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RESEARCH PAPER

A thraustochytrid diacylglycerol acyltransferase 2 with broad substrate specificity strongly increases oleic acid content in engineered *Arabidopsis thaliana* seeds

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Abstract

Diacylglycerol acyltransferase (DGAT) catalyses the last step in acyl-CoA-dependent triacylglycerol (TAG) biosynthesis and is an important determinant of cellular oil content and quality. In this study, a gene, designated *TaDGAT2*, encoding a type 2 DGAT (DGAT2)-related enzyme was identified from the oleaginous marine protist *Thraustochytrium aureum*. The deduced *TaDGAT2* sequence contains a ~460 amino acid domain most closely related to DGAT2s from *Dictyostelium* sp. (45–50% identity). Recombinant *TaDGAT2* restored TAG biosynthesis to the *Saccharomyces cerevisiae* H1246 TAG-deficient mutant, and microsomes from the complemented mutant displayed DGAT activity with C16 and C18 saturated and unsaturated fatty acyl-CoA and diacylglycerol substrates. To examine its biotechnological potential, *TaDGAT2* was expressed under control of a strong seed-specific promoter in wild-type *Arabidopsis thaliana* and the high linoleic acid *fad3fae1* mutant. In both backgrounds, little change was detected in seed oil content, but a striking increase in oleic acid content of seeds was observed. This increase was greatest in *fad3fae1* seeds, where relative amounts of oleic acid increased nearly 2-fold to >50% of total fatty acids. In addition, >2-fold increase in oleic acid levels was detected in the triacylglycerol *sn*-2 position and in the major seed phospholipid phosphatidylcholine. These results suggest that increased seed oleic acid content mediated by *TaDGAT2* is influenced in part by the fatty acid composition of host cells and occurs not by enhancing oleic acid content at the TAG *sn*-3 position directly but by increasing total oleic acid levels in seeds, presumably by limiting flux through phosphatidylcholine-based desaturation reactions.

Key words: *Arabidopsis*, diacylglycerol acyltransferase, fatty acid, oilseed, oleic acid, *Thraustochytrium aureum*, triacylglycerol.

Introduction

Diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) is a key enzyme of acyl CoA-dependent TAG biosynthesis. This enzyme catalyses esterification of the acyl group of acyl-CoAs at the *sn*-3 position of *sn*-1,2-diacylglycerol (DAG) molecules, the final step in the Kennedy pathway

leading to TAG production. In plants, the DAG substrate of DGAT can also be used for the formation of the major endoplasmic reticulum (ER) phospholipid phosphatidylcholine (PC) by the enzymes CDP choline:DAG cholinephosphotransferase (CPT) (Dewey *et al.*, 1994) and PC:DAG

cholinephosphotransferase (PDCT), a recently identified enzyme that catalyses head group exchange between DAG and PC (Lu *et al.*, 2009). Oleic acid linked to PC serves as the substrate for the ER-localized $\Delta 12$ oleic acid desaturase (FAD2), and the PC-linked linoleic acid product of this reaction is the substrate for linolenic acid synthesis via the $\Delta 15$ linoleic acid desaturase (FAD3). As a result, the balance of DGAT, CPT, and PDCT activities not only mediates cellular fatty acid storage and membrane formation, but also influences fatty acid polyunsaturation.

Two major families of DGAT enzymes have been identified, DGAT1 and DGAT2, having similar function but little sequence homology (Shockey *et al.*, 2006; Cao, 2011). DGAT1 belongs to the membrane-bound *O*-acyltransferase (MBOAT) family (Cases *et al.*, 1998), while DGAT2 belongs to the monoacylglycerol acyltransferase (MGAT1) family (Yen *et al.*, 2002). A less studied, soluble (cytosolic) DGAT belonging to the DGAT3 family has been identified in peanut and *Arabidopsis* (Saha *et al.*, 2006; Hernandez *et al.*, 2012). Comparison of the deduced amino acid sequences of 117 DGATs from 70 organisms show them falling into two groups, presumably due to distinct structural differences (Shockey *et al.*, 2006; Cao, 2011). The deduced amino acid sequence of DGAT1 is ~500 amino acid residues while that of DGAT2s is ~350 residues resulting in predicted 20-kDa difference in molecular mass (Cao, 2011). For example, the human DGAT1 is 488 residues (Hiramane and Tanabe, 2011) while the human DGAT2 is 335 residues (Cases *et al.*, 2001). The N and C termini in both DGAT classes from plants extend towards the cytosol while in a mammalian DGAT1 C terminal was reported to be embedded in the ER lumen (Shockey *et al.*, 2006; Stone *et al.*, 2006; McFie *et al.*, 2010). Both types of DGATs contain conserved motifs and residues in their carboxyl-terminal halves that are believed to contribute to catalytic activity (Cao, 2011). Some studies suggested that the highly divergent N-termini of DGAT1s from different species may play a role in regulation of enzyme activity and conformation as well as substrate specificity (Weselake *et al.*, 2000; McFie *et al.*, 2010).

DGATs have received considerable attention as biotechnological targets for enhancement of the TAG content of seeds of crops such as soybean, maize, and rapeseed to meet the growing demand for vegetable oils for food, feed, and biofuel uses (Lung and Weselake, 2006). A number of reports have described the ability to increase total oil content of seeds by transgenic expression of DGAT1 and DGAT2 enzymes of plant and fungal origin (Jako *et al.*, 2001; Lardizabal *et al.*, 2008; Oakes *et al.*, 2011). Seed-specific overexpression of *Arabidopsis* DGAT1, for example, increased oil content by up to 15 and 46% in seeds of transgenic *Brassica napus* and *Arabidopsis*, respectively (Jako *et al.*, 2001; Sharma *et al.*, 2008; Taylor *et al.*, 2009). Small increases in oil content of maize and soybean seeds were conferred by expression of the fungus *Umbelopsis ramanniana* DGAT2 (Lardizabal *et al.*, 2008; Oakes *et al.*, 2011). Moreover a relative increase of 19–26% in kernel oil was observed in transgenic maize lines expressing DGAT2 from the fungus *Neurospora crassa* (Oakes *et al.*, 2011). DGAT2s from plant species such as

Ricinus communis (castor bean) and *Vernonia galamensis* have also been reported to function as specialized enzymes for sequestration of unusual fatty acids (e.g. ricinoleic acid, vernolic acids) into TAGs (Burgal *et al.*, 2008; Li *et al.*, 2010, 2012).

This study isolated, characterized and evaluated a DGAT2-type enzyme from the thraustochytrid *Thraustochytrium aureum*, a marine protist that accumulates high levels of oil that are enriched in the nutritionally important, very-long-chain fatty acid docosahexaenoic acid (DHA) (Qiu *et al.*, 2001; Jeh *et al.*, 2008). *T. aureum* and other thraustochytrids have received increasing attention as platforms for commercial production of DHA-rich oils and as a source of genes for DHA production in recombinant hosts (Qiu *et al.*, 2001; Lee Chang *et al.*, 2012). Given the high oil content of *T. aureum*, which has been reported to be 25–37% of the cell dry weight with DHA accounting for approximately 40% of the oil (Iwao Iida, 1996; Hur *et al.*, 2002; Taoka *et al.*, 2011), this organism is a potentially desirable source of DGAT genes for biotechnological use.

Materials and methods

Growth conditions and generation of *T. aureum* ATCC 34304 cDNA clones

T. aureum ATCC 34304 cells were grown in BY+ medium (Difco, Franklin Lakes, NJ, USA) at 25 °C for 4 days under constant light and agitation at 250 rpm. Biomass was harvested and rinsed in ice-cold RNase-free water followed by lysis in a French press at 69 MPa and transferred into phenol (TE-buffered, saturated pH 6.7–8.0). Total RNA from the aqueous phase was precipitated at –70 °C for 30 minutes in 0.3 M sodium acetate (pH 5.6) and one volume of isopropanol, followed by centrifugation at 15,000 *g* for 30 minutes at 4 °C and treated with DNase. Total RNA was further purified using a RNeasy Maxi kit (Qiagen, Valencia, CA, USA). A *T. aureum* cDNA library was generated by cloning the cDNA into *EcoRI/XhoI*-restricted pBluescript II SK(+) vector, pBluescript II XR library construction kit (Stratagene, La Jolla, CA, USA). For generating a genomic DNA library, genomic DNA was extracted using a genomic DNA extraction kit (Qiagen).

Cloning a putative *TaDGAT2* sequence from *T. aureum* ATCC 34304

Random sequencing of ~5000 cDNA clones was carried out using the T7 promoter primer to generate sequences containing the 5'-end of each cDNA clone. Assembled sequences were then annotated using BLAST2 against the appropriate GenBank divisions and using FASTX against GenPept. Details on the isolation of the 5'- and 3'-ends of the full-length cDNA are described in Supplementary Methods (available at JXB online). The open reading frame designated *TaDGAT2* of 1782 bp, encoding 594 amino acids, was then amplified from *T. aureum* ATCC 34304 cDNA using the gene-specific primers 1424 AT *EcoRI*_FP (5'-GTAGAATTCATGGAGCCCATAGCGTACAAG-3') and 1424 AT *NotI*_RP (5'-ACGGCGGCCGCCTAACCCCTCGGTGTACA G-3').

Phylogenetic analysis

An unrooted phylogenetic tree of *TaDGAT2* deduced amino acid sequences along with other amino acid sequences homologous to

DGAT1 or DGAT2, including several functionally characterized ones, was constructed. The functional and phylogenetic relationships were identified by the neighbour-joining program in MEGA4 (Tamura *et al.*, 2007). The bootstrapping was performed with 1000 replicates.

Yeast and plant vector constructs

The open reading frame encoding for TaDGAT2 was subcloned into *EcoRI/NotI* sites of pESC-URA (Invitrogen) vector generating pESC-TaDGAT2. For expression of TaDGAT2 in *Arabidopsis* seeds, the yeast expression vector pESC-TaDGAT2 was digested with *EcoRI/SpeI* to release the TaDGAT2 gene. The TaDGAT2 *EcoRI/SpeI* fragment was subsequently cloned into the *EcoRI/XbaI* sites of the plant binary expression vector pBinGlyRed3 to generate pBinGlyRed3-TaDGAT2. In this vector, TaDGAT2 is flanked on its 5'-end by the strong seed-specific promoter for the soybean glycinin-1 gene and on its 3'-end by the glycinin-1 3'-untranscribed region. The backbone of this vector is derived from pCAM-BIA0380 and was engineered with the DsRed marker gene under the control of the constitutively expressed cassava mosaic virus promoter for selection of transgenic seeds by fluorescence (Lu and Kang, 2008).

Yeast and *Arabidopsis* transformation and selection

The constructs pESCTaDGAT2, pYAtDGAT1, and pYCrDGAT2 were transformed into *S. cerevisiae* H1246 (W303; MAT α are1- Δ ::HIS3 are2- Δ ::LEU2 dgal1::KanMX4 lro1- Δ ::TRP1 ADE2 met ura3) (Sandager *et al.*, 2002) using the PEG/lithium acetate method (Gietz and Woods, 1998). Yeast cells harbouring the empty pESC-URA or pYes2 vector were used as negative control. Transformants were selected by uracil prototrophy on yeast synthetic medium (YSM) containing 2% (w/v) glucose and lacking uracil (Invitrogen, Carlsbad, CA, USA). For functional expression, YSM containing 2% (w/v) raffinose was inoculated with the yeast transformants and grown at 28 °C for 24 h in a shaker at 350 rpm. For induction, YSM containing 2% (w/v) galactose was inoculated with raffinose-grown cultures to obtain an OD of 0.2 at 600 nm and grown at 28 °C for 48 h. Cells were harvested by centrifugation and used for TAG analysis and microsome isolation.

The binary vector containing the cassette for seed-specific expression of TaDGAT2 was introduced into *Agrobacterium tumefaciens* by electroporation. Transgenic plants were generated by floral dip (Clough and Bent, 1998) of *Arabidopsis* Col-0 or the *fad3fae1* mutant (Smith *et al.*, 2003). DsRed marker was used for selection of transformant seeds which were also PCR confirmed. Expression of transgenes in developing seeds was confirmed by reverse-transcription PCR (Supplementary Methods).

Microscopic imaging of yeast TaDGAT2 transformants

To examine lipid bodies, pellets of transformed yeast cells were washed twice in water and stained with Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) (Sigma, Saint Louis, MO, USA) as described by Kimura *et al.* (2004). Fluorescence microscopic images of the stained cells were recorded as described by Kimura *et al.* (2004) using excitation wavelength at 480–520 nm and emission wavelength at 510 nm.

Preparation of yeast microsomes

Yeast microsomes were prepared from cells from 150 ml stationary phase culture and resuspended in 10 ml lysis buffer A (50 mM HEPES pH 7.5, 50 mM NaCl, 1 mM EDTA, 2 mM DTT, 20% sucrose) containing 100 μ l proteinase inhibitor cocktail (Sigma) and 5 ml of 400 μ m glass beads. The mixture was vortexed vigorously for 30 seconds followed by 30 seconds of incubation on ice between intervals for 10 cycles and centrifuged at 10,000 g at 4 °C for 15 min.

The supernatant was further centrifuged at 4 °C for 2 h at 100,000 g. The pellet was resuspended in buffer B (50 mM HEPES pH 7.5, 50 mM NaCl and 20% glycerol) and stored at –80 °C in 40 μ l aliquots. The concentration of microsomal protein was determined using bovine serum albumin as standard with Bradford reagent (Fermentas, Glen Burnie, MD, USA).

Acyl-CoA synthesis

Synthesis of radiolabelled acyl-CoAs was conducted as previously described (Rajasekharan *et al.*, 1993) (Supplementary Methods). The yield of acyl CoA was 1–2 μ Ci (~50% conversion). The acyl-CoA eluate was determined to be radiopure by analysis on TLC developed in butanol:acetic acid:water (50/20/30, v/v/v).

DGAT assay

The DGAT activity assay was performed using DAG and radiolabelled acyl-CoA substrates in reaction buffer containing 0.2 M Tris HCl (pH 7.4), 60 mM MgCl₂, 40 mM DTT, 120 mM sucrose, 0.02 mM [1-¹⁴C]acyl-CoA, 0.4 mM DAG and 20 μ g of microsomal protein, in a total volume of 200 μ l. DGAT reactions were initiated by adding [1-¹⁴C]acyl CoA. DAG substrates (16:0/18:1, 18:1/18:1, and 18:0/18:2) were purchased from Nu-Chek Prep (Elysian, MN, USA).

The reaction mixture was incubated for 10 min at 30 °C in a water bath. The reaction was stopped by adding 1 ml of heptane/isopropanol (3:2, v/v) and centrifuged for 2 min at 1000 g. The heptane phase (500 μ l) was transferred into a new microcentrifuge tube. The extraction was repeated a second time with 500 μ l heptane, mixed and centrifuged, followed by transfer of 500 μ l heptane phase into the first extract. Each DAG-acyl-CoA combination was assayed three times. The heptane extract was dried under N₂, resuspended in 40 μ l chloroform and spotted on TLC plates (Sigma). The TAG product was resolved by TLC using a solvent system of heptane:diethyl ether:acetic acid (70/30/1, v/v/v). Lipid bands were revealed by staining with iodine vapor. The TAG band was scraped off the plate and measured by liquid scintillation counting. Similar methods were used for measurement of wax synthase activity (Supplementary Methods).

Analysis of the fatty acid composition of lipid classes from *Arabidopsis* lines expressing TaDGAT2

For measurement of *Arabidopsis* seed oil composition and content, ~10 mg of seeds were weighed in a 13 \times 100 mm glass screw-cap test tube. To each tube, 1.5 ml of 2% sulphuric acid in methanol, 400 μ l toluene, and 100 μ l of 10 mg ml⁻¹ triheptadecanoin in toluene (Nu-Chek Prep, Elysian, MN, USA) as an internal standard were added. The tubes were purged with nitrogen, capped, and heated at 90 °C for 1 h. Fatty acid methyl esters (FAMES) generated by the transesterification reaction were extracted by addition of 1 ml H₂O and 1.5 ml heptane to each tube. The heptane layer was recovered and transferred to autosampler vials, following thorough mixing and centrifugation. The extracted FAMES were analysed by gas chromatography using an Agilent 7890 gas chromatograph with flame ionization detection (FID). Oil content was calculated by FID response of sample components relative to 17:0 methyl ester from the internal standard. Stereospecific analyses of TAG and PC were conducted as previously described (Cahoon *et al.*, 2006).

Electrospray ionization MS/MS analysis of PC and TAG molecular species

Mass spectrometry analysis was conducted using an 4000 QTRAP linear ion trap quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) to characterize PC and TAG molecular

species. Seed lipid extraction protocol and detailed instrument analysis conditions are provided in [Supplementary Methods](#).

Results

Isolation of a *T. aureum* DGAT2-related gene

A cDNA library of *T. aureum* ATCC 34304 was generated that contained approximately 2.5×10^6 clones with an average insert size of 700 bp. Random sequencing of 5'-ends of cDNAs from this library resulted in the identification of a 795-bp partial clone, *TA1424*, with homology to known DGAT2-type cDNAs. *TA1424* was subsequently used for isolation of a full-length cDNA designated *TaDGAT2*, encoding a 594 amino acid polypeptide (GenBank accession number JX185322). Homology searches with the coding sequence for amino acids 198–532 revealed closest identity to predicted DGAT2s from *Dictyostelium* species (~48% identity) and slightly lesser identity to fungal DGAT2s from species such as *Umbelopsis ramanniana* and *Ustilago maydis* (40–45% identity) (**Fig. 1**). The 334 amino acid domain encoded by *TaDGAT2* was also related to mammalian DGAT2s (~40% identity) and more distantly related to DGAT2s from green algae and higher plants (~30% identity) (**Fig. 1**). The 197 amino acid N-terminus and 61 amino acid C-terminus of the

TaDGAT2 have no homology to any known polypeptides. Using the SOSUI secondary structure prediction program, the entire *TaDGAT2* polypeptide contains two predicted transmembrane domains encompassing amino acids 153–175 and 219–241. This is similar to plant DGAT2s, which also have two predicted transmembrane domains in the vicinity of their N-termini ([Shockey *et al.*, 2006](#); [Liu *et al.*, 2011](#)). The secondary structure prediction for *TaDGAT2*, like that for plant and *Saccharomyces cerevisiae* DGAT2s, would potentially orient the N- and C-termini of this polypeptide on the cytosolic side of the ER ([Shockey *et al.*, 2006](#); [Liu *et al.*, 2011](#)).

TaDGAT2 is a functional DGAT

To establish the function of the *TaDGAT2* polypeptide, its complete coding sequence was expressed in the TAG-deficient *S. cerevisiae* strain H1246, which contains disruptions of the four acyltransferase genes that contribute to TAG synthesis ([Sandager *et al.*, 2002](#)). For these experiments, *TaDGAT2* was assembled under control of the galactose-inducible *GAL10* promoter in pESC-URA to generate the plasmid pESC-*TaDGAT2*. Large and distinct oil bodies were observed in the Nile red-stained cells harbouring pESC-*TaDGAT2* after 48 h of galactose induction (**Fig. 2A**). Little or no fluorescence

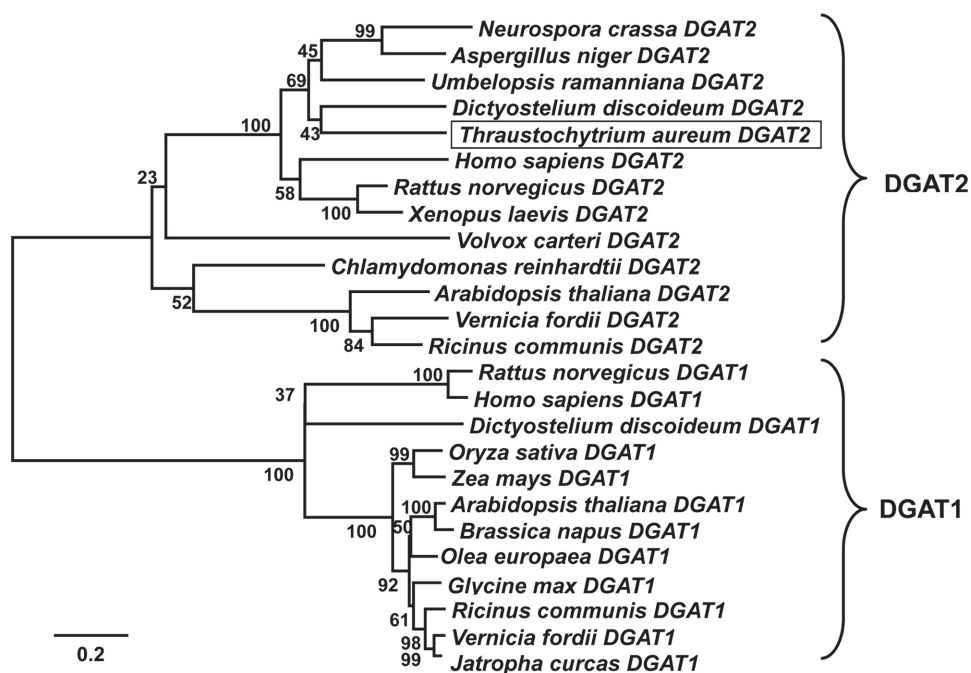


Fig. 1. Unrooted phylogram of *Thraustochytrium aureum* diacylglycerol acyltransferase (DGAT) and other hypothetical or functionally characterized DGATs. The alignment was generated using CLUSTAL W and the unrooted phylogram was constructed by the neighbour-joining method in MEGA4 software ([Tamura *et al.*, 2007](#)). Accession numbers of the sequences are: *A. niger* DGAT2, XP_001396146; *A. thaliana* DGAT1, CAB44774; *A. thaliana* DGAT2, NP_566952; *B. napus* DGAT1, AF164434; *C. reinhardtii* DGAT2, XP_001693189; *D. discoideum* DGAT1, XP_645633; *D. discoideum* DGAT2, XP_635762; *G. max* DGAT1, AAS78662; *H. sapiens* DGAT1, NP_036211.; *H. sapiens* DGAT2, NP_477513; *J. curcas* DGAT1, ABB84383; *N. crassa* DGAT2, XP_965438; *O. europaea* DGAT1, AAS01606; *O. sativa* DGAT1, BAD53762; *R. communis* DGAT1, XP_002514132; *R. communis* DGAT2, XP_002528531; *R. norvegicus* DGAT1, NP_445889; *R. norvegicus* DGAT2, NP_001012345; *T. aureum* DGAT2, JX185322; *U. ramanniana* DGAT2, AAK84179; *V. fordii* DGAT2, XP_002949151; *V. fordii* DGAT1, ABC94471; *V. fordii* DGAT2, ABC94473; *X. laevis* DGAT2, NP_001083204; *Z. mays* DGAT1_2, ABV91586.

was detected in the cells transformed with pESC-URA vector (Fig. 2A). In addition, TLC analysis of extracted lipids from these cells revealed a strong accumulation of TAG that was absent from cells with only the expression vector (Fig. 2B). As a further test of DGAT function, *TaDGAT2*-expression was found to rescue the known toxicity of exogenous oleic acid to the yeast H1246 strain (Siloto *et al.*, 2009a, b). As shown in Fig. 2C, H1246 cells transformed with the empty pESC-URA vector were unable to grow, but these cells were fully viable in cells expressing *TaDGAT2*. These results indicate

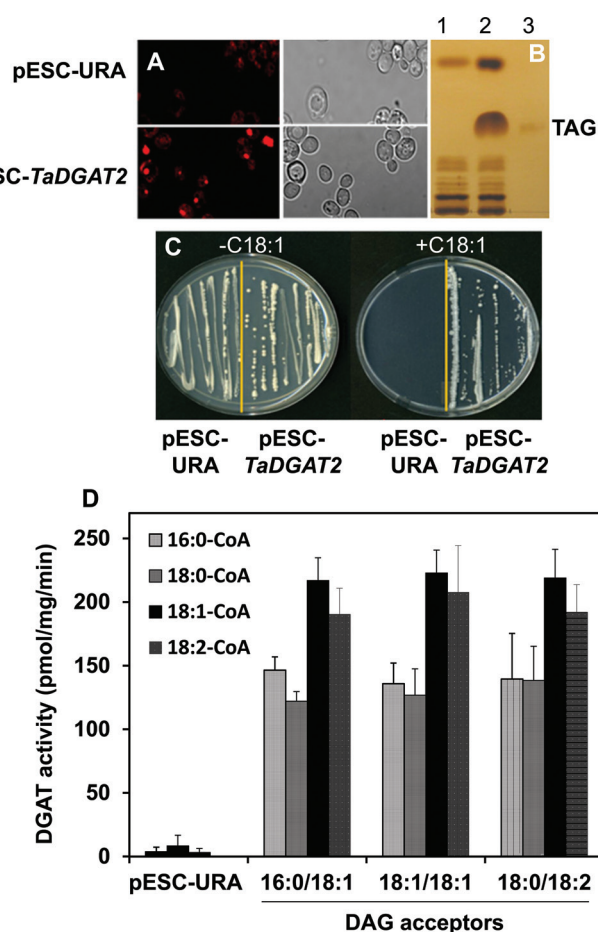


Fig. 2. *Thraustochytrium aureum* diacylglycerol acyltransferase (DGAT) functionally complements the triacylglycerol (TAG)-deficient *Saccharomyces cerevisiae* H1246 mutant and displays broad substrate specificity. (A) Nile red staining (left) and interference contrast imaging (right) of transformants with an empty vector (pESC-URA) and the expression vector harbouring *TaDGAT2* (pESC-*TaDGAT2*) at stationary phase. (B) TLC analysis of neutral lipids from yeast transformants: 1, pESC-URA; 2, pESC-*TaDGAT2*; 3, TAG standard. (C) Rescue of oleic acid (C18:1) toxicity of triacylglycerol-deficient *S. cerevisiae* H1246 cells by expression of *TaDGAT2* (pESC-*TaDGAT2*), but not by the pESC-URA control. (D) DGAT activity in microsomal fractions from yeast cells transformed with pESC-URA (control) or pESC-*TaDGAT2* vectors. Values are mean \pm SD ($n = 3$). Shown for the pESC-URA reactions are results for assays using [14 C]18:1-CoA, and diacylglycerol (DAG) species: 16:0/18:1, 18:1/18:1, and 18:0/18:2.

that *TaDGAT2* has DGAT activity sufficient to restore TAG synthesis to *S. cerevisiae* H1246.

TaDGAT2 is active with structurally diverse substrates

To examine the substrate properties of the *TaDGAT2* enzyme, microsomal fraction from yeast H1246 cells expressing this enzyme was assayed for acyl-CoA dependent DGAT activity by measuring the incorporation of [14 C]acyl-CoA into DAG acceptors. For these studies, radiolabelled acyl-CoA esters of palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), and linoleic acid (18:2) and the 1,2-DAG acceptors 16:0/18:1, 18:1/18:1, and 18:0/18:2 were used as substrates. Microsomes from cells expressing *TaDGAT2* were able to catalyse TAG formation from all three DAG acceptors with each of the four [14 C]acyl-CoA substrates. By comparison, only trace levels of DGAT activity were detected in cells expressing the vector control (Fig. 2D). The endogenous fatty acid profile of H1246 consists mainly of 16:0, 16:1, 18:0, and 18:1. DGAT activity with [14 C]16:0-CoA or [14 C]18:0-CoA was similar with all three DAG substrates tested (130 ± 10 pmol $\text{mg}^{-1} \text{min}^{-1}$) (Fig. 2D). Higher activities were detected with [14 C]18:1-CoA (220 ± 5 pmol TAG $\text{mg}^{-1} \text{min}^{-1}$) and [14 C]18:2-CoA ($190\text{--}208$ pmol TAG $\text{mg}^{-1} \text{min}^{-1}$) with the three DAG acceptors (Fig. 2D). These results indicate that *TaDGAT2* is active with a broad range of saturated, monounsaturated, and polyunsaturated acyl-CoA and DAG substrates. We also assayed microsomes from the recombinant yeast for their ability to use palmitoyl- and oleoyl-fatty alcohols as acceptors for acyl-CoA substrates in a wax synthase-type reaction. Activity was detectable with both fatty alcohol substrates (Supplementary Fig. S1), suggesting that *TaDGAT2* can use substrates other than DAG as acyl-CoA substrates.

T. aureum cells naturally accumulate the long-chain polyunsaturated fatty acid DHA. Although not a direct measure of DGAT activity, this study tested the ability of yeast H1246 cells expressing *TaDGAT2* to incorporate exogenously provided DHA as well as the polyunsaturated fatty acids eicosapentaenoic acid (EPA) and arachidonic acid (ARA) into TAG (Supplementary Methods, Supplementary Fig. S2 and Supplementary Table S1). Cells provided with EPA and ARA accumulated these fatty acids in TAG to amounts of 10–15% of the TAG fatty acids, whereas only low levels of DHA were detected in TAG of cells provided with this fatty acid, likely because of limited uptake of DHA by the H1246 cells. More detailed studies were conducted with *TaDGAT2*-expressing cells provided EPA. For comparison, the studies were also conducted with exogenously provided oleic acid as well as with yeast cells expressing *Arabidopsis DGAT1* (*AtDGAT1*) and *Chlamydomonas reinhardtii DGAT2* (*CrDGAT2*). EPA and oleic acid were readily incorporated into TAG by cells expressing each of these DGATs as determined by GC analysis of isolated TAG. The production of TAG was similar for *TaDGAT2*, *AtDGAT1*, and *CrDGAT2* with oleic acid and EPA (Supplementary Fig. S2). Exogenous oleic acid and EPA were provided in combination to cells expressing each DGAT to gauge selectivity of the expressed DGATs and DAG-generating acyltransferases (e.g. GPAT and LPAT). No

preferential incorporation of oleic acid or EPA was detected in TAG of cells expressing *TaDGAT2*, *AtDGAT1*, or *CrDGAT2* (Supplementary Fig. S2), suggesting that *TaDGAT2* (as well as *AtDGAT1* and *CrDGAT2*) has broad substrate specificity and no detectable selectivity for oleic acid and EPA, which is consistent with results from the *in vitro* assay of *TaDGAT2* with C16 and C18 substrates.

Seed-specific expression of *TaDGAT2* in *Arabidopsis thaliana* seeds enhances oleic acid content

TaDGAT2 was expressed under control of the strong seed-specific glycinin-1 promoter in *A. thaliana* Col-0 to examine its ability to enhance seed oil content. The *Arabidopsis fad3fae1* mutant was initially used for these experiments because the fatty acid composition of its seed oil more closely approximates that of seeds of vegetable oil crops such as maize, low-linolenic acid soybean, safflower, and sunflower. The *fad3fae1* mutant has point mutations that inactivate *FAD3*, encoding the $\Delta 15$ desaturase, and *FAE1*, encoding fatty acid elongase-1 (Cahoon *et al.*, 2006). These mutations result in an enrichment in linoleic acid (18:2) in the seed oil due to the loss of linolenic acid (18:3) and very-long-chain fatty acids ($\geq C20$) (Cahoon *et al.*, 2006). Analysis of seeds from T_2 plants of 15 independent lines expressing *TaDGAT2*, as confirmed by reverse-transcription PCR (Supplementary Fig. S3), revealed little or no increase in total oil content (data not shown). Instead, the more striking phenotype was an increase in oleic acid content of transgenic seeds, with amounts of oleic acid exceeding 50 wt% of the total oil content (Fig. 3A). By comparison, oleic acid levels typically ranged from 26–29 wt% of the total seed oil of non-transformed *fad3fae1* plants (Fig. 3A). This near-doubling of the oleic acid content occurred primarily at the expense of the linoleic acid content of seeds in the total lipids and TAG, which decreased by nearly the same amount as the oleic acid increase in seeds of the top *TaDGAT2*-expression lines (Tables 1 and 2). Oil content was re-evaluated in seeds from T_4 plants of lines 2 and 6, which accumulated the highest levels of oleic acid (Table 1). Seeds from both lines contained absolute amounts of oil 1.5% greater than those of seeds from non-transformed plants grown under identical condition. This oil increase, however, was within the standard error measured for seeds from four independent plants for each line.

The effect of *TaDGAT2* expression was also examined in seeds of wild-type *A. thaliana*. In seeds of five independent lines, increased amounts of oleic acid were detected relative to the seeds from non-transformed plants (Fig. 3B). The highest amounts of oleic acid detected in seeds of these lines was 27.6 wt% of the total fatty acids, an approximately 50% increase relative to the 18 wt% oleic acid in seeds of control plants (Fig. 3B and Table 3). In contrast to the *fad3fae1* background, no significant decrease was detected in linolenic acid content of the transgenic seeds. Instead, the increased levels of oleic acid were accounted for almost entirely in linolenic acid content (Table 3). As with *fad3fae1* plants, no significant change in oil content was detected in seeds of the transgenic wild-type *Arabidopsis* (Table 3).

Strong enhancement of oleic acid content is detected in the *sn*-2 position of TAG and in PC of *TaDGAT2*-expressing *fad3fae1* seeds

To assess the possibility that the increase in oleic acid is restricted to the *sn*-3 position of TAG in *fad3fae1* seeds expressing *TaDGAT2*, stereospecific analysis of the *sn*-2 position was conducted following digestion of isolated TAG with a lipase specific for the *sn*-1 and *sn*-3 positions. Fatty acid analysis of the *sn*-2 monoacylglycerol resulting from this digestion revealed a 2.5-fold increase in the relative content of oleic acid in the *sn*-2 position of TAG from line 6 relative to TAG from seeds of control plants (Table 4). In TAG from the engineered seeds, nearly half of the *sn*-2 fatty acids were present as oleic acid. Based on this finding, it is concluded that the oleic acid increase in *TaDGAT2*-expressing seeds is not due to the exclusive incorporation of oleic acid at the *sn*-3 position via direct catalysis by *TaDGAT2*. Similarly, analysis of the major seed phospholipid PC revealed a 2.4-fold increase in the total oleic acid content of PC in seeds from line 6 relative to PC isolated from non-transformed *fad3fae1* seeds (Table 2). The relative oleic acid content of PC was, in fact, nearly identical to that of TAG in the *TaDGAT2*-expressing seeds. Consistent with this, LC electrospray ionization MS/MS analysis of intact TAG and PC from seeds

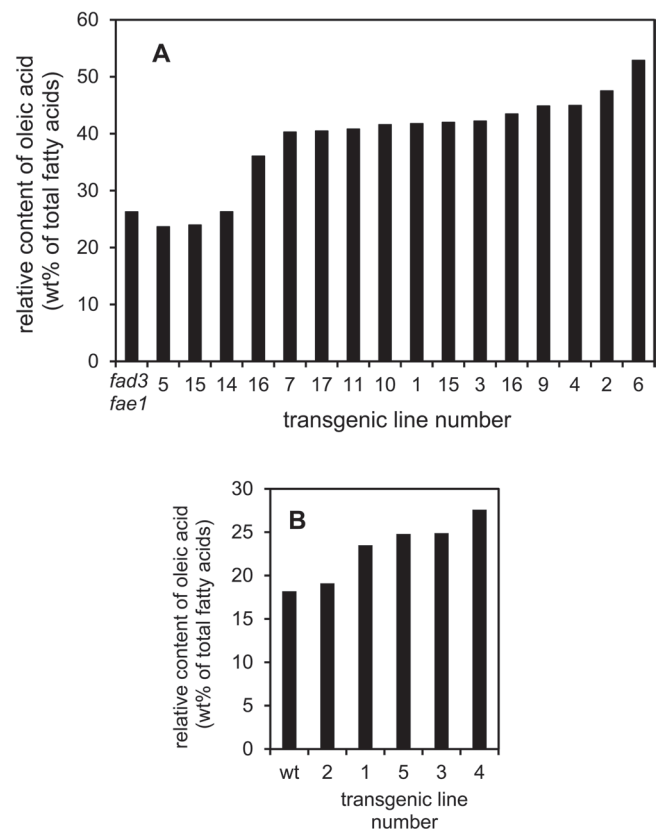


Fig. 3. Distribution of oleic acid content in the total lipids of seeds from transgenic *Arabidopsis fad3fae1* mutant (A) and wild-type (B) backgrounds expressing *TaDGAT2*. The values shown are from seeds of independent transgenic events, with the event number indicated, or from non-transgenic *fad3fae1* or wild-type (wt) controls.

Table 1. Fatty acid composition and oil content of mature seeds from *Arabidopsis fad3fae1* and two independent transgenic *fad3fae1* lines expressing *TaDGAT2* (lines 2 and 6)Values are mean \pm SE ($n = 4$).

Transgenic line	Fatty acid composition (wt% of total fatty acids)						Oil content (% dry weight)
	16:0	18:0	18:1	18:2	18:3	Others	
<i>fad3fae1</i>	8.7 \pm 0.1	4.0 \pm 0.4	28.9 \pm 1.1	54.7 \pm 0.6	1.7 \pm 0.2	2.1 \pm 0.2	28.0 \pm 1.9
<i>fad3fae1</i> line 2	8.1 \pm 0.2	2.7 \pm 0.1	45.1 \pm 1.0	39.8 \pm 0.7	1.9 \pm 0.1	2.5 \pm 0.1	29.5 \pm 1.0
<i>fad3fae1</i> line 6	7.3 \pm 0.1	2.7 \pm 0.1	51.4 \pm 1.6	34.0 \pm 1.6	1.7 \pm 0.1	2.8 \pm 0.1	29.6 \pm 0.6

Table 2. Fatty acid composition of TAG and PC of lipid extracts from mature seeds of *A. thaliana fad3fae1* and the engineered *fad3fae1* line 6Values are mean \pm SE ($n = 4$).

Lipid class	Fatty acid composition (wt% of total fatty acids)					
	16:0	18:0	18:1	18:2	18:3	Others
<i>fad3fae1</i>						
TAG	9.0 \pm 0.6	3.4 \pm 0.0	30.3 \pm 1.2	53.7 \pm 0.7	1.8 \pm 0.2	1.8 \pm 0.3
PC	11.2 \pm 0.4	1.3 \pm 0.0	21.7 \pm 0.8	60.3 \pm 0.4	3.7 \pm 0.2	2.0 \pm 0.1
<i>fad3fae1</i> line 6						
TAG	7.3 \pm 0.3	2.8 \pm 0.1	51.5 \pm 1.2	33.1 \pm 1.9	1.5 \pm 0.1	3.8 \pm 0.7
PC	6.9 \pm 0.5	0.5 \pm 0.1	51.3 \pm 3.0	37.7 \pm 2.3	2.5 \pm 0.1	1.1 \pm 0.1

PC, phosphatidylcholine; TAG, triacylglycerol.

Table 3. Fatty acid composition and oil content of mature seeds from *Arabidopsis Col-0* and two independent transgenic *Col-0* lines expressing *TaDGAT2* (lines 3 and 4)Values are mean \pm SE ($n = 4$).

Transgenic lines	Fatty acid composition (wt% of total fatty acids)									Oil content (% dry weight)
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	Others	
<i>Col-0</i>	7.3 \pm 0.1	3.2 \pm 0.1	18.2 \pm 0.3	30.1 \pm 0.4	14.8 \pm 0.2	2.5 \pm 0.1	19.5 \pm 0.4	1.7 \pm 0.1	2.6 \pm 0.1	28.2 \pm 2.0
<i>Col-0</i> line 3	6.8 \pm 0.2	2.9 \pm 0.1	27.6 \pm 1.1	29.7 \pm 0.6	7.8 \pm 0.2	2.3 \pm 0.1	19.1 \pm 0.2	1.1 \pm 0.1	2.7 \pm 0.1	28.7 \pm 3.3
<i>Col-0</i> line 4	6.9 \pm 0.1	3.1 \pm 0.1	24.4 \pm 0.6	31.4 \pm 0.3	9.0 \pm 0.3	2.4 \pm 0.1	18.7 \pm 0.6	1.2 \pm 0.1	2.7 \pm 0.1	27.5 \pm 0.5

Table 4. Fatty acid species at the *sn*-2 position of TAG isolated from mature seeds of *A. thaliana fad3fae1* and the engineered *fad3fae1* line 6Values are mean \pm SE ($n = 4$). ND, not detected.

Fatty acid	Fatty acid composition (wt% of total fatty acids in <i>sn</i> -2 position)	
	<i>fad3fae1</i>	<i>fad3fae1</i> line 6
	16:0	ND
18:0	ND	ND
18:1	19.5 \pm 0.5	48.5 \pm 1.9
18:2	78.2 \pm 0.4	49.7 \pm 1.9
18:3	2.3 \pm 0.2	1.8 \pm 0.1

uncovered an increase in molecular species containing oleic acid in *TaDGAT2*-engineered plants relative to *fad3fae1* controls (Fig. 4). These alterations included significant increases in relative amounts of triolein and di-oleoyl-PC (Fig. 4B, D). Overall, these findings indicate that expression of *TaDGAT2* enhances the overall oleic acid content of seeds, while

yielding corresponding reductions in polyunsaturated C18 fatty acids.

Discussion

This study isolated a gene, designated *TaDGAT2*, from the marine protist *T. aureum* encoding a polypeptide with a 334 amino acid domain most related to DGAT2s from *Dictyostelium* species. *TaDGAT2* was able to restore TAG biosynthesis to the *S. cerevisiae* H1246 mutant, which contains disruptions of four acyltransferase genes that contribute to TAG synthesis (Sandager *et al.*, 2002). This study also showed that the *TaDGAT2*-encoded enzyme catalyses the esterification of fatty acids from acyl-CoA donors to DAG acceptors in a manner consistent with DGAT activity. In addition, *TaDGAT2* displayed nearly the same activity with ¹⁴C-16:0-, 18:0-, 18:1-, and 18:2-CoAs, and DAG species containing 16:0/18:1, 18:1/18:1, and 18:0/18:1. *S. cerevisiae* H1246 cells expressing *TaDGAT2* were also able to incorporate exogenous DHA into TAG. Similarly, broad substrate specificity has been reported for insect cell-expressed

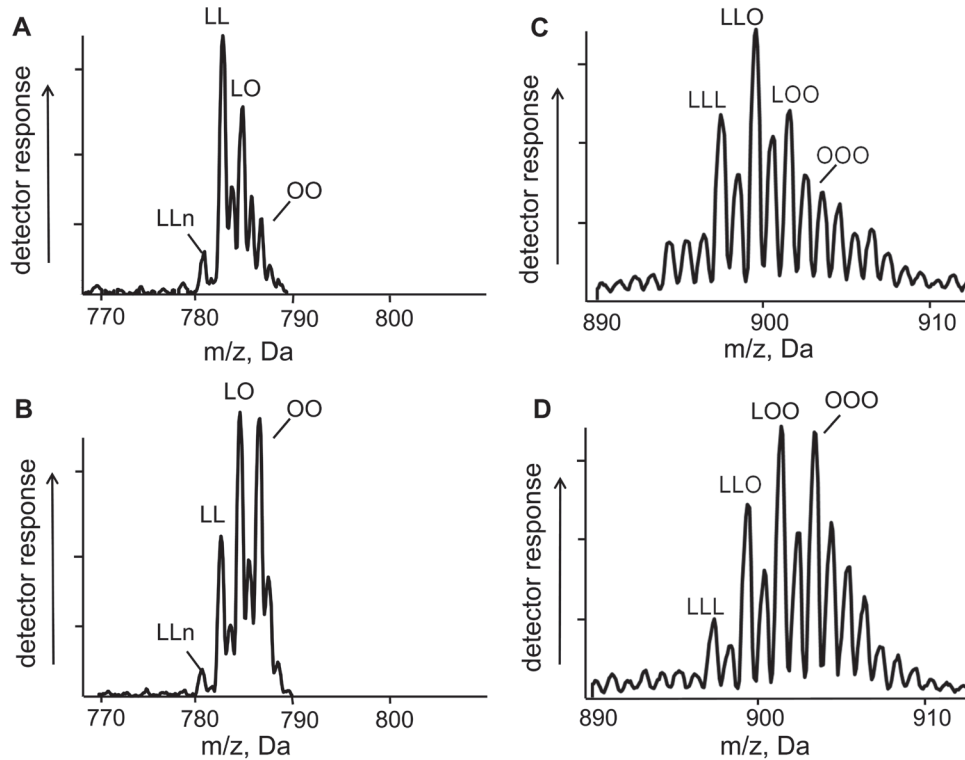


Fig. 4. Electrospray ionization MS/MS scans showing the fatty acid components and relative abundances of intact phosphatidylcholine (PC) and triacylglycerol (TAG) species from *fad3fae1* and *TaDGAT2*-engineered *Arabidopsis* seeds: (A, B) C36 species of PC in *Arabidopsis fad3fae1* background (high linoleic acid) (A) and *fad3fae1* + *TaDGAT2* (B). (C, D) C54 species of TAG in *Arabidopsis fad3fae1* background (C) and *fad3fae1* + *TaDGAT2* (D). Fatty acid components of intact lipid species are indicated by abbreviations Ln (18:3), L (18:2), O (18:1); however, the labels are not intended to indicate the *sn*-1, 2, or 3 positional distribution of the fatty acids.

mammalian DGAT1 and DGAT2 and *Umbelopsis ramanniana* DGAT2A and DGAT2B. In the latter case, *Umbelopsis ramanniana* DGAT2A and DGAT2B were active with an 18:1-CoA substrate and DAG acceptors containing C16-C18 fatty acids (Oakes *et al.*, 2011). Overall, the present data from yeast expression studies conclusively demonstrate that *TaDGAT2* is a functional DGAT. Similar to other DGATs from both classes, *TaDGAT2* does not possess the known ER retrieval motifs (–KXXKXX–COOH or –KKXX–COOH) but in the vicinity of the C-terminus, the motif YKSKW is present, which is similar to a pentapeptide aromatic ER retrieval motif – ϕ –XXK/R/D/E– ϕ , where ϕ represents a large hydrophobic amino acid (McCartney *et al.*, 2004). A notable feature of *TaDGAT2* is a nearly 200 amino acid N-terminal extension that lacks significant homology to any known DGAT2 or any other known polypeptide. A 243 N-terminal extension was also recently identified in a *N. crassa* DGAT2 that lacks identity with any known polypeptide (Oakes *et al.*, 2011). Like the *N. crassa* DGAT2, *TaDGAT2* is predicted using SOSUI and TMPred databases to be largely hydrophilic but contains a transmembrane domain immediately preceding the N-terminus of the DGAT2-related domain. Although the *N. crassa* DGAT2 and *TaDGAT2* extensions share little homology, the function, if any, of this N-terminal extension remains unclear. In the case of the *N. crassa* DGAT2, the complete polypeptide and a truncated form lacking the N-terminal 243-amino acids were compared for their

ability to enhance DGAT activity and increase oil content upon expression in maize seeds (Oakes *et al.*, 2011). In these experiments, the truncated form yielded transgenic maize seeds with higher DGAT activity and oil content (Oakes *et al.*, 2011). This result does not exclude the possibility that the N-terminal extensions of *TaDGAT2* and the *N. crassa* DGAT2 have functional significance in their native hosts.

It is also notable that wax synthase activity is detectable with *TaDGAT2* in *in vitro* assays. Wax synthase activity has also been detected with a number of other DGATs, including mouse DGAT1 and bifunctional wax synthase/DGAT-type enzymes from organisms such as *Acinetobacter calcoaceticus* and *Arabidopsis* (Kalscheuer and Steinbuchel, 2003; Yen *et al.*, 2005; Li *et al.*, 2008). In addition, the human wax synthases AWAT1 and AWAT2 belong to DGAT2 family whose members are known to possess the characteristic HPHG motif which is found in AWAT1 and AWAT2 and is present in *TaDGAT2* (Turkish *et al.*, 2005).

In this study, the seed-specific expression of *TaDGAT2* resulted in no detectable increase in oil content of wild-type *Arabidopsis* seeds. In the *Arabidopsis fad3fae1* background that has seed oil enriched in linoleic acid, an increase in absolute oil content of seeds of 1.5% was detected with the expression of *TaDGAT2*. Although this absolute increase in oil content is similar to that reported for the *Umbelopsis ramanniana* DGAT2 in soybean (Lardizabal *et al.*, 2008), it was not statistically significant in the current study. More rigorous

multigenerational analysis and testing in multiple environments is needed to fully assess the efficacy of TaDGAT2 for generating modest enhancements in seed oil content, as described for the *Umbelopsis ramanniana* DGAT2 in soybean (Lardizabal *et al.*, 2008).

The more striking phenotype associated with the transgenic expression of *TaDGAT2* in wild-type and *fad3fae1* plants was a large increase in oleic acid content. The increase was more pronounced in the *fad3fae1* background where the oleic acid content was increased by ~75% from that in seeds of non-transgenic controls versus a ~50% increase in the wild-type background. The result was the generation of a 'mid-oleic acid' profile in the seed oil of engineered *fad3fae1* background with the oil containing over 50% oleic acid. In the case of seeds of *TaDGAT2* expressed in Col-0 wild-type plants, most of the oleic acid increase was at the expense of linolenic acid (18:3) content, with little or no change detected in relative amounts of linoleic acid (18:2). By contrast, the oleic acid increase in the *fad3fae1* background was almost entirely at the expense of linoleic acid content. Such an increase in oleic acid was not previously reported in DGAT1 or DGAT2 overexpression studies in dicot species, including *Arabidopsis*, *Brassica napus*, and soybean (Jako *et al.*, 2001; Lardizabal *et al.*, 2008; Weselake *et al.*, 2008). Oleic acid increases, however, have been observed in two studies in DGAT overexpression studies in maize (Zheng *et al.*, 2008; Oakes *et al.*, 2011). With overexpression of a high-activity maize DGAT1, oleic acid content of maize seed oil was doubled, resulting in ~45% oleic acid content of the oil of the engineered seeds (Zheng *et al.*, 2008). Similarly, seed-specific expression of the *Umbelopsis ramanniana* and *N. crassa* DGAT2s resulted in as much as a 75% increase in oleic acid content of maize seed oil (Oakes *et al.*, 2011). Interestingly, similar studies conducted with the *Umbelopsis ramanniana* DGAT2 in soybean yielded only small increases in oleic acid content that were within the natural variation detected for non-engineered soybean seeds (Lardizabal *et al.*, 2008). These conflicting results for DGAT-associated oleic acid increases between plants such as maize and soybean may be related to the differences in fatty acid profiles of seeds of these species, based on the current findings in *Arabidopsis*. As has been shown, the oleic acid increase in *TaDGAT2*-engineered plants was larger in the *fad3fae1* mutant, which has a similar fatty acid profile as maize seeds. Soybean seed oil, by contrast, typically contains 7–15% linolenic acid, which is similar to the linolenic acid content of wild-type *Arabidopsis* seeds. From these observations, one hypothesis requiring further examination is that the use of DGAT1/2 expression as a biotechnological approach for oleic acid enhancement is most effective in seeds naturally low in linolenic acid or *fad3* mutants with low linolenic acid oils, such as 'low-lin' soybean mutants that have received recent commercial attention for improved oil-oxidative stability (Graef *et al.*, 1988; Clemente and Cahoon, 2009).

Based on this study's findings, the enhanced oleic acid content of the *TaDGAT2*-engineered seeds does not result from the specific introduction of oleic acid at the *sn*-3 position by TaDGAT2. Although K_m measurements or competition assays were not conducted with different substrates,

these assays indicated that TaDGAT2 is able to use a range of acyl-CoA and DAG substrates and has no apparent strict specificity for oleoyl-CoA or oleic acid-containing DAGs. Moreover, strong enhancement of oleic acid was measured in the *sn*-2 position of TAG and in PC in the top *fad3fae1* lines expressing TaDGAT2. These findings suggest that overall oleic acid content of seeds was increased due to the expression of TaDGAT2. As shown in Fig. 5, DAG is a common substrate for TAG and PC synthesis. Oleic acid entering PC would be available for the desaturation reactions by FAD2 and FAD3, which use PC-linked fatty acid substrates for the synthesis of linoleic and linolenic acids, respectively. It is likely that enhanced flux through DGAT, resulting from strong seed-specific TaDGAT2 expression, pulls DAG toward TAG synthesis and effectively reduces the conversion of oleic acid into polyunsaturated fatty acids via PC. The phenotype observed in the *TaDGAT2*-expressing seeds has similarity to the enhanced oleic acid profile of *Arabidopsis* PC:DAG cholinephosphotransferase (PDCT) mutant seeds, which are blocked in flux of oleic acid-DAG into oleic acid-PC for FAD2- and FAD3-catalysed desaturation (Lu *et al.*, 2009). One possibility is that the presence of an active FAD3 may pull flux more strongly through PDCT. This may explain the differences observed between soybean versus maize and between wild-type *Arabidopsis* versus *fad3fae1* mutant for DGAT-mediated oleic acid enhancement. It is also possible that PDCT is less active in maize seeds compared to soybean seeds, such that DGAT-overexpression more effectively shifts

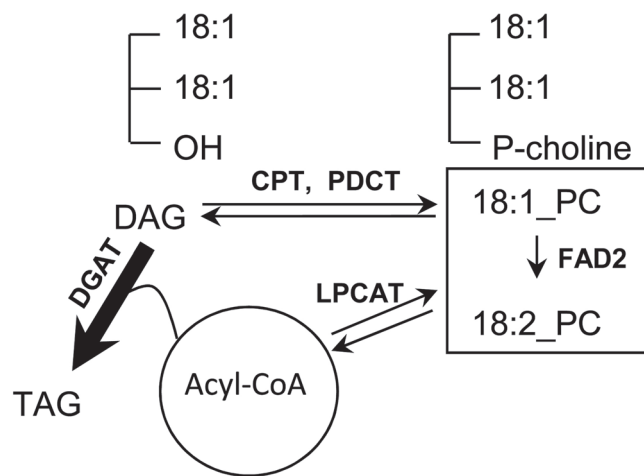


Fig. 5. A model of triacylglycerol (TAG) biosynthesis from diacylglycerol (DAG) in seeds of *Arabidopsis fad3fae1 + TaDGAT2* transgenic lines. As shown, enhanced DGAT flux mediated by TaDGAT2 pulls oleoyl-DAG moieties toward TAG synthesis and away from phosphatidylcholine (PC)-linked desaturation reactions catalysed by $\Delta 12$ oleic acid desaturase (FAD2) and initiated by PC:DAG cholinephosphotransferase (PDCT), which catalyses head group exchange between DAG and PC. DAG is also converted to PC by the enzyme CDP choline:DAG cholinephosphotransferase (CPT). Lysophosphatidylcholine acyltransferase (LPCAT) catalyses the reversible exchange of fatty acids between the *sn*-2 position of PC and the acyl-CoA pool and contributes along with PDCT to PC-linked desaturation of oleic acid.

oleoyl-DAG flux to TAG synthesis in maize seeds. The presence of the enhanced oleic acid phenotype in PC as well as TAG suggests that TaDGAT2 may compete primarily with PDCT for DAG substrates, rather than with CPT (Fig. 5). This would effectively reduce desaturation through PC without affecting bulk PC synthesis by CPT.

Oleic acid enhancement of vegetable oils has been a major biotechnological target. Oils enriched in oleic acid have improved oxidative stability for food processing as well as biofuel and bio-based products. These oils have also been in increasing demand for use in foods with hydrogenated oils that are low in *trans*-fats (Damude and Kinney, 2008a, b). High oleic acid phenotypes have been achieved primarily by seed-specific suppression of FAD2 (Buhr *et al.*, 2002). The current findings combined with those from previous reports suggest that an alternative route to high oleic oils would involve a strategy of seed-specific enhancement of DGAT activity (Zheng *et al.*, 2008; Lu *et al.*, 2009; Oakes *et al.*, 2011), possibly using TaDGAT2 or a fungal DGAT2, and seed-specific suppression of PDCT in a low linolenic acid background, such as maize or low-lin soybean seeds, to shift oleoyl-DAG strongly to the synthesis of TAG and away from PC-based desaturation pathways. Seed-specific suppression of additional enzyme activities such as lysophosphatidylcholine acyltransferase (LPCAT) (Xu *et al.*, 2012) that are associated with oleic acid flux through PC may also contribute to further enhancement of high oleic acid phenotypes in seeds.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Table S1. Arachidonic acid and docosahexaenoic acid content of TAG from *TaDGAT2*- or *AtDGAT1*-expressing yeast H1246 cells.

Supplementary Fig. S1. Wax ester synthase activity in microsomal fractions from yeast cells transformed with pESC-URA or pESC-TaDGAT2 vectors.

Supplementary Fig. S2. Eicosapentaenoic acid and oleic acid content of TAG from *TaDGAT2*-, *AtDGAT1*-, or *CrDGAT2*-expressing yeast H1246 cells.

Supplementary Fig. S3. *TaDGAT2* expression in transgenic *Arabidopsis fad31fae1* plants.

Supplementary Methods. Supplementary experimental procedures.

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Supplementary Material

Preparation of Arabidopsis DGAT1 and Chlamydomonas reinhardtii DGAT2 Yeast Expression Constructs

Arabidopsis DGAT1 (AtDGAT1, Accession# NP_179535) and *Chlamydomonas reinhardtii* DGAT2 (CrDGAT2, Accession# XP_001693189) were subcloned in *Bam*HI/*Xba*I and *Hind*III/*Eco*RI sites of pYes2 vector, yielding *pYAtDGAT1* and *pYCrDGAT2*, respectively. The AtDGAT1 open-reading frame was amplified from an Arabidopsis cDNA library using the forward and reverse oligonucleotides: 5'-CTAGGATCCACCATGGCGATTTTGGATTTC-3' and 5'-ATATCTAGATCATGACATCGATCCTTTTC-3'. The CrDGAT2 open-reading frame was amplified from cDNA using the forward and reverse oligonucleotides: 5'-AGCAAGCTTACCATGGCTAACCAACTAGAG-3' and 5'-GTAGAATTCTCATTGCACGATGGCCAGCG-3'.

Expression analysis of TaDGAT2 in Arabidopsis

Total RNA was extracted from siliques of *Arabidopsis* plants transformed with *TaDGAT2* as described in (Suzuki *et al.*, 2004), 1 µg of DNase treated total RNA was used for first-strand cDNA synthesis using SuperScript VILO (Invitrogen, Grand Island, NY USA). PCR reactions were carried out using the *TaDGAT2* gene specific primers TaDG2f 5'-ATGCTCGGGTACTACATCTGGATC-3' and TaDG2r 5'-GGCCCAACGTGAGCCTGTAG-3' and the positive control *FAD2* (L26296) gene specific primers FAD2f GCATCATGATGTTAACCGTCCAG-3' and FAD2r 5'-CTTTGCCTCCCTATAACATCGCTAC-3'. Amplification for both genes was carried out for 40

cycles (95°/30 sec, 60°C /30 sec and 72°C /1 min) with the final extension step at 72°C for 10 min.

Acyl-CoA Synthesis

Acyl-CoA synthesis was done in 800 µl reaction mixture containing 100 mM MOPS (pH 7.4), 1.2 mM CoA, 2.0 mM ATP, 1 mM MgCl₂, 1 mM DTT, 0.06% Triton X-100, 56 mM [1-¹⁴C] fatty acids (55 mCi/mmol, American Radiolabeled Chemicals, Saint Louis, MO) mixed in 2% Triton X-100 before the reaction mixture was added. Synthesis reactions were conducted with 2.75-5 µCi of [1-¹⁴C] palmitic acid, [1-¹⁴C] stearic acid, [1-¹⁴C] oleic acid, and [1-¹⁴C] linoleic acid. The mixture was sonicated and 0.1 unit of acyl-CoA synthetase (Sigma, Saint Louis, MO USA) was added followed by incubation at 35°C for 1.5 h in a water bath and mixed every 5 minutes. After the incubation 650 µl of 100% methanol was added and the mixture was loaded onto a Supelclean LC-18 SPE Tube (Supelco, Bellefonte, PA USA) preconditioned according to manufacturer's guidelines. Acyl-CoA was eluted with 1 ml of 50% methanol. The eluate was dried with rotary evaporation and dissolved in 100 mM NaC₂H₃O₂ (pH 5.0) and stored at -20°C.

ESI-MS/MS analysis of PC and TAG molecular species

Mass spectrometry analyses were conducted using an Applied Biosystems (Foster City, CA) 4000 QTRAP linear ion trap quadrupole mass spectrometer to characterize PC and TAG molecular species. Total neutral lipids were extracted from 5 mg dry seeds according to the method of (Bligh and Dyer 1959). Total neutral lipids were dissolved in 100 µl of chloroform:methanol (1:1). For phospholipid analysis, a 1:100 dilution of the extract was made in methanol:isopropyl alcohol:water (70:5:25) containing 10 mM ammonium formate (PC buffer A). Fifty µl of the lipid solution was introduced into the mass spectrometer by direct injection followed by a gradient consisting of 100% Buffer A/0% Buffer B (0-6 minutes, 10 µl

per min) followed by 100% PC Buffer B methanol:isopropyl alcohol: water (94:5:1) containing 10 mM ammonium formate to rinse the lines in preparation for the next injection. PC species were monitored by ESI-MS/MS Precursor Ion (184.1 Da) scans set to monitor masses in positive mode from 500 to 1000 m/z with a cycle time of 3 sec. Data is the average of 10 cycles. Instrument settings were: Source temperature 300° C, ESI needle voltage 5.5 kV (positive mode), desolvation potential (DP) 120, Entrance potential (EP) 11, Collision energy (CE) 42, Collision cell exit potential (CXP) 12, CAD gas medium, Curtain gas (CUR) 10, gas1 10 arbitrary units, gas2 90 arbitrary units. For triacylglycerol analysis, the total neutral lipid extract was diluted 1:5000 in water:isopropyl alcohol (60:40) containing 25 mM ammonium formate and directly infused into the mass spectrometer at a rate of 20 µl per minute. A Q1 scan (positive mode) was performed over a mass range of 200 to 1000 m/z with a cycle time of 2 sec. Data was collected for 150 cycles. Instrument settings were: Source temperature 300° C, ESI needle voltage 5.5 kV (positive mode), desolvation potential (DP) 90, Entrance potential (EP) 10, Curtain gas (CUR) 10, and gas 1 (GS1) 50 arbitrary units, gas 2 (GS2) 40 arbitrary units.

TAG analysis of TaDGAT2 yeast transformants fed docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and oleic acid (18:1)

Cultures of the TAG deficient *Saccharomyces cerevisiae* strain H1246 expressing *TaDGAT2*, *AtDGAT1* or *CrDGAT2* under galactose induction was grown as described in the paper. Fatty acid substrates (500 µM DHA, EPA or 250 µM/250 µM EPA/18:1) and Tergitol (1%) were added in 2.5 hrs after the induction of expression in the cultures which had been inoculated to OD₆₀₀ = 0.2. Total neutral lipids were extracted from the pellet of a 30 ml stationary phase culture according to the method described by Bligh and Dyer (1959).

Triheptadecanoyl (100 µg) was used for TAG quantification. Total neutral lipids were dissolved

in 40 μ l of chloroform and spotted on thin layer chromatography (TLC) plates (Sigma, Saint Louis, Missouri USA) using a solvent system of heptane:diethyl ether:acetic acid (70:30:1, v/v/v). The band corresponding to TAG was scraped off the plate and transesterified using 2.5% (v/v) sulfuric acid/methanol at 80°C for 30 min. Fatty acid methyl esters were extracted in 500 μ l of heptane and used for gas chromatography analysis as described in the paper.

In vitro wax ester synthesis

Assay conditions and product analyses for wax ester synthesis were the same as for the DGAT activity assay, except that the diacylglycerol substrate was replaced with oleoyl or palmitoyl fatty alcohols as acceptors for [$1-^{14}\text{C}$] 18:0 or [$1-^{14}\text{C}$] 18:1-CoA substrates.

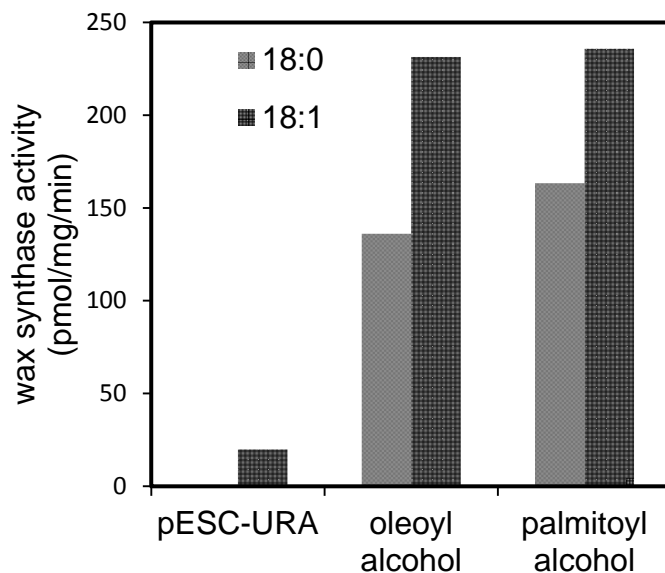
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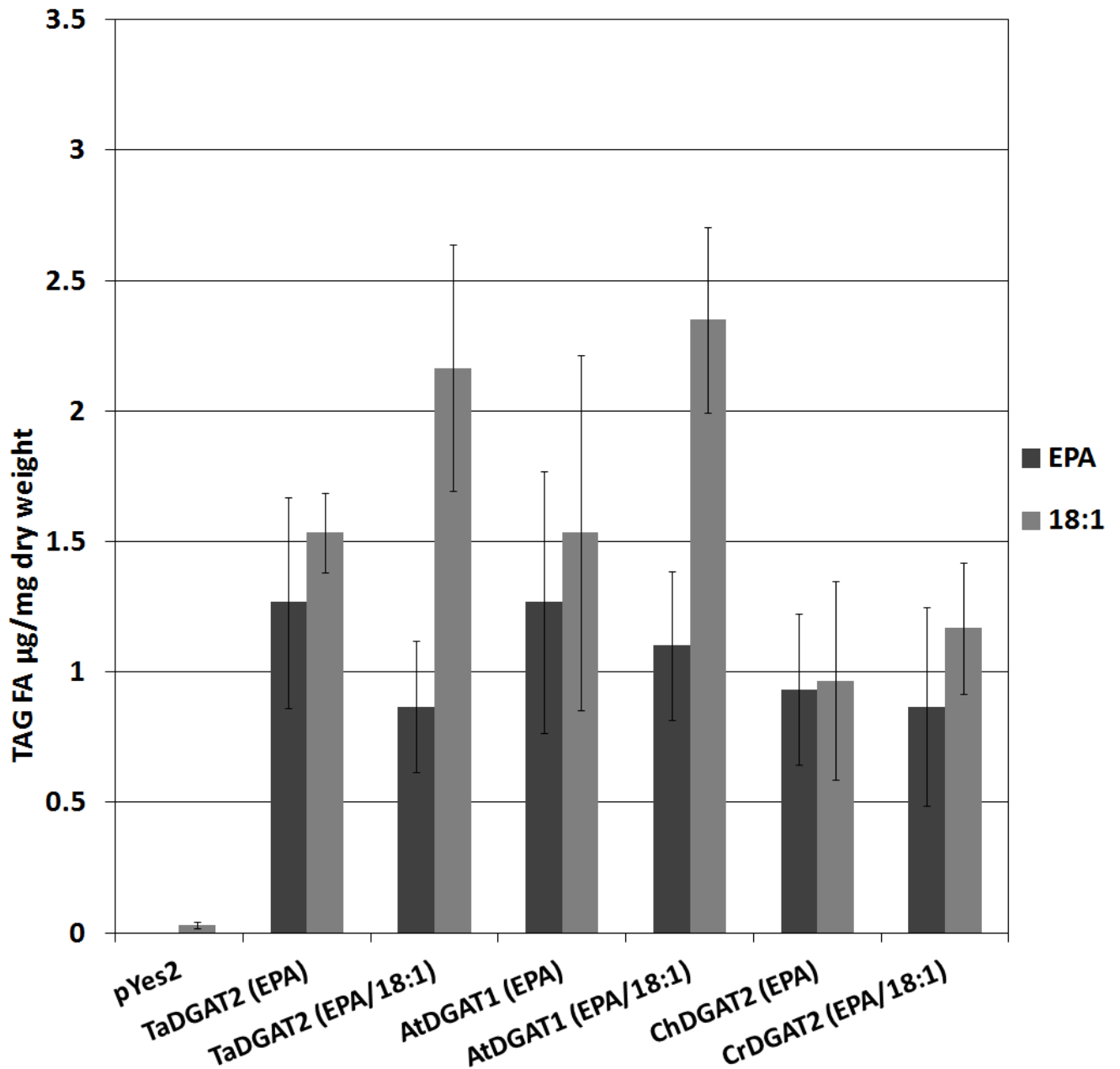
Supplementary Table 1. Arachidonic acid (ARA) and docosahexaenoic acid (DHA) content of TAG from *TaDGAT2*- or *AtDGAT1*-expressing H1246 yeast cells fed ARA or DHA. 250 μ M of ARA or DHA were provided to cells as indicated. TAG was also analyzed from cells expressing *TaGAT2*- and *AtDGAT1*-expressing yeast cells not provided ARA or DHA.

Expressed genes (supplied fatty acid)	Fatty acid composition (wt% of TAG fatty acids)					
	14:0	16:0	16:1	18:0	18:1	DHA
<i>TaDGAT2</i>	4.0	22.0	34.5	11.0	27.0	
<i>AtDGAT1</i>	6.8	21.0	42.0	6.0	17.0	
<i>TaDGAT2</i> (ARA)	3.0	28.0	21.0	14.0	18.0	14.0
<i>TaDGAT2</i> (DHA)	3.5	22.0	31.5	11.0	26.0	3.0
<i>AtDGAT1</i> (ARA)	6.0	24.0	29.7	6.6	12.8	14.8
<i>AtDGAT1</i> (DHA)	6.8	20.0	41.8	6.4	17.0	2.0

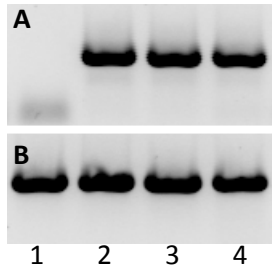
Supplementary Figures



Supplementary Fig. 1. Wax ester synthase activity in microsomal fractions from yeast cells transformed with pESC-URA (control) or pESC-*TaDGAT2* vectors. Shown for the pESC-URA reactions are results for assays using [1-¹⁴C] oleoyl (18:1)-CoA and oleoyl alcohol. Assays were conducted using [1-¹⁴C] oleoyl (18:1)-CoA or [1-¹⁴C] stearoyl (18:0)-CoA and oleoyl alcohol or palmitoyl alcohol.



Supplementary Fig. 2. Eicosapentaenoic acid (E PA) and oleic acid (18:1) content of TAG from *TaDGAT2*, *AtDGAT1*, or *CrDGAT2* expressing H1246 yeast cells fed EPA (500 µM) or EPA+18:1 (250 µM/250 µM). Only trace amounts of 18:1 were observed in TAG from biomass of yeast cells transformed with pYes2 (control). Values are means of three experiments with SD (Bars are averages \pm SD ($n=3$)).



Supplementary Fig. 3. *TaDGAT2* expression in transgenic *Arabidopsis fad3/fae1* plants as assessed by RT-PCR of cDNA from RNA extracted from siliques containing developing seeds. PCR products obtained with gene specific primers for *TaDGAT2* (Panel A) and *FAD2* (Panel B) positive control, *fad3fae1* (Lane 1), *fad3fae1*-Line 2 (Lane 2), *fad3fae1*-Line 4 (Lane 3), and *fad3fae1*-Line 6 (Lane 4).