

Oleosin Is Bifunctional Enzyme That Has Both Monoacylglycerol Acyltransferase and Phospholipase Activities*

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Background: Seeds with high oil content have more oleosins than those with low oil content. However, the exact role of oleosins in oil accumulation is unclear.

Results: We demonstrate that oleosin 3 is involved in diacylglycerol biosynthesis and phosphatidylcholine hydrolysis.

Conclusion: Oleosin, a structural protein, is involved in biosynthesis and mobilization of plant oils.

Significance: This study provides direct evidence for the presence of an alternate route for the biosynthesis of triacylglycerol from monoacylglycerol.

In plants, fatty oils are generally stored in spherical intracellular organelles referred to as oleosomes that are covered by proteins such as oleosin. Seeds with high oil content have more oleosin than those with low oil content. However, the exact role of oleosin in oil accumulation is thus far unclear. Here, we report the isolation of a catalytically active 14 S multiprotein complex capable of acylating monoacylglycerol from the microsomal membranes of developing peanut cotyledons. Microsomal membranes from immature peanut seeds were solubilized using 8 M urea and 10 mM CHAPS. Using two-dimensional gel electrophoresis and mass spectrometry, we identified 27 proteins in the 14 S complex. The major proteins present in the 14 S complex are conarachin, the major allergen Ara h 1, and other seed storage proteins. We identified oleosin 3 as a part of the 14 S complex, which is capable of acylating monoacylglycerol. The recombinant OLE3 microsomes from *Saccharomyces cerevisiae* have been shown to have both a monoacylglycerol acyltransferase and a phospholipase A₂ activity. Overexpression of the oleosin 3 (OLE3) gene in *S. cerevisiae* resulted in an increased accumulation of diacylglycerols and triacylglycerols and decreased phospholipids. These findings provide a direct role for a structural protein (OLE3) in the biosynthesis and mobilization of plant oils.

Triacylglycerol (TAG)² is an important neutral lipid molecule that serves as the primary mechanism of fuel storage in eukaryotes. In eukaryotes, the biosynthesis of TAG is accomplished through two major pathways, the glycerophosphate pathway and the monoacylglycerol pathway (1). In the glycerophosphate pathway, glycerol 3-phosphate is acylated successively to lysophosphatidic acid and phosphatidic acid, which is then dephosphorylated to diacylglycerol (DAG). In the monoacylglycerol (MAG) pathway, which operates predominantly in intestinal cells, DAG is formed directly from monoacylglycerol and fatty acyl-CoA in a reaction catalyzed by MAG acyltransferase (MGAT). The DAG generated by both pathways can be used as a substrate for TAG synthesis by DAG acyltransferase (2, 3).

In plants, TAGs are stored in oleosomes that are spherical (0.6–2 μm in diameter) intracellular organelles surrounded by a monolayer of phospholipids containing embedded proteins that stabilize their structures (4, 5). A predominant protein in the oleosome is oleosin, which prevents the coalescence of oil bodies during seed desiccation and acts as a binding site for lipases during seed germination. Oleosins range in size from 15 to 26 kDa (6, 7). The oleosin protein has an N-terminal amphipathic domain, a conserved central hydrophobic antiparallel/β-strand domain, and a C-terminal amphipathic α-helical domain (4, 8). The central long hydrophobic core has a unique proline knot, PX₅SPX₃P, that is conserved across various species and is used for targeting the protein to oleosomes (9, 10) but is not required for integration into the membrane of the endoplasmic reticulum. A mutation in the conserved residues of the proline knot resulted in inefficient targeting to the oil body; however, oleosin maintained its normal level in seeds (11, 12). Oleosins are not only restricted to oil bodies but are also expressed specifically in the floral tapetum cells of the pollen tube in *Arabidopsis* (13).

The size of oil bodies and the oleosin content in plant seeds are directly correlated (14–16). *Brassica napus* and *Arabidopsis* seeds with high oil content accumulate nearly 20% more oleosin than those with low oil content (17, 18). Overexpression of oleosin in the Pa19 cell culture line of anise (*Pimpinella anisum*) resulted in high oil content (19), whereas oleosin ablation caused an aberrant embryo phenotype with unusually large oil bodies and altered lipid and protein contents in *Arabidopsis*

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² The abbreviations used are: TAG, triacylglycerol; MAG, monoacylglycerol; MGAT, MAG acyltransferase; DAG, diacylglycerol; QM, QM quadruple mutant.

seeds, which caused delayed germination. The aberrant phenotypes were reversed by introducing a recombinant oleosin (18). However, the physiological role of oleosins in seeds has yet to be fully elucidated. In this study, we show that oleosin-3 exists as part of a 14 S multiprotein complex, functioning as both an MGAT and a phospholipase A₂.

EXPERIMENTAL PROCEDURES

Materials—[1-¹⁴C]Oleoyl-CoA, [1-¹⁴C]monooleoyl-*rac*-glycerol, and [2-*palmitoyl*-9,10-³H]phosphatidylcholine were obtained from American Radiolabeled Chemicals. Lipids and acyl-CoAs were obtained from Avanti Polar Lipids. [³²P]Orthophosphate and [1-¹⁴C]sodium acetate were from Board of Radiation and Isotope Technology, Bhabha Atomic Research Center, Mumbai, India. Restriction endonucleases and *Pfu* polymerase were from New England Biolabs. Oligonucleotides, monoclonal antibodies, phosphoamino acids, and all other reagents were obtained from Sigma. Field-grown developing peanut (*Arachis hypogaea* L.) cotyledons (JL-24) were harvested at 20–24 days after flowering (immature).

Preparation of Membranes and Oleosomes—Immature seeds (20 g) were ground in a prechilled mortar and pestle with 2.5 g of acid-washed sand and 50 ml of extraction buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM KCl, 1 mM MgCl₂, 1 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.25 M sucrose (20). The extract was filtered through two layers of cheesecloth, and the filtrate was differentially centrifuged to fractionate the intracellular components. The oleosomes were prepared as described previously (12).

Solubilization of Peanut Microsomes by CHAPS and Urea—Microsomal membranes were washed with 2 M urea containing extraction buffer followed by solubilization with 8 M urea and 10 mM CHAPS at pH 8.0. The mixture was then incubated at 4 °C for 60 min with constant stirring and centrifuged at 150,000 × *g* for 60 min. The enzyme activity present in the supernatant was regarded as “solubilized.” The solubilized preparation was purified by native-PAGE carried out in 1.0-mm slab gels with a discontinuous buffer system at 4 °C.

Sucrose Density Gradient—The solubilized enzyme (2 mg/ml) was layered onto a 10–30% linear sucrose gradient containing 10 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, and 0.1 M NaCl and centrifuged for 18 h at 200,000 × *g* (Beckman SW 41 rotor). Fractions (1 ml) were collected and assayed for acyltransferase activity (21) and protein concentration.

Monoacylglycerol Acyltransferase Assay—Enzyme activity was measured by the independent incorporation of [1-¹⁴C]oleoyl-CoA and [1-¹⁴C]MAG (oleoyl) into diacylglycerol. The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 1 mM MgCl₂, 50 μM MAG (5 μl of sonicated vesicles in 5 mM CHAPS), 10 μM [1-¹⁴C]oleoyl-CoA (55,000 dpm), or 20 μM [1-¹⁴C]MAG (110,000 dpm), and the enzyme source in a total volume of 100 μl. The reaction was initiated by adding enzyme and terminated after 30 min by adding 0.2 ml of acidified water and 0.6 ml of chloroform/methanol (1:2, v/v). The reaction products were separated by TLC and were viewed using a PhosphorImager (20).

Protein Identification by Matrix-assisted Laser Desorption Ionization (MALDI) Mass Spectrometry—The purified complex was separated by two-dimensional gel electrophoresis and visualized by Coomassie Blue staining. Each protein band was excised from the gel, transferred to an acid-washed tube, rehydrated with water, crushed, washed three times for 20 min with 50 mM Tris-HCl (pH 8.0) and 50% acetonitrile, and then dried. The sample was incubated for 6 h at 32 °C with 0.80 ng/ml trypsin to digest the protein. The tryptic fragments were then extracted using 50% acetonitrile and 0.1% trifluoroacetic acid, dried, suspended in 10 mg/ml 4-hydroxycyanocinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid containing angiotensin as an internal standard, and applied to a MALDI sample plate, which was dried and washed with water to remove excess buffer salts. MALDI mass spectrometry analysis was performed on an Ultraflex TOF-TOF Bruker Daltonics instrument equipped with a pulsed N₂ laser and analyzed in the reflectron mode using a time delay of 90 ns with an accelerating voltage of 25 kV in the positive ion mode. Initially, the spectra of 200 laser shots were acquired, and these spectra were calibrated externally to a spectrum of mixed peptides of known masses ranging from 1046 to 2465 Da. The most intense peaks in the spectrum were selected for fragmentation by laser-induced dissociation using the LIFT program of the Ultraflex TOF-TOF instrument. For tandem mass spectrometry, averages of 1000 laser shots were accumulated, and the spectrum was calibrated internally to the precursor ion mass. Peptides were identified by searching the peak list against the mass spectrometry protein sequence database using the Mascot search engine version 2.1.

Cloning and Expression of OLE3—A seed-specific cDNA library of the peanut was constructed in a λ-ZAP II vector (Stratagene, La Jolla, CA). The open reading frame of *OLE3* was PCR-amplified and subcloned into a yeast vector. The primers are listed in Table 3. To overexpress *OLE3* in *Saccharomyces cerevisiae*, the pYES2 construct with full-length *OLE3* was transformed into yeast cells using the lithium acetate method (22), and the transformants were confirmed by colony PCR using *OLE3* sequence-specific forward and reverse primers. The transformed yeast cells were grown to late log phase in SC-U medium containing 2% glucose. The cells were harvested using centrifugation and inoculated at a concentration of A₆₀₀ = 0.1 in an induction medium (SC-U medium containing 2% galactose). The induction was allowed to proceed for 24 h and was confirmed by immunoblotting using anti-*OLE3* antibodies at a dilution of 1:1000 (v/v). Protein concentrations were determined by the protein-dye binding assay using bovine serum albumin as a standard.

Phospholipase Assays—The reaction mixture contained 1 mM sonicated vesicles of dipalmitate and 10 μg of enzyme in a total volume of 100 μl of assay buffer (50 mM Tris-HCl (pH 7.5) and 2 mM DTT). The reaction was carried out at 30 °C for 45 min and terminated by extracting the lipids with butanol. The radiometric assay consisted of 100 μM sonicated vesicles of [2-*palmitoyl*-9,10-³H]phosphatidylcholine (1 μCi/reaction) and 10 μg of enzyme in a total volume of 100 μl of assay buffer. After the reaction, lipids were isolated and analyzed by silica-TLC (23).

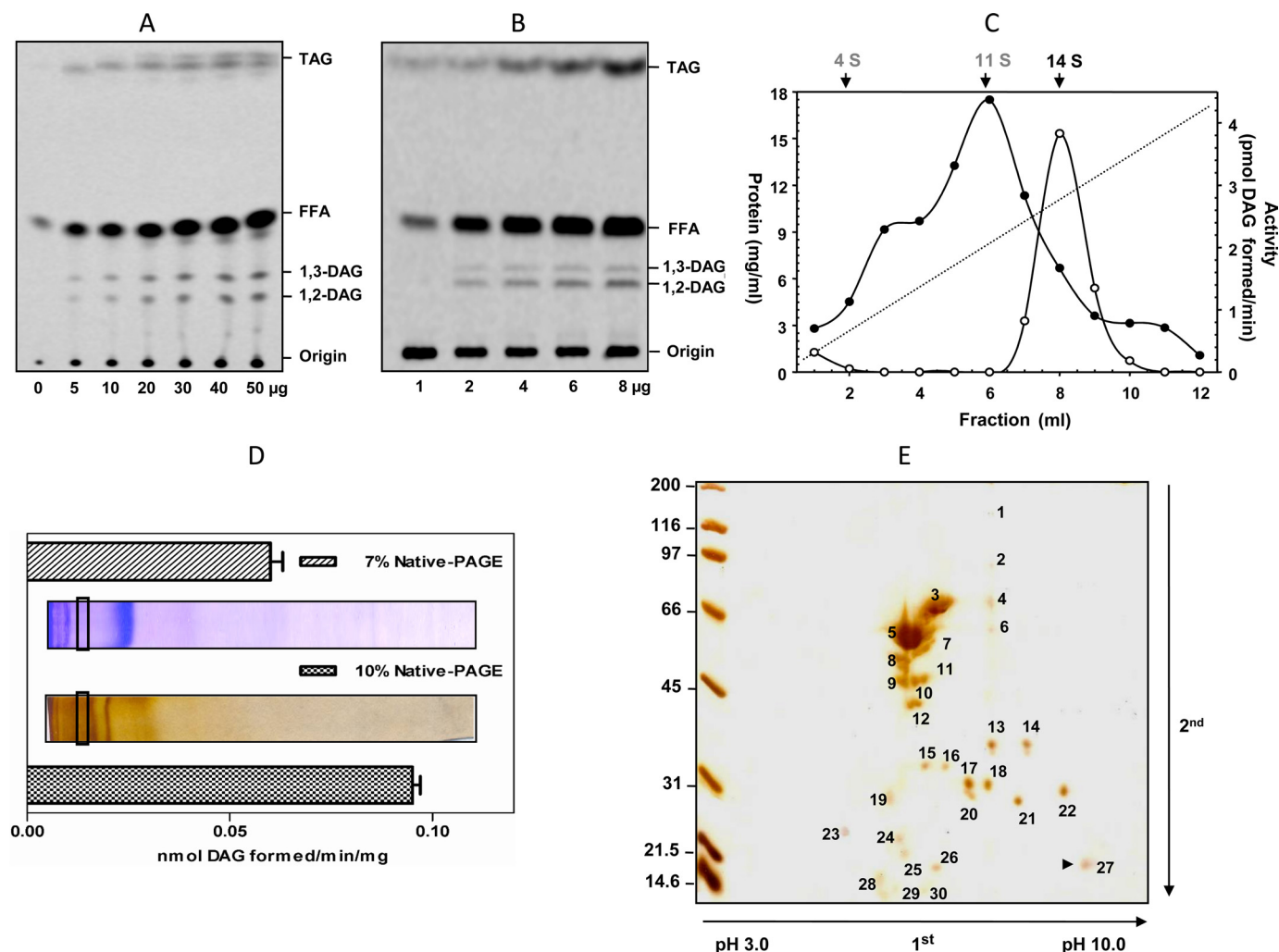


FIGURE 1. MAG acyltransferase exists as a part of a multienzyme complex. Identification of MGAT was done in plant membranes. MGAT activity was measured under standard assay conditions with increasing amounts of microsomal membrane proteins (A) and oil body proteins (B). The enzymatic reaction products were resolved on a silica-TLC using petroleum ether/diethyl ether/acetic acid (70:30:1, v/v) as the solvent system. The enzyme activities were derived from three independent experiments. C, solubilization of MGAT. A mixture of CHAPS (10 mM) and urea (8 M) was used to solubilize the microsomal membranes and loaded onto a linear sucrose density gradient centrifugation. MGAT activity (○) and protein amount (●) were estimated in a 1-ml fraction. The enzyme activity was derived from three independent experiments. D, purification of MGAT. The solubilized microsomal membranes were resolved on a 7% native-PAGE, and active fractions were reloaded onto a 10% native-PAGE. The box denotes the region in which activity was localized. The activities are represented with horizontal bars. E, MGAT activity associated with the multienzyme complex. The purified protein complex was resolved by isoelectric focusing in the first dimension followed by a 12% SDS-PAGE in the second dimension, and proteins were visualized using silver staining.

Incorporation of Radiolabeled Precursors into Lipids—The transformants (pYES2-OLE3 (H(X)₄D), pYES2-OLE3 mutant (A(X)₄A), and pYES2) were grown to late log phase in 5 ml of SM-U containing 2% glucose, and cells ($A_{600} = 0.1$) were then transferred to 10 ml of the fresh media. The cells were grown until the absorbance reached 3. For neutral lipid labeling, $A_{600} = 0.4$ of the cells was inoculated in a fresh medium containing 2% galactose and one of the labeled substances (0.5 $\mu\text{Ci/ml}$ [¹⁴C]acetate; 0.5 $\mu\text{Ci/ml}$ [¹⁴C]oleic acid; 50 $\mu\text{Ci/ml}$ [³²P]orthophosphate) and grown for 24 h. Cells ($A_{600} = 20$) were harvested, followed by the extraction and separation of lipids using petroleum ether/diethyl ether/glacial acetic acid (70:30:1, v/v) for neutral lipids. To resolve the phospholipids, chloroform/methanol/ammonia (65:25:5, v/v) was used in the first dimension followed by chloroform/methanol/acetone/acetic acid/water (50:10:20:15:5, v/v) as the second dimension solvent systems.

RESULTS

Identification of Acyl-CoA:MAG Acyltransferase in Immature Peanut Seed Microsomal Membranes—Microsomal membranes (Fig. 1A) and oleosomes (Fig. 1B) were isolated by differential centrifugation, and the subcellular fractions were assayed for acyltransferase activity using [¹⁴C]oleoyl-CoA as an acyl donor and MAG as an acyl acceptor. The incorporation of the acyl moiety into DAG was observed, suggesting the presence of MAG acyltransferase activity. In Fig. 1A, we show a linear increase in the intensity of the label at the origin and in free fatty acids because of the presence of phospholipid-biosynthesizing acyltransferase and thioesterase, esterase, or phosphoesterase activities in the microsomal membranes. The active microsomal membranes were solubilized using a mixture of 8 M urea and 10 mM CHAPS that did not inactivate the enzyme. To authenticate the solubilization procedure, the frac-

TABLE 1

Purification of membrane-bound MGAT from developing peanut cotyledons

Immature peanut seeds (100 g) were used for the preparation of the total membrane fraction by differential centrifugation.

Purification step	Total protein	Total activity	Specific activity	Purification
	mg	nmol/min	pmol/min/mg	-fold
Microsomal membranes	504	12.84 ± 0.76	25.63 ± 2.81	1.00
10 mM CHAPS + urea-solubilized fraction	254	9.84 ± 0.49	37.09 ± 3.94	1.45
7% native-PAGE	103	6.27 ± 0.37	60.43 ± 8.19	2.36
10% native-PAGE	49.32	4.84 ± 0.35	100.52 ± 9.37	3.92

tion was loaded onto a gel exclusion S-200 FPLC column, and a small amount of MGAT activity (13%) was found in the void volume fraction (data not shown). This finding could be due to the association of nonsedimentable membrane fragments with lipid particles generated during fractionation and solubilization steps. Most of the MGAT activity was eluted between the fractions of 480 to 640 kDa, indicating that many enzymes were either coeluted in the fraction or were present as part of a complex. The sedimentation value of MGAT was estimated by loading the solubilized preparation onto a 10–30% linear sucrose gradient, and the centrifuged fractions were analyzed for acyltransferase activity. The sedimentation value for the active fraction was calculated to be 14 S (Fig. 1C). The solubilized fraction was resolved on a native-PAGE; the proteins were eluted from the gel pieces and assayed for MGAT activity (Fig. 1D). The overall purification procedure is summarized in Table 1. This procedure resulted in a 4-fold purification of MGAT. These results indicated that the MGAT activity exists either as a multienzyme complex or as a multifunctional enzyme.

Identification of Proteins in 14 S Purified Complex—To identify the nature of the proteins in the multiprotein complex, the purified complex was resolved with two-dimensional gel electrophoresis (Fig. 1E). Coomassie Blue-stained protein spots were excised individually and subjected to trypsin digestion to obtain the tryptic peptides of the protein spots. Using MALDI-TOF-MS and matrix science peptide mass fingerprinting analyses in the mascot search, the individual mass values were analyzed, and their peptide mass fingerprint for protein identification, sequence coverage match, score, and theoretical and experimental molecular weight were deduced. All the 27 polypeptides were analyzed and tabulated (Table 2). The acyltransferase and lipase motifs were identified based on search for the motifs in the individual polypeptide sequence. We observed that most of the major proteins and high molecular weight polypeptides in the complex were allergenic storage proteins (*Ara h1*, conarachin, and glycinin). The microsomal multiprotein complex consisted of 33% proteins related to lipid metabolism, 30% hypothetical proteins, 11% transporter proteins, and 26% major storage proteins. Among the 27 polypeptides, 8 have the acyltransferase signature motif. All eight candidate genes were overexpressed in yeast, and the expression was confirmed with His₆ tag monoclonal antibody. The expression analyses revealed that all the candidate proteins were associated with the microsomal membranes of yeast, and the levels of expression were different for each protein. It could be possible that these proteins may have the internal membrane associated or transmembrane signal for targeting to the membranes in the yeast. Of the eight recombinant proteins, only oleosin 3 showed

MGAT activity. These results indicated that oleosin could be the MGAT.

Functional Characterization of OLE3—We used the seed-specific oleosin conserved proline knot sequence (PX₅SPX₃P) to search for oleosins across plant species in the NCBI genomic data base. Multiple sequence alignment of all the retrieved oleosins revealed that this proline knot and an H(X)₄D motif are conserved in the plant kingdom (Fig. 2, A and B). Among the proteins belonging to the oleosin superfamily, OLE3 of *A. hypogaea* has both GXSG lipase and H(X)₄D acyltransferase motifs at its N and C termini, respectively, and OLE3 has a central hydrophobic domain that contains the proline knot that gets anchored in the oil bodies (Fig. 2, C and D). To validate its biochemical function, peanut OLE3 was expressed in a TAG-defective quadruple null mutant of *S. cerevisiae*, and the microsomal membrane fraction was used as the enzyme source in subsequent experiments. *S. cerevisiae* has no intrinsic MGAT activity, and we performed all the experiments in both the wild-type and quadruple mutant strains. The expression of OLE3 was confirmed using immunoblot with anti-OLE3 antibodies (Fig. 3A). The quadruple mutant was preferred for minimizing the competition among acyltransferase (*ARE1*, *ARE2*, *DGA1*, and *LRO1*) activities (24). To determine the acyl acceptor specificity of OLE3, lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, DAG, and MAG were used. We found that OLE3 specifically acylated MAG (Fig. 3B). In addition, MGAT activity was also assayed with either [¹⁴C]oleoylglycerol or [¹⁴C]oleoyl-CoA as the labeled substrates. We found that the enzyme acylated MAG to form DAG (Fig. 3, C and D). The enzyme activity was found to be linear with time (Fig. 3, E and F). Membrane-bound mouse MGAT1 overexpressed in insect and mammalian cells efficiently acylated both *sn*-1-monoacylglycerol and *sn*-2-monoacylglycerol with a similar specific activity (1). Similar to MGAT1, OLE3 also showed no difference in specific activities toward both MAGs (Fig. 3G). These data confirmed that oleosin is capable of acylating MAG using acyl-CoA as the acyl donor and hydrolyzing phosphatidylcholine to lysophosphatidylcholine and free fatty acid.

The histidine and aspartate residues of the H(X)₄D motif are important for acyltransferase activity (25); site-directed mutation of His-125 and Asp-130 to alanine drastically reduced the enzymatic activity (Fig. 3H). Microsomes from cells overexpressing peanut oleosin 1 (OLE1, H(X)₄N) were used as a negative control (Fig. 3D). Apart from the acyltransferase activity, microsomes from cells overexpressing OLE3 were able to hydrolyze phosphatidylcholine in a time-dependent and Ca²⁺-independent manner (Fig. 3I), but it was not able to hydrolyze

TABLE 2

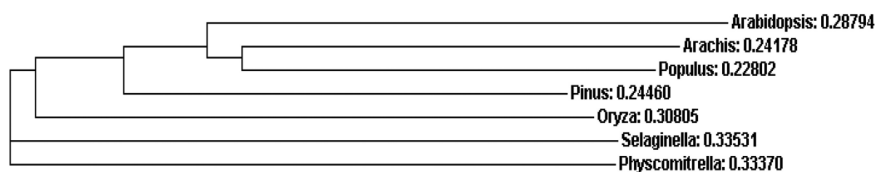
Identification of immature peanut 14 S multiprotein complex proteins by MALDI-TOF MS

Polypeptides from Fig. 1E were trypsin-digested, and the fragments were analyzed by MALDI-TOF-MS. The mass values were analyzed in Mascot peptide mass fingerprint for protein identification, sequence coverage match, score, and theoretical (Thr.) and experimental (Exp.) molecular weight. The acyltransferase and lipase motifs were identified based on the search for these motifs in the individual polypeptide sequence. A, acyltransferase motif (HX₄D); L, lipase motif (GXSG); NS, no signature motifs.

Spot no.	Identification	NCBI accession no.	Coverage %	Score	Thr. <i>M_r</i>	Exp. <i>M_r</i>	Motifs
1	Major allergen Ara h1	Q547W5_ARAHY	47	77	71.3	102.4	NS
2	Serine C-palmitoyltransferase-like protein	Q1SQZ6_MEDTR	44	70	20.6	97.7	NS
3	Peptide ABC transporter	Q1ZMH1_9VR	41	48	37.0	80.3	NS
4	Conarachin	Q6PSU3_ARAHY	49	58	66.5	71.2	A
5	Conarachin	Q6PSU4_ARAHY	49	78	48.0	69.6	A
6	Conarachin	Q6PSU4_ARAHY	49	39	48.0	66.4	A
7	Arachin Ahy-2	Q647H3_ARAHY	16	40	61.4	66.1	A
8	Gly-1 <i>A. hypogaea</i>	Q9FZ11_ARAHY	16	40	60.4	61.6	A
9	3-Oxoacyl-(acyl carrier protein) reductase	Q4WXH3_ASPFU	12	42	40.6	59.5	L
10	Gly-1 <i>A. hypogaea</i>	Q9FZ11_ARAHY	16	46	61.4	55.2	A
11	Conserved hypothetical lipoprotein	Q7VWB9_BORPE	24	42	49.6	54.9	NS
12	Hypothetical protein At2g35070	C84764	28	45	51.2	48.3	NS
13	Choline phosphate cytidyltransferase/predicted CDP-ethanolamine synthase	Q2UG27_ASPOR	29	41	49.7	48.9	NS
14	Lipocalin-type prostaglandin D synthase-like protein	Q8QGV5_BRARE	35	40	20.8	42.1	NS
15	Phosphatidylcholine-2-acylhydrolase	PA2V_AUSSU	100	41	5.3	42.3	NS
16	Hypothetical protein	Q84PY6_ORYSA	75	40	6.5	39.8	NS
17	Hypothetical protein P0614D08	Q5JKK0_ORYSA	87	50	10.6	39.8	NS
18	Acetyl-CoA synthetase	Q7W329_BORPA	39	58	76.3	39.3	NS
19	Transmembrane region, ABC transporter-related precursor	Q3CIB5_THEET	31	50	82.9	35.4	NS
20	Methylmalonyl-CoA mutase	AAZ59635	19	47	120.5	35.8	A
21	Phospholipid/glycerol acyltransferase	AAZ45433	37	46	20.3	35.6	A
22	Molybdate ABC transporter	Q1VI23_9FLAO	49	63	41.6	34.2	NS
23	Putative long chain fatty acid-CoA ligase	Q35FQ5_9BRAD	38	49	71.9	39.2	NS
24	Mitochondrial heat shock 22-kDa protein-like	AAM63747	58	51	23.4	23.8	NS
25	Hypothetical protein	Q2XA59_PSEPU	88	52	4.4	22.1	NS
26	Hypothetical protein	Q9SAL1_ARATH	91	95	20.5	19.5	NS
27	Oleosin-3 (OLE3)	AAU 21501	97	97	16.5	17.5	A, L

A	Selaginella	49	GIPVFLFFSPVLVPL	63	90	YFKGRHPVGS	DKIEAA	109
	Physcomitrella	57	SLPVLIFFFSPILVPL	71	94	YKGGHPVGS	DQVDAA	113
	Oryza	80	LGPIALLTSPWIWVPV	94	121	YFTGRHPVG	ADRVDYA	140
	Arachis	75	ATPLLVIFFSPILVPA	89	116	YVTGKHPAG	SDRLDYA	135
	Populus	54	ATPVLVLSSPILVPA	68	95	YVTGKHPPG	ADKLDYA	114
	Arabidopsis	74	ATPLLVIFFSPILVPA	84	115	YATGEHPQG	SDKLDYA	124
	Pinus	44	ATPVLIFFFSPVLVPE	58	85	YIRGKHPPG	ADKVDYA	104
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B Phylogram



C

OLE1	2	AEALYYGG	9	52	TPLFVIFSPVLVPA	66	99	GKHPPGANQLDTA	111
OLE2	2	AEALYYGG	9	52	TPLFVIFSPVLVPA	66	99	GKHPPGANQLDTA	111
OLE3	11	YGSSYGTS	18	75	TPLLVIFFSPILVPA	89	123	GKHPPAGSDRLDYA	135
		*		***:*****:	***		****: * : : * *		

D



FIGURE 2. **Phylogenetic analysis of seed-specific oleosin.** A, multiple sequence alignment of all seed-specific oleosins across plant species. B, BLAST search was performed using a ClustalW alignment of the close homologues. C, sequence analyses of peanut oleosin genes. Multiple sequence alignment of peanut oleosin 1–3 proteins was performed using sequences from the NCBI database. The conserved H(X)₄D and GXSG motifs are indicated in **boldface** and other conserved residues are shaded in *gray*. Asterisk denotes conserved residue in all sequences in the alignment. D, schematic representation of AhOLE3.

triolein. The N- (1–53 amino acids) and C-terminal domains (96–166 amino acids) and OLE3 (full-length, 1–166 amino acids) were independently cloned, expressed in a bacterial sys-

tem, and purified by Ni-NTA column (Fig. 3f). Primers used in generating deletion mutants are given in Table 3. The expressions of respective OLE3 proteins were confirmed by immuno-

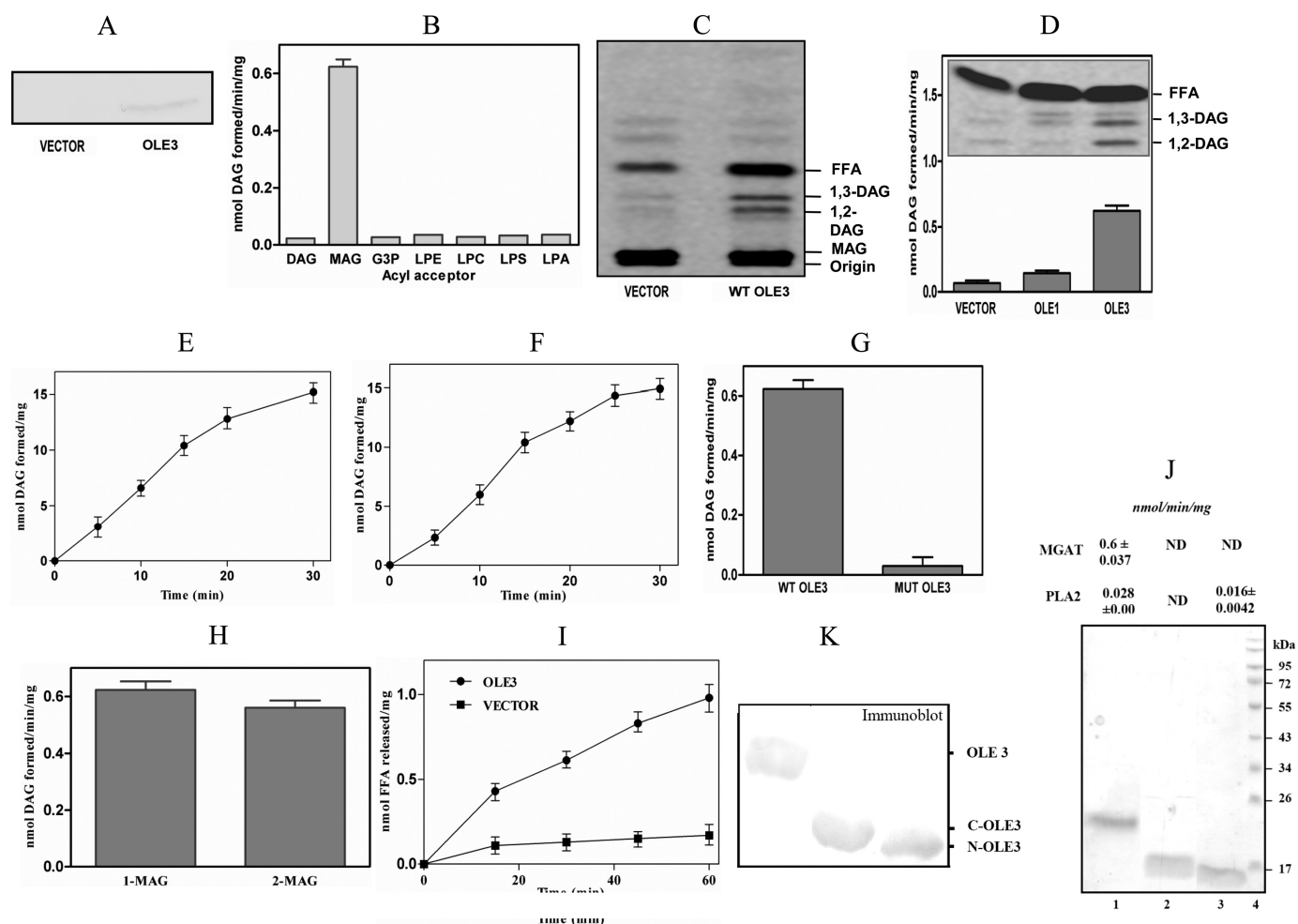


FIGURE 3. OLE3 encodes membrane-bound MGAT and phospholipase. A, immunoblot of OLE3 expression in microsomes of *S. cerevisiae*. The OLE3 overexpressed microsomes were resolved on a 15% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Immunoblotting was carried out using anti-OLE3 antibodies at a dilution of 1:500 (v/v). B, microsomes from cells overexpressing OLE3 isolated from TAG-defective *S. cerevisiae* QM (H1246) were assayed with various acyl acceptors for its substrate preference under the standard assay conditions. LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPA, lysophosphatidic acid; LPS, lysophosphatidylserine; G3P, glycerol 3-phosphate. C, MGAT activity was observed when incubated with *sn*-1-[^{14} C]oleoylglycerol and oleoyl-CoA as substrates in the presence of 5 μ g of microsomal protein from TAG-defective yeast cells. 1st lane, vector control; 2nd lane, overexpressed OLE3. D, reaction product formed by the recombinant OLE3 heterologously expressed in QM. The enzyme (5 μ g of microsomal membrane protein) was assayed using [^{14} C]oleoyl-CoA and MAG. 1st lane, vector control; 2nd lane, overexpressed OLE1; 3rd lane, overexpressed OLE3. The specific activities are represented with an error bar. E, time-dependent reaction was established by incubating 20 μ M [^{14} C]MAG with 10 μ M oleoyl-CoA in the presence of 5 μ g of microsomal protein isolated from cells overexpressing OLE3. F, time-dependent formation of DAG by the expressed OLE3 in *S. cerevisiae* (H1246 strain). The initial rate of the reaction was established by incubating 50 μ M MAG with 10 μ M [^{14} C]oleoyl-CoA at various time intervals in the presence of 5 μ g of microsomal membrane proteins from the cells overexpressing OLE3 as the enzyme source. Enzyme activity was derived from three independent experiments and is shown as mean $\pm$ S.D. G, MAG preference for OLE3. Specific activities were derived from three independent experiments and are shown as mean $\pm$ S.D. H, effect of point mutations in OLE3 on MGAT activity. The purified recombinant wild-type OLE3 (HX₄D) and the mutant (AX₄A) (5 μ g of protein) were assayed for MGAT activity. I, time-dependent hydrolysis of [1,2- ^{3} H]phosphatidylcholine by the microsomal membrane proteins of yeast cells overexpressing OLE3 (20 μ g). Values are means ($\pm$ S.D.) for three independent determinations, and each experiment was performed in duplicate. J, purification of the recombinant N- and C-terminal OLE3. The Ni-NTA affinity-purified full-length (lane 1) and C (lane 2) and N (lane 3) termini of bacterially expressed OLE3 proteins were resolved on a 15% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Specific activity represents the mean $\pm$ S.D. of triplicate measurements. ND represents activity not detected. K, immunoblotting was carried out using anti-His₆ monoclonal antibody at a dilution of 1:5000 (v/v).

TABLE 3

Primers used in this study

FWD OLE3 pYES2	5'-ATATAAGCTTATTATGTTATGTCTGATCAAACAAGGAC-3'
REV OLE3 pYES2	5'-ATATGAATTCTCAATACCCTGGGGTGCCCTC-3'
FWD OLE1 pYES2	5'-ATATAAGCTTATTATGGCTGAAGCACTCACTAC-3'
REV OLE1 pYES2	5'-ATATGAATTCTCAAGAAGCCTGGGCCCCAC-3'
FWD OLE3 pRSET C	5'-ATATCTGCAGATGTCTGATCAAACAAGGAC-3'
REV OLE3 pRSET C	5'-ATATGAATTCTCAATACCCTGGGGTGCCCTC-3'
FWD N-OLE3 pRSET C	5'-ATATCTGCAGATGTCTGATCAAACAAGGAC-3'
REV N-OLE3 pRSET C	5'-ATATGAATTCCAAGAATGAGACACCAATGGTTGA-3'
FWD C-OLE3 pRSET C	5'-ATATCTGCAGGCTGCTGGTGATTGTTTC-3'
REV C-OLE3 pRSET C	5'-ATATGAATTCTCAATACCCTGGGGTGCCCTC-3'
S14A	5'-GGAGGAGGAGGGGCTATGGATCATCC-3'
H125A	5'-GTCACCTGGGAAAGCCCCCTGCTGGCTCTGCTAGGCTTGATTATGC-3'
D130A	5'-GCATAATCAAGCCTAGCAGAGCCAGCAGGGGCTTCCCACTGA-3'

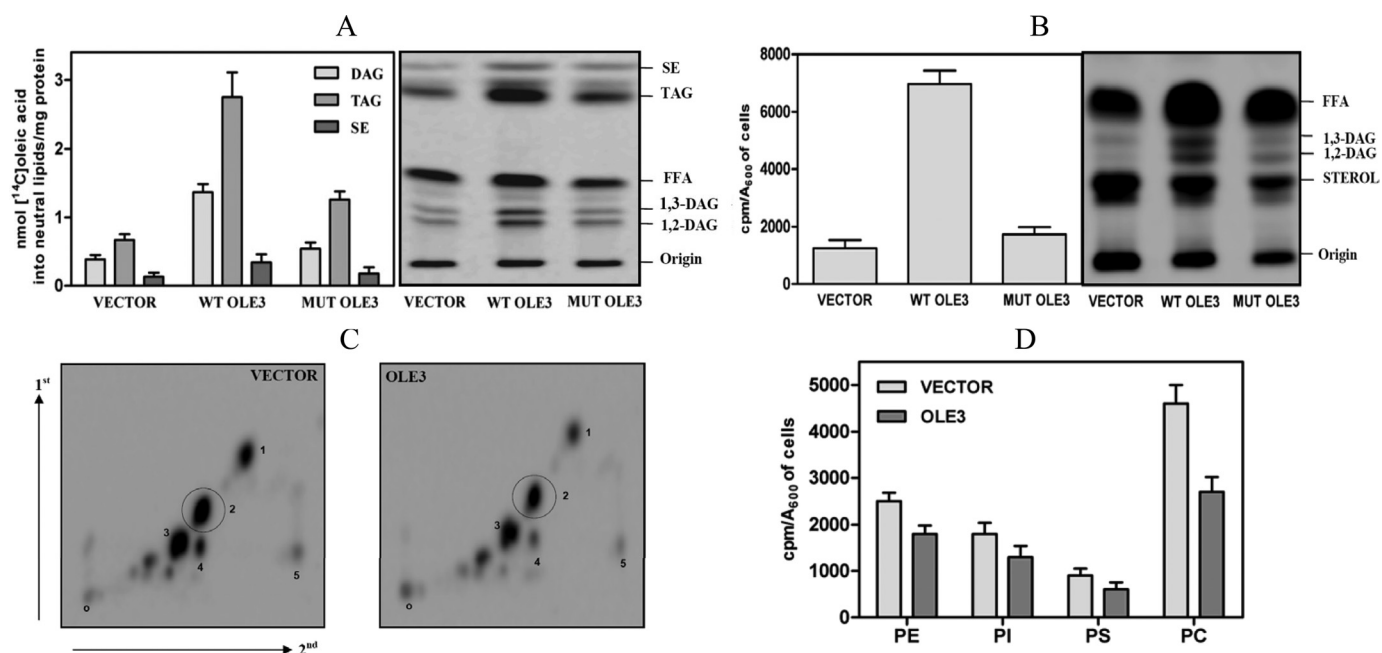


FIGURE 4. *In vivo* functions of OLE3. A, [^{14}C]oleic acid incorporation into lipids in the galactose-induced wild-type yeast cells (BY4741) overexpressing OLE3. B, [^{14}C]acetate incorporation into DAG in galactose-induced H1246 yeast cells overexpressing OLE3. C, incorporation of [^{32}P]orthophosphate (50 μCi) into phospholipids that are indicated as 1, phosphatidylethanolamine (PE); 2, phosphatidylcholine (PC); 3, phosphatidylserine (PS); 4, phosphatidylinositol (PI); 5, phosphatidic acid (PA); o, origin. D, labeling of [^{14}C]acetate (5 μCi) into phospholipids by the OLE3 overexpressing (H1246) yeast cells. Yeast cells were induced with galactose along with radiolabeled substrate overnight, and $A_{600} = 15$ cells were used for lipid extraction followed by silica-TLC. For the quantitation of phospholipids, each value represents the mean ($\pm$ S.D.) of three independent experiments.

blot with anti-His₆ monoclonal antibody (Fig. 3K). The purified recombinant proteins were assayed for MGAT and phospholipase A₂ activities. The full-length oleosin showed both activities, whereas the N-terminal domain showed 58% of phospholipase A₂ activity with no MGAT activity. However, the C-terminal domain showed no acyltransferase and hydrolase activities. The N-terminal domain alone is enough for the phospholipase A₂ activity. The serine residue in the lipase motif (GX¹⁴SXG) was changed to alanine and the site-directed mutant showed a 63% reduction of phospholipase A₂ activity. The site-directed mutation (S14A) caused no significant change in the acyltransferase activity. The full-length acyltransferase site-directed mutant (AX₄A) was not able to acylate MAG, but it hydrolyzed phosphatidylcholine. These data suggested that these two enzyme activities are independent of each other.

The *in vivo* labeling experiments validate the accumulation of DAG suggested by the enzymatic characterization. After examining the incorporation of [^{14}C]oleic acid into the lipids of yeast cells that overexpress OLE3, we found that there was an increase in the accumulation of DAG (Fig. 4A). Similar results were also obtained with [^{14}C]acetate labeling (data not shown). The labeling of [^{14}C]acetate also showed a higher level of DAG (3.87-fold) upon OLE3 overexpression than was showed in the vector control (Fig. 4B). The effect of OLE3 overexpression on the levels of cellular phospholipids also showed a slightly lower level of [^{32}P]orthophosphate incorporation into the phospholipids (specifically phosphatidylcholine) than in the vector control (Fig. 4C). Incorporation of acetate into phospholipids was monitored to study the relative distribution of fatty acids between the neutral lipids (DAG and TAG) and phospholipids.

The fatty acids were channeled more toward neutral lipids than toward phospholipids (Fig. 4D). These results indicate that the overexpression of OLE3 caused an increase in TAG formation and a decrease in phospholipid levels.

DISCUSSION

The biosynthesis of DAG occurs mainly in the microsomal membranes through the sequential acylation of glycerol 3-phosphate to TAG via DAG, unlike in the MAG pathway, where MAG is acylated to DAG by MGAT (26, 27). The membrane-bound glycerol 3-phosphate acyltransferase, AtGPAT1, has a necessary role in pollen development but has no significant effect on the oil content of *Arabidopsis* seeds (28). The overexpression of microsomal lysophosphatidic acid acyltransferase caused an enhanced TAG accumulation in seeds of *B. napus* (29). A soluble MGAT that has a role in TAG accumulation was identified from the immature peanut seeds (20). Gene coexpression network analysis of the transcriptome of developing *Arabidopsis* seeds indicated that the expression profiles of diacylglycerol acyltransferase (DGAT1) and oleosin are similar, suggesting the involvement of these genes in oil accumulation (30). In this study, we purified the microsomal membrane-bound MGAT from immature peanut seeds using a combination of urea (a chaotropic agent) and CHAPS (a zwitterionic detergent) without the loss of enzyme activity. A simple and functionally active multiprotein complex isolation procedure was developed using native-PAGE (31).

The following observations revealed the presence of the multiprotein complex in immature peanut seeds. The proteins in the multiprotein complex could be held together either by protein-protein interactions or by lipid-protein interactions in the

membranes of developing peanut cotyledons. In this study, a 14 S multiprotein complex from the microsomal membranes of immature peanut seeds was purified, and all the polypeptides were identified; the majority of them were storage proteins with the inclusion of a few acyltransferases. The association between storage proteins and acyltransferases is unclear. However, the detergent resistance property of the complex could be due to the presence of allergenic storage protein oligomers that potentially shielded the acyltransferases from denaturing conditions. The MAG acyltransferase was purified to apparent homogeneity by two successive iterations of native-PAGE using solubilized membranes, followed by a final step (10% native-PAGE) yielding a 2.7-fold increase in specific activity. The purified oil bodies showed time- and protein-dependent acylation with a specific activity 2.5-fold higher than the 14 S complex, which clearly indicates the enrichment of the oleosin 3 polypeptide. Oleosins, highly conserved in plant species, stabilize the oleosome. The deletion of the major oleosin resulted in larger oil bodies and the altered lipid content (18). The oleosin genes are classified into different groups based on the tissue-specific expression in *Arabidopsis* (floret tapetum, maturing seeds, and both) and primitive *Phycomitrella* (10, 13).

Across plant species, the seed-specific oleosins have a conserved H(X)₄D motif at their C terminus, which is a signature motif of an acyltransferase. However, their N terminus does not have a consensus conserved motif, with the exception of the GX SXG motif of OLE3, which is unique to the peanut species and acts as a signature motif of phospholipase and lipase activities. Thus far, the plant At4g24160p and yeast Ict1p, members of the α/β -hydrolase family of proteins, have been reported to have both conserved motifs, allowing them to catalyze the hydrolysis of lipids as well as the acylation of lysophosphatidic acid (23). The MBOT family genes from *Arabidopsis* (AtLPLATs) were overexpressed in *S. cerevisiae*, which caused the random selection of lysophospholipids for the synthesis of various phospholipids (32). OLE3 was overexpressed in *S. cerevisiae* (QM) and caused a significant acylation of MAG, which functioned as its acyl acceptor, although none of the other substrates were used as acceptors. We found no specificity or preference for the sn-1 or -2 position of the MAG acyl chain, as is shown from the lack of any apparent change in the K_m values when either 1-MAG or 2-MAG was utilized as a substrate. The overexpression of peanut oleosin 1 was unable to acylate MAG, unlike OLE3, in which the conserved aspartic acid residue is replaced by an asparagine. The specific activity of overexpressed OLE3 showed a 2.4- and 6-fold increase in oil bodies and microsomal membranes, respectively. The point mutations of H125A and D130A abrogate MGAT activity. The histidine in the HX₄D motif abstracts a proton from the hydroxyl group of an acyl acceptor, thereby facilitating the nucleophilic attack on the thioester of an acyl donor (25). The presence of the GX SXG motif at the N terminus of OLE3 suggests the possible lipase activity of the protein. OLE3 has the ability to hydrolyze phosphatidylcholine (Ca²⁺-independent phospholipase A₂), but it does not hydrolyze triolein. The N-terminal domain alone is enough for the phospholipase A₂ activity, but full-length protein is required for the MGAT activity.

Overall, phospholipid levels were lower than in the control vector upon OLE3 overexpression, and there was a significant decrease in the phosphatidylcholine related to the phospholipase A₂ activity of OLE3. OLE3 overexpression caused the metabolic fluxing of fatty acids toward neutral lipids but not phospholipids. Oleosin not only stabilizes the oleosome but also increases the TAG content in oil-accumulating seeds (14–16); it also acts as a surfactant (33). In *S. cerevisiae* (QM) devoid of TAG-biosynthesizing machinery, OLE3 is involved in incorporating labeled oleic acid and acetate mostly into DAG, unlike in the *S. cerevisiae* wild-type BY4741 strain where the incorporation is also seen in TAG. In the plant, overexpression of oleosin not only stabilizes the oleosome but also increases the TAG content in oil-accumulating seeds (16). In the mammalian system, the lipid droplet-associated protein adipophilin stimulates TAG accumulation and lipid droplet formation in murine fibroblast cells, and it is possible that the abundant storage protein may have both a structural and functional role in adiposome assembly (34). Our findings suggest a new role for oleosin in seed cellular physiology (maturation).

It is clear that OLE3 overexpression in *S. cerevisiae* principally affects changes in lipid metabolism, and fatty acids are siphoned off for TAG biosynthesis. A membrane-bound MGAT plays the requisite role in the assimilation of dietary fat in adipose tissues in mice (35), and it does not involve the accumulation of TAG. In contrast, OLE3 is involved in TAG biosynthesis during seed maturation. Furthermore, the identification of an lysophosphatidic acid phosphatase from oil-accumulating seeds denotes the existence of the MGAT pathway in plants (36), and it is indispensable in the biosynthesis and accumulation of TAG, given that MAG is sequentially acylated to DAG and TAG by MGAT and DAG acyltransferase, respectively. An alternative phosphatidic acid-independent pathway for TAG biosynthesis is proposed in this study (the MAG pathway). The regulation of MGAT activity remains an unanswered question in the field of lipid (TAG) accumulation. Thus, MGAT (OLE3) plays a pivotal role during seed maturation for synthesis and stores its future energy in the form of TAG.

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