Oleosin Is Bifunctional Enzyme That Has Both Monoacylglycerol Aeryltransferase 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 lyso phosphatidic 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, PX₅SP₅P, 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.
seeds, which caused delayed germination. The aberrant pheno-
types 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. Oligonu-
cleotides, monoclonal antibodies, phosphoamino acids, and all
other reagents were obtained from Sigma. Field-grown devel-
oping peanut (Arachis hypogaea) cotyledons (IL-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, 50 μM acetyl-CoA, 1 mM β-mercaptoethanol, 0.1 mM phenylmethyl-sulfo-
nyl fluoride, and 0.25 M sucrose (20). The extract was filtered
through two layers of cheesecloth, and the filtrate was differen-
tially 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 contain-
ing 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 contain-
ing 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]ole-
oyl-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 Phos-
phorImager (20).

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Protein Identification by Matrix-assisted Laser Desorption
Ionization (MALDI) Mass Spectrometry—The purified com-
plex 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, rehy-
drated 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-hydroxyxyocanocimnic acid in
50% acetonitrile and 0.1% trifluoroacetic acid containing ang-
iotensin as an internal standard, and applied to a MALDI sam-
ple 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 exter-
nally 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 deter-
mined 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).
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 (A600/H11005 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, A600/H11005 0.4 of the cells was inoculated in a fresh medium containing 2% galactose and one of the labeled substances (0.5 μCi/ml [14C]acetate; 0.5 μCi/ml [14C]oleic acid; 50 μCi/ml [32P]orthophosphate) and grown for 24 h. Cells (A600 = 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/glacial 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 mM urea and 10 mM CHAPS that did not inactivate the enzyme. To authenticate the solubilization procedure, the frac-
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 glycadin). 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 (PX5-PX5-P) 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 GXXG 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-monoyacylglycerol 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
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 system, and purified by Ni-NTA column (Fig. 3J). Primers used in generating deletion mutants are given in Table 3. The expressions of respective OLE3 proteins were confirmed by immuno-

**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 (H\(X_4D\)); L, lipase motif (G\(X_3G\)); NS, no signature motifs.

| Spot no. | Identification | NCBI accession no. | Coverage | Score | Thr. \(M_r\) | Exp. \(M_r\) | Motifs |
|----------|---------------|-------------------|----------|-------|------------|-------------|--------|
| 1        | Major allergen Arah1 | 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    | 57.0       | 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       | 64.4 A       |       |
| 7        | Gly-1A hypogaea | Q9FZ11_ARAHY | 16       | 40    | 61.4       | 66.1 A       |       |
| 8        | Gly-1A. hypogaea | 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-1A. hypogaea | Q9FZ11_ARAHY | 16       | 46    | 61.4       | 55.2 A       |       |
| 11       | Conserved hypothetical lipoprotein | Q7VWB9_BORFE | 24       | 42    | 49.6       | 54.9 NS     |       |
| 12       | Hypothetical protein A02G3070 | C94764 | 28       | 45    | 51.2       | 48.3 NS     |       |
| 13       | Choline phosphate cytidylyltransferase/predicted CDP-ethanolamine synthase | Q2UG27_ASPO | 29       | 41    | 49.7       | 48.9 NS     |       |
| 14       | Lipocalin-type prostaglandin D synthase-like protein | Q8QGV3_BRARE | 35       | 40    | 20.8       | 42.1 NS     |       |
| 15       | Phosphatidylcholine-2-acylhydrolase | PA2V_AUSU | 100      | 41    | 42.0       | 42.1 NS     |       |
| 16       | Hypothetical protein | Q6P4Y6_ORYSA | 75       | 40    | 6.5        | 39.8 NS     |       |
| 17       | Hypothetical protein P0614D08 | Q5IKK0_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 | AAZ29635 | 19       | 47    | 120.5      | 35.8 A       |       |
| 21       | Phospholipid/glycerol acyltransferase | AAZ4533 | 37       | 46    | 20.3       | 35.6 A       |       |
| 22       | Molybdate ABC transporter | Q1V123_9FLAO | 49       | 63    | 41.6       | 34.2 NS     |       |
| 23       | Putative long chain fatty acid-CoA ligase | Q3SFQ5_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 | Q2XAZ9_PSEPU | 88       | 52    | 4.4        | 22.1 NS     |       |
| 26       | Hypothetical protein | Q9SAI1_ARATH | 91       | 95    | 20.5       | 19.5 NS     |       |
| 27       | Oleosin-3 (OLE3) | AAU21501 | 97       | 97    | 16.5       | 17.5 A, L   |       |

**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 G\(X_3G\) 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.
TABLE 3

| Primers used in this study | Primer sequence (5’-3’)|
|---------------------------|------------------------|
| FWD OLE3 pYES2            | 5’-ATATAAGCTTATTATATGCTGATCAAAACAAGGAC-3’ |
| REV OLE3 pYES2            | 5’-ATATGAAATTCTCAAATACCCCTGGGGTGCCCTC-3’ |
| FWD OLE1 pYES2            | 5’-ATATGAAATTCTCAAATACCCCTGGGGTGCCCTC-3’ |
| REV OLE1 pRSET C          | 5’-ATATCTGCAGAGATGTCTGATCAAAACAAGGAC-3’ |
| REV OLE3 pRSET C          | 5’-ATATGAAATTCTCAAATACCCCTGGGGTGCCCTC-3’ |
| FWD OLE3 pRSET C          | 5’-ATATGAAATTCTCAAATACCCCTGGGGTGCCCTC-3’ |
| REV OLE3 pRSET C          | 5’-ATATGAAATTCTCAAATACCCCTGGGGTGCCCTC-3’ |
| S14A                      | 5’-GGAGGGAGGAGGGCTTATGAGACACCCAG-3’ |
| H125A                     | 5’-GTCAAATGCGACAGAGGACGAGGAGGCTTCCAGA-3’ |
| D130A                     | 5’-GTAAATGCGACAGAGGAGGACGAGGAGGCTTCCAGA-3’ |
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The purified recombinant proteins were assayed for MGAT and phospholipase A_2 activities. The full-length oleosin showed both activities, whereas the N-terminal domain showed 58% of phospholipase A_2 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 A_2 activity. The serine residue in the lipase motif (GX_{14}G) was changed to alanine and the site-directed mutant showed a 63% reduction of phospholipase A_2 activity. The site-directed mutation (S_{14}A) caused no significant change in the acyltransferase activity. The full-length acyltransferase site-directed mutant (A_{X_{4}}A) 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, A_{t}GPAT1, 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 GXSXG 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 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 a 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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