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Expression of a type 2 diacylglycerol acyltransferase from *Thalassiosira pseudonana* in yeast leads to incorporation of docosahexaenoic acid β-oxidation intermediates into triacylglycerol

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Glycerolipids of the marine diatom *Thalassiosira pseudonana* are enriched particularly with eicosapentaenoic acid (EPA), and also with an appreciable level of docosahexaenoic acid (DHA). The present study describes the functional characterization of a type 2 diacylglycerol acyltransferase (DGAT2, EC 2.3.1.20) from *T. pseudonana*, designated TpDGAT2, which catalyzes the final step of triacylglycerol (TAG) synthesis. Heterologous expression of this gene restored TAG formation in a yeast mutant devoid of TAG biosynthesis. TpDGAT2 was also shown to exert a large impact on the fatty acid profile of TAG. Its expression caused a 10–12% increase of 18:1 and a concomitant decrease of 16:0 relative to that of AtDGAT1 (*Arabidopsis thaliana*). Furthermore, in the presence of the very-long-chain polyunsaturated fatty acids (VLCPUFA) EPA and DHA, TAG formed by TpDGAT2 displayed three- to six-fold increases in the percentage of VLCPUFA relative to that of AtDGAT1 even though TpDGAT2 conferred much lower TAG-synthetic activities than Arabidopsis DGAT1. Strikingly, when fed DHA, the yeast mutant expressing TpDGAT2 incorporated high levels of EPA and DHA isomers derived from DHA β-oxidation. In contrast, no such phenomenon occurred in the cells expressing AtDGAT1. These results suggested that, in addition to the role in breaking down storage lipids, yeast peroxisomes also contribute to lipid synthesis by recycling acyl-CoAs when a fatty acyl assembly system is available to capture and utilize the fatty acyl pools generated via β-oxidation. Our study hence illustrated a case where the efficiency and substrate preference of an acyltransferase can elicit far reaching metabolic adjustments that affect TAG composition.

Introduction

Diacylglycerol acyltransferase (DGAT) mediates the final step of the glycerolipid synthesis pathway for the production of triacylglycerol (TAG), the major storage lipid in most eukaryotes [1]. In eukaryotic cells, three

Abbreviations
DAG, diacylglycerol; DGAT, acyl-CoA:diacylglycerol acyltransferase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ER, endoplasmic reticulum; SC-ura, synthetic complete medium lacking uracil; TAG, triacylglycerol; VLCPUFA, very-long-chain polyunsaturated fatty acids.

types of DGATs have been identified: the endoplasmic reticulum (ER) localized DGAT1, DGAT2 and a soluble cytosolic DGAT3. The *Arabidopsis DGAT1* gene was cloned in 1999 by several groups [2–4], and homologous *DGAT1* genes from other plants have been cloned subsequently [5–10]. The first type 2 DGAT was identified in the oleaginous fungus *Mortierella ramanniana* [11], and later DGAT2s from human and several plant species were characterized [10,12,13]. DGAT3 is a family of soluble DGATs, which was initially isolated from developing peanut cotyledons (AhDGAT) through protein purification [14] and recently from *Arabidopsis* [15]. Although they catalyze the same reaction, the three types of DGAT belong to different gene families and share little structure and sequence similarity [11,14,16].

Studies on DGAT2 suggested that in plants containing unusual fatty acids DGAT2 may play an important role in channeling unusual fatty acids into seed storage oils [10,13,17]. Transcript analysis through real time RT-PCR in castor bean tissues revealed that *DGAT2* was highly elevated (18-fold) in seeds versus leaves, while the expression of *DGAT1* stayed at a similar low level regardless of developmental stages, hence suggesting that DGAT2 is the major DGAT in TAG biosynthesis in castor seeds, which accumulate high levels of the unusual fatty acid ricinoleate [13]. Accordingly, expression of castor bean DGAT2 in Arabidopsis results in significant increases in ricinoleate in seed oil [17]. The tung tree DGAT2 has also been shown to be much more specific in production of TAG-containing eleostearic acid in seeds than DGAT1 [10]. Additionally, a type 2 DGAT from the fungus *Claviceps purpurea* (CpDGAT2) was recently shown to be involved in the preferential assembly of ricinoleic acid, a hydroxyl fatty acid of industrial importance, into triglycerides [18].

Aside from their differences in substrate specificities, DGAT2 and DGAT1 also differ by their locations in cellular membrane systems. As shown by expressing tung tree (*Vernicia fordii*) DGAT1 and DGAT2 in a tobacco cell line, DGAT1 and DGAT2 reside in distinct punctuated areas of the ER, suggesting that the enzymes are in different ER subdomains [10]. DGAT1 and DGAT2 are also thought to be part of distinctive multiprotein complexes of TAG synthesis [19]. Nevertheless, it should be pointed out that individual DGATs may have multiple subcellular localizations as is the case for mammalian DGAT2, which not only is localized in the ER but also is dynamically associated with lipid droplets, mitochondria-associated membranes and mitochondrial compartments [20].

In the present study, we characterized a type 2 diacylglycerol acyltransferase (TpDGAT2) from the eukaryotic diatom *Thalassiosira pseudonana*. Its TAG-forming activity was verified through heterologous expression in a *Saccharomyces cerevisiae* mutant deficient in TAG synthesis. TpDGAT2 was also shown to have greater preference for very-long-chain polyunsaturated fatty acids (VLCPUFA) than Arabidopsis DGAT1. In feeding experiments with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), TAG formed by TpDGAT2 showed three- to six-fold increases in the percentage of these two fatty acids compared with Arabidopsis DGAT1. Furthermore, the intermediates of DHA breakdown via β-oxidation were found to accumulate at high levels in TAG molecules isolated from the mutant cells expressing TpDGAT2, whereas such phenomenon did not occur in the cells expressing Arabidopsis DGAT1. Our results indicate the existence of a fatty acyl pool originating from the intermediates of β-oxidation in the process of TAG synthesis and also raise the prospects that DGAT1 and DGAT2 may prefer different acyl-CoA pools for TAG synthesis and that coordinated regulation of these two enzymes in eukaryotic cells is critical for maintaining appropriate rates of TAG synthesis as well as balancing fatty acyl-CoA levels in different compartments.

Results

Isolation and characterization of a *TpDGAT2* cDNA from *Thalassiosira pseudonana*

To identify DGAT2 enzyme(s) from *T. pseudonana*, the v3.0 draft genome of this diatom was searched for DNA sequence(s) encoding peptide(s) similar to Arabidopsis DGAT2 and yeast DGA1. Three homologous sequences were identified, and one of them, designated *TpDGAT2*, encodes a peptide with relatively high similarity to known DGAT2s. To dissect the properties of *TpDGAT2,* its full-length cDNA clone was amplified by PCR from a cDNA library of *T. pseudonana*. It contains an open reading frame of 1371 bp, which encodes a polypeptide of 456 amino acids with a calculated molecular mass of 51.5 kDa.

In contrast to the fact that only a single copy of *DGAT2* was identified from other eukaryotes studied, most algal species appeared to have multiple copies of putative *DGAT2s* [21]. For example, three *DGAT2* genes have been identified from the microalga *Ostreococcus tauri*, named *OtDGAT2A*, *OtDGAT2B* and *OtDGAT2C* respectively [22]. The amino acid sequence of TpDGAT2 exhibits 20–30% identity with

OtDGAT2A, OtDGAT2B and OtDGAT2C. TpDGAT2 also shares limited identity (approximately 15–20%) with other DGAT2s from various plant species, such as AtDGAT2 (*Arabidopsis thaliana*, GenBank AT3g51520), OsDGAT2 (*Oryza sativa*, GenBank NP_001057530), VfDGAT2 (*V. fordii*, GenBank ABC94473) and RcDGAT2 (*Ricinus communis*, GenBank AAY16324). The similarity between TpDGAT2 and other DGAT2s from fungi and animals was found to be much lower. Based on phylogenetic analysis using the GENEIOUS4.6.4 software, TpDGAT2 is clustered with the algal OtDGAT2A, and this group is distantly separated from two other clades of DGAT2s (Fig. 1).

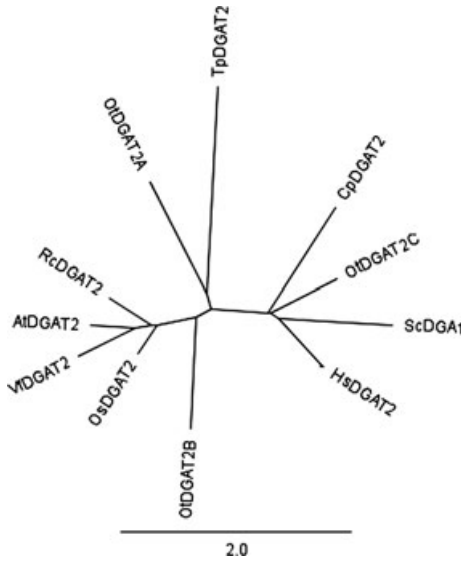

Fig. 1. Phylogram of the phylogenic tree of TpDGAT2 and diacylglycerol acyltransferases from other species: OtDGAT2A (*O. tauri*, GenBank CAL54993), OtDGAT2B (*O. tauri*, GenBank CAL58088), OtDGAT2C (*O. tauri*, GenBank CAL56438), AtDGAT2 (*A. thaliana*, GenBank AT3g51520), OsDGAT2 (*O. sativa*, GenBank NP_001057530), VfDGAT2 (*V. fordii*, GenBank ABC94473), RcDGAT2 (*R. communis*, GenBank AAY16324), HsDGAT2 (*Homo sapiens*, GenBank NP_940914), CpDGAT2 (*C. purpurea*, GenBank ADF29677) and DGA1 (a type 2 DGAT from *S. cerevisiae*, GenBank NP_014888. This phylogenic tree was generated using GENEIOUS4.6.4 program.

Heterologous expression of TpDGAT2 in the yeast TAG deficient mutant

To determine biochemical functionality of TpDGAT2, it was heterologously expressed in the *S. cerevisiae* quadruple mutant strain H1246, which lacks all four genes contributing to TAG synthesis, *ARE1*, *ARE2*, *DGA1* and *LRO1* [23]. The full-length coding region of *TpDGAT2* was cloned into the yeast expression vector pYES2.1/V5-His-TOPO under the control of the galactose-inducible *GAL1* promoter.

The Arabidopsis *AtDGAT1* gene inserted into the same vector was used as a positive control. These two recombinant vectors as well as the empty vector were introduced into H1246, and the transgenic strains harboring *TpDGAT2* and *AtDGAT1* are herein designated TpDGAT2/quadruple and AtDGAT1/quadruple, respectively. As shown by TLC analysis (Fig. 2A), TAG was at undetectable levels in the H1246 strain bearing the empty pYES2.1 vector, whereas expression of *TpDGAT2* and *AtDGAT1* genes restored TAG synthesis in this mutant. The amounts of TAG formed by the two genes, however, were evidently different. The

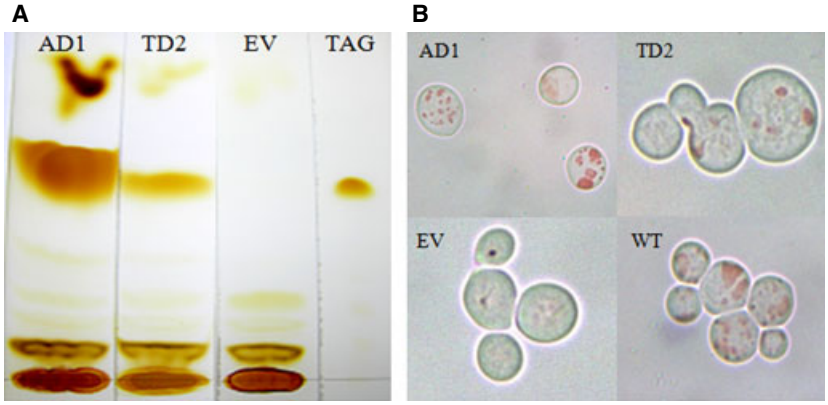

Fig. 2. Expression of TpDGAT2 in the yeast quadruple mutant restored the TAG synthesis. (A) *In vitro* DGAT assays were performed on cell lysates of the yeast quadruple mutant transformed with *TpDGAT2*, *AtDGAT1* (positive control) and the empty vector pYES2.1/V5-His-TOPO in the presence of ^{14}C-labeled acyl-CoAs and *sn*-1,2-diolein (18:1 DAG). (B) Lipid bodies of the quadruple yeast mutant cells expressing TpDGAT2 or AtDGAT1 were stained with Oil Red O and were visualized by bright-field microscopy: AD1, AtDGAT1/pYES2.1; TD2, TpDGAT2/pYES2.1; EV, empty pYES2.1 vector; WT, untransformed wild-type.

TpDGAT2/quadruple strain produced much less TAG than the AtDGAT1/quadruple strain (Fig. 2A).

To visualize the lipid bodies generated by the expression of these two genes, the two yeast transformants were stained with Oil Red O (Fig. 2B). While both TpDGAT2 and AtDGAT1 could rescue the formation of lipid bodies in the quadruple mutant, expression of TpDGAT2 resulted in fewer lipid bodies than that of AtDGAT1. To further assess the capacity of TpDGAT2 for TAG deposition, we compared *in vivo* TAG- forming activities between TpDGAT2 and AtDGAT1 by conducting a ^{14}C-glycerol labeling experiment with yeast cells harboring *TpDGAT2* and *AtDGAT1*, respectively. Incorporation of ^{14}C-label in different lipid classes was determined by scintillation counting, and the ratio of the neutral lipid TAG to phospholipids was calculated. The results from this analysis showed that the *TpDGAT2* transformants had much lower levels of incorporation of ^{14}C-glycerol into TAG than did the *AtDGAT1* transformants (Table 1). Taken together, the TAG-synthetic activity resulting from expression of TpDGAT2 is much lower than that conferred by AtDGAT1 in this heterologous system.

Fatty acid substrate preferences of TpDGAT2

To assess the acyl-CoA substrate preferences of TpDGAT2, microsomal membrane fractions from the TpDGAT2-expressing yeast cells were assayed for DGAT activity using fatty acyl donors of various chain lengths. The AtDGAT1-expressing cells were used as control. AtDGAT1 seemed to utilize a broad range of acyl-CoA donors, whereas TpDGAT2 showed much less preference for 16:0. Nevertheless, both the enzymes displayed a trend of higher activity toward fatty acyl donors with increasing chain length (Fig. 3).

Fatty acid profile in the TpDGAT2/quadruple strain expressing TpDGAT2

We investigated whether expression of TpDGAT2 has any effects on the fatty acid composition of cellular lipids. The AtDGAT1/quadruple and TpDGAT2/quadruple strains were incubated for 3 days at 28 °C in the presence of galactose. The total lipids were extracted and then separated into TAGs and phospholipids by TLC. As shown in Table 2, the expression of TpDGAT2 in the quadruple mutant resulted in differential fatty acid composition of both TAG and phospholipids compared with the strain expressing AtDGAT1. An approximate 12% decrease in 16:0 with a concomitant increase in 18:1 was detected in the TAG species formed by TpDGAT2 relative to those resulting from AtDGAT1 expression. This result is in agreement with the previous finding from an *in vitro* activity assay showing that TpDGAT2 possesses low substrate preference for 16:0. Nevertheless, we cannot rule out the possibility that the decrease in 16:0 was partly attributable to the relatively low DGAT activity of TpDGAT2, which allowed for 16:0 to be elongated and desaturated yielding 18:1. In addition, it was observed that phospholipids from the TpDGAT2-expressing cells had slight increases in 18:1 and decreases of 16:0 (Table 2).

Table 1. Ratio of TAG to phospholipids (PL) in yeast transformants fed ^{14}C-glycerol.

	Ratio TAG/PL	
	^{14}C-glycerol	^{14}C-glycerol + 100 μM DHA
TpDGAT2	0.08 ± 0.012	0.06 ± 0.005
AtDGAT1	1.30 ± 0.128	1.53 ± 0.165
Empty vector	0.004 ± 0.001	0.004 ± 0.001

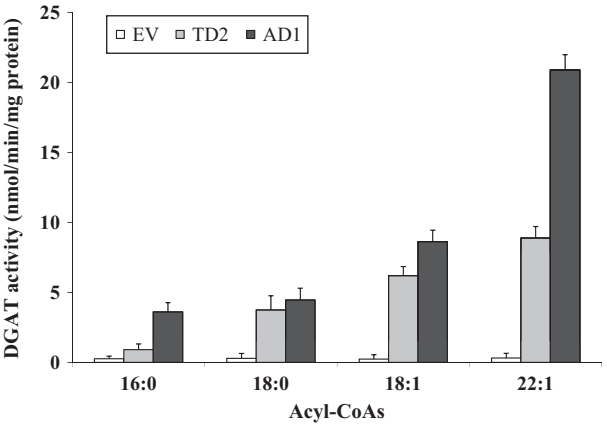

Fig. 3. Substrate preference of TpDGAT2. *In vitro* DGAT assays were performed on microsomal protein of the yeast quadruple mutant transformed with TpDGAT2, AtDGAT1 (positive control) and pYES2.1/V5-His-TOPO plasmid only in the presence of *sn*-1,2-diolein (18:1 DAG) and ^{14}C-labeled 16:0-, 18:0-, 18:1- or 22:1-CoA. AD1, AtDGAT1/pYES2.1; TD2, TpDGAT2/pYES2.1; EV, empty pYES2.1 vector.

Incorporation of EPA, DHA and DHA β-oxidation products into TAG in the mutant strain expressing TpDGAT2

Since glycerolipids of *T. pseudonana* contain a high percentage of the VLCPUFAs EPA and DHA, we tested whether the expression of the *TpDGAT2* gene could lead to increased accumulation of EPA and DHA in yeast glycerolipids. Under galactose induction conditions, the TpDGAT2/quadruple and AtDGAT1/

Table 2. Fatty acid composition of the lipids from the yeast quadruple mutant transformed with *TpDGAT2* or *AtDGAT1*.

	Proportion of fatty acid (mol%)				
	16:0	16:1	18:0	18:1	Unsaturated
TAG					
TpDGAT2	7.80 ± 1.74	31.56 ± 1.38	14.34 ± 1.62	46.31 ± 1.73	77.87 ± 1.96
AtDGAT1	20.75 ± 1.97	30.12 ± 0.50	13.68 ± 2.10	35.45 ± 1.22	65.57 ± 0.54
Phospholipids					
TpDGAT2	7.19 ± 0.69	33.94 ± 0.79	10.30 ± 1.96	48.60 ± 1.03	82.5 ± 1.72
AtDGAT1	12.63 ± 2.33	33.97 ± 2.01	9.24 ± 1.65	44.16 ± 2.12	78.13 ± 1.96

Table 3. Incorporation of EPA and DHA into TAGs in the yeast quadruple mutant (*dga1Δlro1Δare1Δare2Δ*) transformed with the *TpDGAT2* or *AtDGAT1*.

		Mol% of fatty acids in TAGs		
Feeding	Construct	EPA (20:5)	DHA (22:6)	DHA isomer (22:6)
EPA	*TpDGAT2*	3.21 ± 0.65		
	AtDGAT1	0.95 ± 0.20		
DHA	*TpDGAT2*	0.93 ± 0.04	4.75 ± 0.29	10.41 ± 0.71
	AtDGAT1		2.21 ± 0.15	

quadruple strains were cultured in the presence of EPA or DHA. TAGs from 3-day cultures were extracted and analyzed for fatty acid composition. As shown in Table 3, TAG formed by TpDGAT2 had much higher levels of VLCPUFAs compared with AtDGAT1. In the presence of exogenous EPA, EPA accounted for 3.21% (molar percentage) of the total fatty acids in TAG species isolated from the TpDGAT2-expressing strain versus less than 1% for the AtDGAT1-expressing strain. When the cells were fed DHA, a more than two-fold higher incorporation of DHA into TAGs was found in the TpDGAT2/quadruple cells (4.75%) in comparison with the AtDGAT1/quadruple cells (2.21%).

We further discovered that when the TpDGAT2/quadruple strain was fed with DHA, the resulting TAG had two extra peaks next to the regular signal of DHA on the GC data file (Fig. 4A). Repeated experiments confirmed that these peaks were absent in TAG molecules from the AtDGAT1/quadruple sample, indicating that they were not contaminants of the DHA used in the present study. The smaller peak to the left of DHA, representing 0.93% of the total fatty acids, had the same GC retention time as EPA. The peak on the right of DHA did not match any fatty acid standards used in the assay but constituted 10.41% of the total fatty acids of the TAG fraction (Table 3).

GC-MS analysis was conducted to determine the molecular weight of these peaks. As shown in Fig. 4B, the mass spectrum shows diagnostic ions at $m/z = 316$ and 342. The molecular weight is given by (M-CH3) atomic mass units. Since EPA ($C_{20}H_{30}O_2$) and DHA ($C_{22}H_{32}O_2$) have molecular weights of 302 and 328 respectively, the 316 and 342 peaks should correspond to EPA ($C_{20}H_{30}O_2$-CH_3, molecular weight 302 + 15−1 = 316) and DHA ($C_{22}H_{32}O_2$-CH_3, molecular weight 328 + 15−1 = 342) respectively. Thus, this result, along with the finding that the corresponding peak had the same GC retention time as EPA, indicates that the peak on the left of DHA is EPA. The major peak adjacent to the right of DHA has the same molecular weight as DHA-CH3 and hence is most probably a DHA isomer with one double bond at a different position (Fig. 4B). As shown in Fig. 4C, β-oxidation of DHA-CoA (22:6 – 4, 7, 10, 13, 16, 19) starts with an oxidization step catalyzed by acyl-CoA oxidase. The auxiliary enzyme 2,4-dienoyl-CoA reductase and an enoyl-CoA isomerase further modify the DHA-CoA for the general β-oxidation reaction, which produces two DHA isomers with one double bond at different positions (B and C, shown in Fig. 4C). The product observed in our experiment could be one of them. After one circle of β-oxidation reaction, a two carbon unit is removed, producing EPA-CoA (20:5 – 5, 8, 11, 14, 17) (Fig. 4C).

Examination of DHA β-oxidation in yeast β-oxidation mutant

To further prove that TAG of the TpDGAT2/quadruple strain contained intermediates of fatty acid β-oxidation, we introduced TpDGAT2 into the yeast β-oxidation mutant strain *fox1*. Fatty acyl-CoA oxidase FOX1 catalyzes the first step of β-oxidation by creating a double bond between the C-2 and C-3 positions. When TpDGAT2 was expressed in the *fox1* mutant, neither the previously identified EPA nor DHA isomer was detected in the *fox1* cells fed the same batch of DHA as used in other studies (data not shown). This result suggested that the EPA and DHA isomer found in TAG species of the TpDGAT2-expressing quadruple mutant fed DHA were generated via β-oxidation of DHA molecules, thereby providing

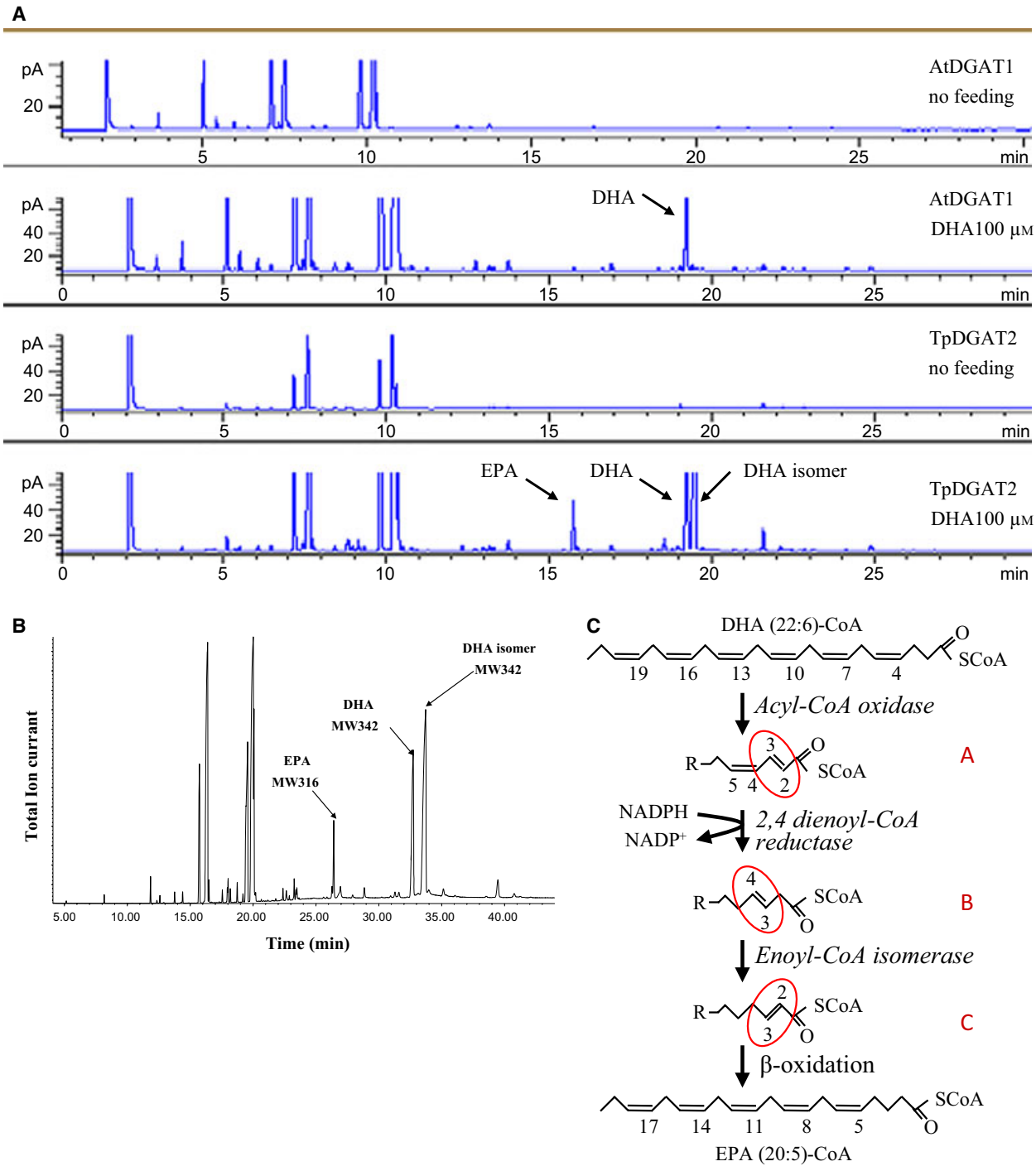

Fig. 4. Fatty acid composition of HPLC purified TAG. (A) GC analysis: 1, AtDGAT1, no feeding; 2, AtDGAT1, DHA (100 μM) feeding; 3, TpDGAT2, no feeding; 4, TpDGAT2, DHA (100 μM) feeding. (B) GC-MS analysis on HPLC purified TAG from the yeast mutant expressing TpDGAT2 in the presence of 100 μM DHA. (C) β-oxidation of docosahexaenoyl-CoA (C22:6 DHA) in yeast peroxisome. (Based on [27].)

an additional line of evidence supporting our conclusion that TpDGAT2 is capable of incorporating intermediates of β-oxidation into TAG.

Discussion

A type 2 DGAT, designated TpDGAT2, was identified from the marine diatom algae *T. pseudonana*. Our results show that this enzyme is likely to play a crucial role in determining fatty acid composition in *T. pseudonana*. Overexpression of TpDGAT2 in a yeast mutant strain lacking TAG synthesis was capable of restoring TAG production, modifying yeast fatty acid composition and incorporating EPA and DHA into TAGs. Although TpDGAT2 exhibited a much lower activity based on either *in vitro* enzyme assays or *in vivo* TAG accumulation compared with AtDGAT1, it showed higher preference for EPA and DHA than did AtDGAT1. Interestingly, TpDGTA2 behaved similarly to the DGAT2 from the microalga *O. tauri*, which exhibits low DGAT activity when expressed in yeast [22]. While factors governing this phenomenon are unclear, it would be critical to improve TpDGAT2 activities in heterologous expression systems for future use to enhance EPA and DHA assembly in plants or other species.

Results from our *in vitro* enzyme assays suggested that TpDGAT2 preferred long-chain fatty acids. The lack of commercially available EPA-CoA and DHA-CoA prevented us from directly examining preferences towards these VLCPUFAs. Hence, we resorted to *in vivo* feeding of DHA to assess the properties of TpDGAT2. This led to a surprising finding that, in addition to DHA, a significant amount of VLCPUFAs in the TAG fraction was in the form of a DHA isoform and EPA. In contrast, TAG containing these DHA derivatives was not detected when TpDGAT2 was expressed in wild-type yeast, nor was it generated when AtDGAT1 was expressed in the quadruple yeast mutant. Given that the most direct way for production of the DHA isoform and EPA from DHA is through β-oxidation and that fatty acid β-oxidation is restricted to peroxisomes in yeast cells [24], it is reasonable to propose that yeast cells may respond to inadequate assembly of fatty acids into TAG by recycling fatty acids via β-oxidation for subsequent incorporation into TAG. This notion is supported by the previous findings that in the developing seeds of the Arabidopsis *tag1* mutant deficient in DGAT1, an increased flow of fatty acids toward β-oxidation occurred and that, even in a plant tissue primarily devoted to the accumulation of storage lipids, a considerable flow toward β-oxidation appeared [25].

The general β-oxidation machinery is composed of acyl-CoA oxidase, multifunctional protein (2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-hydroxyacyl-CoA epimerase), 3-ketoacyl-CoA thiolase and Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase. However, the breakdown of unsaturated fatty acids requires auxiliary enzymes in addition to the enzymes necessary for the β-oxidation of saturated fatty acids [26,27]. DHA-CoA (22:6 – 4, 7, 10, 13, 16, 19) is first oxidized by acyl-CoA oxidase, and then the auxiliary enzyme 2,4-dienoyl-CoA reductase and an enoyl-CoA isomerase are involved to modify the DHA-CoA for the general β-oxidation cycle, and after one circle of β-oxidation reaction a two carbon acetyl-CoA group is removed, producing EPA-CoA (20:5 – 5, 8, 11, 14, 17) (Fig. 4C). Since in the yeast β-oxidation occurs solely in the peroxisome organelle and the peroxisomal membrane is a permeability barrier for a wide variety of metabolites [28] including acyl-CoAs [29], the question now arises how the intermediates of DHA oxidation including EPA become accessible to TpDGAT2 for TAG formation. In higher eukaryotes, there are two mechanisms by which fatty acids can exit the peroxisome: a carnitine-dependent route and a thioesterase-dependent route [30]. In yeast, evidence of involvement of PTE1, the peroxisomal acyl-CoA thioesterase, in transferring acyl moieties out of peroxisomes was recently reported [31]. Hence, the DHA β-oxidation intermediates could be transported out of peroxisomes through either carnitine acetyltransferase (Cat2p), or the thioesterase (PTE1) dependent route, or both. Extensive studies of DHA and EPA metabolism in higher eukaryotes indicated that DHA and EPA possess different metabolic properties [32,33]. DHA is a poor substrate for both mitochondrial and peroxisomal β-oxidation [33], while EPA can be oxidized and to a much greater extent than DHA [34,35]. We propose a model for the TpDGAT2-mediated incorporation of β-oxidation intermediates of DHA into TAG (Fig. 5). The excess DHA from the exogenous feeding was channeled into peroxisomes under the circumstance where the fatty acid assembly system was weak, and the β-oxidation intermediates (EPA and a DHA isomer) were transported out of the peroxisomes. The fatty acids would be activated to acyl-CoAs again in the cytosol to be incorporated into lipids. The synthesis of acyl-CoA could take place in the lipid body where acyl-CoA synthase is localized [36].

A possibility may exist that the peroxisome-derived acyl-CoA pool is in close proximity to the site of TAG synthesis catalyzed by TpDGAT2. This is because one of the β-oxidation intermediates, a DHA isomer (10.41%), was found to be more enriched in TAG than DHA (4.75%), suggesting that the peroxisome-derived acyl-CoAs were more easily accessible to TpDGAT2 than those from the general acyl-CoA pool. DGAT2

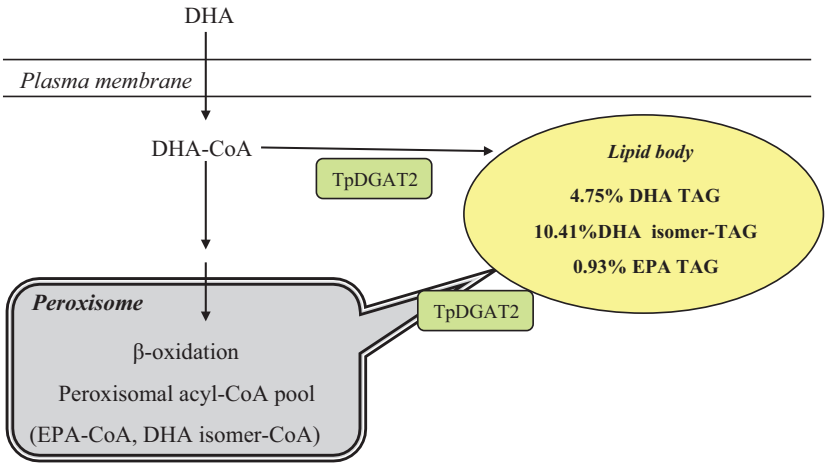

Fig. 5. Efficient incorporation of β-oxidation products into TAG suggests a close connection between peroxisome and lipid body. TpDGAT2-mediated incorporation of β-oxidation intermediates of DHA into TAG, including 0.93% of EPA and 10.41% of a DHA isomer. The higher level of DHA isomer than DHA (4.75%) suggests that these peroxisome-derived acyl-CoAs are more accessible to TpDGAT2 than those from the general acyl-CoA pool.

and DGAT1 are generally believed to be localized in the ER [10], but evidence of their localization in the oil body has also been reported [11,37]. Peroxisomes were found to adhere stably to lipid bodies, and they can even extend into lipid body cores. Furthermore, evidence has been documented that proteins involved in β-oxidation (e.g. acyl-CoA oxidase) are localized close to the inner surface of the peroxisomal membrane at sites of contact with the oil body [38]. A number of studies have suggested that such close associations may also exist in oilseed cells between oil bodies and peroxisomes [39–41]. Although the major function of β-oxidation is to break down fatty acids for energy production, evidence that β-oxidation may play multiple roles in both catabolism and metabolism in plants has been well documented [42–46]. Thus, our finding of the incorporation of peroxisomal β-oxidation intermediates of DHA into TAG provides evidence that DGAT2 is a participant in such paradoxical metabolic interaction.

Materials and methods

Isolation of a DGAT2 cDNA from *Thalassiosira pseudonana*

The draft genome of the diatom *T. pseudonana* was searched using Arabidopsis *DGAT2* and yeast *DGA1* as query. One homologous nucleotide sequence, designated *TpDGAT2*, was retrieved and amplified by PCR as described below. Plasmid from a cDNA library of *T. pseudonana* was used as template. A 50 μL PCR reaction containing 50 ng of plasmid DNA, 20 pM of each primer (5′-GAAATGACAACAAA GAAGCG-3′ and 5′-GTTTGTGCCTGTGTGGTCTG-3′) and 1 μL of BD Advantage™ 2 Polymerase Mix (Clontech Laboratories Inc., Mountain View, CA, USA) was incubated for 30 cycles of the following thermocycle program: 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min 30 s. The PCR product was purified and subsequently cloned into the pYES2.1/V5-His-TOPO expression vector (Invitrogen, Carlsbad, CA, USA).

Heterologous expression of TpDGAT2 in yeast

The TpDGAT2 in the pYES2.1/V5-His-TOPO plasmid was transformed into the yeast quadruple mutant *H1246MATα* (*dga1Δ lroΔ are1Δ are2Δ*) which is deficient in oil synthesis [23] using the S.c. EasyComp TM Transformation Kit (Invitrogen). Yeast cells transformed with Arabidopsis AtDGAT1/pYES2.1 construct and an empty pYES2.1 /V5-His-TOPO plasmid were used as controls. Yeast transformants were selected by growth on synthetic complete medium lacking uracil (SC-ura), supplemented with 2% (w/v) glucose. The colonies were transferred into liquid SC-ura with 2% (w/v) glucose and grown at 28 °C overnight. The overnight cultures were diluted to $A = 0.4$ in induction medium (SC-ura + 2% galactose + 1% raffinose) and were induced by incubating at 28 °C overnight [47]. The yeast cells were collected and broken using glass beads. The protein concentrations in the lysates were normalized using the biorad assay [48] and then assayed for DGAT activity.

Enzyme assays

DGAT assays were conducted at pH 7.4, with shaking at 100 r.p.m. in a water bath at 30 °C for 10 min. Assay mixtures (0.5 mL final volume) contained 100 μg protein lysate normalized as described above, 90 mM HEPES/

NaOH, 200 μM *sn*-1,2 diolein in 0.02% Tween-20, and 18 μM 18:1-CoA (1-^{14}C oleic) (specific activity 2 nCi·nmol^{-1}) as the acyl donor. The ^{14}C-labeled TAGs were isolated by TLC on silica gel G plates developed in hexane : diethyl ether : acetic acid (70 : 30 : 1 v/v/v), the radiolabeled TAG bands were visualized on a Bioscan AR-2000 radio-TLC scanner using Win-Scan 2D© software (Bioscan Inc., Washington, DC, USA) and the bands were scraped and quantified as described by Taylor *et al*. [49].

Lipid body staining with Oil Red O

Lipid bodies of yeast quadruple mutant cells expressing TpDGAT2 or AtDGAT1 were stained with Oil Red O (Sigma-Aldrich) as described previously [38]. Yeast cells were first grown in SC-ura with 2% (w/v) glucose, and then were transferred into 10 mL induction medium (SC-ura + 2% galactose + 1% raffinose, starting at A_{600} = 0.4) and grown at 28 °C overnight. One milliliter of overnight yeast cultures were pelleted by centrifugation at 1200 ***g*** for 5 min, and then washed with 1 mL water and resuspended in freshly filtered Oil Red O solution (one A_{600} unit of yeast per 200 μL of filtered Oil Red O solution) in a 1.5 mL microfuge tube. The tube was vortexed briefly and incubated in the dark at room temperature for 10 min. The stained yeast was washed twice with 0.5 mL of water, resuspended in 50 μL of water and mounted on slides for microscopy. The Oil Red O image was captured by bright-field microscopy.

^{14}C-glycerol feeding

The yeast quadruple mutant cells harbouring TpDGAT2 or AtDGAT1 (TpDGAT2/quadruple or AtDGAT1/quadruple) were first grown in SC-ura with 2% (w/v) glucose at 28 °C overnight and were then transferred into 10 mL induction medium (SC-ura + 2% galactose + 1% raffinose, starting at A_{600} = 0.4) in a 50 mL tube. A mixture of 20 μL ^{14}C-glycerol (specific activity 130 nCi·nmol^{-1}) and 100 μL 3 mM unlabeled glycerol was added to each sample. These yeast cultures were incubated at 28 °C for 4 h, then cells were collected by centrifugation and washed once with fresh medium, and the lipids were extracted using the procedure of Schneiter and Daum [50]. The neutral lipids, TAG and phospholipids were separated by TLC on Silica Gel 60 plates as described above. The TAG and phospholipid bands were scraped off the TLC plates and the total incorporation of label was determined by scintillation counting.

Yeast DHA/EPA feeding and total lipid analysis

Yeast cultures were grown at 28 °C in the presence of 2% (w/v) raffinose and 1% (w/v) Tergitol NP-40 (Sigma, St Louis, MO, USA). Expression of the transgene was induced at an A_{600} of 0.2–0.3 by supplementing galactose to 2% (w/v). At that time, the appropriate fatty acids were added to a final concentration of 100 μM. Yeast cells (20 mL) were harvested after a 3-day incubation, and total lipids from yeast homogenates were extracted by the method of Schneiter and Daum [50]. Separation of neutral lipids and polar lipids was performed by running TLC plates (Si 250-PA, Baker, Phillipsburg, NJ, USA) in a developing solvent of hexane : ether : acetic acid (70 : 30 : 1, v/v/v) and identified by co-migration with known standards. Spots corresponding to TAG and phospholipid were scraped off the TLC plates and were transmethylated with methanolic HCl and quantified by gas chromatography as described previously [51].

GC-MS analysis of DHA β-oxidation products

Neutral lipid TAG was extracted from the TpDGAT2/quadruple yeast strain, and the fatty acids fractions were purified by HPLC. GC-MS analysis was performed using a 2 μL HPLC purified sample with a 40 : 1 split injection on an Agilent 6890 GC with oven temperature set to ramp from 125 °C to 300 °C at 5 °C·min^{-1} and equipped with a DB-5MS column (30 m, 0.25 mm ID, 0.25 μm film thickness, Agilent Technologies), coupled to an Agilent 5973 mass selective detector set to scan between 50 and 700 atomic mass units, under standard electron ionization conditions (70 eV). Compounds were identified by comparison of retention time and mass spectra to standard compounds and quantified by their relative single-ion responses.

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