous FAs.

7.2.2.2. Retinol metabolism. The storage form of retinol (vitamin A_1) are retinyl esters which are predominantly stored in hepatic stellate cells with smaller amounts present in hepatocytes [709,710]. Two pathways catalyze the synthesis of retinyl esters. In the acyl-CoA-independent pathway, lecithin:retinol acyltransferase uses phosphatidylcholine as an acyl donor. The acyl-CoA-dependent pathway esterifies retinol using acyl-CoA as the acyl donor. The identity of lecithin:retinol acyltransferase has remained elusive. However, since DGAT1 also catalyzes the acyl-CoA-dependent synthesis of retinyl esters it has been proposed to have a role in hepatic retinyl ester formation [83,711,712]. Unexpectedly, total hepatic retinyl ester levels were not decreased in *Dgat1* knockout mice [713]. Instead, hepatic stellate cells from *Dgat1* knockout mice had increased retinyl ester content, relative to hepatocytes. The authors of this study concluded that DGAT1 does not make a significant contribution to retinyl ester synthesis in either stellate cells or hepatocytes in the liver [713]. Instead, DGAT1 may play an indirect role in retinyl ester storage by modulating the distribution of retinyl esters between hepatocytes and hepatic stellate cells

7.2.3. Small intestine

7.2.3.1. Role of DGAT1 and DGAT2 in dietary fat absorption. Due to its hydrophobic nature, TAG cannot be absorbed intact from the intestinal lumen. Instead, dietary TAG is hydrolyzed to *sn*-2-MAG and FAs by gastric and pancreatic lipases [714,715]. Unesterified FAs and *sn*-2-MAG are taken up by enterocytes where they are used as substrates by the MGAT pathway to resynthesize TAG. MGAT catalyzes the re-acylation of *sn*-2-MAG producing DAG, which is then used by DGAT to generate TAG. TAGs are then packaged into chylomicrons that are released into the circulation for transport of dietary TAG to other tissues [716]. Although the Kennedy pathway is present in enterocytes, the MGAT pathway accounts for ~75% of TAG biosynthesis in the small intestine [657,717]. The liver and adipose tissue also have robust MGAT activity, but the role of the MGAT pathway in these tissues is poorly understood. There is some evidence linking MGAT activity in these tissues to FA recycling, glucose metabolism and TAG biosynthesis [718,719].

DGAT1 and/or DGAT2, which catalyze the terminal step of TAG biosynthesis for the both the Kennedy and MGAT pathways, would be expected to play significant roles in this process. Indeed, the rate of dietary fat absorption was reduced in *Dgat*1-deficient mice with TAG accumulating in cytosolic LDs in intestinal epithelial cells [720]. However, TAG absorption was quantitatively normal occurring more distally in the small intestine. It was speculated that DGAT2, which is also produced in mouse intestine, could partially compensate. However, the contribution of DGAT2 to dietary fat absorption is less clear as a mouse model lacking this enzyme in the small intestine has not been generated. In humans, DGAT2 is not required for fat absorption as the encoding gene is not expressed in the small intestine [116].

Not surprisingly, postprandial TAG absorption was restored when *DGAT1* was expressed only in the small intestine of global *Dgat1*-deficicent mice [721]. However, these mice had increased amounts of TAG in adipose tissue and liver and were no longer resistant to diet-induced

obesity even though DGAT1 was absent from these tissues. This highlights the ability of DGAT2 to compensate in the absence of DGAT1 in mice.

The role of DGAT1 in dietary fat absorption was further explored in mice lacking DGAT1 only in the intestine. Like the global knockout mice, mice lacking DGAT1 only in the small intestine were protected from diet-induced obesity [722]. TAG secretion was also delayed and chylomicrons were reduced in size by ~20% [722–724]. Plasma cholesterol levels were also lower due decreased secretion from the intestine. This was attributed to alterations in chylomicron assembly caused by the absence of DGAT1 in the intestine. It was proposed that the smaller chylomicrons are more efficiently removed from the circulation leading to reduced weight gain [722].

7.2.3.2. Congenital diarrhea and DGAT1 deficiency in the small intestine. Several instances of congenital diarrhea have been linked to mutations in *DGAT1*. Congenital diarrhea is a group of rare early onset diseases associated with nutrient malabsorption that can be life threatening if untreated [725]. Initially, a homozygous recessive mutation in *DGAT1* was identified in three children from a single family that had severe diarrhea [726]. A single base pair change in *DGAT1* altered a splice donor site that led to skipping of exon 8 that encoded 25 amino acid residues. Although the mutated gene was expressed, DGAT1 protein could not be detected as it was unstable and rapidly degraded. In a second study, a different homozygous recessive missense mutation was identified in twin patients that resulted in a leucine to proline residue change (L105P) [175]. In this case, DGAT1 protein could be detected but at reduced levels leading to partial loss of DGAT1 function, but with less severe diarrhea occurring. Other studies have identified additional *DGAT1* mutations in congenital diarrhea patients that include insertion/deletions, nonsense mutations and alterations in splice sites [727,728]. Collectively, these mutations lead to a reduction, or the complete absence, of DGAT1 protein and decreased TAG production in organoids derived from intestinal samples [728].

It is unclear how the absence of DGAT1 in the small intestine leads to diarrhea. It has been proposed that there is altered signaling in the gut due to the accumulation of DGAT1 substrates (DAG and FAs). However, although TAG levels were reduced in patient organoids, DAG levels appeared to be unaffected [728]. Alternatively, *Dgat1*-deficient cells may be more susceptible to lipotoxic stress leading to cellular dysfunction. *Dgat1*-deficient organoids from patients undergo cell death in response to exogenously added oleate [728]. Similar results were obtained with *Dgat1*-deficient mouse embryonic fibroblasts which underwent cell death when incubated with oleate [729]. In contrast, wild-type fibroblasts, that readily synthesize TAG, were able to tolerate a high concentration of exogenous oleate.

7.2.4. DGAT1 and DGAT2 in the heart

FAs are the main source of energy utilized by the heart for contraction [730]. Non-esterified FAs in the circulation, or FAs released from TAG carried in VLDL or chylomicrons, are taken up by cardiac myocytes and can be esterified to TAG [731,732]. These FAs can subsequently be released from this TAG storage pool and undergo β-oxidation to generate ATP [733]. The futile cycling between FAs and TAG prevents unesterified FA levels rising to toxic levels. However, the accumulation of TAG in the heart is also thought to promote cardiac dysfunction [734].

That both *DGAT1* and *DGAT2* are expressed in the heart, suggests that one, or both DGAT enzymes has a role in the cycling of cardiac TAG [39,116]. However, hearts from *Dgat1*-deficient mice only had modest changes in TAG levels, and although they were enlarged, heart function was normal [735]. Several factors could be responsible for the lack of decrease in TAG levels. Despite the reduced DGAT activity in *Dgat1*-deficient hearts, *DGAT2* is expressed at WT levels and is likely at the least partially compensating for the absence of DGAT1. However, in this study, DGAT assays on cardiac tissues were performed with 150 mM $MgCl_2$ which would completely inhibit DGAT2, so it is not entirely clear what enzyme is contributing to this residual acyltransferase activity [116,736]. Another finding was that both FA oxidation and lipid uptake were reduced in *Dgat1*-deficient hearts which would prevent induction of lipotoxic stress pathways. Collectively, these metabolic modifications appeared to have a protective effect as they likely prevented lipids from reaching toxic levels in cardiac myocytes.

While global *Dgat1*-deficient mice did not have any apparent cardiac abnormalities, mice with *DGAT1* disrupted specifically in cardiomyocytes had increased mortality and features found in human heart failure (i.e., increased DAG and ceramide levels, decreased *DGAT1* expression) [737]. Again, although DGAT activity was reduced by ~40%, cardiac TAG levels were not affected. Heart failure in these mice could be reversed by disruption of *DGAT1* in the small intestine. This cardioprotective effect was attributed to delayed dietary fat absorption due to the absence of DGAT1 in the small intestine which appeared to reduce the availability of lipids to the heart [720].

In a separate study, DGAT1 was disrupted specifically in the heart using an inducible system [738]. These mice only had a small reduction in heart TAG and cardiac function was unaffected. Presumably in this case, DGAT2 was able to compensate for the lack of DGAT1. Similarly, pharmaceutical inhibition of DGAT2 also had little effect on TAG synthesis indicating that DGAT1 is compensating. Therefore, it appears that both DGAT1 and DGAT2 contribute to TAG synthesis in the heart to some extent.

Interestingly, although over-expression of *DGAT1* in the hearts of mice, using *α-MYOSIN HEAVY CHAIN* promoter, doubled TAG levels in this tissue, these transgenic mice also had normal cardiac function [739]. This provides further evidence that toxic molecules, like FAs acids and DAGs, can be sequestered as TAG, which is relatively inert [740,741].

8. Three-dimensional structure of DGAT1

8.1. Structural and functional insights into plant and human DGAT1

Our understanding of the enzymatic mechanism for TAG biosynthesis has been greatly enhanced from recent structural analysis of MBOAT enzymes. Although DGAT1 was known to be an integral membrane protein that resides in the ER membrane, the number of helices and how these are assembled in the membrane to form an active enzyme to modify lipids, was elusive. Mammalian and plant DGAT1 have been shown to oligomerize into dimer and tetramers in a process involving the NTD [51,52,54,55,111,742]. As discussed in section 6.2.2.2, the NTD of *B. napus* DGAT1 has been shown to be largely unstructured, containing an IDR, along with a non-catalytic allosteric acyl-CoA/CoA-binding site [54,111,523,524]. These studies have also shown that the NTD is involved in the allosteric regulation of *B. napus* DGAT1 activity with acyl-CoA and CoA acting as positive and negative effectors, respectively. Other unanswered questions remained. How is the protein assembled? What is the architecture of the active site and how do substrates assemble in the membrane protein to form the products of the enzyme-catalyzed reaction?

Recent analysis of the crystal structure of bacterial DltB provided the first insights into the mechanism of an acyltransferase from an MBOAT family member [93]. Shortly thereafter, in 2020, cryo-electron microscopy (cryo-EM) structures for human DGAT1 were reported from two independent groups with a resolution of 3 Å [51,52]. Both studies reported a dimeric structure for human DGAT1, that has apparent pseudosymmetry, with an alignment showing very good structural agreement with a root mean square deviation of 0.69 Å. Each protomer consisted of nine transmembrane segments that formed a loosely assembled helical bundle consisting of long and short helices. The dimer forms a concave shape, with a wide and shallow cytosolic-facing cavity located beneath the plane of the ER membrane, and a smaller cavity facing the ER lumen (Fig. 14A). The enzyme was shown to form a unique

dimer interface facilitated by domain swapping of the NTDs [51,52]. The NTD of human DGAT1 exhibits a unique extended trajectory in the structure; amino acid residues 1-65 are unresolved while residues 66-77 are unstructured. This latter uncoiled region is located in the cytoplasmic face of the enzyme where it interacts with the neighboring protomer, and facilitates dimerization (Fig. 14.B, C). Amino acid residues 77-100 are located in the core of protein at the dimer interface, while residues 101 – 126 wrap around the helical bundle perpendicular to the plane of the membrane. The interface is stabilized by an extensive network of hydrogen bonds and hydrophobic interactions (Fig. 14.C). In solution, this domain was shown to be largely unstructured in plant DGAT1 [54,524]. Given this large area of interaction, it is not surprising that these amino acid residues have been shown to be important for enzymatic function. Structural analysis of the N-terminal acyl-CoA binding segment of *B. napus* DGAT1 (BnaC.DGAT1.a $_{81\text{-}114}$) using nuclear magnetic resonance spectroscopy has shown that arginine 96 and arginine 97 interact with CoA [54]. In human DGAT1, the equivalent residues are lysine 66 and arginine 67; these residues face towards the cytosolic cavity. Binding of CoA to these residues could alter the interaction of the NTD with the active site region, possibly regulating the binding of the acyl donor (e.g., oleoyl-CoA).

This large area of interaction, along with analysis of the activity of N-terminal truncations, also supports a mechanism for human DGAT1 being a cooperative dimer [51,52] as was shown for *B. napus* DGAT1 [54]. For membrane-embedded enzymes, this is a means to quickly enhance activity in the bilayer [743]. Furthermore, in a crowded environment like the lipid bilayer, this interaction would likely enhance stability of this membrane-embedded protein, which has a largely unstructured NTD that is functional [744,745].

Cryo-EM structures of human DGAT1 incubated with oleoyl-CoA

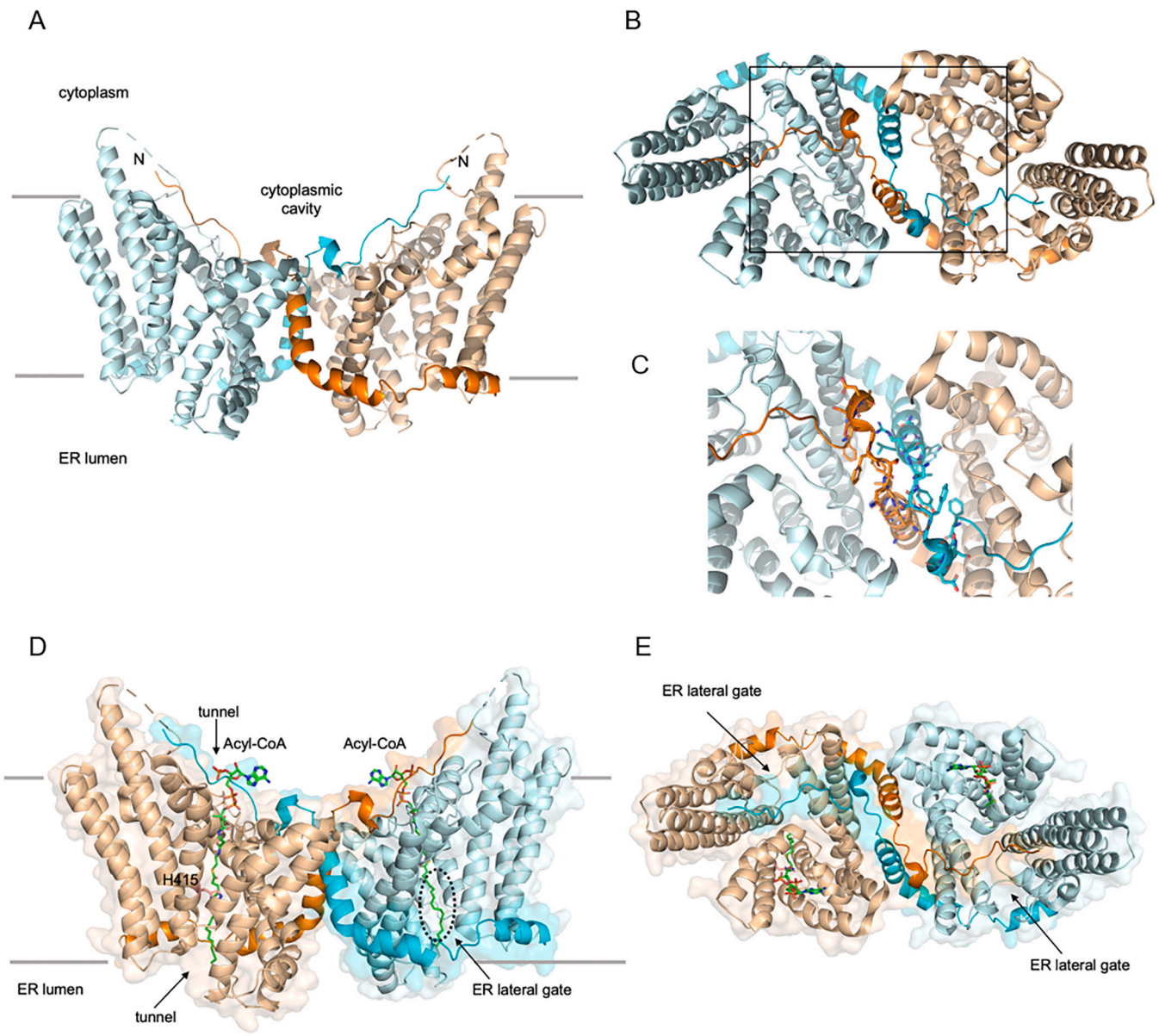

Fig. 14. Cryo-electron microscopy structure of the full length human diacylglycerol acyltransferase 1 (DGAT1) (A). Dimeric structure of human DGAT1 (blue and orange protomers indicated), with an aqueous cavity on the cytoplasmic face of the bilayer (6ZV1.pdb). (B). The N-terminal domain of human DGAT1 (in dark orange) participates in domain swapping between the protomers. (C). The dimer interface is supported by both H-bonds and hydrophobic interactions. (D). An acyl-CoA (green sticks) is bound in each promoer of human DGAT1 in a tunnel, which is accessible in the cytoplasmic cavity and endoplasmic reticulum lumen face. (E). Cartoon representation indicates that the oleoyl-CoA is bound in each protomer.

resolved the fatty acyl donor bound within each protomer in the dimer (Fig. 14.D, E). The hydrophobic fatty acyl chain is located within the bundle of helices and is coordinated by extensive hydrophobic interactions. The charged CoA moiety of oleoyl-CoA is essential for the catalytic function of this enzyme [51,52]. The structures reveal the CoA is coordinated by the charged phosphate groups, which hydrogen bond with the side chain nitrogen atoms of glutamine 375 and arginine 404, and the hydroxyl group of tyrosine 390 (Fig. 15.A, B).

The cryo-EM structures also provided information regarding the residues involved in the catalytic activity of the human DGAT1 enzyme. The active site of human DGAT1 is comprised of a conserved motif ^{411}SXXXHEY^{417} that contains the catalytic histidine 415 to carry out

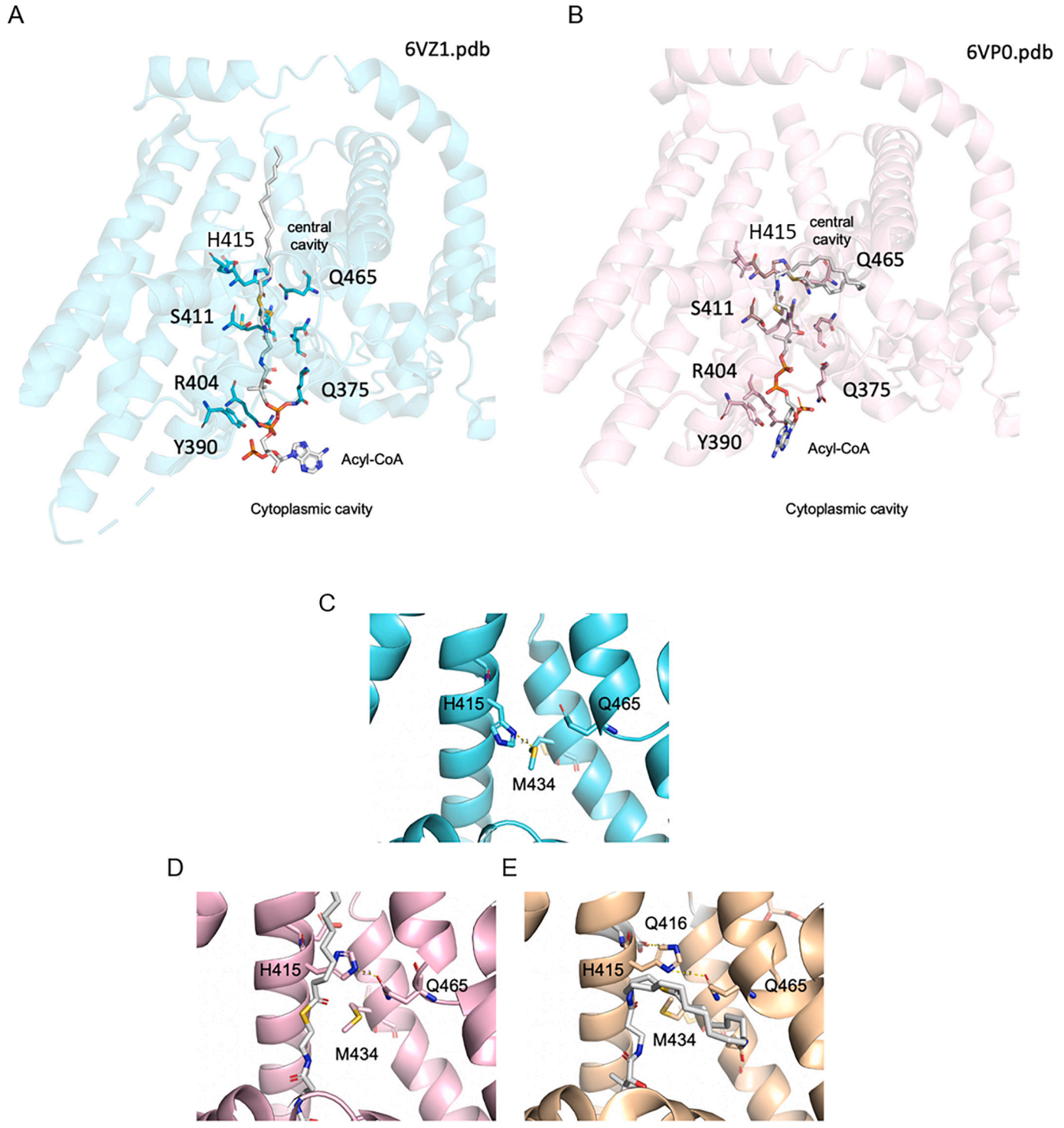

Fig. 15. Analysis of amino acid residues involved in the catalytic activity of the human diacylglycerol acyltransferase 1 (DGAT1). Acyl-CoA bound in the enzyme showing an (A) elongated acyl chain and (B) a curved acyl chain. Residues involved in lipid coordination are shown including the catalytic H415, and residues coordinating the charged the CoA moiety, Q375 and R404, are indicated. (C). In the absence of lipid, the catalytic H415 for an H-bond with M434 (6VYI.PDB). (D). When an oleoyl-CoA is bound in the enzyme, the H415 rotates and H-bonds with Gln465 (6VZ1.PDB). (E). In the presence of a curved lipid, the H415 is also proposed to interact with Gln416 (6VP0.PDB) [51,52].

cleavage of the thioester bond in acyl-CoA to allow for transfer of the fatty acyl moiety to DAG (Fig. 15.A, B). The active site histidine 415 is buried in the central cavity in each protomer. For DGAT1 to generate a nucleophilic histidine residue, typically a H^+ is abstracted by a nearby acidic residue, creating a charge relay system. The current structures provide differing scenarios how this might happen in DGAT1. In the absence of lipid, histidine 415 is hydrogen bonded to methionine 434 (Fig. 15.C). In the presence of oleoyl-CoA, the side chain of histidine 415 rotates and forms a hydrogen bond with glutamine 465 and is also positioned within hydrogen bonding distance to the thioester group of the acyl-CoA. In this structure the fatty acyl chain is extended in the tunnel (Fig. 15.D) [52]. In a separate structure with oleoyl-CoA, the investigators proposed that the nearby glutamine 416 creates a charge relay system to generate a catalytic histidine residue [51]. However, the distance between these side chains is approximately 5.0 Å and flexibility would be needed in this region for a charge relay to be established (Fig. 15.E). Overall, it appears there is a strong network of charged residues in the central cavity that support the activation of the catalytic histidine 415 in the presence of lipid in the cavity. Upon removal of the proton from histidine 415, this nucleophilic residue is then able to abstract a proton from the hydroxyl group of DAG which can then attack the carbon-sulphur bond of oleoyl-CoA [51]. A loss in DGAT activity occurred when histidine 415 or asparagine 478 was substituted for an alanine residue, and also when methionine 434 was substituted with an isoleucine residue [52], highlighting the importance of these residues for catalysis.

The structures of human DGAT1 also provided insight into substrate access to this active site buried in the central cavity of this membrane-embedded enzyme. The electron density for the acyl-CoA indicated a long tunnel in association with the catalytic residue, which is accessible from both the cytoplasm and ER lumen (Fig. 14.D, 15.A) [52]. In a separate structure with oleoyl-CoA, the acyl chain does not extend the length of the tunnel, but rather is curved in a hydrophobic central cavity with the CoA moiety also in the cytoplasm (Fig. 15.B). It is proposed that the acyl-donor enter this extended tunnel from the cytosol. The lateral gate located on the luminal side of the lipid leaflet, perpendicular to this primary tunnel would allow for DAG access to the active site, (Fig. 14) [52]. This lateral gate provides access to the hydrophobic lipid environment allowing direct deposition of TAG in the lipid bilayer. The curved nature of this lateral gate could accommodate a flexible lipid like DAG, but not a rigid lipid such as cholesterol [52]. The newly formed TAG would likely add constrained steric bulk to the lateral gate, thus allowing for release of the lipid into the bilayer; however, such conformational dynamics remain to be validated. The bacterial DltB [93], which does not catalyze the acylation of DAG but rather extracellular teichoic acid, has no need for a lateral gate. This feature is missing from the DltB structure, and indirectly supports the hypothesis that this lateral gate binds DAG in human DGAT1. Substitution of a bulky residue at the entrance to this lateral tunnel (L346W) resulted in a significant loss of activity [93] further supporting a role for this lateral gate in substrate accessibility.

The length of the tunnel may be related to the substrate preference of human DGAT1. Substrates with longer fatty acyl chains would result in a hydrophobic mismatch with energetically unfavorable interactions. This theme has been identified with other lipid modifying enzymes including PhoPQ-activated gene P which is a bacterial outer membrane acyltransferase that acylates toxins to assist with pathogen evasion of host immune responses [746]. PhoPQ-activated gene P has been shown to have a hydrophobic tunnel with preference for palmitoyl chains, which is regulated by residues in the lipid donor tunnel. In contrast, human DGAT1 has an open tunnel at the end supporting diverse fatty acyl chain lengths. Of note, oleoyl-CoA fits perfectly within the lipid tunnel, with no hydrophobic mismatch (Fig. 14; [52]). Electron density, representing lipids, was also identified at the lipid interface between the dimer in both cryo-EM structures (Fig. 16). In 6VP0.pdb, several lipid and detergent molecules are observed at the dimer interface and outer perimeter of the DGAT1, which was likely preserved during extraction of the protein from lipid bilayer. This has been observed with other membrane proteins and is known to contribute to protein stability and function [745].

Although the two cryo-EM structures for human DGAT1 have been nicely resolved, they are, however, static images. It is likely that the TMD helix with the catalytic histidine 415 rotates to accommodate lipids (Fig. 15.C,D,E). This could be assessed using double electron paramagnetic resonance spectroscopy [747]. Furthermore, other studies have shown that mammalian DGAT1 can also form tetramers [55,742]. Evidence in support of tetramerization has also been reported for plant DGAT1 [111,522]. Although this aspect was alluded to in the cryo-EM structural analyses [51,52], it was not pursued since the tetramer was not a predominant species. Thus, this represents an area for future investigation.

8.2. *Modeling of the putative three-dimensional structure of plant DGAT1*

We have generated a molecular model for the BnaDGAT1 structure (isoform BnaC.DGAT1.a, AFM31259.1), residues 60-501, using the program AlphaFold [748]. AlphaFold is an advanced program that uses deep learning artificial intelligence to accurately predict protein structures. The first 60 amino acid residues of BnaC.DGAT1.a were omitted from the modeling since this area was not observed from the cryoEM structures of the closest structural homolog human DGAT1 and would not be modeled accurately. Peptide bond geometry was optimized in the model using the Amber force field option in AlphaFold. The modeling run resulting in a predicted model with a homodimer configuration similar to that of the current structures for human DGAT1, with a bundle of helices and domain sharing with the N-terminal segment (Fig. 17.A-D). The Local Distance Different TEST is over 80 for the length of the core protein suggesting no clashes within the model structure. The location of benchmark residues further allows us to assess the validity of this model: The BnaC.DGAT1.a catalytic histidine 428 and glutamine 482 are located on the identical TMD in human DGAT1 corresponding to histidine 415 and glutamine 465, respectively. Furthermore, residues arginine 96 and arginine 97, previously shown to interact with CoA [54], are located in the cytoplasmic cavity (Fig. 17.C).

The 3-D model of plant DGAT1 can also be used to gain insight into amino residues known to enhance the performance of the enzyme. As previously discussed in section 6.2.3., A directed evolution approach was used to generate variants of BnaC.DGAT1.a having amino acid substitutions that enhance activity and/or polypeptide accumulation thereby resulting in increased TAG production [94–96]. Nineteen single amino acid substitutions were identified that were linked to increased TAG production. Here, the top five amino acid residue substitutions are mapped. The substitutions are located in different regions of the enzyme, indicating separate regions of the enzyme can influence function (Fig. 17.E). When recombinantly produced in H1246 yeast, the substitution of phenylalanine 473 for a leucine residue in BnaC.DGAT1.a resulted in the highest level of TAG production, approximately twice that of the WT BnaC.DGAT1.a [95]. This amino acid residue is located in the back of the lateral gate that allows access of lipid substrates from the luminal leaflet of ER into the central cavity (Fig. 17.F). Altering the bulky phenylalanine 473 to a less bulky but still hydrophobic leucine side chain would allow more room for fatty acyl chains to be accommodated in the central cavity. Residues in the NTD also influenced TAG production in yeast. A114P, S112R and I108T are TAG-enhancing substitutions clustered at the dimer interface near the cavity where the CoA moiety binds (Fig. 17.G), further supporting the role of the NTD in FA recognition [54]. In particular, the substitution S112R would attract the negatively charged CoA of the fatty acyl-CoA. Substitution L136F also enhanced TAG production. This residue is located at the top of the central TMD helix near the ER lumen (Fig. 17.H). This bulky hydrophobic substitution is more difficult to rationalize for enhancement of TAG production; however, it be can speculated that this substitution

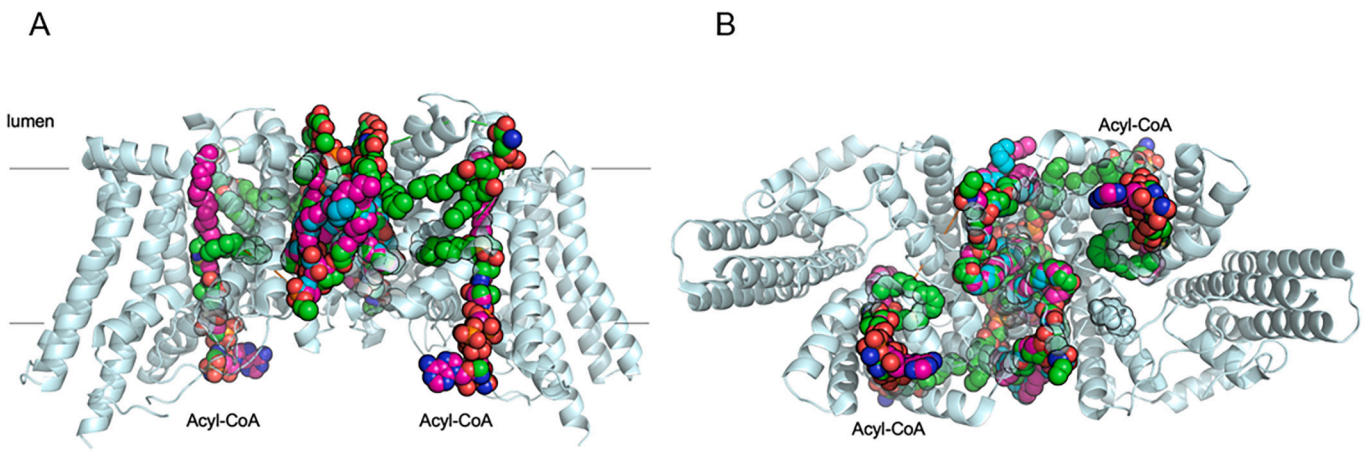

Fig. 16. Lipids support the structure of human diacylglycerol acyltransferase 1 (DGAT1). (A) A side view, and (B) view from the cytoplasmic face of the cryo-electron microscopy structure of the full length human DGAT1 reveals several lipids and detergent molecules were observed at the interface between the protomers in the dimer (6VP0.pdb). These are separate from acyl-CoA found in the channel of each protomer.

may likely affect positioning of the helices in the bilayer, since lipid and detergent molecules were observed in this region of human DGAT1 (Fig. 16. Two mutations in BnaC.DGAT1.a that were examined further in both yeast and tobacco expression systems that resulted in higher TAG production were L441P and I447F [95]. L441P caps the end of a helix that is adjacent to the active site histidine and could affect positioning of active site residues in the central cavity. In contrast, L447F points directly into the hydrophobic central cavity and this substitution likely influences the affinity of the enzyme for lipid substrates in the reaction center.

In 2018, the crystal structure of a bacterial WSD provided insight into how WEs were made from alcohols and FAs [296]. Similar to models for human and *Brassica* DGAT1, the investigators found a catalytic histidine residue in the active site. Predictions were made to determine which amino residues assisted with making the catalytic histidine nucleophilic. With this structural information they were able to generate a mutation in WSD that allowed the enzyme to utilize shorter chain fatty acyl moieties. With our new structural model of BnaC. DGAT1.a (Fig. 17), similar targeted mutations can be made to tailor for enhanced TAG biosynthesis in plants.

9. DGAT as target in metabolic engineering of triacylglycerol biosynthesis

ME can be viewed as the intentional modification of cellular networks to enhance production of native molecules or macromolecules or to produce new bioproducts [749–751]. Synthetic biology expands the repertoire of tools for ME through the design and creation of new biological pathways by using synthetic DNA and genetic circuits [750,752,753]. “Proof-of-concept” and optimization of a metabolically engineered process follows a cycle of “design/build/test/learn” (DBTL) [751,754]. In addition, to optimizing the production of a target biomolecule(s), the DBTL cycle can provide valuable insights into the genetic and biochemical regulation of pathways and even lead to new opportunities for ME.

ME strategies involving the DGAT-catalyzed reaction in bacteria, yeast, plant-like protists and higher plants are discussed in sections 9.1 to 9.4 of this review. Given the plethora of investigations, only a limited number of examples are discussed. Both increasing TAG content and/or altering the FA composition of TAG have been pursued. In many cases, other metabolic steps have been modified in conjunction with manipulation of DGAT, thus contributing to synergistic strategies for modifying TAG biosynthesis. Both improvement of edible oils and development of oils for industrial applications, such as production of biodiesel and oleochemicals, have been investigated. In some instances, endogenous *DGAT* genes or modified *DGAT* genes were over-expressed. Other strategies have involved the heterologous expression of *DGAT* genes from other organisms. For example, plant *DGAT* cDNAs have been used to modify TAG biosynthesis in yeasts. In other cases, modification of DGAT action has been a part of an overall strategy to produce other bioproducts (other than TAG) in the metabolically engineered organism.

9.1. Metabolic engineering of bacteria

9.1.1. Modification of triacylglycerol biosynthesis in Escherichia coli

Most bacterial species effectively regulate cellular composition and cell growth, and non-oleaginous species generally do not allow the accumulation of excess storage lipids beyond their metabolic demands [755,756]. There has been a keen interest in producing FAs, TAGs and lipid-related products in the fast-growing model species *E. coli*, where genetic tractability, growth conditions, biochemical and physiological properties, and lipid metabolism have been well characterized and many genetic manipulation tools have been well developed [756–758]. While *E. coli* has been used as the host in the functional characterization of bacterial WSDs, the current section will focus on the use of bifunctional WSDs and other enzymes in *E. coli* for production of TAG and acyl esters.

TAG accumulation has been reported in *E. coli* with the over-expression of *ScoWSD1* and deletion of *E. coli diacylglycerol kinaseE* (also referred as *dgk*) [38], co-expression of *ScoWSD1* and *S. coelicolor PAP* (*Lppβ*; *ScoPAP*)[248], and co-expression of *AbaWSD* and *E. coli PAP* (also named *pgpB*) [195]. WSDs from different strains were not equally as effective in promoting TAG accumulation. For instance, when *ScoWSD1*, *MhyWSD1*, *MhyWSD2*, *RopWSD1* and *RopWSD2* were individually expressed in a *dgk* mutant of *E. coli*, respectively, only ScoWSD1 and MhyWSD1 led to TAG accumulation, wherein *MhyWSD1* expression resulted in TAG produced at 4.9% of the biomass [307]. In addition, the expression of *WSD* without *PAP* over-expression or *dgk* deletion did not result in TAG production in *E. coli*. Although *E. coli* can synthesize DAG, the increase in DAG content was essential for TAG production in the transgenic *E. coli*.

Various studies have explored the effect of different gene combinations to increase TAG content in *E. coli*. These studies have included the comparison and selection of *WSDs* and other genes for over-expression, targeted deletion of *E. coli* genes, and in some cases, fine-tuning of gene expression such as changing promoters and plasmids. Although various TAG contents have been attained in the ME of *E. coli* strains in different studies, TAG contents and WSD performance could not be directly compared because different expression techniques, gene combinations, genetic backgrounds and fermentation conditions of the applied strains had a strong influence on TAG production.

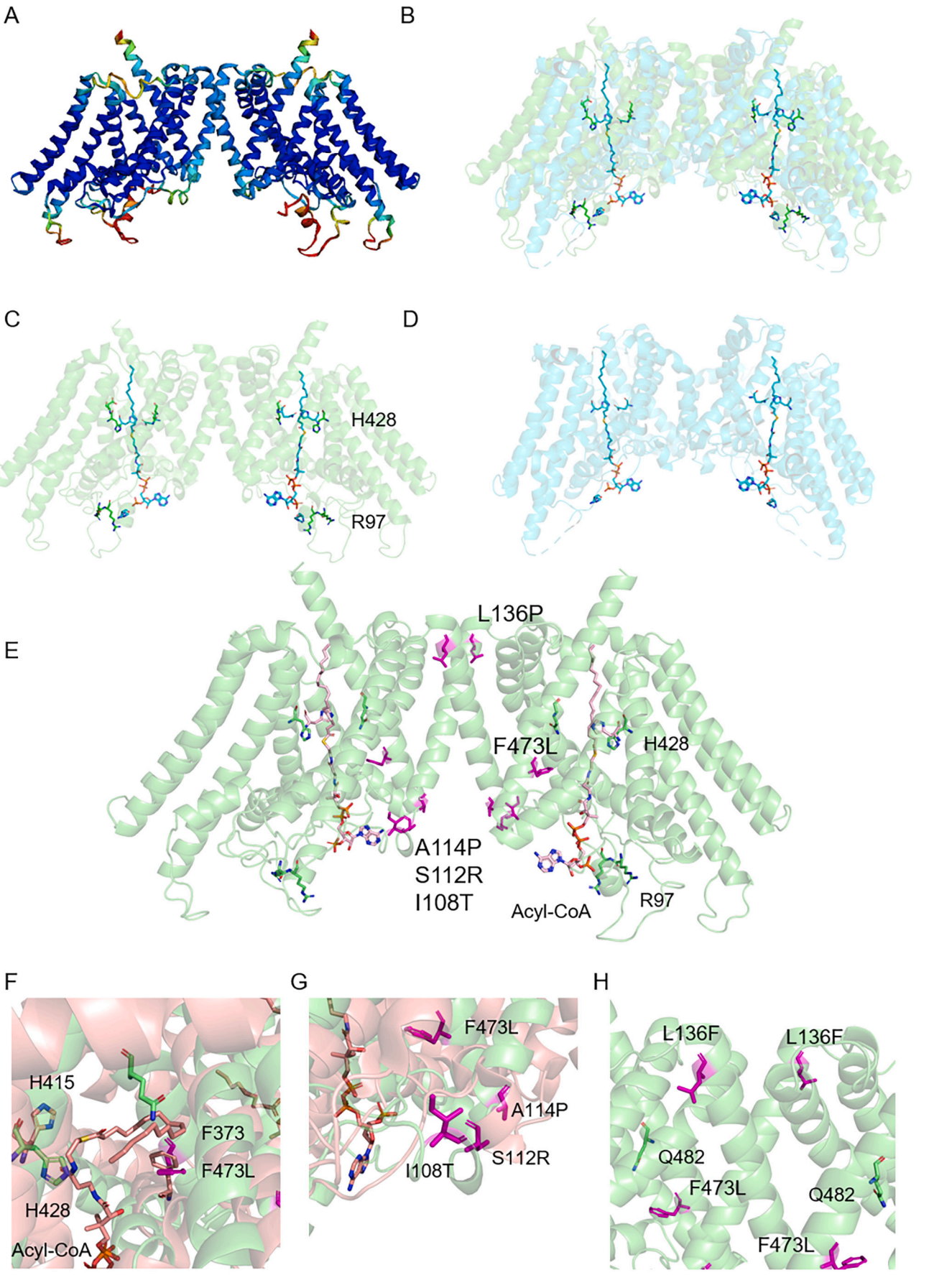

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Fig. 17. Molecular model of *Brassica napus* diacylglycerol acyltransferase 1 (isoform BnaC.DGAT1.a). (A). model of BnaC.DGAT1.a colored according to confidence for the model (Local Distance Different TEST) with dark blue being >90% confidence, light blue 80%, green 70%, yellow 60% and red <50%. A structural alignment of the BnaC.DGAT1.a in green is superimposed with the human DGAT1 structure (6VZ1.PDB). (B). The model of BnaC.DGAT1.a (green) overlaid with human DGAT1 (blue). (C). The model of BnaDGAT1 (green) with key residues including the catalytic H428 shown. (D). For comparison, the human DGAT1 structure (6VZ1.PDB) is presented alone. (E). The model of BnaC.DGAT1.a (green) with amino acid substitutions known to increase triacylglycerol (TAG) production shown in magenta. (F). Zoomed in region of substitution F473L which is located at the back on the lateral gate in the pocket occupying a curved lipid in 6VP0.pdb. (G). Zoomed in region showing substitutions I108T, S112R, and A114P in the N-terminal region residues that enhance TAG production. (H). Substitution L136F is located on the end of a helix at the endoplasmic reticulum lumen side and likely influences positioning of the enzyme in the bilayer.

When *E. coli fadD* and two copies of *AbaWSD* were overexpressed in the *E. coli dgk* mutant, TAG accounted for 8.5% of the cellular DW after fermentation optimization [759]. In another study from the same lab, the over-expression of *AbaWSD*, *E. coli fadD, fadR* (encoding a central transcriptional regulator positively regulating FA production), *pgpB* (*PAP*), *plsB* (*GPAT*) and *tesA* (*thioesterase A*) in the *E. coli* strain BL21 (DE3) resulted in a similar level of TAG accumulation [760]. Interestingly, *E. coli* C41 (DE3) with the same gene combination produced a lower amount of TAG than the transgenic *E. coli* BL21 (DE3) strain. In addition, the knockout of *dgk* from *E. coli* BL21 (DE3) equipped with the same plasmids did not increase the TAG content further [760]. The study also reported that *AbaWSD* expression led to greater TAG accumulation than *ScoWSD1* expression when the same gene combinations were used [760]. In contrast, *ScoWSD1* expression led to greater TAG accumulation than *AbaWSD* expression, where the modifications included the deletion of *E. coli dgk* and *fadE* (*acyl-CoA dehydrogenase*) and over-expression of *ScoPAP*, *ScoWSD1* and *ScoACCase* [761]. In addition, co-expression of *C. reinhardtii PDAT, AbaWSD* and *R. opacus PAP* (*RopPAP*) in *E. coli* led to higher TAG content than the strain only expressing *AbaWSD* and *RopPAP*, indicating a synergist effect on TAG accumulation between the acyl-CoA-independent pathway for TAG synthesis mediated by *CrPDAT* and the acyl-CoA-dependent pathway involving *AbaWSD* [762].

Medium-chain FAs such as C10:0, C12:0, and C14:0, are of particular interest in the oleochemical industry. Therefore, *E. coli* has also been metabolically engineered to produce TAG rich in medium-chain FAs [308,763]. A stepwise approach started with the comparison of *RopPAP* and *R. jostii PAP* in *E. coli* BL21 (DE3) expressing *AbaWSD*. Expression of *RopPAP* led to a greater accumulation of TAG. In contrast, expression of *R. jostii PAP* led to a higher amount of FAEEs and unesterified FAs [308]. Six bacterial WSDs were then compared by co-expression with *RopPAP*, respectively, and their performance on TAG accumulation were shown as *TcWSD* > *AbaWSD* > *AbaWSD* (codon optimized) > *RjoWSD8*, while *RopWSD1* and *RopWSD2* led to no TAG accumulation. Therefore, *E. coli* over-expressing *RopPAP* and *TcWSD* was chosen as the host to compare *R. opacus acyl-CoA synthetases* (*RoFadD1 and RoFadD2*) in the next step. Co-expression of *RoFadD1* resulted in higher TAG content than for co-expression of *RoFadD2*; therefore, *RoFadD1* was selected and added to the combination. The new *E. coli* strain was used to compare three *R. opacus TetRs* (homologs of the transcriptional regulator *fadR* in *E. coli*). The best combination for TAG production included *TcWSD*, *RopPAP*, *RoFadD1* and *RoTetR2*. When the genes were arranged in one vector and expressed in *E. coli*, the transgenic *E. coli* could produce 175.7 mg/L TAG (approximately 5.4% of the cellular DW) before fermentation optimization. Lastly, six medium-chain FA-specific acyl-ACP thioesterases were individually expressed in this strain for comparison. The co-expression of castor *thioesterase* led to the highest content of medium-chain FAs in TAG, but the total TAG content was reduced. The final strain produced 699.4 mg/L of TAG with 43.8% medium-chain FA after fermentation optimization [308].

9.1.2. Acyl ester production in Escherichia coli via metabolic engineering

The WS activity and broad substrate specificity of WSD has also been used to produce FAEEs in *E. coli*. When *Zymomonas mobilis pdc* (*pyruvate decarboxylase*) and *Z. mobilis adhB* (*alcohol dehydrogenase*) and *AbaWSD* were co-expressed in *E. coli*, 0.26 g/L FAEE were produced. The FAEE were mainly ethyl oleate with minor amounts of ethyl palmitate and ethyl palmitoleate, when grown under aerobic cultivation in the presence of glucose and sodium oleate [764]. These results indicated that engineered *E. coli* could produce ethanol and convert exogenous oleic acid to oleoyl-CoA, which was then used by AbaWSD to produce ethyl oleate. The minor amount of ethyl palmitate and ethyl palmitoleate indicated that FAs derived from *de novo* FA biosynthesis could also be used in FAEE biosynthesis [764]. This proof-of-concept study paved the way to improve FAEE production in *E. coli*.

Similar to TAG production in *E. coli*, combinatorial approaches have been used to increase FAEE content in *E. coli* via ME. An engineered *E. coli* strain was generated that could produce 427 mg/L of FAEES in 72 hours with glucose as the sole carbon source. FAEE evaporation was prevented with an overlay of dodecane [765]. Modifications introduced into *E. coli* included the over-expression of *Zmpdc* and *ZmadhB* for ethanol biosynthesis, overexpression of *tesA* and *fadD* and deletion of *fadE* to increase acyl-CoA content, and the overexpression of two copies of *AbaWSD* to catalyze the production of FAEE [765]. Subsequently, a dynamic sensor-regulator system was developed and used to balance the metabolism of host cells and improve the stability of the FAEE biosynthetic pathway; this increased the FAEE titer to 1.5 g/L [766].

WSDs have been modified to change their substrate preference and activity, and the performance-enhanced WSDs were used to improve FAEE production in *E. coli* [291,296]. Since a single amino acid residue substitution in AbaWSD (G355I) shifted its substrate preference to shorter chain alcohols, the functions of AbaWSD-G355I in FAEE production in transgenic *E. coli* was tested [760]. The gene combinations included the over-expression of *fadD, fadR, tesA, Zmpdc* and *ZmadhB*, and *AbaWSD/AbaWSD-G355I*. The WT AbaWSD led to the production of both FAEE and TAG, whereas AbaWSD-G355I improved FAEE synthesis by approximate 40% on a relative basis. However, there were no TAGs produced. These results indicated that the amino acid residue substitution at G355 might block the enzyme's ability to bind a bulky substrate like DAG [760].

Due to the WS and DGAT activities and their broad substrate specificities, bacterial WSDs have also been used in the ME of *E. coli* to synthesize other acyl esters, including WEs [266,765], mixed FA short-chain esters [767], glycidyl acyl esters [768], FA isopropyl esters [769], FA isobutyl esters [770], FA branched-chain esters and branched FA branched-chain esters [771]. With the publication of the crystal structure of bacterial WSD [296] and rapid development of protein structure prediction, it would be attractive to modify WSDs for the production of designer acyl esters in *E. coli* and other microorganisms.

9.1.3. Modification of triacylglycerol and acyl ester biosynthesis in other bacterial species

Corynebacterium glutamicum is a robust species for fermentation and has been used in large-scale industrial production of amino acids [772,773]. With the well-developed tools for its ME, this species has potential to be developed for the production of other bioproducts [772,773]. Although WT *C. glutamicum* can only synthesize membrane PLs, but not TAGs, a combinatorial ME approach has been used to develop a final strain with a 7.5% yield of total FAs (2.38 g/L intracellular FAs and 0.64 g/L extracellular FAs, including both unesterified FAs and esterified lipids including TAG) when grown in flasks with glucose as the carbon source. This corresponds to 17.8% of the cellular DW [774]. The modifications included (1) over-expression of *RopWSD1* and *RopWSD1* from *R. opacus* PD630 and *E. coli pgpB* (the *PAP* gene) to complete the Kennedy pathway for TAG biosynthesis; (2) over-

expression of *tadA* from *R. opacus* PD630 to facilitate LD maturation; (3) over-expression of *E. coli thioesterase* (*tesA*) and *acyl-CoA synthetase* (*FadD*) to increase the fatty acyl-CoA pool; (4) deletion of *dgk* and four genes encoding TAG lipases to reduce TAG degradation; (5) deletion of the TF *fasR* to enhance FA synthesis; (6) deletion of the genes encoding pyruvate quinone oxidoreductase and a lactic dehydrogenase to eliminate the biosynthesis of lactic acid and acetic acid (by-products); and (7) shortening of the lag phase in flask incubation with one round of cell adaption [774].

A polyhydroxyalkanoate-producing *Cupriavidus necator* (also known as *Ralstonia eutropha*) strain has also been engineered for FA production. This strain produced 60.64 mg/g DW of total FAs in a one-step fermentation [775].The combinatorial modification included the disruption of the *phaC1* gene encoding polyhydroxyalkanoate synthase and over-expression of genes encoding endogenous ACCase, endogenous cytoplasmid thioesterase, *C. glutamicum* type I polyketide synthase and *C. glutamicum* holo-ACP synthase [775]. It would be interesting to determine if *DGAT* overexpression could further increase the total FA content in this strain.

There has also been considerable interest in the production of FAs and their derivatives in cyanobacteria, which have very high photosynthetic yields and simple cellular architecture [220,776,777]. In a recent study, *A. baylyi AbaWSD* was expressed in the mutant strain, *Synechocystis sp. PCC 6803*, in which the *recJ* gene was disrupted. The *recJ* gene encodes a single-stranded DNA-specific exonuclease that decreases transformation efficiency [219,778]. The transgenic strain accumulated 0.508 nmol/ml/OD730 of TAG (OD730 was used to measure cell density) after one week of incubation under high light, which corresponded to 0.245% of TAG (DW basis) [219]. Since various gene combination strategies improved the TAG content of *E. coli* and many other microorganisms, it would be interesting to test similar approaches in cyanobacteria for increasing TAG productivity.

WSD has also been used in FAEE production in *Rhodococcus* spp, which has the remarkable ability to accumulate large amounts of TAG (e.g., 80% of cellular DW in some strains under optimized culture conditions); generic tools have been developed for applications in ME [779–788]. In a recent study, TAG production in *R. opacus* PD630 was increased to 82.9 g/L via fermentation optimization. This modified strain was then further engineered to produce FAEEs [779]. *R. opacus* PD630 was first allowed to accumulate TAG. The strain was then induced so that the fatty acyl chains contained in the TAGs were used for FAEE synthesis. Genes facilitating the release of unesterified FAs from TAG, fatty acyl-CoA synthesis from unesterified FAs, ethanol synthesis from acetyl-CoA, and FAEE synthesis from acyl-CoA and ethanol (i.e., WSD), were over-expressed under acetamide-inducible promoters. Moreover, the endogenous promoter of *acyl-CoA synthetase* was replaced with an acetamide-inducible promoter to control the initiation of fatty acyl-CoA synthesis for FAEE formation. Seven major *acyl-CoA dehydrogenases* were also deleted from this strain to prevent acyl-CoA degradation. The final strain produced 21.3 g/L FAEEs from glucose in a one-step fermentation without ethanol supplementation [779]. *MhyWSD1* and *AbaWSD* were individually expressed in the strains and *MhyWSD1* expression was more effective at promoting FAEE production [779].

9.2. Metabolic engineering of fungi

9.2.1. Modification of triacylglycerol biosynthesis in non-oleaginous yeasts

S. cerevisiae normally produces about 5-7% lipid on a per cell DW basis [360] with a TAG content of about 1% [789,790]. Given the importance of *S. cerevisiae* in the food and beverage industry and as a cell factory for producing bioproducts, there has been keen interest in increasing TAG content and modifying the FA composition of TAG in this organism [751,790–792]. In addition, lipid pathways in *S. cerevisiae* have been studied in detail (see Fig. 6; [793]). Genetic interventions to alter TAG biosynthesis in *S. cerevisiae* could also provide insight into the regulation of lipid biosynthesis in this well studied organism. In addition, the quadruple mutant H1246 is often the 'go to' host in terms of functionally characterizing putative *DGAT* genes from various sources. Indeed, H1246 yeast have also proven to be a useful biological system for assessing the potential of lipid biosynthetic genes from higher plants and plant-like protists for applications in ME [794].

Transposon mutagenesis experiments with *S. cerevisiae* have revealed that the mutant Δ*snf2* produces about 3-fold more TAG than the WT after 7 days of cultivation [789]. The effect was further enhanced under conditions of nitrogen limitation. *SNF2* encodes a DNA-dependent ATPase which is associated with the switching/sucrose-non-fermenting chromatin remodeling complex [795] which has been shown to regulate PL biosynthesis in *S. cerevisiae* [796]. Over-expression of *DGA1*, encoding ScDGAT2, with expression of *LEU2*, encoding a leucine biosynthetic enzyme, has been shown to increase lipid content in *S. cerevisiae* to approximately 30%, essentially converting the organism into an oleaginous yeast [114]. Exogenous leucine was also effective in boosting lipid content in association with *DGA1* over-expression in *S. cerevisiae*. Interestingly, leucine has also been associated with regulation of lipid biogenesis in *Y. lipolytica* in ME studies involving over-expressing *DGA1* [367,368]. In a follow up study, it was shown that an N-terminal segment of ScDGAT2 was cleaved off in the Δ*snf2* mutant resulting in activation of the acyltransferase thereby leading to increased TAG content [360]. Production of recombinant N-terminally truncated ScDGAT2 in the Δ*dga1* mutant has been shown to result in further increases in cellular lipid content to 45% [797]. This high level of lipid production was achieved without a decrease in cell DW. The expression of *ESA1*, which encodes a histone acetyltransferase, was decreased at early stages of culture suggesting an involvement in the effect of *dga1* disruption on TAG accumulation catalyzed by the N-terminally truncated ScDGAT2. Other research, however, has shown that the *S. cerevisiae* mutant Δ*snf2* exhibits severe growth defects on galactose which render it unsuitable for a *GAL1/GAL10* expression system which is a commonly used inducible expression system in yeast [798].

S. cerevisiae containing 8.2% TAG has been generated via over-expression of a gene (*GUT1*) encoding glycerol kinase combined with over-expression of *DGA1* and *LRO1* [793]. The yeast was grown on medium containing glycerol and the study represented the first report on production of TAG from glycerol in this organism. The study has further practical implications in that glycerol is a by-product of biodiesel production [21,793].

In a recent study, alteration of the expression of several endogenous genes, including *DGA1*, has been shown to result in an *S. cerevisiae* strain producing 25% of the dry cell mass as TAG [791]. In addition to over-expressing *DGA1*, the investigators also over-expressed a gene (*ACCase1*$^{S659A/S1157A}$) encoding a deregulated ACCase 1 and a gene (*PAH1*) encoding a PAP together with alteration of TAG lipase action, β-oxidation, peroxisomal FA transport and utilization of G3P. In a follow up study, TAG content was further increased in this strain through the heterologous production of proteins (human and fungal perilipins) known to be involved in LD biogenesis and deletion of genes known to induce an ER stress response and stimulate LD formation [799].

Studies of stress conditions that regulate lipid accumulation in algae such as *C. reinhardtii* have revealed gene expression regulators and enzymes which are involved. Two TFs (NRRI [403] and PSR1 [800]) have been identified and a recent study has shown that MYB1 is a novel regulator of TAG production under N depletion conditions [801]. When recombinantly produced in WT *S. cerevisiae*, the TF Aureochrome-1 (AUERO1), from the alga *Nannochloropsis gaditana*, has been shown to increase lipid content by 1.6-fold [802]. AUREO1 upregulated the expression of *ACCase1* and *DGAT2* but repressed LACS expression. The promoter of these genes contained the target sequence TGACGT which was recognized by AUREO1. It was hypothesized that upregulation of *ACCase1* and *DGAT2* enhanced lipid synthesis whereas downregulation of *LACS* repressed the catabolism of long chain FAs.

The expression of *AthDGAT1* in WT *S. cerevisiae* is an early example of the heterologous expression of a *DGAT* cDNA resulting in increased TAG content in yeast [131]. Three of the transformed yeasts exhibited 25-80 nmols TAG per mg DW compared to 9 nmols for the void plasmid transformed yeast. Since then, several other studies involving heterologous expression of *DGAT* genes in *S. cerevisiae* have been conducted.

Expression of *BnaDGAT1* (gene form *BnaA.DGAT1.b*) in WT *S. cerevisiae* has been shown to increase TAG content by 50% relative to cultures expressing the *LacZ* control [476]. Interestingly, the incorporation of an N-terminal tag into the enzyme further increased TAG content to 6.4% of the DW of the cells. Yeast cultures expressing modified *B. napus* DGATs produced up to the equivalent of about 0.45 g FA/L. Relative to the LacZ control, cultures producing recombinant N-terminally tagged BnaA.DGAT1.b generated TAG with 6.4% less unsaturated FAs. For the isoform BnaC.DGAT1.a, both untagged and tagged versions of the enzyme did not significantly affect FA composition when recombinantly produced in *S. cerevisiae*. N-terminal tagging had no effect on the growth of the yeast. The N-terminal tag appeared to increase DGAT1 accumulation by masking deleterious polymorphisms present at the +5 nucleotide position.

DGAT2 from *C. reinhardtii* has also been used to increase TAG biosynthesis in *S. cerevisiae* [433,434]. In addition, production of a recombinant *R. fluvialis* DMKU DGAT2 in *S. cerevisiae* has also been shown to increase TAG content [352]. *R. fluvialis* is an oleaginous yeast strain. Over-expression *R. fluvialis DGA1* was more effective in increasing TAG content then over-expression of *S. cerevisiae DGA1*.

DGAT over-expression has also been incorporated into strategies to modify the FA composition of TAG in *S. cerevisiae*. Considerable research has focused on the development of microbial and plant oils which are enriched in bioactive FAs. In this regard, palmitoleic acid ($16{:}1\Delta^{9}$) and stearidonic acid ($18{:}4\Delta^{6,9,12,15}$) can be considered bioactive FAs providing health benefits. Palmitoleic acid has been associated with increased membrane fluidity, a reduction in inflammation, protection of the cardiovascular system and anti-cancer effects [803]. In the ω-3 pathway for production of PUFAs in humans, stearidonic acid is more effectively converted to EPA then α-linolenic acid due to limitations in Δ-6 desaturation of 18:3, and thus dietary intake of stearidonic acid may provide some of benefits of oils enriched in EPA [804,805]. Stearidonic acid-enriched flax oil has also been shown to reduce the growth of human breast cancer *in vitro* and *in vivo* [806].

Palmitoleic acid is one of the few long chain FAs normally produced by *S. cerevisiae*. As previously mentioned in this section, the *S. cerevisiae Δdga1* mutant producing a recombinant N-terminally truncated ScDGAT2 has a high oil content [797]. In a further investigation with this high oil strain, it was shown that the addition of high concentrations of methionine and cultivation at low temperatures (20-25°C) increased the palmitoleic acid content of the yeast by 2.5-fold with the greatest enrichment occurring in TAG. This increase appeared to require active TAG accumulation.

The high oil *S. cerevisiae* mutant Δ*snf2* over-expressing *DGA1* and expressing *LEU2* [114] has been further transformed with a cDNA encoding rat Δ-6 desaturase which utilizes acyl-CoA as a substrate [807]. When the transformed yeast was grown in the presence of 0.7 g/L of 18:3 for seven days, 400 mg of 18:3/L and 44 mg of stearidonic acid/L were produced, mainly as TAG. A further increase in stearidonic acid was achieved by introducing histidine into the growth medium [808].

The supply of cocoa butter has become limited due to decreasing cultivation of the cocoa (*T. cacao*) tree and the increasing global demands of the food and cosmetics industries [792,809,810]. There is a strong interest, however, in developing other sources of cocoa butter-like lipids (CBLs) for use in the chocolate industry, with stearoyl-oleoyl- stearoyl (18:0-18:1-18:0) being a key component [792,810]. Many yeasts, including *S. cerevisiae*, are generally recognized as safe (GRAS) and thus can be implemented by the food and cosmetic industries for production of CBLs [792]. A combination of cDNAs encoding GPAT, LPAAT and DGAT from cacao was shown to be effective in producing CBLs in *S. cerevisiae* [482,483]. Following expression of cacao cDNAs in WT *S. cerevisiae*, the best producer exhibited a CBL content 8-fold higher than the control strain [482]. Expression of cacao TAG biosynthetic genes in an *S. cerevisiae* mutant lacking the equivalent endogenous TAG biosynthetic genes indicated that the cacao cDNAs encoded functional enzymes [483]. Shea (*V. paradoxa*) butter extracted from shea fruit kernels can be used as cocoa butter substitute [810]. A recent study involving transcriptomic analysis of TAG biosynthetic genes from developing shea fruit kernels, combined with heterologous expression in *S. cerevisiae*, identified two functional DGATs which may be useful in engineering shea butter production in the yeast [499].

S. cerevisiae has also been metabolically engineered to produce punicic acid ($18{:}3\Delta^{9cis,11trans,13cis}$) [812], which is an edible conjugated FA normally found at a high concentration in pomegranate (*Punica granatum*) seed oil [813]. Punicic acid has been shown to have nutraceutical properties including anti-cancer, anti-obesity and anti-inflammatory effects along with potential for use in the oleochemical industry [813]. Punicic acid is produced at the level of PC from 18:1 through the sequential action of FAD2 and a specialized FADX which catalyzes the conversion of 18:2 to punicic acid [813]. Recently, combinations of *PgDGAT2* and *PgLPCAT*, *PgDGAT2* and *PgPLA$_2$* or *PgDGAT2* and *PgPDCT* have been expressed in *S. cerevisiae* BY4741 co-expressing *PgFAD2* and *PgFADX* [812]. Co-expression of *PgFAD2* and *PgFADX* in *S. cerevisiae* BY4741 resulted in a punicic acid content of 0.5% of the total FAs; however, the three additional gene combinations, together with *PgFAD2* and *PgFADX* co-expression, each resulted in an approximate doubling of the punicic acid content.

Numerous studies have also focused on the ME of *S. cerevisiae* to produce biofuels and other bioproducts. The reduced viscosity and improved cold temperature properties of acetyl-TAG, with an acetate group esterified to the *sn*-3 position, has the potential to be used directly as a diesel fuel and for use in other applications, including plasticizers and lubricants [19,82,814]. In a proof-of-concept study, acetyl-TAGs have been shown to be produced in a *S. cerevisiae* strain which was engineered to grow on carbohydrates derived from lignocellulosic feedstock [82]. Acetyl-TAG production was achieved through the introduction of recombinant EaDAcT, the specialized DGAT previously shown to be responsible for formation of acetyl-TAG in developing seeds of *E. alatus* [81].

DGA1 expression has also been altered in numerous studies aimed at producing bioproducts other than TAG in *S. cerevisiae*. Non-esterified FAs are often desired due to their low cost of production and harvest, and ease of conversion into a range of oleochemicals and biofuels [765]. In a multi-pronged strategy, *DGA1* was over-expressed, with several other genetic interventions, to produce 2.2 g/L of extracellular non-esterified FAs [815]. Other interventions included disruption of β-oxidation and inactivation of selected LACS along with over-expression of cDNAs encoding TAG lipases. Enhancing carbon flux into non-polar LD formation and degradation, via DGAT and TAG lipase action, were critical in achieving high levels of extracellular non-esterified FA production.

Long-chain fatty alcohols have broad applications in the production of detergents, surfactants, cosmetics, pharmaceuticals and as biofuels [798,816,817]. Multiple interventions, guided by quantitative analysis of global protein production and flux modeling, led to a strain producing 1.2 g/L of fatty alcohol from media containing glucose [818]. Deleting the competing reaction catalyzed by endogenous DGAT2 was integral in the development of the fatty alcohol-producing strain. The ME strategy also involved the heterologous expression of an improved variant of *M. musculus FATTY ACID REDUCTASE 1*. Use of lignocellulosic feedstock, instead of glucose, resulted in the production of 0.7 g/L of fatty alcohol. Another fatty alcohol-producing strategy, involving deletion of endogenous *DGA1* in *S. cerevisiae* and utilizing a *Mycobacterium marinum* carboxylic acid reductase, along with several other interventions, increased supply of C8 to C19 non-esterified FAs [819]. In this case, fatty alcohols were produced to levels of about 0.3 g/L.

FAEEs represent a potential replacement for diesel fuel [817]. Biodiesel, or alkyl esters of vegetable oil or animal fat, are commonly produced through transesterification in a process which utilizes TAG, methanol, and a strong base [19,21]. In this case, transesterification results in the production of FAMEs. Although methanol is cheaper than ethanol, methanol is toxic and thus production of FAEE-based biodiesel would be more sustainable [299,764]. Acyl acceptor feeding experiments with *S. cerevisiae* producing recombinant AbaWSD have suggested that this bacterial enzyme can use a broad range of long and short chain alcohols [178]. Thus, *AbaWSD* has been used in ME strategies to produce FAEEs in *S. cerevisiae* [299,817,820]. The ability to produce FAEEs, however, was based on the WS activity of this bifunctional enzyme. About 0.52 g/L FAEEs have been produced in ethanol tolerant *S. cerevisiae* in a ME strategy involving heterologous expression of the bacterial *WSD* and utilization of glycerol in the growth medium. FAEE synthesis utilized endogenously produced ethanol.

Triterpenes have been shown to exhibit anti-oxidative, anti-inflammatory and anti-cancer properties [821] and are widely used in the pharmaceutical, nutraceutical, fragrance, and food industries [811,822]. The acyclic triterpene, squalene, is naturally produced in *S. cerevisiae* and sequestered along with TAG and SE in LDs [823]. Squalene production was increased over 250-fold in a multi-intervention ME strategy involving over-expression of *DGA1* and enhancement of squalene biosynthesis [811]. Squalene was produced at a level of about 0.45 g/L in nitrogen-limited minimal medium. In a recent study involving *DGA1* over-expression, the plant triterpene, α-amyrin, was produced in *S. cerevisiae* at a level of about 1.1 g/L under batch fed fermentation conditions [822].

The methylotrophic yeast *P. pastoris*, which is not oleaginous, has been gaining traction as a cell factory for production of chemicals and industrial enzymes [824,825]. *P. pastoris* can oxidize methanol for energy production and use the alcohol as its sole carbon source, but the organism can also utilize glucose and glycerol [824]. Although unlike *S. cerevisiae*, PDAT (Lro1p) is the major TAG synthase in *P. pastoris* [6,351], various *DGAT* genes can be functionally expressed in this yeast species to modify its lipid content and composition. A *DGAT1* gene cloned from microspore-derived cell suspension cultures of *B. napus* and *C. purpurea DGAT1* have been functionally expressed in *P. pastoris* [355,475]. The *BnaDGAT1* gene expressed in *P. pastoris* was shown later to correspond to the gene form *BnaA.DGAT1.b* [476]. A truncated form of *BnaA.DGAT1.b* was also functionally expressed in *P. pastoris* [475].

A haploid WT strain (GS115) of *P. pastoris* has been shown to produce oleic acid (~51%) and linoleic acid (~23%) as the dominant molecular species of FA [355]. When the gene encoding the endogenous Δ12 desaturase was disrupted, about 78% oleic acid was produced. The enrichment in oleic acid rendered the yeast more suitable for production of ricinoleic acid. Subsequently, the investigators co-expressed *CpFAH* and *CpDGAT1* in the haploid knockout strain. Although TAG containing ricinoleoyl moieties was produced, the bulk of the ricinoleic acid was present as unesterified FA, with very little in the PL fraction. Ricinoleic acid represented about 56% of the total FAs and lipid content was increased by about 76% relative to the control at day three after induction of expression. The findings agreed with the fact that *P. pastoris*, unlike *S. cerevisiae*, was very tolerant to non-esterified FAs. The study also indicated that transformed *P. pastoris* had an efficient system for removing ricinoleate from the PL fraction.

P. pastoris has also been engineered to produce FA branched-chain esters which have improved low-temperature properties and higher energy densities compared to more conventional biodiesel [771]. AbaWSD was one of the components of the engineered pathway. The study utilized a multiple gene expression vector under the control of a powerful *GAP* promoter, with integration into the yeast chromosome. Fatty acid branched-chain esters were produced to a level of about 0.17 g/L.

9.2.2. Modification of triacylglycerol biosynthesis in oleaginous fungi

As indicated previously, oleaginous yeasts produce over 20% of their cellular DW as lipids [356,357]. *Y. lipolytica* is recognized as a model for oleaginous yeast with large amounts of data available for its lipid metabolism and genetics, and like *S. cerevisiae*, it is has received GRAS status [356,791,826,827]. Under certain growth conditions, *Y. lipolytica* can form about 50% lipid per cell DW [356,828] and can utilize hydrophobic substrates such as alkanes and FAs [829]. Compared to other oleaginous yeasts, *Y. lipolytica* accumulates a high proportion of linoleic acid (about 50%) with lesser proportions of oleic acid (28%), palmitic acid (11%) and palmitoleic acid (6%) [356]. Only very small amounts of stearic acid and α-linolenic acid are present. There has been great interest in further increasing the lipid content of *Y. lipolytica* and other oleaginous yeasts.

DGAT over-expression has been used in numerous ME studies aimed at further increasing lipid content in *Y. lipolytica* [367,511,830–840]. Using an intron-enhanced gene expression platform, transformation of *Y. lipolytica* with tandem gene construct of *ACCase1* and *DGA1* resulted in about 62% lipid content after 120 hours in a 2 L bioreactor [830]. Since *AACase1* affects FA supply, this aspect of the strategy can be considered the "push" whereas TAG assembly catalyzed by DGAT2 can be thought of as the "pull". In another study, stearoyl-CoA desaturase (SCD) has been shown to be an important factor in the regulation of lipogenesis in mammalian lipid storing tissue [831]. Enrichment of intracellular monounsaturated FAs benefited cell growth and sequestration into NPLs. In a variation of the push-pull approach, *SCD*, *ACCase1* and *DGA1* were simultaneously over-expressed in *Y. lipolytica*. The altered strains exhibited improved growth over the WT and a high lipid titer of about 55 g/L.

A combinatorial approach has been used to develop a *Y. lipolytica* strain accumulating about 90% lipid and lipid titer of 25 g/L [367]. Modifications included over-expression of *DGA1* and the deletions Δ*pex10* and Δ*mfe1*. The deletion Δ*pex10* prevented peroxisome biogenesis (Peroxisome Biogenesis Factor 10) whereas Δ*mfe1* prevented β-oxidation through inactivation multifunctional enzyme 1 (Mfe1p).

Recently, a strategy was developed which involved co-expression of *DGA1* and the cDNA (*GPD1*) encoding glycerol-phosphate dehydrogenase [837]. The genes were expressed in a strain of *Y. lipolytica* which was engineered to be impaired in the mobilization of accumulated lipids. When grown on glucose-containing medium, lipid content increased from 18 to 55%.

A *Y. lipolytica* strain has recently been developed which over-expressed *DGA1*, *ACCase1*, *Δ12 DESATURASE* and a *Δ15 DESATURASE* from flax along with the deletions *Δpex10* and *Δmfe1* [839]. A lipid content of about 78% was reached along with a lipid titer of 50 g/L, with glucose-containing medium, using a 5 L stirred-tank bioreactor during the stationary phase.

In a study involving the heterologous expression of *DGAT*, various *DGA1* and *DGA2* genes from other oleaginous yeasts, producing 50% or more lipid, were first screened for their effectiveness in increasing lipid content in *Y. lipolytica* [834]. Combined heterologous expression of *Rhodosporidium toruloides DGA1* and *C. purpurea DGA2*, along with the deletion of a gene (Δ*tgl3*) encoding a native TAG lipase, resulted in a lipid content of 77% in batch glucose fermentation. In fed-batch glucose fermentation, 85 g/L lipid was produced.

There has also been a strong interest in developing high oil strains of *Y. lipolytica* to grow on industrial by-products and waste. When oleaginous microorganisms are grown under conditions of excess carbon, combined with nitrogen limitation, carbon is channeled into lipid storage [841]. A strain of *Y. lipolytica* has been developed for growth on molasses, which is an agro-industrial by-product enriched in sucrose [832]. A quadruple deletion strain (Δ*dga1*, Δ*dga2*, Δ*lro1*, Δ*are1*), defective in storage lipid accumulation was transformed with three copies of *DGA2*. The yeast was further modified by introducing a gene (*SUC2*) encoding an invertase from *S. cerevisiae*, thereby imparting a sucrose hydrolysis capability to *Y. lipolytica*. During growth on medium

containing sucrose, at a carbon to nitrogen ratio of 80, the modified yeast (Q4-*SUC2 DGA2* x 3) accumulated more than 40% lipid, which corresponded to a 2-fold increase. When grown on 8% (v/v) molasses, the strain accumulated more than 30% lipid after three days. In a further modification, the gene encoding the second enzyme of the β-oxidation pathway was knocked out. Although the resulting strain produced 49% lipid in the sucrose-containing medium and similar lipid content when grown in molasses, the production of biomass was somewhat less.

Metabolically engineered *Y. lipolytica* has also been grown on glycerol, which is a by-product of the transesterification of vegetable oil to produce biodiesel. A Q4 strain of *Y. lipolytica* over-expressing *DGA2* (JMY3580) has been shown to accumulate 40% lipid when grown on glycerol-containing medium at a carbon to nitrogen ratio of 90 [835]. This represented a substantial improvement over the WT strain that only produced 20% lipid when grown on glycerol-containing medium. A further increase in lipid content to 50% was achieved using strain JMY3580 when glycerol was added during cultivation in fed-batch grown cells.

In a more recent ME study with *Y. lipolytica*, simultaneous over-expression of *DGA1* and a cDNA (*TKL1*) encoding transketolase of the pentose phosphate pathway has been shown to result in a 40% increase in TAG content over the control strain (*DGA1* over-expression) when grown in medium with glycerol as the sole carbon source. Previously, it was demonstrated that the pentose phosphate pathway is a source of reducing agent for lipid synthesis from glucose in *Y. lipolytica* [833]. The addition of *TKL1* over-expression appears to have improved lipid synthesis in *Y. lipolytica* culture in medium containing glycerol.

Fermentation using lignocellulosic biomass has also been explored using oleaginous yeast. Over-expression of *SCD1* and *DGA1* in *Y. lipolytica* engineered to digest cellulose has been shown to result in the accumulation of 14% lipid content along with consumption of 12 g/L of cellulose under batch conditions with minimal medium [836]. Lipid content was further enhanced by adding glucose or increasing cellulose consumption using a commercial cellulase cocktail.

The production of lower-viscosity TAG containing an acetyl moiety at the *sn*-3 position has also been explored in *Y. lipolytica*. Recently, *DAcT* from *Euonymus europaeus* was over-expressed in strain of *Y. lipolytica* that was capable of storage lipid accumulation [511]. The engineered strain accumulated 20% intracellular lipids of which 40% were conventional TAGs and 20% were *sn*-3 acetyl-TAGs. The FA composition, in terms of long-chain FAs, was similar for both TAGs and *sn*-3 acetyl-TAGs.

In addition to *S. cerevisiae*, *C. purpurea FAH* has also been used to produce ricinoleic acid in *Y. lipolytica* [836,842]. *LRO1* and *CpFAH* have been over-expressed in a *Y. lipolytica* strain which was defective in β-oxidation along with the knockout of genes encoding endogenous Dga1p, Dga2p, Lro1p and Δ12 desaturase (Fad2p) [842]. Δ12-desaturase activity was eliminated to increase the amount of oleoyl moieties for the hydroxylation reaction catalyzed by CpFAH. When using batch cultures, the engineered strain accumulated ricinoleic acid up to 43% of the total lipids and over 60 mg/g cell DW. The recombinant PDAT (Lro1p) appears to have increased removal of ricinoleic acid from its site of synthesis in PC and increased TAG synthesis. Interestingly, co-expression of *RcDGAT2* or *CpDGAT2* with *CpFAH* had negative effects on the accumulation the hydroxy FA.

ME of oleaginous yeast to secrete FAs offers the advantage of saving on energy-intensive costs associated with harvest, drying and lipid extraction [827,843]. Ledesma-Amaro et al. (2016) have developed two ME strategies for secretion of unesterified FAs from *Y. lipolytica* [843]. In the most effective strategy, *DGA2* was over-expressed in a background where LACS activity and β-oxidation were impaired (deletions Δ*faa1* and Δ*mfe1*) and TAG lipase activity increased through over-expression of genes *TGL4* from *Y. lipolytica* and *TGL3* from *Kluyveromyces lactis*. When grown on 120 g/L glucose, the engineered strain secreted up to 4.3 g/L unesterified FAs with total lipid accumulation (intracellular and extracellular) of about 92%. When cultured in presence of 15% dodecane, FA secretion increased to 10.4 g/L with total lipid content reaching 120.4%, essentially overcoming the limitations of biomass.

Over-expression of endogenous *DGAT* genes or heterologous expression of *DGAT* genes from other sources has also been used in various ME strategies to increase lipid content and/or modify FA composition in various other oleaginous fungi [352,434,493,507,844–849]. The yeast *Candida phangngensis* [846] and filamentous fungus *Ashbya gossypii* [847] have also been engineered to utilize products of industrial waste. In terms of tropical/subtropical oleaginous plants, palm kernel oil is enriched in lauric acid which has many industrial uses, including soap production [850]. Oil palm (*E. guineensis*) *DGAT1-1* has been shown to be expressed at the beginning of lauric acid accumulation in the seed endosperm [591]. cDNAs encoding *AthDGAT1* or oil palm DGAT1-1 has been heterologously expressed in *Y. lipolytica* devoid of TAG biosynthesis [493]. When cultured in lauric acid-supplemented medium, the oil palm *DGAT1-1* expressing strain selectively accumulated medium-chain TAGs. In contrast, the *AthDGAT1*-expressing strain resulted in accumulation of TAGs consisting of long-chain fatty acyl moieties. The results suggested that oil palm *DGAT1-1* may be useful in producing medium-chain TAGs in oleaginous yeasts. A survey of various oleaginous yeasts, grown on nitrogen-limiting medium, has identified *Trichosporon oleaginous* as having the highest potential for CBL production [851]. Thus, it might of interest to use cocoa genes, including *DGATs*, to produce CBLs in this oleaginous yeast.

9.3. Metabolic engineering of plant-like protists

9.3.1. Nutrient stress

For the last twenty years or so, it has been known that algae, when nutrient-stressed, will accumulate TAG. Several nutrients will cause this effect, but the macronutrients nitrogen and phosphorous are the most important. For many diatoms, silicon is also critical [378]. Freshwater green algae, such as *Chlorella*, show growth reductions for nitrogen levels below 32 mg/L and for phosphorous below 11 mg/L [852]. In addition, different algae have different growth characteristics but the maximum TAG accumulation for *C. vulgaris* is when the nitrogen level is below 0.5 mg/L [853]. Nitrogen is the usual nutrient stressor that is used [374]. Typically, accumulation of TAG in algae will be in the range 20-50 % of DW [375]. Phosphorus deficiency will also have major effects on lipid metabolism and lead to TAG accumulation [854–856]. As a generalisation, the accumulation of TAG is caused by a combination of catabolism of chloroplast lipids combined with *de novo* synthesis (e.g., [857]).

In diatoms, nutrient stress is the most commonly applied factor to enhance TAG productivity, particularly nitrogen deficiency (e.g., [856,858,859]). Any reduction in chloroplast membrane lipids will require the induction of catabolic enzyme activities such as those catalyzed by galactolipases or phospholipases [378]. It is against this background that any research on *DGAT* expression, and hence DGAT activity, must be considered.

Early work to study the details of TAG accumulation following nitrogen deficiency used *C. reinhardtii*. Boyle et al. (2012) [403] identified *DGAT1* and *DGTT2B* induction in response to nitrogen stress and the data for *DGAT1* expression were quickly confirmed [432]. The latter group also examined the five *DGAT2* genes and identified *CrDGAT2-1* and *CrDGAT2-5* as contributing significantly to TAG formation under nitrogen stress. Msanne et al. (2012) also worked with *C. reinhardtii* and *Coccomyxa* sp. C-169 [428]. In both species, the accumulation of TAG induced by nitrogen deficiency was accompanied by a decrease in thylakoid components. For *C. reinhardtii*, after nitrogen stress, the investigators reported increased expression of DGAT2s, especially of *CrDGTT2B*, but no change in *CrDGAT1* expression [428].

For *Chlorella pyrenoidosa*, nitrogen-, phosphorous- and iron-deficiency stresses were tested. The most marked TAG accumulation was with nitrogen deficiency, giving a doubling of total lipid (per

cellular DW). Gene expression analysis showed significant increases in the expression of three *DGATs* [860]. Similarly, under nitrogen deficiency, several green algae were found to accumulate more than 35% of their DW as TAGs. These organisms included *C. vulgaris*, *C. zofingiensis*, *Neochloris oleoabundans* and *Scenedesmus obliquus* [861]. For *C. zofingiensis* (now called *Chromochloris zofingiensis*; [416]), there are two *DGAT1* genes and eight *DGAT2* genes. Marked up-regulation of *CzDGAT1A* and *CzDGTT5* were noted following nitrogen stress [417]. For the ARA-producing alga, *L. incisa*, nitrogen stress caused significant increases in the expression of *LiDGAT1* which was dependent also on light intensity [413,862]. The research also revealed putative phosphorylation sites on the LiDGAT1 which may be involved in regulation of its activity and, perhaps, could be connected to signaling pathways [413].

The eustigmatophyte *Nannochloropsis* has also been examined following nitrogen stress. *N. gaditana* was shown to contain 38% of TAG on a DW basis. Most of this TAG was formed by *de novo* synthesis rather than recycling of membrane lipids and, in starved cells, chloroplast integrity was kept at a level to maintain photosynthesis [857]. In *N. oceanica*, nitrogen deficiency caused an increase in TAG from less than 10 mg/g up to 230 mg/g. Under these conditions, expression of *NoDGAT1A* increased 4-fold [422].

In the diatom, *P. tricornutum*, nitrogen deficiency caused a large increase in TAG, which was enriched in 16C fatty acids. Nitrogen stress caused thylakoid senescence and growth was arrested [856]. The changes induced in *P. tricornutum* by nitrogen deficiency have been well-discussed [378] and the role of betaine lipids as a major source for TAG formation under these conditions has been noted [858]. When another diatom, *T. pseudonana*, was investigated, it was noted that its response to nitrogen starvation was different to that of *C. reinhardtii* and closer to that of the cyanobacterium *Prochlorococcus marinus* [863].

9.3.2. Changing algal DGAT expression levels

A recent review summarized the ME of microalga for enhanced lipid production in general [864]; the current section focuses on the ME strategies for microalgae involving DGATs. For a general review discussing over-expression of *DGAT* see [375], for diatoms specifically [378], and for DGAT itself [31].

For green algae, experiments using *C. reinhardtii* were the first to be conducted. Deng et al. (2012) used artificial silencing of the five DGAT2s (*CrDGAT2-1* to *CrDGAT2-5*). Their results suggested that reduction of *CrDGAT2-1* and *CrDGAT2-5* were especially effective; thus, the investigators over-expressed these genes. Over-expression caused a 27% and 48% increase in lipid content on a relative basis, respectively, and the increases were also found under nitrogen-limited conditions [432]. In contrast, over-expression of three *DGAT2* genes in *C. reinhardtii* did not boost TAG levels under standard growth conditions or after nutrient stress [431]. Nevertheless, knockdown experiments targeting *CrDGAT2B*, *CrDGAT2E* or *CrDGAT2D* resulted in a 20-35% decrease, on a relative basis, in TAG content [434], which agreed with the work of Deng et al. (2012) [432].

Moreover, when *CrDGAT2B* was expressed in another green alga, *S. obliquus*, it enhanced lipid content nearly two-fold. Furthermore, the recombinant strain was successfully grown under commercial conditions, such as in open ponds [865]. In addition, the overexpression of *CrDGAT2B* significantly increased EPA production in *N. oceanica* [866].

Another green microalga, *H. pluvialis*, contains five putative DGAT-encoding genes (one *DGAT1* and four *DGAT2s*); however, one of the latter failed to complement TAG synthesis in a yeast mutant. Further investigation showed that *HpDGTT2* actually encoded a LPAAT and, when expressed in *C. reinhardtii*, it retarded growth but enhanced TAG accumulation to a similar degree to transgenic strains overexpressing *CrLPAAT* [427].

N. oleoabundans is of commercial interest because of its high lipid content. When a *NeoDGAT2* was over-expressed, it increased total lipid 1.6-2.3-fold to yield 75% of DW. The accumulating lipid had an altered FA composition with saturated FAs increased [867]. The effect of single (*GPAT*, *LPAAT*, *DGAT*) or combined expression of genes from *Acutodesmus obliquus* in *N. oleoabundans* was studied. Strains expressing single genes increased TAG content approximately 1.4-fold. Interestingly, combined expression of these genes had less of an effect and also slowed growth rate [868]. In another study, the over-expression of *NeoLPAAT1* and *NeoDGAT2* resulted in 1.6- and 2.1-fold increase of total FAs and TAG, respectively [869].

The oleaginous microalga, *N. oceanica*, has two purported *DGAT1* genes and 11-12 *DGAT2* genes. When a *DGAT2* was over-expressed in the same organism, TAG biosynthesis was increased and NPLs were elevated by 69%. At the same time, the FA composition was altered, but no change in growth parameters were seen [440]. Similarly, over-expression of *NoDGAT1A* substantially increased TAG levels under nitrogen-deficiency. Over-expression also raised TAG content (2.4-fold) under nitrogen-replete conditions, without compromising growth [422]. Ziekiewicz et al. (2017) over-expressed six of the *DGAT2s* in *N. oceanica* [441]. Of these, *NoDGTT5* gave levels of TAG, under nitrogen-replete conditions, that were equivalent to those found under nitrogen stress. In addition, as mentioned in section 5.2, the large number of *DGAT2* genes in *N. oceanica*, together with their different substrate preferences, provides opportunities to develop strains that are potentially useful for either the health food or the biodiesel markets [442,443].

Various diatoms have also been studied [378], although attention has been focused on *P. tricornutum*. Over-expression of *PtDGAT1* led to more than two-fold increase of TAG and total lipid content [409]. Over-expression of *PtDGAT2A* caused more LDs to be formed and increased NPL content by 35% with a significant increase in PUFAs, especially EPA [446]. When different DGAT2s from *P. tricornutum* under nitrogen stress were studied, only the expression of *PtDGAT2D* was up-regulated. Therefore, this isoform was examined in over-expressing strains and it gave rise to a 2-fold higher total lipid content with only a small decrease in growth compared to the WT [870]. In another study, co-expression of *GPAT* and *DGAT2* under a strong constitutive promoter led to 2.6-fold increase of total lipid content [871]. Because *P. tricornutum* accumulated significant n-3 PUFAs, it has been subject to manipulation experiments to increase EPA or DHA levels. Although this work has concentrated on FA desaturation and elongation pathways, manipulation of other genes, including *DGAT*, has been considered [377,872]. The over-expression of *PtDGAT2B* in *P. tricornutum* resulted in higher TAG content compared to the WT, and the co-expression of *PtDGAT2B* and *O. tauri Δ5-ELONGASE* produced a significant increase in DHA-containing TAG [873]. *P. tricornutum* contains a WSD (see section 5.1). When the gene encoding this dual-function enzyme was over-expressed in *P. tricornutum*, it increased levels of TAG, regardless of nitrogen availability [79]. TAG accumulation in another diatom *T. pseudonana* over-expressing a *DGAT2* homologue has been studied. TAG accumulation (as measured with Nile Red) was raised by up to 2.3-fold without compromising growth. Increased percentages of PUFAs were also seen [447].

9.3.3. Using DGAT genes from other sources to engineer plant-like protists

Because of the similarities in lipid metabolism between higher plants and algae (especially green algae; see section 5.1), some research has focused on using higher plant *DGAT* genes to increase TAG content and/or to modify the FA composition of TAG in plant-like protist. As an example, over-expression of a truncated *DGAT1* from *B. napus* produced significantly higher lipid amounts in *C. reinhardtii* [874]. The truncated gene was inadvertently referred to as a *DGAT2*. Moreover, the heterologous expression of *H. lacustris*, *DGAT2D* in *C. reinhardtii* increased the TAG content and raised the PUFA% [439]. For the marine green microalga, *Tetraselmis chui*, expression of either *E. pitardii DGAT1* or *S. cerevisiae DGAT2* increased TAG levels up to double compared to WT [875]. Furthermore, expression of yeast *ScDGAT1* in *P. tricornutum* also doubled TAG levels under nitrogen replete conditions [876]. Algal DGATs have also been used to manipulate lipid biosynthesis and

accumulation in higher plants, as discussed in section 9.4.1.

Knowledge of the structure/function of DGATs in other eukaryotes has facilitated the generation of high-performance enzymes and their subsequent application in biotechnology (refer to section 6.2.3 for details). Although structure/function studies of algal DGATs are still in their infancy as mentioned in section 5.2, the results may provide a means for improving algal DGATS for biotechnology applications. For instance, several algal DGAT1s contain a Pleckstrin homology domain motif (section 5.2) and that in the *L. incisa* enzyme has been shown to be important. Deletion of the Pleckstrin homology domain led to decreased TAG accumulation and lowered the proportion of ARA [413], pointing to its potential for manipulation.

9.4. Metabolic engineering of higher plants

9.4.1. Increasing seed triacylglycerol content and modifying fatty acid composition

As the final reaction in the Kennedy pathway, it might have been surmised that DGAT-catalyzed reaction could be important in controlling TAG formation. Indeed, in early biochemical experiments examining TAG accumulation in *B. napus*, it was observed that DAG accumulated to appreciable levels during periods of rapid oil synthesis [877,878]. This was interpreted to indicate that DGAT activity was limiting TAG production. Further experiments (also in maturing *B. napus* seeds) have shown that DGAT activity was the lowest of the four enzymes of the Kennedy pathway and that its substrate, DAG, accumulated to the highest levels of all the intermediates in the pathway during peak rates of oil biosynthesis [455]. In addition, earlier studies with maturing seeds of *B. napus*, safflower, soybean and castor have shown that high DGAT activity is associated with the rapid phase of oil accumulation [479,879–881]. Further evidence for a key role for DGAT-catalyzed reaction in regulating TAG biosynthesis during seed development in plants came from the observation that the *Arabidopsis TAG1* mutant had a mutation in a *DGAT* gene and reduced TAG content [474,514]. Molecular genetic analyses have suggested that DGAT1 is the major TAG-biosynthetic enzyme for seed oil accumulation in *Arabidopsis* [410,474,514,618], soybean [410] and *B. napus* [11].

The first demonstration of *DGAT* over-expression leading to increased seed oil content was in *Arabidopsis* where over-expression of *AthDGAT1* was shown to result in an increase in both seed oil content and seed weight [619]. The application of *DGAT* over-expression to significantly increase seed oil content in crops was demonstrated in *B. napus* [23,179,530,882,883], maize [492] and soybean [340]. The investigations with *B. napus* involved over-expression of *AthDGAT1*, *B. napus BnaA.DGAT1.b* or garden nasturtium *DGAT1* [179,530,882,883]. Using *B. napus* L. cv Quantum and a double haploid breeding line (DH12075) of *B. napus*, DGAT1 over-expression resulted in seed oil content increases ranging from 2.5% to 7%, on an absolute bases, in both greenhouse and field trials [883]. The maize study involved the ectopic expression of a high-oil *DGAT1-2* allele [492] whereas the soybean study involved the heterologous expression of a codon-optimized fungal *DGAT2A* [340]. In addition, to increasing maize kernel oil content, the *DGAT1-2* allele was associated with a substantial increase in oleic acid content [492]. Plant oils enriched in oleic acid have a protective effect for cardiovascular disease and other disorders, and these oils, comparing to the ones with high content of PUFAs, also exhibit increased stability during frying [18]. In the soybean study [340], a significant absolute increase of 1.5% in seed oil content was achieved without a major impact on protein content or seed yield. Over-expression of a *DGAT* can also lead to other non-intended effects in the transgenic plant. For example, the seed-specific over-expression of *AthDGAT1* during seed development in *B. napus* L. cv Quantum has been shown to be associated with transcriptional and growth regulator changes that are not limited to storage lipid accumulation [620].

In relation to *DGAT* over-expression in higher plants, MCA has emerged as a technique to guide the ME of oil crops. As a quantitative way of measuring the amount of regulation an individual enzyme could have in a particular pathway, Harwood's group used MCA to examine lipid synthesis for the first time [884]. Initially, the technique was applied to oil palm and olive [885–888] and, later, to canola-type *B. napus* [530,889] and soybean [890–893]. When applied to the important oil crop, *B. napus*, MCA revealed that TAG assembly was more important than FA formation [530]. Furthermore, when *DGAT1* was over-expressed, the control exerted by the TAG assembly reactions decreased from 69% to 50%, thus confirming the importance of DGAT in regulating TAG accumulation. As predicted, over-expression of *DGAT1* increased TAG accumulation in both greenhouse and field trials [530,883]. In addition, *DGAT1* over-expression has been shown to reduce the penalty on seed oil content caused by drought stress [530].

Thus, MCA is acknowledged to have made a substantial contribution to our understanding of the regulation of lipid biosynthesis [894]. Indeed, initial studies using this method (e.g., [530,886]) have proven useful in helping to guide later research. For example, expression of a *T. majus LPAAT* during seed development in *B. napus* has also been shown to contribute to an increase in seed TAG content despite the low intrinsic control coefficient for this reaction [457]. As mentioned previously, however, this effect may have been associated with increased PA exerting a stimulatory effect on endogenous DGAT1 activity.

Since the initial demonstration of the effect of *DGAT* over-expression on seed oil content in *Arabidopsis* [619], the model plant has been used to functionally characterize *DGAT* genes from various other sources via heterologous expression of the encoding cDNAs. In many of these studies, the intent was to demonstrate proof-of-concept in *Arabidopsis* with the long-term goal of transferring the biotechnology to a commercially relevant crop. These numerous studies, using *Arabidopsis* as a host for expression, have included *DGAT* genes from castor [481], upland cotton (*Gossypium hirsutum*) [645], *E. alatus* [81,895], *J. curcas* [896], *L. chinensis* [490], sesame (*Sesamum indicum*) [599], soybean [140], the marine plant-like protist *Thraustochytrium aureum* [897], tung tree [507] and developing embryos of *X. sorbifolia* [510]. Constitutive over-expression of either soybean *DGAT1A* or *DGAT1B* in *Arabidopsis* resulted in increased seed oil content and reduced protein content [140]. Over-expression of *DGAT1A*, however, also increased seed weight.

Tobacco has also been used as a model system for the heterologous expression of plant *DGAT* genes. *Jatropha DGAT1* or *DGAT2* have been functionally assessed using tobacco [488]. Although each DGAT could boost seed oil content in tobacco and yeast strain H1246, *Jatropha DGAT2* displayed a preference for the incorporation of substrates containing 18:2. Constitutive silencing of endogenous *DGAT1* in tobacco has been shown to result in decreased seed oil content and seed weight, whereas protein and sugar content increased [898].

In further engineering studies with soybean, expression of a sesame *DGAT1* has been shown to lead to a mean increase in absolute seed oil content of about 1.4% in T_3 homozygous seeds [599]. Given the relatively high seed oil content of sesame seed (~55%), it was hypothesized that a recombinant DGAT1 from this source may prove particularly useful in boosting seed oil in soybean. *DGAT1* has also been cloned from the high oil hazelnuts (60%) of American hazelnut shrub [165]. Four variants of hazelnut DGAT1 with different kinetic properties were generated through directed evolution and the encoding cDNAs were expressed in soybean somatic embryos to assess their potential for increasing seed oil content [165]. The affinity for 18:1-CoA was highly correlated with oil content. Information on the amino acid residue substitutions in the best hazelnut DGAT1 variants was used to guide the engineering of a soybean DGAT1 variant with 14 amino acid residue substitutions. In highly replicated field trials, seed-specific expression of the cDNA encoding the soybean DGAT1 variant resulted in an average increase in seed oil content of 3% on an absolute basis (16% relative increase) with a significant reduction in soluble carbohydrate, and increased protein content in some cases. The seed oil from transgenic soybean exhibited a significant increase in 18:1 content. In field trials, heterologous expression of *V. galamensis DGAT1A* in soybean has been

shown to result in a seed oil content increase of 4% on an absolute basis without a reduction in protein content or yield per unit land area [899,900]. The transgenic lines exhibited significant increases in the proportions of 18:0 and 18:1 and decreases in the proportions of 18:2 and 18:3 without negative influences on genotypes, seed growth and final soybean yield [899–901].

A candidate gene approach has been used to identify associations of mutations within three *DGAT1* and *DGAT2* soybean isoforms with seed oil content, protein content and seed yield in a recombinant inbred line population stemming from varieties with moderately high seed oil content [602]. An insertion/deletion mutation in the soybean *DGAT2B* gene was significantly associated with decreased seed oil content across three environments. Recently, the seed specific over-expression of soybean *DGAT2A* in soybean has been shown to lead to relative increases in seed oil content of as high as 9.4% with increases in 18:2 content of the oil of 20.4% [503]. The enhancement in 18:2 content was presumably associated with the high specificity of recombinant soybean DGAT2 for 18:2-CoA. Based on growth in different regions, most agronomic traits did not appear to be affected. Interestingly, however, the transgenic higher oil lines exhibited enhanced radicle lengths (5-day-old-plants) relative to the WT. Presumably, enhanced radicle development in the transgenic lines was associated with the increase in TAG storage reserves.

Recently, the simultaneous down-regulation of three *DGAT1* genes in soybean decreased seed oil content by 8.5% with a concomitant increase in protein content of 11% [902]. In terms of FA composition of the seed oil, there was an increase in the proportion of 18:1 (up to 47.3%) and a decrease in 18:2 [902]. Given the relatively small decrease in seed oil content observed, the investigators suggested that down-regulation of *DGAT1* genes in soybean may represent a way of improving the FA composition of soybean oil and increasing protein content. The relatively small decrease in seed oil content observed was surprising considering that previous expression studies suggested that *DGAT1* was the main enzyme responsible for seed oil accumulation in soybean [410,501]. In another previous study, down-regulation of *DGAT1* in canola-type *B. napus* was shown to also lead to an increase in 18:1 with a reduction in 18:3 [623]. The observed effects of *DGAT1* down-regulation on seed oil FA composition in soybean and canola-type *B. napus* were opposite to what was observed with the *Arabidopsis* AS11 *dgat1* mutant where a large increase in 18:3 was observed along with a decrease in 18:1 [514,618]. Seed oil contents were reduced by 25-30% and 30%, respectively, in the *AthDGAT1* mutant and *B. napus* antisense line [474,623]. It was suggested that the large increase in the 18:3 content of the *AS11* Arabidopsis *dgat1* mutant may have been attributable to more 18:1 being available for desaturation [514].

In further ME studies with *B. napus*, increases in seed oil content up to about 15% have been achieved through seed-specific over-expression of a native *DGAT1* in combination with genes encoding native GPAT, and the yeast genes encoding *sn*-glycerol-3-phosphate dehydrogenase (GPDH) and LPAAT [903]. The yeast *LPAAT* (*SLC1-1)* and *GPDH* (*GPD1*) open reading frames were synthesized and codon-optimized for expression in *B. napus*. A cDNA encoding *GPDH* was included to increase the supply of G3P for the Kennedy pathway. A previous study demonstrated that seed-specific over-expression of *S. cerevisiae GPDH* (*GPD1*) during seed development in *B. napus* resulted in an increase in seed oil content [452]. Recently, the heterologous expression a *PDAT1* gene from the woody oil tree, *Sapium sebiferum*, in *B. napus* has been shown to lead to seed oil content increases up to about 11% [904].

The knowledge acquired about *DGAT* genes also has been shown to be useful in breeding programs aimed at increasing seed oil content. Expression analysis of the four highly homologous *DGAT1* genes in 34 inbred lines of *B. napus* has revealed variations in expression levels among the genes depending on the line [594]. High oil lines were associated with high expression levels for all four genes whereas in lower oil lines some of the genes were weakly expressed or silenced. The investigators suggested that examining the extent of *DGAT1* expression might be a useful approach to develop high oil lines of *B. napus*.

Kim et al. (2016) have shown that seed specific over-expression of *Camelina DGAT1B* in *Camelina* has been shown to lead to a relative increase in seed oil content of about 25% [598]. Seed size and seed weight were also increased. *DGAT1B* was selected for over-expression due to its high level of expression during seed development in WT *Camelina*. Another investigation on *DGAT* and *PDAT* manipulation in *Camelina*, however, failed to reveal any increase in seed oil content through seed specific over-expression of *Camelina DGAT1* [561]. The *DGAT1* homolog used in the more recent study differed in only one amino acid residue (serine- 32 to threonine 32). In a more recent report, seed-specific co-expression of *S. cerevisiae GPDH* (*GPD1*) and *AthDGAT1* has been shown to result in relative increases of up 13% and 52%, respectively, of seed oil content and seed mass [905]. The oil harvest index (grams oil per gram total dry matter) was two-fold greater than for the WT and lines expressing *GDP1* or *DGAT1* alone. Within this study, the effect of substituting serine 205 in AthDGAT1 for an alanine residue was also examined. Previously, it was demonstrated that the equivalent residue in *T. majus* DGAT1 was a phosphorylation site for SnRK1; phosphorylation of this site was associated with down-regulation of the enzyme [179]. As mentioned previously, direct proof for down-regulation DGAT1 by SnRK1-catalyzed phosphorylation was demonstrated by Caldo et al. in 2018 [523]. Expression of the modified *Arabidopsis DGAT1* was only slightly more effective in increasing seed oil content in *Camelina* [905]. Further metabolic constraints on TAG accumulation in transgenic lines of *B. napus* heterologously expressing *GDP1*, *DGAT1* or a combination of the two genes have been identified through comparative transcriptomics and metabolomics [906]. Among the limiting factors identified was a decrease in the levels of TFs regulating FA biosynthesis and increased activity of lipases/hydrolases eroding the TAG pool.

The woody perennial plant, *Jatropha*, has been emerged as important crop for domestication and expanded cultivation as a biofuel crop [539]. Increased seed oil content and optimization of FA composition for biodiesel production are among the traits targeted for improvement. Ectopic expression of *AthDGAT1* in *Jatropha* has been shown to result in a relative increase in seed oil content of 20-30% [907]. Oil content was also increased in leaves and there was an increase in mean plant height, seeds per tree, seed mass and seed size. In a more recent report, ectopic over-expression of either *Jatropha DGAT1* or *DGAT2* in *Jatropha* resulted in a relative increase in seed oil content, respectively of about 25% or 30%, and leaf TAG content also increased [601]. Seeds from transgenic plants also exhibited a decrease in protein content and soluble sugars. Analysis of the FA composition of seed oil suggested that each *Jatropha DGAT* exhibited an enhanced preference for substrates containing 18:2.

ME studies, involving heterologous expression of *DGAT* genes, have also been used in the modification of FA composition of seed oil with the long-term goal of developing designer oil crops for various nutritional and industrial applications. Medium-chain FAs (C10-C14) are useful in the production of soaps and other industrial products [850]. *Cuphea* species accumulate oils containing saturated medium-chain FAs, mainly caprylic (8:0), capric (10:0) and lauric (12:0) [908]. Earlier research with microsomes from developing seeds of *Cuphea* species have indicated increased substrate specificities for medium-chain acyl-CoAs and DAG containing medium-chain FAs [98]. In a multi-gene strategy, a *Cuphea DGAT1* was co-expressed with cDNAs encoding a *Cuphea* thioesterase and *Cuphea* 10:0-CoA-specific LPAAT [485]. Seed from transgenic plants exhibited TAG with up to 25 mol % 10:0.

ME studies involving *DGAT* have also resulted in increased oleic acid content of seed TAG. As mentioned previously, ectopic expression of the high-oil maize *DGAT1-2* allele in maize was shown to lead to both an increase in seed oil content and oleic acid [492]. Seed-specific expression of *T. aureum* (a marine protist) *DGAT2* in high 18:2 *fad3fae1 Arabidopsis* mutant resulted in a two-fold increase in the oleic acid content of the oil, with this FA representing > 50% of the total FAs [897]. DGATs from other algal species have also been used in manipulating TAG content and fatty acid profile. For example, The *NoDGTT5* was able to

successfully rescue an *Arabidopsis tag1-1* mutant by restoring the TAG content in seeds [441]. When a *DGAT1* gene from the green microalga, *C. ellipsoidea*, was expressed in either *Arabidopsis* or *B. napus* it increased seed oil content by 8-37% and 12-18%, respectively. Seed weights also went up so the total lipid content/1000 seed was raised by 25-50% [420]. Heterologous expression of *HlDGAT2D* in *Arabidopsis* increased both TAG content and PUFA% [439].

As mentioned previously, there is an interest in the ME of high yield oil crops to produce TAG enriched in hydroxy FAs [470,909]. Several proof-of-concept studies have explored the ME of *Arabidopsis* to produce oils enriched in hydroxy FAs (e.g., [467,468,481,910,911]). In the first of these series of investigations, expression of castor *DGAT2* in *Arabidopsis* expressing castor *FAH12* resulted in an increase in hydroxy FAs from 17% to about 30% [481]. Further genetic interventions involved strategies to overcome the negative effects of expressing castor *FAH12* on subsequent seedling establishment in transgenic *Arabidopsis*. Recently, Shockey et al (2019) reported the effects of various gene combinations (including cDNAs encoding DGAT and other acyltransferase) on the accumulation of hydroxy FAs and other unusual FAs in transgenic *Arabidopsis* [912]. The incremental increases in hydroxy FA content as result of various genetic interventions suggested that we have long way to go in developing a high yielding oil crop producing TAG highly enriched in ricinoleic acid.

ME experiments have also focused on the production of vernolic acid in soybean oil [545,546]. Co-expression of a cDNA clone (*SIEPX*) encoding a *S. laevis* Δ-12 epoxygenase with *V. galamensis DGAT1* or *V. galamensis DGAT2* in soybean, respectively, was shown to result in about 15% or 26% vernolic acid content in the seed oil [545]. *VgDGAT2* was more effective than *VgDGAT1* in increasing the vernolic acid content of soybean oil. Sole expression of *SIEPX* led to phenotypic alterations including reduced oil and protein content; these alterations were largely overcome in *DGAT1*-co-expressing lines [546].

There has also been interest in the ME of *Camelina*, soybean and pennycress to produce acetyl-TAG for industrial applications where low viscosity oils are required [895,913–915]. These advances were dependent on the heterologous expression of the cDNA encoding *E. alatus* DAcT. Initial proof-of-concept experiments were conducted with WT *Arabidopsis* where expression of *DAcT* resulted in accumulation of 45 mol% acetyl-TAGs [81]. *Camelina* or soybean transformed with EaDAcT resulted in seed oils with 70 mol% acetyl-TAG [895]. Seed germination was like the WT and acetyl-TAGs were effectively mobilized. Later, a multi-faceted strategy involving *EaDAcT* was used to engineer *Camelina* to produce seed oil enriched 77 mol% acetyl-TAG which also contained medium-chain FAs; however, seed oil content was reduced [914].

In ME situations where a *DGAT* gene is heterologously-expressed during seed development to produce a desired FA, such as ricinoleic acid, it has proven useful to reduce interference (or competition) by endogenous DGATs and/or other endogenous lipid biosynthetic enzymes using a gene-silencing approach [910,914]. Silencing approaches involving oil crops with more complex genomes represent a challenge due to an increased number of closely related genes encoding DGATs and other acyltransferases (e.g., [137,153,156,916]). Aznar-Moreno and Durrett (2017) have developed a CRISPR/Cas genome editing system to install mutations in all three *Camelina DGAT1* or *PDAT1* genes [916]. Therefore, genome editing represents a useful way to alter endogenous lipid biosynthesis in polyploidy species such as *Camelina* and *B. napus*. The use of genome editing in ME of oil crops has been increasing steadily, especially the application of loss-of-function approaches [565].

The *AthDGAT1* mutant (AS11) exhibited decreased seed oil content due to the absence of DGAT1 activity [474,514,618]. Expression of *AthDGAT1* in the AS11 mutant was later shown to lead to a restoration of WT seed oil content and composition [619]. In this regard, as examples, the AS11 mutant has proven useful in ME to increase the acetyl-TAG content of *Arabidopsis* seed oil [895], and in the functional characterization of *BnaDGAT1* genes [594], *Camelina DGAT1* genes [598], garden nasturtium *DGAT1* [179], soybean *DGAT1* [599] and *L. chinensis DGAT1* and *DGAT2* [490].

Other ME strategies have involved altering the regulation of *DGAT* expression during seed development. TFs affecting *DGAT* expression were discussed in section 6.2.7. The strong effects of the TF MYB96 on TAG accumulation suggested that this TF could be used to enhance TAG biosynthesis in both seed and vegetative tissues [614]. The seed-preferred gene *GmZF351*, which encodes a tandem CCCH zinc finger TF that has been selected during soybean domestication [611]. Constitutive over-expression of *GmZF351* in *Arabidopsis* or soybean, respectively, increased the TAG content of up to 29% or 38% [611]. Another CCCH-type TF, BnZFP1, has been shown to be associated with the high oleic trait in *B. napus* [612]. Over-expression of *BnZFP1* in *B. napus* was shown to increase the 18:1 content of seed oil by about 19%, with about a 4% increase in seed oil content. It was suggested that BnZP1 up-regulates the synthesis of DGAT1 that is known to exhibit a high selectivity for 18:1-CoA during seed development [153]. In another study involving ME, over-expression of the gene encoding TF, BnaWIN1, has been shown to result in an 8% relative increase in seed oil content in *B. napus* [615]. Aoyagi et al. (2018) have designed a chimeric promoter which expresses early in seed development with high-level expression still maintained at later stages [917]. The chimeric promoter was generated by fusing the promoter of the *BIOTIN CARBOXYL CARRIER PROTEIN2* (*BCCP2*) gene, encoding the BCCP2 subunit of ACCase, to the promoter of the *FAE1* gene of *Arabidopsis*. When this chimeric promoter was ligated upstream of the *AthDGAT1* gene and introduced into *Arabidopsis*, relative increases in seed oil content among independent transgenic lines ranged from 18-73%. Thus, the modification of *DGAT1* expression via various TFs offers a new suite of tools for further enhancement of seed TAG content in oil crops.

There has also been considerable interest in the ME of cereals to increase their oil content and modify FA composition resulting in grains with increased nutritional value [918,919]. In cereal kernels, oil is mainly associated with the embryo and with some in the endosperm [918], although oat (*Avena sativa*) represents an exception where up to 18% of the DW of the endosperm is accounted for by oil [920]. In a recent study, transcript profiling of the developing starchy endosperm of wheat (*Triticum aestivum*) indicated that the Kennedy pathway was responsible for TAG accumulation in this tissue [921]. In addition to the study with the high oil maize *DGAT1-2* allele [492], other approaches for increasing kernel oil content in maize have involved the heterologous expression of fungal *DGAT2s* [922] and the combined constitutive expression of *AthDGAT1*, *WRI1* and *OLEOSIN* [923]. In the latter study, FA compositional changes associated with human health benefits were also observed. In more recent research, a multi-gene approach involving heterologous expression of *DGAT* has been applied to rice [924]. Combined expression of *AthDGAT1*, *PDAT*, *WRI1* and *OLEOSIN* resulted in a 70% increase in grain oil content and 28% increase in the oleic acid content of the oil. In previous breeding research with rice, a strong QTL for oleic acid and linoleic acid has been shown to be associated with a ortholog of a gene encoding a DGAT1 [925]. It was suggested that this information may be useful in improving rice quality using marker-assisted selection.

9.4.2. Increasing the triacylglycerol content of non-seed tissue

For centuries, seed and mesocarp tissues have served as the primary sources of vegetable oils. Global vegetable oil production for 2020/2021 was about 210 million metric tons with global utilization close behind at about 200 million metric tons (www.statista.com). In the future, as the global population continues to grow, with increasing demands on plant oils for both edible and biofuel applications, there will likely to be a shortage unless other sources of TAG can be generated. Current advances in the ME of microorganisms and plant-like protists to produce TAG along with boosting the TAG content of seeds from oleaginous crops suggests that these sources may contribute some additional TAG in the future, but even larger gains may come from the reprogramming of

vegetative tissue to produce TAG (for reviews see [25,56,571,926,927]). It has been suggested that biomass crops, such as *Miscanthus* sp. and switchgrass (*Panicum virgatum*), hold great potential as enormous sources of plant oil if these crops can be metabolically engineering to produce substantial amounts of TAG in their vegetative tissues [928].

Over-expression of *DGAT*, alone, or in combination with other interventions, has been shown to be a useful in increasing oil accumulation in vegetative tissues. A pioneering study has shown that constitutive expression of *AthDGAT1* in tobacco resulted a 7-fold increase in leaf TAG content [131]. Observation using light microscopy indicated that the TAG appeared as cytoplasmic LDs. A further increase in tobacco (*Nicotiana tabacum*) leaf TAG to 20-fold was achieved using *AthDGAT1* under the control of a strong promoter for the *RIBULOSE-BISPHOSPHATE CARBOXYLASE SMALL SUBUNIT* [929].

As discussed previously (in Section 9.2.), the terms 'push' and 'pull' have been used in characterizing ME strategies to increase oil content in yeast, wherein 'push' refers to *de novo* FA biosynthesis and 'pull' refers to TAG assembly. In the case of a 'pull'-only strategy, *DGAT* overexpression and increased TAG accumulation would provide an increased demand on FA biosynthesis. These terms have also been used to describe engineering strategies in plant vegetative tissues [930]. In a more recent example of a 'pull' only strategy, plant ACBP was fused to algal DGAT1 to create a more effective TAG-biosynthetic enzyme [419]. In this study, fusion of the *A. thaliana* ACBP6 (see [392]) to the N-terminus of full-length CzDGAT1B gave rise to higher oil accumulation in tobacco leaves compared to expression of the unmodified full-length CzDGAT1B. Further analysis indicated that the N-terminal fusion of AthACBP6 substantially increased the production of the recombinant protein and its affinity to acyl-CoA and therefore led to higher oil accumulation, though the DGAT1 activity of the fused protein was lower than WT DGAT1 [419]. This approach was also shown to be effective in increasing oil content in yeast [419].

Eventually, the terms 'package' and 'protect' were introduced wherein 'package' refers to storage of TAG as a stable cytoplasmic LD and 'protect' involves minimizing TAG turnover via down-regulation of TAG lipase and/or key reactions in ß-oxidation [25]. 'Pull' involving *DGAT* expression is common to a multitude of strategies aimed at increasing the oil content of vegetative tissues. Several examples of various strategies involving *DGAT* expression are presented in Table 3. Combining different molecular genetic interventions has been shown to lead to synergistic effects in boosting TAG content. For example, transient co-expression of *AthWRI1* and *AthDGAT1* in tobacco was shown to result in a leaf TAG content of 2.48% (DW basis) [930]. In contrast, expression of *WRI1* or *DGAT1* alone resulted in TAG content of 0.57% or 0.45%, respectively. In a later study, performance enhanced BnaC. DGAT1.a – L441P, generated through directed evolution, was shown to be even more effective than AthDGAT1 when introduced in combination with AthWRI1 ([95]; Table 3).

In an example involving 'pull-package', AthDGAT1 with an alanine residue substituted for serine residue at position 205 and cysteine-oleosin were introduced into *Arabidopsis* [931] and later into perennial ryegrass (*Lolium perenne*) [932,933]. The modification to AthDGAT1 was intended to block down-regulation of the enzyme by phosphorylation, as first described for the *T. majus* DGAT1 [179]. Cysteine-oleosin represents a polymer of oleosin which was generated by introducing cysteine residues at critical positions in a 15-KDa sesame oleosin resulting in poly-oleosin 'cages', with inter-oleosin disulfide bonds, for increased LD stability. In addition to a 50% increase in *Arabidopsis* leaf biomass, the FA content of mature leaves, senescing leaves and roots increased by 2-, 3- and 5-fold, respectively [931]. In the same study, this strategy was also shown to increase lipid content in yeast. Later, the same strategy, when applied to perennial ryegrass, resulted in increases in biomass and plant performance [932,933]. The 'pull-package' strategy increased carbon demand leading to leaf level changes resulting in an increase the rate of photosynthesis and growth [933]. In an example of a 'push-pull-package' strategy, *AthWRI1* and *AthDGAT1* were co-expressed with sesame *OLEOSIN* in tobacco resulting in > 15% TAG (DW basis) in vegetative tissue [934]. Later, this strategy was applied to increasing the TAG content of vegetative tissues from maize [923], *Sorghum* [935], potato (*Solanum tuberosum*) [936] and rice [924].

The 'push-pull-package' approach, involving co-expression of *WRI1*, *DGAT1* and *OLEOSIN*, has also been applied to mutants of *Arabidopsis* which were defective in carbohydrate synthesis and sucrose transport [938,937]. The mutant *adg1suc2* is defective in the first reaction of starch biosynthesis and unloading of sucrose from the apoplast to the phloem [937]. Leaf FA content increased by 2-fold and the level of TAG increased to 2.3% DW, which was 4.6-fold higher than what could be achieved by implementing the 'push-pull-package' approach in the WT. In addition, the proportion of 18:2 increased while 18:3 decreased. Further increases in *Arabidopsis* leaf oil content were achieved by co-expression of *AthWRI1*, *AthDGAT1* and *CYSTEINE-OLEOSIN* in a *gbss1* mutant [938]. The *gbss1* mutant was defective in the gene encoding granule-bound starch synthase 1 (GBSS1) which was responsible for catalyzing amylose synthesis. Presumably, disruption of the gene resulted in more carbon being available for TAG synthesis. Negative effects on plant growth were overcome by using ethanol-induced gene expression.

A 'push-pull-protect' approach involving co-expression of *AthWRI1* and *AthDGAT1* in *Arabidopsis* mutant *sdp1* has been shown to result in TAG contents ranging from 5% to 8% of DW in roots, stems and leaves [939]. *SUGAR-DEPENDENT1* (*SDP1*) encodes a TAG lipase [940]; therefore, silencing of *SDP1* would be expected to decrease TAG turnover resulting in a 'protection' effect.

Transcriptome and biochemical analyses of tobacco lines expressing *AthWRI1*, *AthDGAT1* and sesame *OLEOSIN* have identified a futile cycle of lipid synthesis and degradation which probably limited further accumulation of TAG [941]. A 'push-pull-package-protect' strategy involving co-expression of *AthWRI1*, *AthDGAT1* and sesame *OLEOSIN* in tobacco, with RNAi-mediated silencing of *SDP1*, has been shown to reduce lipid turnover resulting in leaf tissue with TAG content of 33% of DW [941], essentially doubling the level of TAG achieved without silencing of *SDP1* [934]. This level of TAG accumulation was similar to the accumulation levels of this storage lipid in the seeds of many oil crops. Similar enhancement of TAG content in tobacco leaf tissue was achieved via expression of embryogenic *TF LEC2* in combination with co-expression of *AtWRI1*, *AtDGAT1* and sesame *OLEOSIN* [941].

A 'push-pull-package-protect' approach, involving *DGAT* expression, has also been applied to increasing TAG content in the vegetative tissues of high-biomass perennial C4 sugarcane (*S. officinarum*) [942]. The study involved co-expression of *AthWRI1*, maize *DGAT1-2* and *AthOLEOSIN* combined with co-suppression of a gene encoding a subunit of peroxisomal membrane transporter1 (PXA1) and a gene encoding ADP-glucose pyrophosphorylase catalyzing the first reaction in starch synthesis. Here, the 'protect' aspect was addressed through down-regulation of *PXA1* which encodes a protein participating in lipid transport across the peroxisomal membrane for β-oxidation. The added down-regulation of starch synthesis presumably made more carbon available for FA synthesis and storage lipid accumulation. TAG accumulation in leaves or stems of transgenic sugarcane increased by 95- or 43-fold to 1.9% or 0.9% of DW, respectively. The investigators calculated that each percentage of TAG accumulated in sugarcane was equivalent to the entire oil yield from the same land area of canola-type *B. napus*.

In 'pull' strategies involving only *DGAT* expression, changes in the FA composition of leaf TAG may be influenced by the substrate selectivity properties of the introduced DGAT and/or decreased desaturation capacity in the ER due to overall increased lipid production [929,937,942]. Heterologous expression of *AthDGAT1* in tobacco or *Jatropha* has been shown to enhance the proportion of 18:1 content and decrease the proportion of 18:3 [907,929]. DGAT2-2 appears to be a major contributor to the high proportion of 18:1 and total oil in yellow nutsedge tubers [586]. Indeed, expression of yellow nutsedge *DGAT2-2*

Table 3
Examples of metabolic engineering strategies involving *DIACYLGLYCEROL ACYLTRANSFERASE* (*DGAT*) expression to increase the triacylglycerol (TAG) content of vegetative tissues.

Strategy	Intervention(s)	Effects	References
Pull	Constitutive expression of *AthDGAT1* in tobacco	• TAG content increased 7-fold (DW basis)	[131]
Pull	Expression of *AthDGAT1* in tobacco under the control of a strong *RUBISCO SMALL SUBUNIT* promoter	• TAG content increased 20-fold • Increased 18:1 • Decreased 18:3	[929]
Pull	Fusion of the *A. thaliana* ACBP6 to the N-terminus of full-length *C. zofingiensis* CzDGAT1B	• Increased TAG content	[419]
Pull	Expression of *C. reinhardtii DGAT2* in *Arabidopsis*	• TAG content increased 25-fold in 15-day-old seedlings • Enhanced VLCFA content in TAG • Increased biomass 4-8 weeks after germination	[570]
Pull	Expression of *AthDGAT1* in tobacco with *MGDG SYNTHASE* suppressed	• TAG content increased 5.8 to 9.7-fold • 18:2 increased 41-58% • 18:3 decreased 29-33% • FAs de-esterified from chloroplast membrane galactolipids redirected to TAG • *LIPID TRANSFER PROTEIN* expressed in transgenic lines	[573]
Pull	Xylem-specific expression of *AthDGAT1* in tobacco	• FA content of stems increased 63% • Enhanced TAG content • Increased 16:0, 18:0, 18:2 and 18:3 in TAG	[1095]
Pull	Expression of *AthDGAT1* in *Jatropha*	• Two-fold increase in leaf lipid content • Increased 18:1 • Decreased 18:3 • No penalty on growth rate or plant development	[907]
Pull	Expression of *C. esculentus DGAT2-2* in tobacco	• Leaf oil content increased 7.2-fold • Increased 18:1 • No effect on growth rate or germination	[586]
Push-pull	Transient co-expression of *AthWRI1* and *AthDGAT1* in tobacco	• TAG content increased almost 100-fold • 18:1 increased at the expense of PUFAs	[930]
'Push-pull'	Transient co-expression of *AthWRI1* and *BnaC.DGAT1.a-L441P* (encoding an improved BnaDGAT1 isoform)	• TAG content doubled over what could be achieved with co-expression of *AthDGAT1*	[95]
Pull-package	Co-expression of *AthDGAT1-S205A* and *CYSTEINE-OLEOSIN* in *Arabidopsis*	• FA content increased 2-, 3- and 5-fold in mature leaves, senescing leaves and roots, respectively • Increased leaf 18:1 and 18:2 • Decreased leaf 16:0, 16:1, 16:3 and 18:3 • Substantially increased CO_2 assimilation rate per unit leaf area • Leaf biomass increased 50%	[931]
Pull-package	Co-expression of *AthDGAT1-S205A* and *CYSTEINE-OLEOSIN* IN perennial rye grass	• Increased growth rate, specific leaf area and photosynthetic rate per unit leaf area • Stabilized micro-sinks of cysteine-oleosin encapsulated LDs with reduced feedback inhibition of photosynthesis drive increased carbon capture	[932] [933]
Push-pull-package	Co-expression of *AthWRI1*, *AthDGAT1* and sesame *OLEOSIN* in tobacco	• TAG content > 15% in vegetative tissue at the seed-setting stage • Increased 18:1 and 18:3 • Decreased 18:3 • No major negative phenotype	[934]
Push-pull-package	Co-expression of *AthWRI1*, *AthDGAT1* and *AthOLEOSIN1* in maize	• Total leaf oil content increased 79% • Decreased saturated FAs • Increased 18:1 and 18;2	[923]
Push-pull-package	Co-expression of *AthWRI1*, *AthDGAT1* and *AthOLEOSIN1* in *Arabidopsis* mutant *adg1suc2*; ADG1 is the small subunit of ADP-glucose pyrophosphorylase which catalyzes the initial reaction in starch synthesis; suc-proton symporter 2 (SUC2) facilitates sucrose loading from leaves into phloem	• TAG and total FA contents increased to 2.3% and 11% (DW) compared with 1% and 8.3% TAG and total FA contents in *adg1suc2* • TAG 4.6-fold greater than what could be achieved with co-expression of the three genes in WT *Arabidopsis* • Increased 18:2 • Decreased 18:3	[937]
Push-pull-package	Co-expression of *AthWRI1*, *AthDGAT1* and *CYSTEINE-AthOLEOSIN1* in an *Arabidopsis* mutant defective in the gene encoding granule-bound starch synthase1 (GBSS1)	• Increased oil content in mature leaves to 2.3% (DW); 15-fold higher than the WT	[938]
Push-pull-package	Co-expression of maize *WRI1*, *U. ramannia DGAT2A* and sesame *OLEOSIN-L* in the high biomass C4 monocot *Sorghum*	• TAG content increased 3 to 8.4% (DW) depending on the leaf and plant developmental stage	[935]
Push-pull-package	Co-expression of *AthWRI1*, *AthDGAT2* and sesame *OLEOSIN* in potato; *WRI1* was driven by a senescence-induced promoter; *DGAT1* and *OLEOSIN* were driven by a *Cauliflower Mosaic Virus 35S* promoter and *RIBISCO SMALL SUBUNIT* promoter, respectively	• TAG content of senescent leaves increased about 30-fold	[935]
Push-pull-package	Co-expression of *AthWRI1*, *AthDGAT1*, *AthPDAT1* and *AthOLEOSIN* in rice	• Leaf oil increased from 1.88% to 2.3% (22.5% relative increase)	[924]
Push-pull-protect	Co-expression of AthWRI1 and AthDGAT1 in *Arabidopsis* mutant *sdp1*; *SUGAR-DEPENDENTI* (*SDP1*) encodes a TAG lipase	• TAG contents of roots, stems and leaves ranged from 5% to 8% of DW • Provision of 3% (w/v) exogenous sucrose increased root TAG to 17% of DW	[939]
	Co-expression of *AthWRI1*, maize *DGAT1-2* and *AthOLEOSIN1* in sugarcane wherein a *SUBUNIT OF PEROXISOMAL MEMBRANE TRANSPORTER1* and *ADP-*	• TAG accumulation in leaves or stems increased by 95- or 43-fold to 1.9% or 0.9% of DW, respectively	[942]

(*continued on next page*)

Table 3 (*continued*)

Strategy	Intervention(s)	Effects	References
Push-pull-package-protect	*GLUCOSE PYROPHOSPHORYLASE* were suppressed using RNAi; *AthWRI1*, *ZmDGAT1-2* and *AthOLEOSIN1* were codon-optimized for expression in sugarcane; *AthWRI1* and *ZmDGAT1-2* expressions were driven by a *RICE UBIQUITIN 3* promoter and *MAIZE UBIQUITIN* promoter, respectively; *AthOLEOSIN1* expression was driven by the 35S promoter	• In leaves of transgenic plants, 18:2, 18:3 and 18:1 accumulated at the expense of saturated FAs which decreased to half of the WT • TAG of mature leaf or stem of WT sugarcane consists of about 70% 16:0 and 30% 18:0	
Push-pull-package-protect	Co-expression of *AthWRI1*, *AthDGAT1* and sesame *OLEOSIN* in tobacco with RNAi silencing of *SDP1* expression	• Leaf tissue TAG content of about 33% of DW • Variations in plant height and overall development observed	[941]

Other abbreviations: DW, dry weight; FA, fatty acid; LD, lipid droplet; PUFA, polyunsaturated fatty acid; VLCFA, very-long-chain fatty acid; WT, wild type.

in tobacco has also been shown to enhance the proportion of 18:1 in leaf oil [586]. In further support of increases in 18:1 content, high levels of TAG accumulated in H1246 yeast expressing the recombinant yellow nutsedge DGAT2-2 when the yeast were cultured in medium containing 18:1 [586]. The reasons underlying changes in FA composition, however, become less clear as other molecular genetic interventions are introduced. For example, transient expression of *AthDGAT1* in tobacco resulted in 18:1 increasing from 3.9 to 6.3% in leaf TAG whereas expression of *AthWRI1* alone resulted in a similar increase [930]. Co-expression of *AthWRI1* and *AthDGAT1*, however, resulted in 18:1 increasing to 21.1%, indicating that the effect on enhancement of 18:1 content was not additive, but synergistic, as was also the case for the increases observed in leaf TAG content.

There have also been advances in the ME of leaf tissue to produce industrially useful FAs such as medium-chain FAs and α-eleostearic acid [943–945]. Reynolds et al. (2017) have heterologously introduced a complete acyl-CoA-dependent pathway for biosynthesis of medium-chain FA-enriched leaf oil using a tobacco transient expression system [944]. Their ME strategy involved expression of *DGAT1* from oil palm. In another study aimed at production of *Arabidopsis* leaf oil containing α-eleostearic acid, co-expression of tung *FADX* and tung *DGAT2* was shown to result in a synergistic increase in leaf NPLs and α-eleostearic acid content. Fifteen-day-old transgenic seedlings exhibited about 10 mol% of α-eleostearic acid. The specialized tung FADX catalyzes the conversion of 18:2 into α-eleostearic acid at the *sn*-2 position of PC [43,541]. Evidently, the transgenic plants possessed a mechanism(s) for liberating α-eleostearoyl moieties from PC and making this unusual FA available to tung DGAT2 in the form of a DGAT substrate.

An ME strategy involving heterologous *DGAT* expression has also been used to engineer tobacco accumulating the hydrophobic sesquiterpene, *α*-bisabolol, in leaf tissue [946]. Plant sesquiterpenes are useful in the production of pharmaceuticals, cosmetics and flavouring agents [946]. The ME strategy involved co-expression of *Artemisia annua α-BISABOLOL SYNTHASE* with added expression of *AthDGAT1*, castor *WRI1* and castor *OLEOSIN1* [946]. The α-bisabolol content of mesophyll tissue increased > 17-fold indicating that LDs can be used for intracellular storage of hydrophobic sesquiterpenes.

A 'push-pull-package' approach has also been used in the ME of potato to increase the lipid content of tubers [947]. Co-expression of *AthWRI1* and sesame *OLEOSIN* was driven using a tuber-specific promoter whereas *AthDGAT1* was constitutively expressed using the 35S promoter. The resulting tubers from transgenic plants exhibited a 100-fold increase in TAG accumulation up to 3.3% of the tuber DW. This was accompanied by a significant reduction in starch content and increase in soluble sugar content. LDs were found in close association with ER and mitochondria suggesting the interactive involvement of these organelles during lipid biosynthesis and turnover. Thus, potato tubers show potential for development of yet another high biomass tissue for production of high-energy storage lipids.

10. Intentional down-regulation/inhibition of mammalian DGAT1 and DGAT2

10.1. Inhibition of DGATs to treat diseases of excess triacylglycerol accumulation

The excessive accumulation of TAG in adipose tissue can lead to obesity, which is a risk factor for type 2 diabetes, cardiovascular disease, non-alcoholic fatty liver disease (NAFLD), arthritis, some cancers and sleep apnea [948–950]. Accumulation of TAG in non-adipose tissues, such as the liver can also lead to NAFLD, which is a progressive disease that is often accompanied by insulin resistance and hypertriglyceridemia [951–953]. It is estimated that 25% of the adult population world-wide has NAFLD which can lead to liver failure with a liver transplant being the only option for a patient's survival [954]. Because DGAT1 and DGAT2 catalyze the final step of the TAG biosynthetic pathway, these enzymes have been attractive pharmaceutical targets for developing therapeutic strategies to reduce TAG in tissues to treat obesity and associated metabolic disorders. Several DGAT1 and DGAT2 inhibitors have been developed. Key findings will be discussed.

10.1.1. Inhibition of DGAT1

Mice in which *Dgat1* was genetically disrupted had a marked reduction in adiposity, were resistant to diet-induced obesity and had improved glucose metabolism [696,955]. This enhanced metabolic phenotype indicated that small molecule DGAT1 inhibitors could be a promising treatment for reducing obesity. In rodent studies, highly selective DGAT1 inhibitors have been well tolerated and found to be effective at reducing plasma TAG levels after an oral fat challenge [149,956–961]. Inhibition of DGAT1 in the small intestine by these inhibitors interfered with the resynthesis of dietary TAG in intestinal epithelia cells reducing its secretion into the circulation. DGAT1 inhibition resulted in delayed fat absorption in a manner analogous to that observed in *Dgat1*-deficient mice [720]. Body weight was also decreased and was accompanied by improved glucose metabolism. Other studies have shown than intragastric infusion of a DGAT1 inhibitor enhanced FA oxidation in the small intestine, specifically in the jejunum [962].

Several DGAT1 inhibitors (pradigastat, AZD7687, PF-04620110, GSK3008356) have been tested in human clinical trials on healthy and overweight/obese subjects. These inhibitors were generally very effective at reducing postprandial TAG after a high fat meal [958,960,963–965]. However, the gastrointestinal side effects (nausea, vomiting and diarrhea) observed at doses effective at reducing plasma TAG were intolerable and potentially limits the utility of DGAT1 inhibition as an effective therapy [960,964,965]. This contradicts findings from studies in rodent models where DGAT1 was inhibited, or genetically disrupted, without any apparent adverse gastrointestinal affects. One possible explanation is that in rodents, *DGAT2* is also expressed in the small intestine and compensates for the lack of DGAT1. However, in humans, only DGAT1, and not DGAT2, is present in the small intestine [39,116]. It is not clear how inhibition of DGAT1 results in diarrhea. It has been proposed that the accumulation of DGAT1 substrates (DAG and FAs) is toxic to enterocytes which negatively effects gastrointestinal function [726]

Pradigastat has been tested on patients with familial chylomicronemia syndrome (FCS) [966]. FCS is an autosomal recessive disease caused by mutations either in lipoprotein lipase, or in proteins that regulate lipoprotein lipase activity (apoCII, apoAV, GPIHBP1, and LMF1) [967–969]. The loss of lipoprotein lipase activity impairs chylomicron clearance from the circulation. Instead, chylomicrons accumulate in the circulation resulting in severe hyperchylomicronemia. In FCS patients given pradigastat, both postprandial and fasting TAGs were markedly reduced. The reduction in TAG in the circulation was attributed to decreased chylomicron secretion from the small intestine because of intestinal DGAT1 inhibition. Pradigastat was well tolerated by the subjects in this study with only mild adverse gastrointestinal effects reported. This was attributed to the very low-fat diet (<10%-15% of total daily energy intake) recommended as treatment for FCS patients [970].

10.1.2. Inhibition of DGAT2

In stark contrast to disruption of *Dgat1*-deficient, *Dgat2*-deficicent mice died only a few hours after birth [694]. This finding suggested that there would be a narrow therapeutic window for inhibition of DGAT2. However, more recent studies demonstrated that the lethality observed in global *Dgat2*-deficient mice was due to the absence of DGAT2 in the skin, but not in other tissues, such as the liver and adipose tissue [677,678]. Furthermore, inhibition of DGAT2 in mice with antisense oligonucleotides reduced hepatic TAG and VLDL secretion, reversed hepatic steatosis and improved glucose metabolism [705,706,971]. These findings have recently been recapitulated in DGAT2 liver-specific knockout mice [677].

Several DGAT2 inhibitors have been developed and tested on rodent models. The greatest effect was observed on liver lipid metabolism where hepatic TAG and VLDL secretion were both reduced in a dose-dependent manner [670,972–974]. Inhibition of DGAT2 also improved steatosis and reduced fibrosis in a mouse model of NAFLD. Interestingly, inhibition of DGAT2 also resulted in the suppression of several lipogenic genes (*Srebp1c*, *Acc1, Fads*, *Fasn*, and *Scd1*) which likely indirectly contributed to the reduction in hepatic TAG. This is consistent with observations made when DGAT2 in murine primary hepatocytes was inhibited pharmacologically [669].

In contrast to the studies in rodents, both chronic and acute inhibition of DGAT2 in non-human primates had little to no effect on hepatic TAG, plasma TAG or hepatic VLDL secretion [670]. The explanation given for the discrepancy between rodent and non-human primates is that DGAT2 in mouse liver is expressed at much higher levels than DGAT1 and therefore has a more prominent role in hepatic TAG metabolism. In non-human primates, *DGAT1* and *DGAT2* expression are more balanced. In the latter case, DGAT1 is able compensate adequately when DGAT2 is inhibited.

In phase 1 clinical trials, the DGAT2 inhibitor, PF-06427878, also had no appreciable effect on plasma TAG levels, which is consistent with the findings in non-human primates [670,974]. However, liver fat in healthy subjects, assessed by magnetic resonance imaging, was reduced by ~30% after only two weeks of treatment. Unlike DGAT1 inhibition, PF-06427878 was well tolerated with no adverse gastrointestinal effects. Another clinical trial using a DGAT2 antisense inhibitor (IONIS-DGAT2$_{Rx}$) reported similar findings [706,975,976]. This inhibitor was also well tolerated with negligible adverse effects. Liver fat was reduced by ~25% in subjects with type 2 diabetes mellitus receiving weekly subcutaneous injections of the DGAT2 inhibitor for 13 weeks. In contrast, the DGAT1 inhibitor, pradigastat, only reduced liver fat by ~3% [977]. Again, the modest effect of DGAT1 inhibitors on liver TAG may be attributed to low *DGAT1* expression relative to *DGAT2* in this tissue in humans.

10.2. DGAT inhibition and cancer

Various types of cancer cells have reprogrammed energy metabolism and have up-regulated FA synthesis and uptake to increase FA supply [978,979]. These FAs are presumably used to generate ATP via β-oxidation to meet the high energy demands required for their rapid proliferation but can also lead to lipotoxicity [980]. To prevent this, cancer cells can store excess FAs in LDs as TAG, which can be utilized when needed. Many cancer cells in fact have increased LDs compared to normal cells. Furthermore, both *DGAT1* and *DGAT2* have been found to be over-expressed in several different types of cancer [981–985].

In cancer cells where *DGAT1* has increased expression, pharmacological inhibition of DGAT1 or knockdown with RNA interference has had beneficial effects [982,983,986]. Generally, the inhibition of TAG synthesis reduced LDs and FA synthesis and was accompanied by decreased proliferation of cancer cells and the activation of cell death pathways. It appears that when DGAT1 is inhibited, FAs are redirected into the mitochondria due to upregulation of CPT1, which is the rate-limiting enzyme for β-oxidation [985]. This increased movement of FAs into mitochondria promotes the generation of reactive oxygen species that causes mitochondrial damage and apoptosis. *In vivo* studies have shown that DGAT1 inhibition reduces tumor growth suggesting that reducing *DGAT1* expression maybe an effective cancer therapy [985,986].

Much less is known about the role of DGAT2 in cancer cell metabolism. As mentioned previously, *DGAT2* is over-expressed in many cancers. However, its expression is reduced instead in hepatocellular carcinoma tumor tissue [987]. Over-expression of *DGAT2* in either Hep3B or Huh7 cells, which are used as models of hepatocellular carcinoma, reduced cell proliferation. It was proposed that DGAT2 controlled proliferation of these two cell types by indirectly modulating the expression of cell cycle genes. *In vivo*, tumors produced from implanted Hep3B cells over-expressing *DGAT2* were smaller than tumors produced from control cells. From this single study, it was concluded that DGAT2 is a suppressor of hepatocellular carcinoma.

10.3. DGAT1 inhibition and viral infection

Millions of people globally are infected with hepatitis C virus (HCV) every year which can lead to severe liver damage. HCV is a positive strand RNA virus that encodes a single large polyprotein that undergoes co-translational and post-translational proteolytic processing producing 10 individual viral proteins [988]. Half of these are non-structural proteins that are involved in viral replication. Other viral proteins have structural roles or are required for viral assembly.

New viral particles were thought to be produced at the ER and exit infected cells via the secretory pathway. More recent findings have demonstrated that viral replication is intricately linked to host lipid metabolism [989]. Specifically, that the HCV capsid protein (Core) and non-structural proteins, NS3 and NS5A, associate with LDs, while other viral proteins are present at the ER [990–992]. Core appears to recruit non-structural proteins (i.e., NS5A) to LDs where they participate in the assembly of viral particles. Mutations in Core that prevented its association with LDs also impaired viral production [990,991].

The interaction of Core with LDs was found to be dependent on DGAT1 [993]. Ablation of DGAT1, either by RNA interference or with a small molecule inhibitor, prevented Core from interacting with LDs which was instead retained in the ER. Consequently, HCV particle production was impaired markedly. Interestingly, knockdown of DGAT2 had no effect on the interaction of Core with LDs suggesting that DGAT1 has a unique role in this process. Further investigation revealed that DGAT1 interacted directly with Core directing it to LDs and that HCV particle formation is dependent on LDs produced by DGAT1, but not DGAT2.

The utilization of LDs for viral particle production is not unique to HCV as LDs are utilized by several other viruses, including SARS-CoV-2 [994–996]. SARS-CoV-2, a positive stranded RNA virus, also modifies host cell lipid metabolism to promote its perpetuation. Treatment of cells infected with Sars-CoV-2 with the DGAT1 inhibitor, A922500, blocked LD formation and viral replication [997].

Because DGAT1 appears to be closely linked to HCV particle formation, inhibition of DGAT1 may be a viable treatment of HCV and the accompanying hepatic steatosis which is seen in approximately half of HCV-infected individuals [998]. Expression of Core alone in cells can cause an accumulation of TAG. Hepatic accumulation of TAG is dependent on DGAT1 since livers from *Dgat1*-deficient mice were resistant to Core-induced steatosis [999]. However, Core is not an activator of DGAT1. Instead, TAG accumulation was caused by decreased TAG turnover, possibly by alterations in liver lipid lipase activities.

The DGAT1 inhibitor, pradigastat, is a potent DGAT1 inhibitor. It was effective at reducing plasma TAG levels and decreased liver TAG content in NAFLD patients [966,977,1000]. However, its use was accompanied by a high number of adverse gastrointestinal effects previously described [977,1001]. In a separate study exploring the possible use of pradigastat to treat HCV, the DGAT1 inhibitor impaired HCV production and release from infected Huh7.5 cells [1002]. However, in a randomized clinical trial, there was no reduction in viral load in HCV-infected adults after 14 days of daily pradigastat treatments [1002]. Plasma pradigastat levels were reported to be high enough to adequately inhibit DGAT1 [1002]. However, liver pradigastat levels may have been lower than expected leading to insufficient hepatic DGAT1 inhibition and no reduction in HCV release.

11. *DGAT* as a marker for improvement of production traits of farmed animals

Numerous studies have focused on exploring the association of *DGAT* genes, and genes encoding other lipid biosynthetic enzymes and factors regulating adipogenesis, to farm animal production traits such as milk fat content and carcass quality parameters. Associations between *DGAT* gene polymorphisms and production traits can provide useful genetic tools for marker-assisted selection in breeding programs targeted at improvement of these traits in farmed animals. Although most studies on bovine DGAT have been related to trait improvement, there have, however, been a few studies in developing insights into structure/function in bovine DGAT1. These investigations have examined the interaction of synthetic peptides, representing segments of the bovine DGAT1 polypeptide, with the enzyme's substrates, along with membrane interactions at the putative substrate binding sites [1003–1005]. The following two sections deal with *DGAT* as a marker in the improvement of milk and carcass production traits.

11.1. Milk production traits

It has been shown that mice lacking both copies of *DGAT1* are unable to lactate [689] and have impaired mammary gland development [1006]. Mapping studies have placed the *DGAT1* gene close to a QTL, near the centromeric region of chromosome 14, associated with variations in milk fat content [562,563]. As mentioned in section 6.2.3, a non-conservative substitution of an alanine (A) residue for a lysine (K) residue at position 232 in bovine DGAT1 resulted in an allele (*K* allele) associated with increased milk fat content and yield [562,563]. The *K* allele was also associated with increased protein percentage and decreased milk volume and protein yield [562,1007]. Further investigation has shown that the *K* allele encodes an enzyme form with higher apparent V_{max} than the form encoded by the *A* allele [1008]. The identity of this causal mutation resulted in a valuable marker-assisted selection approach for improving the milk fat content trait. It has been suggested that diagnostics of *DGAT1* gene variants in individual animals may be useful for further investigating the biochemistry and physiology of lactation in addition to studying possible interactions with other causal genes [1009]. The DGAT1 K232A effect on milk fat content has been shown, however, to differ in magnitude between different breeds of dairy cattle [1007,1009,1010]. A study of *DGAT1* allele frequency involving 38 breeds of *Bos taurus* and *Bos indicus* from 13 different countries has revealed a range of variation from fixation of $DGAT1^A$ to fixation of $DGAT1^K$ [1010]. $DGAT1^K$ appeared to be the ancestral allele, with the substitution at position 232 occurring after separation of the *B. taurus* and *B. indicus* lineages over 200,000 years ago [1011]. The highest frequency of $DGAT1^K$ occurred in *B. indicus* [1010]. Microarray analysis of mRNA from the bovine mammary gland has shown that the expression of 30 annotated genes related to cellular growth, development, cell-signaling and the immune response are affected due to the substitution of an alanine for a lysine residue at position 232 in DGAT1 [1012].

Co-linearity analysis has shown that the *DGAT* gene families are homologous between cattle and buffalo (*Bubalus bubalis*) [1013]. Studies with various types of buffalo have also shown that the *K* allele is fixed in this species [1014–1016]. Indeed, buffalo are known to produce higher milk fat than cattle [1015].

The literature suggests that genetics, diet and the rumen microbiome have a collective influence on the FA composition of fat in cow's milk [1017,1018]. The two bovine *DGAT1* alleles have also been associated with changes in the FA composition of milk fat [1016, 1017, 1019-1024]. The milk fat from *KK* homozygous dairy cattle has been shown to have an increased saturated FA to unsaturated FA ratio [1016]. The 16:0 and 18:0 content increased whereas 14:0 and C18 unsaturated FA content decreased [1016]. Decreases in conjugated linoleic acid content have also been observed [1017,1021]. In contrast, *AA* homozygous cattle produced milk fat with increased n-3 PUFAs [1022].

The *K* allele in cattle has also been shown to be associated with lower lactose yields and higher lactose content [1025]. More favorable milk coagulation properties have been also associated with the *K* allele [1026].

Other studies have shown that the DGAT1 K232A substitution is not the only milk production QTL on bovine chromosome 14 [1010,1026,1027]. Some of this variation has been associated with a polymorphism in the promoter of *DGAT1* when investigated using homozygous *AA* German Holstein cattle [1027]. Other polymorphisms affecting milk production traits have been identified in *DGAT1* from cattle [1028], buffalo [1029,1030] and dairy sheep (*Ovis aries*) [1031,1032]. The effects of a single-nucleotide polymorphisms (SNPs) have been studied in a population of 312 Chinese Holstein cattle [1028]. The SNP, 1801116A/G, in the *DGAT1* gene was significantly associated with milk fat percentage. Interestingly, this SNP was also associated with mastitis resistance phenotypic traits including a low somatic cell score. Mastitis, which is an inflammatory disease of the mammary gland, is known to be a costly disorder affecting dairy cattle [1033]. The *DGAT1* SNP associated with mastitis resistance [1028] is interesting to consider in the light of the DGAT1 K232A substitution being associated with changes in the expression of immune response genes [1012]. A lower somatic cell score, reflecting better mastitis resistance, has also been reported for the K232A substitution in North American Holstein cattle [1034]. Other studies involving the K232A substitution in dairy cattle, however, have shown that somatic cell score is not affected [1025,1026].

Research on possible associations between *DGAT2* polymorphisms and milk production traits in farmed animals has been more limited. Winter et al. (2003) first reported on the cloning, physical mapping and sequence analysis of DGAT2 for *B. taurus* [1035]. *DGAT2* has been shown to be expressed along with *DGAT1* in the bovine mammary gland [1036,1037]. An association between intronic SNPs in *DGAT2* and milk yield has been reported in Iraqi Holstein cattle [1038]. A polymorphism in the 3' untranslated region of the *DGAT2* gene from Sarda dairy sheep has been associated with changes in the conjugated linoleic acid content of milk fat and milk yield [1039]. In addition, polymorphisms in the *DGAT2* gene associated with milk yield and fat percentage have also been identified the goat (*Capra aegagrus hirus*) [1040].

11.2. Carcass production traits

Carcass production traits for cattle include marbling score, intramuscular (IM) fat content and backfat thickness. Marbling refers to the white visible depots of IM fat which occur between bundles of muscle fibers in beef [1041–1043]. In the United States, marbling is subjectively assessed in the *longissimus* muscle at the interface between the 12th and 13th rib [1041]. Higher marbling scores have been positively associated with better palatability [1044,1045]. In some grading systems, such as those in the United States and Canada, higher quality grades are given to carcasses with higher marbling scores. Efforts to reach higher levels of marbling often result in over-fattening of other fat depots, including subcutaneous and intermuscular fat (seam fat) depots [169]. The IM fat, as recognized by the beef industry, are the adipocytes between the muscle fibers and should not be confused with the intramyocellular TAG of the muscle fibers [736,1046]. The TAG within the muscle cells, however, is not considered to be a major contributor to the marbling trait [1042]. Numerous studies, however, have referred to IM fat as the total fat in beef tissue regardless of the extent of marbling. Thus, the TAG in these analyses can come from both IM adipocytes and muscle cells.

Some studies on identifying potential markers for the marbling trait have focused on determining tissue activities of enzymes, including DGAT, involved in TAG metabolism [169–171,1047,1048]. In one earlier study using *pars costalis diaphragmatis* muscle (skirt muscle) samples from various crosses of Wagyu cattle, it has been shown that DGAT total activity in IM adipose tissue was strongly correlated with muscle DGAT total activity, which suggested a coordination of DGAT activity between the two tissues [169]. There was also an inverse correlation between the DGAT specific activity of IM adipose tissue and the lipid content of the muscle suggesting that DGAT activity was down-regulated as IM adipose tissue became filled with fat. During investigations on the possible association between DGAT activity and the extent of marbling, it has also been shown that DGAT activity was stable in dehydrated bovine muscle tissue stored at room temperature for two to three weeks [171]. These results suggested that dehydration may be an effective method for shipping and storing muscle biopsy samples which are destined for assessment of DGAT activity. In addition, the dehydration approach might also prove useful for storing other biological samples containing DGAT activity, such as plant vegetative tissue and yeast cells.

The *K232A* polymorphism has also been examined in relation to IM fat content and other carcass traits in cattle [1009,1049–1056]. In German Holstein cattle, the *DGAT1* polymorphism has been shown to have a significant effect on the IM fat content of the *semitendinosus* muscle [1009]. In another study involving Holstein and Charolais bulls, the DGAT activity of *longissimus dorsi* muscle from individuals with the *KK* genotype was about five-fold greater than for either the *KA* or *AA* genotypes [1052]. This observation agrees with the higher activity of the enzyme form with the lysine residue at position 232. In an investigation with a Hungarian Angus population, it has been shown that a sunflower diet combined with selection for the *K* allele resulted in increased fat content in both the *longissimus* and *semitendinosus* muscles [1055]. The *K232A* polymorphism in a commercial population of Aberdeen Agnus-sired beef cattle has also been shown to be associated with changes in sirloin weight after maturation and sirloin fat depth [1053]. In another study involving 243 bulls from five different breeds, the *KA* genotype was associated with a higher IM fat content and marbling score than for the *AA* genotype [1056]. Other studies with cattle, have not identified a significant relationship between the IM fat content and the *K232A* polymorphism and other carcass traits, including backfat thickness [1049–1051,1054]. Investigations involving other polymorphisms in bovine *DGAT1* [1057–1059] and bovine *DGAT2* [1060] have also been linked to changes in IM fat content. In addition, polymorphisms in *DGAT1* associated with meat tenderness have been identified in Ginnan yaks (*Bos grunniens*), a long-haired bovine species [1061].

There has also been an interest in the probing the level of expression of various genes, encoding enzymes and other proteins involved in fat deposition, as a means of predicting IM fat content in cattle [1062,1063]. Gene expression abundance for *DGAT1* and *DGAT2* has been shown to exhibit a significant positive correlation with the IM fat content of *longissimus dorsi* muscle [1063]. In another study aimed at increasing the TAG content of mouse skeletal muscle, bovine *DGAT1* was expressed under the control of a muscle *CREATINE KINASE* promoter [1064]. The TAG content of the *posterior tibial* muscle was about four-fold greater in transgenic mice than for WT mice. DGAT1 polypeptide abundance was increased by 50%. The investigators suggested that over-expression of *DGAT1* may be a useful approach to generating transgenic cattle with increased TAG content in the muscle. A previous study on over-expression of mouse *DGAT1* in mouse skeletal muscle also showed an increase in intramyocellular TAG content [736].

Other *DGAT* polymorphisms associated with carcass traits have been identified in studies with sheep [1065, 1066, 1097] and goats [1067]. In one study with Chinese indigenous sheep breeds, a *DGAT1* polymorphism in exon 17 was significantly associated with higher marbling score and higher IM fat content [1097]. In another investigation with pure bred Texel sheep from Uruguay, an intronic SNP in *DGAT1* was shown to be highly associated with fat thickness [1066]. Genetic variations of the *DGAT2* gene have been examined in 299 goats from three breeds [1067]. A polymorphism in exon 3, resulting in a change of a lysine to an arginine residue, showed a relationship with various growth traits.

DGAT gene polymorphisms affecting carcass traits have also been examined in the monogastric pig (*Sus scrofa domesticus*) [1068–1071]. A base variation in the 3' untranslated region of porcine *DGAT2* has led to genotypes with significant associations with backfat between the 6th and 7th ribs [1068]. A 13-base pair insertion/deletion polymorphism, also in the 3' untranslated region of porcine *DGAT2*, was shown to be associated with backfat thickness and lean percentage [1070]. In another study, a SNP in exon 9 of porcine *DGAT2* was associated with changes in *DGAT2* expression affecting muscle FA composition [1071]. The allele resulting in increased *DGAT2* expression produced muscle with more C14 and C16 FAs at the expense of C18 FAs. There was also an increase in palmitoleic acid relative to palmitic acid. The results suggested that porcine *DGAT2* allele, resulting in higher expression, encodes an enzyme form which prefers substrates containing shorter fatty acyl chains, especially if they are monounsaturated.

Several studies with pigs have also examined the expression levels of fat deposition genes in relation to carcass lipid traits (e.g., [1070,1072–1075]). In a study with three breeds of pigs, the expression level of *DGAT1* was positively correlated with backfat thickness whereas *DGAT2* expression level was positively correlated with IM fat content [1072]. Another investigation with a Chinese indigenous pig breed also showed that the expression level of *DGAT2* was positively correlated with IM fat content [1074]. Over-expression of porcine *DGAT1* in mice under the control of a muscle *CREATINE KINASE* promoter has also been shown to result in increased TAG content in skeletal muscle [1076]. It was suggested that this study served as proof-of-concept for eventually developing transgenic pigs with higher IM fat content and improved pork quality.

Genetic variations in the *DGAT2* gene of the domestic pigeon (*Columba livia*) associated with changes in carcass and meat quality traits have also been identified [1077]. It was suggested that two SNPs, one in exon 5 and the other in exon 6, may be useful as genetic markers for marker-assisted breeding in pigeons.

12. Investigations of DGAT in various other organisms

DGATs have also been studied in a few other organisms. Some of these investigations have involved animal-like protists. Earlier studies with protozoan parasites producing TAG as a major NPL have focused on *DGAT1* genes [1078–1080]. The involvement of DGAT action in TAG

production in these protozoans suggested that useful therapeutic treatments for parasite-born diseases such as malaria could be developed. *Toxoplasma gondii* DGAT1 has been characterized and was established as the first endogenous marker for the ER in this organism [1080]. A combination of prediction and experimental data indicated that the C-terminus of TgDGAT1 resides in the lumen of the ER. Disruption of the *DGAT1* genes in *Plasmodium falciparum* and *T. gondii* were lethal suggesting that the enzyme was essential for the growth of the organism [1079,1080].

In a study aimed at identifying novel genes for ME of plants to produce WEs, four genes encoding DGAT2 isoforms have been characterized in *Tetrahymena thermophila,* a unicellular protozoan belong to the phylum Ciliophora [1081]. The four DGAT2 forms were designated TtWS1, TtWS2, TtWS3 and TtWS4 given their involvement in WE synthesis. TLC analysis suggested that recombinant TtWS2, TtWS3 and TtWS4 were able to restore TAG biosynthesis in a yeast mutant devoid of TAG biosynthesis whereas recombinant TtWS1, TtWS2 and TtWS4 appeared to produce WEs if supplied with exogenous FA alcohols. Substrate specificity studies were then conducted with yeast membranes harboring each TtWS. TtWS1 catalyzed WE as the main product, with the most effective substrate combination being 10:0-OH and 14:0-CoA; DGAT activity, however, was relatively low. TtWS2 and TtWS3 exhibited high WS and DGAT activity with a broad range of saturated and monounsaturated FA alcohols and saturated acyl-CoAs. 18:1-CoA was also an effective acyl donor for TtWS3. In contrast, the WS activity of TtWS4 was high with straight-chain alcohols (9C to 14C) along with methyl-branched alcohols. The proportions of WE and TAG produced via the catalytic action of TtWS4 varied depending on the acyl-moiety of the thioester, as also seen for TtWS3.

DGAT1 and DGAT2 have been investigated in the amoeba (*Dictyostelium discoideum*) [1082]. DdDGAT1 localized to the ER whereas DGAT2 was associated with LDs. In the amoeba, expression of *DGAT2* in vegetative cells was shown to be about 16-fold less than expression of *DGAT1* (http://dictyexpress.biolab.si/). DGAT1 provided the main activity in support of TAG formation and could also catalyze the synthesis of non-polar ether lipids in the amoeba and WEs when recombinantly produced in H1246 yeast cultured in the presence of long-chain alcohols. The *dgat2*$^{-}$ knockout had essentially no effect on TAG accumulation. When *DGAT2* was expressed in a *dgat1*$^{-}$ knockout or *dgat1*$^{-}$/*dgat2*$^{-}$ knockout, however, TAG production was rescued, but the cells did not produce ether lipid.

Moving up the evolutionary scale, there have been a couple of reports regarding DGAT in the model nematode worm, *Caenorhabditis elegans*. *DGAT1* was the first *DGAT* gene cloned (and functionally expressed) from an invertebrate [1083]. *C. elegans* has been used to demonstrate that the LACS, known as FATP1, and DGAT2 are components of a TAG biosynthesis complex which facilitates expansion of LDs [674]. Loss of FATP1 or DGAT2 activity blocked LD expansion in *C. elegans*. FATP1 and DGAT2 were also shown to interact when recombinantly produced in mammalian cells and act synergistically to promote LD expansion.

DGAT has also been studied in insects including *Drosophila melanogaster* (e.g., [1084,1085]), the silk moth (*Bombyx mori*) [1086] and tobacco hawk moth (*Manduca sexta*) [1087]. Gene expression analysis of a putative *DGAT2* from *B. mori* has shown that transcript levels peak in pheromone glands during a key stage for sex pheromone production in newly emerged females [1086]. Further experiments involving decapitation and application of a juvenile hormone analog suggested that DGAT2 was involved in the regulation and release of sex pheromone in *B. mori*. Another study involving *M. sexta* has suggested that insects only have a single *DGAT1* gene but that they also have a single *MGAT* gene, exhibiting some homology to the *MGAT2* gene of vertebrates [1087]. *MsMGAT* and *MsDGAT1* were expressed in the fat body, midgut and ovaries. The relative expression levels of *MsMGAT* and *MsDGAT1* were positively correlated with relative rates of FA utilization for the biosynthesis of DAG and TAG, respectively. This suggested that regulation of the expression of *MsMGAT* and *MsDGAT1* determined whether the fat body secretes DAG or stores FAs as TAG. Overall, the observations implied a role for the MAG pathway in the biosynthesis and degradation of DAG in the *M. sexta* fat body. The study involving *M. sexta* also suggested that the putative *DGAT2* gene identified in *B. mori* [1086] encodes an MGAT2-like protein [1087].

Drosophila (the common fruit fly) has become one of the model systems for lipid metabolism research [1085]. In this organism, the *midway* gene (*mdy*), which encodes DGAT1 [1084], has been shown to be expressed in the fat body and other adult organs, including the ovaries [1085]. Mutants of the *mdy* gene with reduced DGAT1 activity have been shown to be female sterile because of reduced lipid deposition in the developing oocyte and subsequent degeneration of the egg chamber [1084,1085]. Expression of *mdy* in cultured insect cells resulted in increased DGAT activity [1084]. Several studies with *Drosophila* have probed mechanisms underlying LD growth and utilization [1085,1088–1091]. Inactivation of *mdy* in embryonic *Drosophila* tissue culture cells has been shown to reduce storage lipid synthesis [1089] and adult flies with impaired *mdy* gene function had one fourth of the body fat of control flies [1092]. Three other genes, arranged in tandem, encoding proteins with possible DGAT activity have been identified [1085]. ER to LD targeting of GPAT4 and other LD-associated TAG biosynthetic enzymes has been shown to be required for LD growth in *Drosophila* [1091]. A smaller class of LDs, without GPAT4, was identified during culturing in oleate-containing medium. Inactivation of *mdy* in *Drosophila* cells resulted in reduced TAG levels and the absence of smaller LDs. Thus, DGAT1 was needed for formation of small LDs. Inactivation of *mdy* also affected the overall quantity of LDs because some of the LDs, originally formed via DGAT1 action, expand later.

The goby (*Gymnogobius isaza*) is a freshwater fish endemic to Lake Biwa in Japan [1093]. Lipid analysis data has suggested that some of the goby's n-3 long-chain PUFAs are synthesized *de novo* in addition to being obtained from dietary sources [1093]. n-3 PUFAs accounted for 27% and 22% of the total FAs in muscle and liver, respectively, with EPA being the most abundant PUFA. EPA was also enriched at the *sn*-1 and *sn*-3 positions which contrasted with marine fish where this PUFA is enriched at the *sn*-2 position. In addition, the EPA content of many other species of freshwater fish was considerably lower than what was observed in the goby lipids. When goby *DGAT1* or *DGAT2* was expressed in TAG-deficient yeast, feeding experiments with exogenous PUFAs revealed that recombinant goby DGAT1 produced TAG with a high proportion of EPA. In addition, the ratio of expression of *DGAT1/DGAT2* in muscle was greater than in liver, and the incorporation of EPA in muscle TAG was 1.3-fold higher than in liver. The results suggested that DGAT1 is involved in the biosynthesis of EPA-enriched TAG in the freshwater goby.

Amphibians are considered the first vertebrates to have transitioned from water to land [1094]. Recently, DGAT2 has been shown to be a major protein in the skin secretions of the amphibian, *Phyllomedusa distincta*, and proposed to be the key enzyme catalyzing TAG accumulation in support of dehydration control in this organism [1094]. The protein was highly purified from skin secretions and analyzed by SDS-PAGE. Using a proteomic approach, the major polypeptide of about 45 kDa was subjected to LC-MS/MS and identified as a DGAT2. Polyclonal antibodies were also raised against the highly purified DGAT2 and used in the immunohistochemical analysis of DGAT2 localization in the skin of different species of amphibians, along with mouse and human skin. Staining was demonstrated in the epidermal surface, lipid glands, periphery of the poison glands and blood vessels of different species of *Phyllomedusa.* There was, however, no immuno-recognition when using samples prepared from mouse or human skin. The investigators suggested that *P. distincta* DGAT2 had a different spatial structure than mammalian DGAT2 which interfered with antigen recognition. Skin secretions used in this study were collected by mechanically stimulating the surface of the amphibian's skin in the presence of deionized water. The soluble nature of PdDGAT2 in water, however, is puzzling since

DGAT2 is well known as an integral membrane protein.

13. Closing comments

TAGs have a crucial role in functioning as major source of stored metabolic energy. Unraveling the complex processes involved in the biosynthesis of storage lipids in a range of organisms has been a monumental task. The identification of the *DGAT1* gene in 1998 and the subsequent identification of *DGAT2* shortly thereafter laid the groundwork for understanding the roles that DGAT1 and DGAT2 enzymes have in TAG metabolism in a wide variety of species. This initial research was followed by the discovery of other enzymes with DGAT activity including the bi-functional WSDs from bacteria, higher plant DAcT and soluble DGATs from yeast, plant-like protists and higher plants.

Progress in DGAT research is dependent on suitable enzyme assays, many of which have been adapted to HTP applications. Direct assays, quantifying TAG produced, which are based on the use of radiolabeled substrates, represent the gold standard for assaying DGAT activity. Both direct assays and indirect assays quantifying CoA, however, have been used to determine DGAT activity. Several well thought out methods have been developed for determining substrate selectivity, especially in studies with DGATs from higher plants. Although cell-based assays have provided insight into DGAT action under physiological conditions, they can be subject to interference by other cellular processes. Cell-based assays, however, have proven useful in characterizing the effects of mammalian DGAT inhibitors and in the directed evolution of DGATs from higher plants and bacteria. The directed evolution studies have led to the generation of high-performance enzyme variants with potential biotechnological applications.

WSDs are the major DGAT in bacteria and this class of bi-functional enzymes, in general, have broad substrate preferences. The recently reported crystal structure of a representative WSD opens the door to explain their reaction mechanism and other possible functions, as well as to design WSDs rationally for biotechnological applications. Some bacterial WSD deletion mutants still have DGAT activity, but the corresponding enzymes have yet to be identified. The study of bacterial DGATs may also lead to treatments for diseases caused by bacteria. For example, *M. tuberculosis* causes tuberculosis and infects more than one quarter of the world's population. This pathogen accumulates large amounts of TAG, which is essential for its long-term survival and has a role in its drug resistance. Therefore, DGAT might be an appropriate target for developing novel drugs to combat tuberculosis. In addition, the identification of the first enzyme with DGAT activity in cyanobacteria should lead to new insights on DGAT action in these bacterial species with high photosynthetic capacity.

Most of our insight into fungal DGATs has been based on studies with the yeast *S. cerevisiae* and the oleaginous yeast, *Y. lipolytica*. In particular, the genetic and genomic resources and other tools available for *S. cerevisiae* have made this a very useful model system for understanding human lipid metabolism. Since many yeasts have GRAS status, these microorganisms are well accepted by the food and cosmetic industries. For the first time, non-acyl-CoA-dependent TAG biosynthesis via PDAT action was also identified in *S. cerevisiae*. In addition, the development of an *S. cerevisiae* strain (H1246) devoid of TAG biosynthesis has proven to be a valuable tool for the investigation of DGATs from a plethora of organisms.

Studies of TAG biosynthesis and DGAT action in plant-like protists, such as the microalgae, have become prominent in recent years, although the research is not as advanced as for DGAT research in bacteria, yeast and higher plants. In many cases, multiple forms of DGAT1 and DGAT2 have been identified in plant-like protists along with WSDs and soluble DGATs. Much of the recent research on plant-like protist DGATs has been driven by the need to develop alternative sources of biologically derived oil for industrial applications.

The biochemical and physiological properties of higher plant DGATs, and the expression of their encoding genes, have been extensively investigated, especially for DGAT1 and DGAT2. Research towards understanding higher plant DGAT action has been largely driven by the need to increase seed oil content and modify FA composition of TAG for both edible and industrial applications. A few notable advances within the last decade include the experimental determination of the topology of DAcT, determining role of the hydrophilic NTD of BnaDGAT1 in the allosteric regulation of the enzyme, identification of a polypeptide region in BnaDGAT2 which is linked to acyl-CoA specificity, identification of TFs regulating the expression of *AthDGAT1* and evidence for DGAT1 and DGAT2 operating within an interactome involving interaction with other transferases participating in storage lipid biosynthesis. In addition, a BnaDGAT1 variant developed through directed evolution was shown to be both more active and resistance to substrate inhibition by higher concentrations of acyl-CoA. This mounting knowledge about the characteristics of higher plant DGATs strongly suggests that it may soon be possible to generate customized DGATs, with higher levels of associated gene expression and enzyme activities, possessing strict substrate selectivities. In cases where the DGAT is allosterically regulated, it may also be possible to desensitize the enzyme to the down-regulating effects of negative effectors to further increase enzyme performance. Indeed, with global production of plant oil being very close to utilization of plant oil, it is becoming increasing clear that producing adequate amounts of plant oil, with suitable properties, for future generations, will be an enormous challenge. ME strategies involving DGAT manipulation have already been extensively applied to oil crops, yeast, bacteria and plant-like protists. Within this context, highly customized DGATs would be valuable tools in ME strategies aimed to increase seed content and with oil enriched in specific FAs. In oil crops, control analysis, involving analysis of DGAT activity, has emerged as a useful tool in helping to guide ME to increase seed oil content. The use of DGAT in multi-pronged strategies to increase the TAG content of vegetative tissues represents an advantageous technology which could potentially lead to massive increases in the global supply of vegetable oil. Thus, highly customized DGATs may also prove useful in this context. Furthermore, the emerging role of higher plant DGAT1 in various abiotic stresses, such as low temperature and drought stress, suggests that ME strategies involving manipulation of the enzyme may lead to the development of crops exhibiting improved performance under conditions of environmental stress.

Within the last decade, our insight into the action of soluble DGAT3 from an oleaginous yeast, plant-like protists and higher plants has also increased. The soluble DGAT3 may also prove to be a useful biotechnological tool for increasing the oil content and modifying the FA composition oils of higher plants, yeast and plant-like protists, In higher plants and plant-like protists, the possible role of DGAT3 in both the cytosol and plastid needs to be further explored. Furthermore, the soluble nature of DGAT3 enzymes suggests that they may be more amenable to crystallization than the membrane bound DGAT1 and DGAT2 enzyme forms.

Although major advances have been made in our knowledge of how mammalian DGAT1 and DGAT2s function, it has become very apparent that these enzymes are not redundant, with each enzyme serving specific biological functions. Furthermore, the complexity of how DGAT1 and DGAT2 participate in a variety of biological processes in organisms has become more appreciated. One of the major advances in the research of TAG biosynthesis has been the recent determination of the 3-D structure of human DGAT1 and the determination of its reaction mechanisms. These discoveries should facilitate drug design for inhibitor development for treating human metabolic diseases. It remains to be seen if DGAT2 possesses a similar structure and reaction mechanism despite its differences to DGAT1 with respect to its amino acid sequence and membrane topology.

The recently reported 3-D structure of human DGAT1 has also increased our insight into DGAT1 from higher plants. Recently, several high performance BnaDGAT1 variants, many with single amino acid residue substitutions, generated through directed evolution, were

characterized. The modeling of the 3-D structure of BnaDGAT1, guided by the 3-D structure of human DGAT1, has shed light on underlying effects of specific amino acid residue substitutions on possible polypeptide conformational changes linked to enhanced BnaDGAT1 activity.

Finally, the genetics of *DGAT1* and *DGAT2*, has proven to be useful in the selection of farmed animals with improved production trails. In terms of milk production in dairy cattle, a single amino acid substitution in DGAT1 has been linked to increased milk fat and yield. In addition, both *DGAT1* and *DGAT2* genes have been implicated in influencing carcass traits including the extent of IM fat deposition. In the future, it may be possible to intentionally engineer livestock, by altering DGAT action, to produce animals with improved production traits.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plipres.2022.101181.

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