Defective in Cuticular Ridges (DCR) of Arabidopsis thaliana, a Gene Associated with Surface Cutin Formation, Encodes a Soluble Diacylglycerol Acyltransferase

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A key step in the triacylglycerol (TAG) biosynthetic pathway is the final acylation of diacylglycerol (DAG) by DAG acyltransferase. In silico analysis has revealed that the DCR (defective in cuticular ridges) (At5g23940) gene has a typical HX4D acyltransferase motif at the N-terminal end and a lipid binding motif VX3GF at the middle of the sequence. To understand the biochemical function, the gene was overexpressed in Escherichia coli, and the purified recombinant protein was found to acylate DAG specifically in an acyl-CoA-dependent manner. Overexpression of At5g23940 in a Saccharomyces cerevisiae quadruple mutant deficient in DAG acyltransferases resulted in TAG accumulation. At5g23940 rescued the growth of this quadruple mutant in the oleate-containing medium, whereas empty vector control did not. Lipid particles were localized in the cytosol of At5g23940-transformed quadruple mutant cells, as observed by oil red O staining. There was an incorporation of 16-hydroxyhexadecanoic acid into TAG in At5g23940-transformed cells of quadruple mutant. Here we report a soluble acyl-CoA-dependent DAG acyltransferase from Arabidopsis thaliana. Taken together, these data suggest that a broad specific DAG acyltransferase may be involved in the cutin as well as in the TAG biosynthesis by supplying hydroxy fatty acid.

Triacylglycerol (TAG), an acyl ester glycerol, is the most efficient storage form of energy in eukaryotic cells (1, 2). In plants, TAG is the major component of the seed oils and is mainly deposited during seed and fruit development. Plant oils have a tremendous socioeconomic value in the food industry as well as in the production of lubricants and biofuels. They are also becoming increasingly important raw materials in the petrochemical industry as a substitute for non-renewable crude oil reserves (3). Thus, numerous conventional and molecular genetic strategies have been explored in attempts to increase the TAG content and modify the fatty acid composition of the plant seed oils. Isolation of the genes involved in oil biosynthesis offer exciting opportunities for overexpressing the desired gene of interest so as to redesign the plant oil metabolism for greater yields (3).

TAG is usually synthesized by the sequential incorporation of acyl groups to glycerol 3-phosphate by substrate-specific acyltransferases to form lysophosphatidic acid and phosphatidic acid, respectively (4, 5). The synthesized phosphatidic acid is dephosphorylated to diacylglycerol (DAG). The last step in this pathway is catalyzed by acyl-CoA:DAG acyltransferase (DGAT; EC 2.3.1.20) that transfers an acyl group to DAG to form the TAG. DAG lies at the branch point between phospholipid and storage lipid biosynthesis. Therefore, much research has been focused on the acyl-CoA-dependent reaction catalyzed by the DAG acyltransferase, which is an integral endoplasmic reticulum protein (6) and has also been shown to be present in the oil bodies (7) and plastids (8). Several lines of evidence suggest that the TAG biosynthesis and DGAT activity are not restricted to the embryo and anthers. There are reports of having DGAT activity in leaves (8) and cotyledons of the germinating seeds (9). TAG is also formed in the plants via two different acyl-CoA-independent pathways, catalyzed by phospholipid: DAG acyltransferase (10) and DAG transacylase (11). Initially, a gene encoding DGAT was identified in mice (DGAT1) (12) that is homologous to acyl-CoA:cholesterol acyltransferase. The isolated gene shared a significant sequence homology with Arabidopsis thaliana, and the sequence information was used to isolate DGAT1 from A. thaliana (13–15) and Brassica napus (16). Acyl-CoA:cholesterol acyltransferase-unrelated DGAT2 genes have been isolated from Saccharomyces cerevisiae (17) and Mortierella ramanniana (18). A novel bifunctional DGAT/wax ester synthase was described in Acinetobacter calcoaceticus (19). All of these acyltransferases are membrane-bound, and we have isolated a novel DGAT from developing peanut (Arachis hypogaea) cotyledons (20) and named it DGAT3; unlike all of the above reported DGAT’s, DGAT3 is soluble.

Every plant aerial organ is largely made of cutin, which consists of fatty acids, glycerol, and aromatic monomers. This is the basic adaptation developed by the plants to prevent the water loss by controlling epidermal permeability. Cutin, a polymer of hydroxy fatty acids, forms the covalent linkage to...
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the cell wall. Cutin attaches to the epidermis with a pectinaceous glue layer. Disruption of the pectinaceous glue that attaches the cuticle to the epidermal layer by enzymes or chemicals is required to release the cuticular layer (21). α,ω-Dicarboxylic fatty acid and in-chain hydroxy fatty acids have been reported to be the characteristic monomers of cutin. ω-hydroxy fatty acids are one of the components in lipid polyester but are rarely found in seed oils.

Recently, it has been shown that DCR (defective in cuticular ridgets)-deficient plants have the characteristics of defective cuticle with altered epidermal cell differentiation (22). Defective cuticle in reproductive and vegetative tissues was correlated with the low abundance of 9(10),16-dihydroxyhexadecanoic acid in the cutin polymer of DCR (At5g23940)-deficient plants. DCR mutant lines exhibited more susceptibility to water deprivation, salt, and osmotic stresses that is due to the lack of intact cuticle responsible for protection against abiotic stresses. DCR is also involved in growth, development, post-genital organ fusion, and interactions within the plant organs. However, the authors were unable to elucidate the specific role of At5g23940. Resolving this uncertainty will require definite biochemical characterization.

In the present study, we describe the acylation of DAG to form TAG by the recombinant At5g23940. The gene At5g23940 has a conserved HX₄D motif with no transmembrane domains. Hence, we investigated the gene for the presence of an acyltransferase activity. When the gene was expressed in Escherichia coli, it produced a soluble protein with DGAT activity. The purified recombinant protein is specific for 1,2-DAG and oleoyl-CoA. Overexpression of At5g23940 in S. cerevisiae quadruple DGAT mutant (H1246) resulted in TAG formation. The At5g23940 gene was able to rescue the growth of the mutant in oleate-containing medium. To evaluate the role of this gene in cuticle formation, a precursor (23) (16-hydroxyhexadecanoic acid) was fed to the quadruple mutant transformed with At5g23940, and it was observed that there was a 2-fold increase in the incorporation of hydroxy fatty acid in the total lipids compared with vector control. The hydroxy fatty acid was found to be incorporated into the storage TAG of the quadruple mutant transformed with At5g23940. Taken together, these data strongly support that DCR is a DAG acyltransferase playing a significant role in cutin biosynthesis.

EXPERIMENTAL PROCEDURES

Materials—[1,14C]Oleoyl-CoA (54 mCi/mmole), [1,14C]palmitoyl-CoA (54 mCi/mmole), and [14C]acetate (51 mgCi/mmole) were purchased from PerkinElmer Life Sciences. Peptide, yeast extract, tryptone, yeast nitrogen base, and drop out were obtained from Difco. Silica gel 60 F₂₅₄ TLC plates were from Merck. Oligonucleotide primers, TAG, DAG, other lipid standards, oil red O, BF₃-methanol, 3,3′-diaminobenzidine, and solvents were purchased from Sigma-Aldrich. Acyl-CoAs, acyl acceptors, and hydroxy fatty acids were obtained from Avanti Polar Lipids. Polyclonal antibodies were raised against the nickel-nitritolriacetic acid affinity column-purified recombinant protein as described (24). The At5g23940 clone was obtained from the Arabidopsis Biological Resource Center.

Strains and Culture Conditions—The quadruple mutant S. cerevisiae strain H1246 (MATa are1-Δ::HIS3 are2-Δ::LEU2 dga1-Δ::KanMX4 tpo1-Δ::TRP1 ADE2), which was kindly donated by Dr. S. Stymne (25), and the wild type S. cerevisiae strain (BY4741: MATa; HIS3A1; LEU2Δ0; MET15A0; URA3Δ0) were used as heterologous hosts to study the expression of At5g23940. Transformed yeast cells were cultivated at 30 °C on minimal medium (synthetic dropout medium) lacking uracil and including 2% glucose or galactose.

Prediction of Conserved Protein Domains—Conserved protein domains were examined using the conserved domain data base at NCBI and the pfam data base (both available on the World Wide Web). The multiple sequence alignment was carried out by using ClustalX.

Microarray Expression Analysis of At5g23940—The GENEVESTIGATOR online search tool Meta-Analyzer method was used to retrieve the levels of gene expression during various conditions, such as growth, stimuli, and mutations (26). The expression levels are given as a ratio of expression and microarray signal intensity.

Cloning and Expression of At5g23940—Open reading frame At5g23940 in the pUNI vector was used as a template for amplification of the gene by using forward primer (5′-ATAATG-GATCCATGAAGATAAAGATTATGAGCAA-3′) and reverse primer (5′-ATAATGAATCTCAAAACCCATTG- GCCATTTCC-3′). The PCR mixture consisted of 100 ng of template, 10 pmol of sense and antisense primers, 0.2 mM dNTPs, 1.5 mM MgCl₂, 1 unit of Pfku polymerase (Bangalore Genei, India), and 1 X reaction buffer. PCR conditions used were as follows: initial denaturation of the template at 94 °C for 4 min, followed by 30 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at 53 °C, and 1.5 min of elongation at 72 °C. The final extension was carried out at 72 °C for 10 min. The purified PCR product and pRSET A (Invitrogen) vector were digested with BamHI and EcoRI and ligated directionally. The construct was transformed into E. coli BL21 (DE3) cells and induced with 1 mM isopropyl β-d-thiogalactopyranoside for 4 h at 37 °C. The cell pellet was resuspended in lysis buffer containing 50 mM Tris-HCl (pH 8.0) and 300 mM NaCl. Cells were disrupted by sonication. The sonicated sample was subjected to high speed centrifugation (10,000 × g) for 20 min at 4 °C. The supernatant containing the recombinant protein was allowed to bind to the Ni²⁺-NTA matrix. The column was washed with lysis buffer containing 25 mM imidazole. The bound protein was eluted with 250 mM imidazole in lysis buffer. Fractions (1 ml each) were collected and analyzed on 12% SDS-PAGE, followed by Coomassie Brilliant Blue staining. The purified protein was subjected to dialysis and then used for the DGAT assay. For overexpression of At5g23940 in S. cerevisiae, full-length At5g23940 cDNA was subcloned from pRSET A into pYES2 vector at the same BamHI-EcoRI sites with Kozak consensus sequence in the forward primer (ATTATGG) and transformed into yeast cells by the lithium chloride method (27). Transformants were selected on synthetic minimal medium devoid of uracil (SM-U) containing 2% dextrose and were grown to late log
phase. The change in the carbon source in the medium from glucose (medium YNBD) to galactose (YNBG) induces the expression of a heterologous gene. Galactokinase (GAL1) promoter is present in pYES2 for the induction. The cells were harvested by centrifugation and washed twice with water to remove the residual dextrose from the cells. Washed cells were inoculated at an A_{600} of 0.4 in SM-U medium containing 2% galactose and grown for 24 h. To confirm the protein expression, cells (A_{600} = 5) were resuspended in 50 mM Tris-HCl (pH 7.5) and 2% SDS and then disintegrated using glass beads. The proteins were separated on 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The overexpression of Atg5g23940 was confirmed using anti-Atg5g23940 polyclonal antibodies that were raised against the recombinant protein at a dilution of 1:5,000 (v/v). 3,3'-Diaminobenzidine and H_2O_H_2O were used as substrates for developing immunoblot. Prestained protein molecular weight marker was used to determine the molecular size of the protein.

**DAG Acyltransferase Assay**—The purified recombinant enzyme (1–5 mg) was incubated with 10 mM [1-14C]oleoyl-CoA (110,000 dpm/assay), and 50 mM 1,2-DAG in assay buffer in a total volume of 100 l. The incubation was carried out at 30 °C for 15 min and stopped by the addition of CHCl_3/CH_3OH in a 1:2 ratio (v/v). Lipids were separated by silica TLC using petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v) as a solvent system. Lipids were visualized with iodine vapor and identified by their migration with standards. The spots of TAG were scraped off from TLC for determination of radioactivity by liquid scintillation counting. Control incubations were carried out for zero time and in the absence of enzyme. The control values were subtracted from the actual assay value, and enzyme activity was calculated after correction.

**Incorporation of [1-14C]Acetate into TAG**—E. coli cells (A_{600} = 0.6) were induced with isopropyl β-D-thiogalactopyranoside, incubated with [1-14C]acetate in Luria-Bertani medium, and grown for 4 h at 37 °C. Cells were harvested by centrifugation, and the cell pellet was washed twice with ice-cold water. To the pellet, chloroform/methanol (1:2, v/v) was added, and the lipids were extracted. The extracted lipids were separated on a silica TLC using petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v) as the solvent system. Lipids were identified by their migration with standards. The TLC plate was exposed to a phosphorimaging screen, individual spots were scraped off from the plate, and the radioactivity was measured in a liquid scintillation counter. In the case of yeast cells, transformants (pYES2-Atg5g23940 and pYES2) were grown to late log phase in 5 ml of SM-U containing 2% glucose and then transferred to 10 ml of the fresh medium with an absorbance of 0.1. The cells were grown until the absorbance reached 3. For neutral lipid labeling, A_{600} = 0.4 absorbance of the cells were inoculated in a fresh medium containing 2% galactose and 4 μCi/ml [1-14C]acetate and grown for 24 h. The cells (A_{600} = 10) were harvested, and the lipids were extracted and separated on a silica TLC using petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v) as the solvent system.

**Preparation of Yeast Cell Lysate**—To measure the enzymatic activity of *S. cerevisiae* overexpressing Atg5g23940 and the vector control, the cell extracts were prepared by lysis with glass beads, followed by centrifugation at 1000 × g to remove glass beads and unbroken cells. Cell-free extract was again subjected to high speed centrifugation at 10,000 × g for 30 min, and the supernatant obtained was used as an enzyme source for the DGAT assay.

**Oil Red O Staining**—Yeast cells grown in synthetic medium (devoid of uracil) containing dextrose as the carbon source were washed and inoculated in synthetic medium containing galactose for 24 h. Cells were washed with phosphate-buffered saline, resuspended in lytic buffer (1.2 M sorbitol, 50 mM MgCl_2, 40 mM KH_2PO_4), and used to prepare smears of cells. The smears were stained with oil red O stain (0.3% oil red O, 24% triethyl phosphate) for 30 min and air-dried. Microscopic analysis was performed on a Leica DC 350F microscope, using a 100 × oil immersion objective. Fluorescence emission was detected between 596 and 620 nm.

**Rescue of Quadruple Mutant Subjected to Lipotoxicity of Oleate by Atg5g23940**—Quadruple mutant (pYES2 and Atg5g23940 overexpressed) cells were grown in synthetic medium agar without uracil, having galactose for the induction of the gene. Oleate was supplemented to the medium at a concentration of 0.1 mM (in absolute ethanol). The yeast cells were serially diluted and spotted on the agar plates.

**Analysis of Hydroxy Fatty Acid Uptake**—Quadruple mutant *S. cerevisiae* overexpressing Atg5g23940 was induced in synthetic medium containing galactose, along with 0.1% Triton X-100 and 0.5 mM 16-hydroxyhexadecanoic acid for 24 h. Cells were pelleted and washed twice with distilled water. Lipid extraction was carried out by using methanol, chloroform, 2% acidified water (2:1:0.8, v/v/v) and incubating for 1 h at room temperature. Later, the ratio was restored to 1:1 (v/v/v). Lipids were extracted from the lower chloroform layer after vortexing and spinning at 12,000 rpm for 5 min. Subsequent extractions were performed with chloroform to ensure the completion of total lipid extraction. The chloroform layer was washed with 2% orthophosphoric acid and was dried in a vacuum concentrator.

For analysis of each fraction of lipid in H1246 cells (transformed with vector and gene), the total lipids were separated on a TLC using petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v) as the solvent system. Each lipid fraction on the TLC was scraped, and lipids were re-extracted from them using the above mentioned protocol. Each fraction of lipid was subjected to fatty acid methyl ester (FAME) preparation. The protocol for FAME preparation is as follows. The lipid sample was treated with boron trifluoride methanol and incubated in a 65 °C water bath for 50 min. The reaction mixture was cooled in ice for 5 min, and to this 1 ml of distilled water was added. The FAMEs were extracted from the reaction mixture using HPLC grade hexane. The reaction mixture was subjected to an additional round of hexane extraction, and both of the hexane fractions were pooled together. The pooled hexane fraction containing FAMEs was dried in a layer of sodium sulfate and subjected to gas chromatography-mass spectrometry analysis. Heptadecanoic acid (100 μg) was used as an internal standard for each reaction. Capillary gas chromatography analysis of FAMEs was performed on an HP-5MS...
column (Agilent Technologies) whose dimensions were 30-m length, 0.25-μm film thickness, and 0.25-mm inner diameter. The method used for separation of FAMEs on the GC system was as follows: 40 °C for 3 min, and then the temperature was raised to 150 °C at the rate of 10 °C/min and thereafter raised to 230 °C at the rate of 5 °C/min. The column was further maintained at 230 °C for 5 min, and then the temperature was raised to 300 °C at the rate of 30 °C/min and maintained at 300 °C for 5 min. The total run time was 53 min.

Isolation of 10,16-Dihydroxyhexadecanoic Acid from Tomato Peel—Isolation and purification of 10,16-dihydroxyhexadecanoic acid were performed as described (28). Briefly, commercially available tomatoes (5.5 kg) were cut and then the temperature was further maintained at 230 °C for 5 min, and then the temperature was raised to 300 °C at the rate of 30 °C/min and maintained at 300 °C for 5 min. The total run time was 53 min.

Isolation of 10,16-Dihydroxyhexadecanoic Acid from Tomato Peel—Isolation and purification of 10,16-dihydroxyhexadecanoic acid were performed as described (28). Briefly, commercially available tomatoes (5.5 kg) were cut and soaked in boiling water for 40–45 min. The peels were separated and washed with water followed by methanol and air-dried. Dried and crushed tomato peels (13.6 g) were exhaustively extracted with methanol (200 ml) with refluxing for 6 h. After filtration, the peels were further extracted with chloroform/methanol (1:1) for 4 h. The remaining peels were treated with 0.15 M methanolic KOH (120 ml) and left overnight at room temperature. It was filtered and washed with methanol. The filtrate was evaporated to dryness to obtain crude fatty acid methyl esters.

Purification of 10,16-Dihydroxyhexadecanoic Acid Methyl Ester (Compound 1) (see Structure 1)—The crude mass (1.75 g) was charged on a silica gel (90 g, 100–200 mesh, 700 × 36 mm) column and eluted successively with hexane (500 ml), chloroform/hexane (20–80%, 500 ml each), chloroform (1000 ml), acetone/chloroform (1–5%, 500 ml each), and methanol/chloroform (1–6%, 500 ml each). Similar fractions were pooled on the basis of TLC profile. Fractions 118–121 contained pure fractions of 10,16-dihydroxyhexadecanoic acid methyl ester eluted at 2–3% methanol in chloroform.

Yield = 364 mg, m.p. = 48–49 °C [47–49 °C]; 1H NMR (300 MHz, CDCl3): δ 1.17–1.53 (24H), 1.29–2.24 (t, 3H, CH2- COO-), J = 6.6 Hz), 2.64 (bs, 2H, exchangeable, 2xOH), 3.49–3.53 (bt, 3H, CH-O and CH2-O, J = 6.6 Hz), 3.58 (s, 3H, -COOCH3); 13C NMR (75 MHz, CDCl3): δ 25.24, 25.39, 25.42, 25.55, 28.89, 28.98, 29.19, 29.27, 29.43, 32.41, 32.49, 37.10, 37.22, 51.27, 62.39, 71.54, 174.26; electrospray mass (MeOH): 303 [M + H]+.

Hydrolysis of Ester to 10,16-Dihydroxyhexadecanoic Acid—Compound 1 (100 mg) was taken in 20 ml of 10% aqeous methanolic (1:5) KOH. It was heated at 80 °C for 1 h. Upon completion of the reaction, it was cooled and acidified with dilute HCl (5%, 5 ml). The reaction mixture was extracted with ethyl acetate (3 × 20 ml). The combined organic layer was washed with water, dried over anhydrous sodium sulfate, and evaporated in vacuum. The free acid (compound 2) was obtained as a light yellow solid. Yield = 89 mg (93%), m.p. = 78–80 °C (77–79 °C); 1H NMR (300 MHz, CDCl3): δ 0.96–1.53 (m, 24H), 1.75–1.77 (d, 3H, exchangeable, 10-C- OH), 1.98–2.17 (t, 3H, CH2-COO-, J = 6.6 Hz), 3.26–3.31 (bt, 3H, CH2-O and CH-O, J = 6.6 Hz). 13C NMR (75 MHz, CDCl3 + CD3OD): δ 25.18, 28.54, 28.65, 28.84, 28.93, 29.08, 31.81, 33.58, 36.54, 36.62, 61.59, 70.94, 176.24; electrospray mass (MeOH): 287 [M − H]+; negative ESI mass: 287 [M − H]−.

Silylation of the Purified 10,16-Dihydroxyhexadecanoic Acid—To enable the detection of polyhydroxy fatty acids by GC-MS, the most commonly followed protocol is silylation (29). Briefly, the polyhydroxy fatty acid is first subjected to a round of methylation, which converts the fatty acid to its respective FAME derivative. Then the FAME is dissolved in 2 ml of hexane (HPLC grade). To this, 100 μl of 1:1 N,O-bis(trimethylsilyl)acetamide/pyridine is added and incubated at 72 °C for 40 min. The reaction is cooled on ice for 5 min and then dried in a vacuum concentrator to remove hexane. The methylated and silylated polyhydroxy fatty acid is dissolved in methanol (HPLC grade) and then subjected to GC-MS analysis.

RESULTS

At5g23940 Encodes a Soluble Protein—The multiple sequence alignment revealed a conserved HX3D motif in At5g23940 (Fig. 1A). The gene, At5g23940, possesses a lipid binding (VX3GF) motif (Fig. 1B). Hydropathy plot of At5g23940 predicted the absence of any transmembrane domain (Fig. 1C). At5g23940 was cloned into the plasmid pRSET A and expressed in BL21 (DE3) cells (Fig. 1D). Immunoblot analysis using anti-His6 antibody confirmed the expression of At5g23940 (data not shown). Immunoblot analysis was also conducted using anti-At5g23940 polyclonal antibodies for both the cell lysate and the purified protein (Fig. 1E). Recombinant protein in the soluble fraction was purified using nickel-nitrirotiacetic acid column chromatography (Fig. 1F).

E. coli Cells Expressing At5g23940 Synthesize TAG—E. coli cells overexpressing At5g23940 incorporated radiolabeled acetate into the TAG, whereas the control did not (Fig. 2A). To assess the acyl acceptor specificity, monoacylglycerol, glycerol, lysophosphatidylcholine, lysophosphaticidylserine, and lysophosphatidylethanolamine, and lysophosphatidylserine were used. There was no significant incorporation of oleate into these substrates, but there was a substantial amount of activity with DAG (Fig. 2B). Diacylglycerol with acyl groups at sn-1 and sn-2 positions yielded enhanced TAG formation compared with 1,3-DAG (Fig. 2C). To assess the preference of acyl-CoA by the enzyme, the assays were performed using, oleoyl-, palmitoyl-, and stearoyl-CoAs as acyl donors and DAG as an acyl acceptor. Higher activity was observed with oleoyl-CoA (18 nmol of TAG formed/min/mg of protein) (Fig. 2D) as compared with palmitoyl-CoA (10.11 nmol of TAG formed/min/mg of protein) (Fig. 2E). When

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stearoyl-CoA was used as an acyl donor, activity was found to be 8.9 nmol of TAG formed/min/mg of protein under the standard assay conditions (Fig. 2). These data suggest that At5g23940 is an acyl-CoA-dependent DAG-specific acyltransferase, with greater preference for oleoyl-CoA.

Characterization of Recombinant At5g23940—The enzyme showed a time-dependent (Fig. 3A) and protein-dependent (Fig. 3B) increase in the incorporation of [14C]oleoyl-CoA into DAG to form the TAG. There are two major types of DGATs, DGAT1 and DGAT2. Although they catalyze same reaction, they differ in co-factor dependence. Whereas DGAT2 is an Mg$^{2+}$-dependent enzyme, DGAT1 does not require Mg$^{2+}$ for its activity (supplemental Table 1). The effect of Mg$^{2+}$ on DGAT activity by At5g23940 was investigated. Inhibition of DAG acyltransferase activity with varying concentrations of Mg$^{2+}$ up to 1 mM was observed (Fig. 3C). Saturation kinetics for TAG formation with varying concentrations of 1,2-dioleoylglycerol was performed. The purified recombinant At5g23940 (0.5 μg) was added to 10 μM oleoyl-CoA with varying concentrations of diacylglycerol (10, 20, 40, 50, and 100 μM). Data represent mean ± S.D. for triplicate samples. Calculated kinetic values were found to be $K_m = 22.76 ± 4$ μM and $V_{max} = 16.09 ± 0.8$ nmol/min/mg (Fig. 4, A and B). Based on the facile purification of recombinant

**FIGURE 1. Purification of recombinant At5g23940.** A, multiple-sequence alignment of At5g23940 with DGATs of other organisms showing a conserved acyltransferase motif. B, lipid binding motif is shown in At5g23940 at the middle of the sequence. C, Kyte-Doolittle hydropathy plot of At5g23940. D, At5g23940 was overexpressed in E. coli BL21 (DE3) cells, and the proteins were resolved on 12% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, uninduced lysate; lane 2, induced lysate. E, immunoblot analysis with anti-At5g23940 polyclonal antibodies was carried out. Lane 1, induced lysate; lane 2, the purified recombinant protein. F, Ni$^{2+}$-NTA-purified protein fractions of At5g23940. M, prestained molecular weight marker. *, “conserved identical residue” among the given species; :, “conserved substitutions”; ., “semi-conserved substitutions.”
At5g23940 and the above results, we conclude that At5g23940 is a soluble DGAT.

**At5g23940 Overexpression Enhances Triacylglycerol in S. cerevisiae**

Yeast is able to import exogenous fatty acids and convert them to their respective acyl-CoA derivatives. Supplementation of oleate in the medium can further enhance the TAG accumulation in yeast cells by expressing functional DGAT genes. The quadruple knock-out yeast strain H1246, in which all four DGATs (Dga1p, Lro1p, Are1p, and Are2p) have been knocked out is suitable for expression and characterization of extraneous DGAT genes because it is devoid of inherent DGAT activity and does not accumulate TAG. The quadruple mutant was transformed with pYES2 and pYES2-At5g23940. Immunoblotting with anti-At5g23940 antibodies confirmed the overexpression of the protein (Fig. 5A) in the mutant. To understand the fate of neutral lipid biosynthesis, a [14C]acetate labeling study was performed with pYES2- and pYES2-At5g23940-transformed mutant cells. As expected, the At5g23940-expressing cells yielded TAG but
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FIGURE 4. Saturation kinetics for triacylglycerol formation with varying concentrations of acyl acceptor and acyl donors. A, the purified recombinant At5g23940 protein (0.5 µg) was added to 10 µM oleoyl-CoA with varying concentrations of 1,2-dioleoylglycerol, and the reaction mixture was incubated at 30 °C for 15 min. Lane 1, 10 µM DAG; lane 2, 20 µM DAG; lane 3, 40 µM DAG; lane 4, 50 µM DAG; lane 5, 100 µM DAG. Data represent means ± S.D. (error bars) for triplicate samples. B, Lineweaver-Burk plot showing the triacylglycerol formation with varying concentrations of 1,2-dioleoylglycerol. UN, unknown; FFA, free fatty acids.

not the cells having vector alone (Fig. 5B). Cell-free extracts of the transformants (pYES2 and pYES2-At5g23940) were prepared and assayed for DAG acyltransferase activity that showed acylation of 1,2-DAG when compared with vector control (Fig. 5C), confirming that At5g23940 is a DGAT.

At5g23940 Expression Rescues Yeast DGAT Mutant from Fatty Acid Toxicity—Rescue of mutant cells in oleate medium is used for the validation of DGAT function. Quadruple mutant in the absence of any fatty acid in the medium grows normally and is used as a control. However, reduced growth of quadruple mutant cells was observed in the medium containing oleate. This is because of the fatty acid toxicity observed in the absence of all four DGATs. To assess the DGAT activity in vivo, we have compared the oleic acid toxicity in the absence and presence of the At5g23940 gene. We inoculated H1246 cultures expressing pYES2 and At5g23940 on YNBG solid medium with and without fatty acid supplement. The dilutions used were 10, 10^2, 10^3, and 10^4. At 0.1 mM oleic acid, the H1246 cells did not survive, whereas H1246 cells overexpressing At5g23940 survived, suggesting the DAG acyltransferase activity of At5g23940 (Fig. 6A). This clearly establishes that exogenously supplied free fatty acid is removed effectively from its intracellular sites of accumulation by the activity of At5g23940.

Oil Red O Staining of Lipid Particles in Yeast Cells—Lipid particles are abundantly present in wild type and completely absent in quadruple DGAT-disrupted strain (H1246). Overexpression of At5g23940 in yeast H1246 cells leads to TAG accumulation, as observed in the [14C]acetate labeling and in vitro DGAT assays. Cytoplasmic lipid droplet formation was observed in quadruple DGAT-disrupted cells transformed with At5g23940 when compared with cells transformed with pYES2 alone (Fig. 6B).

Isolation and Purification of 10,16-Dihydroxyhexadecanoic Acid—10,16-Dihydroxyhexadecanoic acid was isolated and purified as described under “Experimental Procedures.” The purified 10,16-dihydroxyhexadecanoic acid was confirmed by NMR and by ESI-MS (data not shown). The purified 10,16-dihydroxyhexadecanoic acid was subjected to silylation and then analyzed by gas chromatography-mass spectrometry (supplemental Fig. S2). These spectral analyses confirmed the successful isolation of 10,16-dihydroxyhexadecanoic acid.

Feeding Yeast Cells with 10,16-Dihydroxyhexadecanoic Acid—Due to the commercial unavailability of 10,16-dihydroxyhexadecanoic acid, which is reported as the substrate for At5g23940 (22), we have isolated the 10,16-dihydroxyhexadecanoic acid from tomatoes and used it for feeding studies and monitored the incorporation by GC-MS. The yeast cells were unable to take up and incorporate the dihydroxy fatty acid into lipids (data not shown). However, under the same conditions, 16-hydroxyhexadecanoic acid was incorporated into TAG. We then tried to synthesize 10,16-dihydroxyhexadecanoyl-CoA chemically and enzymatically, but we were not successful. In vitro incorporation of dihydroxy fatty acid into TAG was also performed using recombinant yeast cell free extract, DAG, ATP, MgCl2, and CoA, which showed that there was no incorporation into lipid. This could be due to the inherent difficulties with the hydroxy fatty acid and its fatty acid activating enzyme.

GC-MS Analysis by Feeding the Cells with 16-Hydroxyhexadecanoic Acid—Analysis of the total cellular fatty acids in yeast cells (pYES2- and At5g23940-transformed) treated with 16-hydroxyhexadecanoic acid revealed the increase in uptake of hydroxy fatty acids in At5g23940-transformed S. cerevisiae quadruple mutant cells when compared with the pYES2-transformed mutant cells (Fig. 7, A and B). Further analysis of the lipids of H1246 yeast cells (transformed with pYES2 and At5g23940) fed with the hydroxy fatty acid revealed its incorporation only into the TAG fraction of At5g23940-transformed S. cerevisiae quadruple mutant cells (Fig. 7C). The fraction incorporating 16-hydroxyhexadecanoic acid in At5g23940-transformed S. cerevisiae quadruple mutant cells was further confirmed to be TAG by ESI-MS (data not shown). This suggests the possible role of At5g23940 as an enzyme that effectively utilizes hydroxy fatty acids to incorporate them into the neutral lipid fraction, TAG. The incorpora-
tion of 16-hydroxy fatty acid into major phospholipid fractions was also investigated. However, we did not observe the incorporation of hydroxy fatty acids into any of the phosphatidylcholine and phosphatidylethanolamine fractions (supplemental Fig. S1, A and B). These data suggest that the yeast cells have evolved mechanisms to exclude unusual fatty acids from the membrane lipids.

**DISCUSSION**

**DCR Is DGAT**—Diacylglycerol acyltransferase catalyzes the formation of triacylglycerol from diacylglycerol using acyl-CoA as the acyl donor. The following findings suggest that DCR is a DGAT. (i) A typical acyltransferase motif (HX_D) and a lipid binding motif were present. (ii) Because *E. coli* cells do not synthesize TAG, *At5g23940*-transformed *E. coli* cells showed a significant amount of TAG. (iii) To validate further, quadruple mutant (H1246)-overexpressing *At5g23940* was able to incorporate the [14C]acetate into the TAG fraction. (iv) A high level of DGAT activity was observed in the cell lysate of *At5g23940*-transformed *S. cerevisiae* quadruple mutant. (v) Accumulation of lipids was seen in the *At5g23940*-transformed quadruple mutant cells as observed by fluorescence microscopy.

Triacylglycerol is the major storage lipid in seeds and the TAG is involved in seed development and embryo maturation. Seeds of the DCR mutant display surface alterations, including a defect in mucilage extrusion and germination capacity (21). Shrunken seeds are a characteristic feature of lack of accumulation of TAG, which also leads to the reduction in germination efficiency. DCR is ubiquitously expressed in all integumenta layers in the young seeds and is specifically expressed in the inner integumenta of the mature seeds. Recently, it has been shown that acyltransferases *PDAT1* and
DGA1 and DGA2 have overlapping functions that are essential for normal pollen and seed development of Arabidopsis (30), and the absence of these genes leads to the pollen shrinkage. Our in vitro functional analysis of At5g23940 suggested that the gene is involved in triacylglycerol formation and also in cutin biosynthesis.

Unusual fatty acids occur mainly in storage triacylglycerols of certain oilseed species but are excluded from polar glycerol-
DCR Is Diacylglycerol Acyltransferase

lipids and consequently from the membranes of cells. Presumably, the accumulation of unusual fatty acids (hydroxy fatty acids) in membrane lipids would perturb the integrity of the bilayer and pose deleterious effects to the cell (31). Thus, plants have developed processes to screen out unusual fatty acids from membrane lipids. Phospholipases and acyltransferases contribute to the strong fatty acid bias observed between the storage and the membrane glycerolipids (32). In this regard, *At5g23940* functions as an acyltransferase and plays a key role in the accumulation of hydroxy fatty acids into storage TAG and does not let them into the membranes.

**DCR Is Involved in Cutin Biosynthesis**—The gene expression data of *At5g23940* was retrieved from the GENEVESTIGATOR using Meta-Analyzer, which showed that the gene is highly expressed in inflorescence and cotyledons. Analysis of the gene expression during various growth stages suggested that the gene is expressed highly during rosette and flowering stages. The co-expression data of *At5g23940* using GENECAT shows that pectinase genes are up-regulated along with this gene. This shows the homeostasis maintained by the cell. Cutin, a major component of the cuticle, is a polyester mostly consisting of C16 and C18 fatty acids and other aromatic acids like ferulic and coumaric acid. The presence of hydroxylated fatty acids, ATP, and coenzyme A together is the primary prerequisite for successful cutin polymer biosynthesis (33). These fatty acids are linked to glycerol by different acyltransferases. Environmental stress, such as wounding, leads to the chemical depolymerization of the polyester, yielding the fragments containing monoacylglycerols, diacylglycerols, glycerol, and hydroxy fatty acids. This indicates that the cutin contains DAG and hydroxy fatty acids in its structure. It also indicates that the enzymes involved in esterification of fatty acids or their derivatives must exist in cutin biosynthesis (22, 31, 34). Recently, two membrane-bound acyl-CoA:glycerol-3-phosphate acyltransferases (*GPAT4* and *GPAT8*) have been identified from *A. thaliana*. *GPAT4* and *GPAT8* were shown to be important for cutin biosynthesis (34), whereas *GPAT5* is shown to be involved in suberin biosynthesis (35, 36).

**Hydroxy Fatty Acid Containing TAG Is the Precursor for Cutin Biosynthesis**—Based on the data that were obtained in the current study, we have proposed a model for explaining cutin biosynthesis (Fig. 8). In plants, two possibilities could exist. (i) The mono- and dihydroxy fatty acids could be esterified and incorporated directly into cutin by *At5g23940* activity (22). (ii) We propose that the mono- or dihydroxy fatty acids are acylated to glycerol 3-phosphate by *GPAT4*, *GPAT6*, and *GPAT8* to form lysophosphatidic acid, which in turn dephosphorylated to MAG by the same enzymes (37), and the synthesized MAG could be further acylated to DAG by MGAT (38). Alternatively, the synthesized hydroxy fatty acid containing lysophosphatidic acid is dephosphorylated to MAG by lysophosphatidic acid phosphatase (39), and the MAG is acylated to DAG by MAG acyltransferase (38), that was reported earlier from our laboratory. However, the role of these enzymes in cuticle biosynthesis is not clearly established. The synthesized DAG containing esters of hydroxy fatty acid in turn is acylated to TAG by incorporating another hydroxy fatty acid in the presence of *At5g23940*. The OH-

![FIGURE 8. A schematic model showing the proposed biosynthetic pathway for cutin.](image)

**TAG that is formed becomes hydrolyzed to free hydroxy fatty acids by lipase, a BODYGUARD (BDG) gene product.** BDG is an epidermis-specific extracellular α/β-hydrolase fold-containing protein that is essential for the formation of the cuticle (40). Then the free hydroxy fatty acids are esterified and polymerized into cutin by cutin synthase or polyester synthase. Alternatively, hydroxy fatty acid containing TAG itself could be used to form cutin by cutin synthase or polyester synthase, as shown in Fig. 8. From this it is concluded that *At5g23940* is a broad specificity acyltransferase that can utilize non-hydroxy, mono, and dihydroxy fatty acids for its activity. As the evolution progressed, nature might have selected the same enzyme that had been used as DGAT to take part in cutin biosynthesis as well (41). Depolymerization of cutin by CaO-methanolysis releases monomers that include C16 and C18 ω-hydroxyacids, fatty acids with mid-chain oxygenated substitutions, namely epoxy and hydroxyl groups, as well as small quantities of 1- and 2-monoacylglycerol esters of ω-hydroxyacids (42). The non-prediction of TAG in the depolymerization data may be due to the quick hydrolysis of TAG into MAG containing hydroxy fatty acids. The production of MAGs with acyl chains in the internal or external position of the glycerol and of free fatty acids could also be due to the action of lipase and phosphatase of the epidermis. It is also possible that the presence of hydroxy fatty acid-specific TAG lipase may give rise to MAG.

**Utilization of Hydroxy Fatty Acid by Yeast**—All of the higher plants have the ability to synthesize various hydroxy fatty acids that are the basic elements of cutin. Fatty acids
with a hydroxyl group on the terminal carbon are the common components in lipid polyester. There is a strong interest in producing these fatty acids in seed oils of crop species. Much effort has been devoted to understanding the enzymes involved in the synthesis of oxygenated fatty acids and to express them in the seeds of the model plant Arabidopsis or crop species. Many efforts from both industrial and academic laboratories have been directed toward producing oxygenated fatty acids in oilseed crops. Current oilseed crops, such as soybean, rapeseed, and sunflower, do not produce hydroxy fatty acids or any other oxygenated fatty acid naturally (43). Yeast cells expressing At5g23940 were grown in the medium containing hydroxy fatty acid, which resulted in the incorporation of fatty acid into TAG. This clearly indicates the utilization of hydroxy fatty acid by At5g23940 into the storage lipids under the null DGAT background. We have also carried out a similar kind of 16-hydroxyhexadecanoic acid feeding studies in wild type yeast cells. There was no detectable amount of hydroxy fatty acid in the yeast cells (supplemental Fig. S3A). When the wild type cells were grown in 16-hydroxyhexadecanoic acid-containing medium, the cells did take up the fatty acid (supplemental Fig. S3B) but did not incorporate them into TAG (supplemental Fig. S3C). These data indicate that membrane-bound DGATs were unable to utilize hydroxy fatty acid.

The present study for the first time deals with the identification, purification, and characterization of At5g23940, a member of the α/β-hydrolase fold-containing protein family of acyltransferases required for incorporation of the most abundant monomer into the polymeric structure of cutin. In addition, the present study highlights the existence of a cytosolic DAG acyltransferase in plants and provides an insight into the molecular and biochemical function of the DCR gene At5g23940.

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