

Gene Family of Oleosin Isoforms and Their Structural Stabilization in Sesame Seed Oil Bodies

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Oleosins are structural proteins sheltering the oil bodies of plant seeds. Two isoform classes termed H- and L-oleosin are present in diverse angiosperms. Two H-oleosins and one L-oleosin were identified in sesame oil bodies from the protein sequences deduced from their corresponding cDNA clones. Sequence analysis showed that the main difference between the H- and L-isoforms is an insertion of 18 residues in the C-terminal domain of H-oleosins. H-oleosin, presumably derived from L-oleosin, was duplicated independently in several species. All known oleosins can be classified as one of these two isoforms. Single copy or a low copy number was detected by Southern hybridization for each of the three oleosin genes in the sesame genome. Northern hybridization showed that the three oleosin genes were transcribed in maturing seeds where oil bodies are being assembled. Artificial oil bodies were reconstituted with triacylglycerol, phospholipid, and sesame oleosin isoforms. The results indicated that reconstituted oil bodies could be stabilized by both isoforms, but L-oleosin gave slightly more structural stability than H-oleosin.

Key words: oil body; oleosin isoform; seed; sesame; structural stability

Plant seeds store triacylglycerol (TAG) as energy sources for germination and growth of seedlings. Storage TAG is confined to discrete spherical organelles called oil bodies.¹⁾ The oil bodies are 0.5–2.5 μm in diameter,²⁾ and their size is probably affected by the nutritional status and environmental factors.³⁾ An oil body contains a TAG matrix surrounded by a monolayer of phospholipid (PL) embedded with structural proteins termed oleosins and some minor proteins of higher molecular mass.⁴⁾ Oil bodies are maintained as individual small organelles even after long storage in plant seeds.⁵⁾ This stability is a consequence of the steric hindrance and electronegative repulsion provided by oleosins on the

surface of oil bodies.⁶⁾ Oleosins seem to cover the entire surface of an oil body so that the compressed oil bodies in the cells of a mature seed never coalesce or aggregate.⁷⁾

Oleosins are alkaline proteins with molecular masses of 15 to 24 kDa depending on the plant species.⁸⁾ Two immunologically distinct oleosins are present in seed oil bodies of diverse angiosperm species, and have been classified as high and low- M_r isoforms (H- and L-oleosin) depending on their relative molecular masses in the plant species in question.⁹⁾ Immunofluorescence labeling has shown that both oleosin isoforms coexist in oil bodies.¹⁰⁾ The physiological significance of there being two isoforms is unknown. In some species, three or more oleosins are found in the oil bodies. Whether these oleosins are isoforms other than H- and L-oleosin classes is not sure. In contrast, only one L-oleosin is present in gymnosperms.¹¹⁾ This finding leads to the inference that L-oleosin is the more primitive isoform, with H-oleosin derived from L-oleosin before the divergence of monocot and dicot species during evolution.

An oleosin molecule has been proposed to comprise three structural domains: an N-terminal domain, a central hydrophobic domain, and a C-terminal domain.¹²⁾ The central hydrophobic domain of oleosin is highly conserved among species. However, controversial secondary structures of this central hydrophobic domain have been proposed.^{6,13–15)}

In this study, we completed the cDNA sequencing of the oleosin gene family and confirmed the existence of two H-oleosins and one L-oleosin in sesame seed oil bodies. The difference between the two oleosin isoforms was investigated. The results showed that the main difference was an additional 18-residue fragment in the C-terminal domain of H-oleosins, and that all known oleosins can be grouped into these two classes. Copy numbers and expression patterns of the three sesame oleosin genes were identified. Ar-

The nucleotide sequences reported in this paper have been submitted to the GenBank Database under accession numbers AF091840 (15-kDa oleosin) and AF302807 (17-kDa oleosin).

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Abbreviations: DEPC, diethyl pyrocarbonate; IPTG, isopropyl-beta-D-thiogalactopyranoside; PL, phospholipid; TAG, triacylglycerol

tificial oil bodies were reconstituted with TAG, PL, and various sesame oleosin isoforms, and their structural stability was compared.

Materials and Methods

Plant materials. Mature and fresh maturing sesame (*Sesamum indicum* L., Tainan-1) seeds were obtained from the Crop Improvement Department, Tainan District Agricultural Improvement Station. The mature seeds were soaked in water for 10 min before preparation of oil bodies. Fresh maturing seeds 24 days were harvested after flowering of the plant for the construction of a cDNA library.

Purification of oil bodies. Oil bodies were extracted from sesame seeds and further purified by a protocol described before.¹⁶⁾ The method involved two-layer flotation by centrifugation, detergent washing, ionic elution, treatment with a chaotropic agent, and integrity testing with hexane.

Isolation of total RNA and poly(A)⁺ RNA. Total RNA extracted from the maturing seeds (24 days after flowering) was ground in liquid nitrogen using the phenol-SDS method.¹⁷⁾ Poly(A)⁺ RNA was isolated with Dynabeads (Dyna) following the manufacturer's instructions. The isolated poly(A)⁺ RNA was dissolved in water containing diethyl pyrocarbonate (DEPC) and then measured in terms of the A_{260} .

cDNA library construction, screening, and sequencing. cDNA was synthesized from poly(A)⁺ RNA by the protocol described by the manufacturer (cDNA synthesis, ZAP-cDNA synthesis, and ZAP-cDNA Gigapack III Gold Cloning kits were from Stratagene). A cDNA library of 10⁶ plaques was constructed with 5 µg of poly(A)⁺ RNA. A cDNA sequence encoding the central hydrophobic domain of sesame 15.5-kDa oleosin (an H-oleosin, accession no. U97700) or the entire rice sequence of 16-kDa oleosin (an L-oleosin, accession no. U43930) was ³²P-labeled and used as a probe to screen for sesame 17- or 15-kDa oleosin genes in the cDNA library as described elsewhere.¹⁸⁾ In the screening with the sesame 15.5-kDa oleosin probe, plaques with weak rather than strong signals were collected. We then excised the pBluescript phagemid from the Uni-ZAP XR vector *in vivo* from the plaques with weak signals on hybridization by the manufacturer's instructions. The potential clones were sequenced with an automatic sequencer. Sequences were compared in GenBank by the Blast program.¹⁹⁾ Sequence similarity was calculated by the Megalign program of DNASTar.

Overexpression of sesame oleosin clones in *Escherichia coli*. A full-length cDNA clone of sesame

17-kDa oleosin was constructed in the fusion expression vector pET29a (+) (Novagen) with an *Nco*I and a *Xho*I site in the polylinker of the vector. The recombinant fusion protein comprised an N-terminal appendix of 27 amino acid residues. The recombinant plasmid was used to transform cells of *Escherichia coli* strain BL21 (DE3). Overexpression was brought about with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) in a bacteriophage T7 RNA polymerase-promoter system. Three hours after this treatment, the *E. coli* cells were harvested, lysed by sonication in 10 mM Tris-HCl, pH 7.5, and then analyzed by SDS-PAGE and western blotting.

A 435-bp PCR product of a sesame 15-kDa oleosin clone with an engineered start codon but no stop codon was constructed in the same expression vector with an *Nco*I site in the polylinker of the vector. The recombinant fusion protein comprised an N-terminal appendix of 27 amino acid residues and a C-terminal appendix of 30 amino acid residues. The recombinant plasmid was overexpressed in *E. coli* and analyzed by SDS-PAGE and western blotting under the same conditions as above.

SDS-PAGE, antibody preparation, and western blotting. Proteins were resolved by Tricine SDS-PAGE.²⁰⁾ The sample was mixed with an equal volume of 2× sample buffer as suggested in the Bio-Rad instruction manual, and the mixture was boiled for 5 min. The electrophoresis system consisted of 12.5% and 4.75% polyacrylamide in the separating and stacking gels, respectively. After electrophoresis, the gel was stained with Coomassie Blue R-250 and destained.

Sesame 17- and 15-kDa oleosin were eluted separately from the SDS-PAGE gels and used to elicit antibodies in chickens.²¹⁾ Immunoglobulins were purified from egg yolks for the immunoassays. In the immunoassays, proteins in the gel for SDS-PAGE were transferred onto nitrocellulose membranes in a Bio-Rad Trans-Blot system by the manufacturer's instructions. The membrane was treated with secondary antibodies conjugated with horseradish peroxidase (Sigma, St. Louis, MO), and was then incubated with 4-chloro-1-naphthol containing H₂O₂ for color development as described before.²²⁾

Isolation of genomic DNA and Southern blotting. Genomic DNA was isolated from sesame leaves by the protocol of Sambrook *et al.*²³⁾ Isolated genomic DNA of 10 µg was digested with *Bam*HI and *Hind*III at 37°C overnight, and the resulting fragments were resolved in a 0.8% agarose gel and transferred onto a piece of blotting membrane (Sartorius, Göttingen, Germany). The blotted membrane was UV-cross-linked and hybridized with a ³²P-labeled probe containing a clone of sesame 17-, 15.5-, or 15-kDa oleo-

sin at 65°C for 14 h. The membrane was washed four times at 65°C by the manufacturer's instructions, and used to expose an X-ray film.

Isolation of RNA and northern blotting. Total RNA from seeds of various stages of maturity was extracted in liquid nitrogen by the phenol-SDS method.¹⁷⁾ The isolated RNA was dissolved in water containing DEPC and then measured in terms of the A_{260} . Total RNA of 5 µg was resolved in a 1% formaldehyde-agarose gel, transferred onto a piece of blotting membrane (Sartorius), and then treated by northern hybridization with the same probes as for Southern hybridization.

Reconstitution of artificial oil bodies. Three essential constituents (TAG, PL, and oleosin) of oil bodies were used to reconstitute artificial ones. TAG was extracted from sesame oil bodies; the PL dioleoyl phosphatidylcholine was purchased from Sigma. Three oleosin isoforms were eluted separately from SDS-PAGE and precipitated with an equal amount of acetone chilled at -20°C. The acetone mixture was kept at -20°C for 30 min and then centrifuged at 10,000 × g for 30 min to give a protein pellet. The pellet was suspended in 500 µl of 0.1 M sodium phosphate buffer, pH 7.5. The acetone precipitation was repeated two more times to remove SDS. After the removal of SDS, the insoluble protein pellets were sonicated in 500 µl of the sodium phosphate buffer before being used for reconstitution of artificial oil bodies.

For reconstitution, a 1-ml suspension containing 15 mg of TAG, 150 µg of PL, and 225 µg of oleosin was prepared in a 1.5-ml Eppendorf tube.¹⁰⁾ PL dissolved in chloroform was placed at the bottom of the Eppendorf tube, and the chloroform was allowed to evaporate in a chemical hood overnight. After evaporation, TAG and oleosin were incorporated, followed by sonication with a 3-mm-diameter probe in an ultrasonic processor GE 601 at an amplitude of 30% for 20 s. The sample was then cooled in an ice bucket for 5 min. The sonication was repeated two more times to generate artificial oil bodies.

Turbidity test. Oil bodies or reconstituted oil bodies in a suspension of 0.1 M sodium phosphate buffer, pH 7.5, floated to the top of the mixture. As a consequence, the suspension below the floating oil-body layer had decreased turbidity and was measured at intervals by the following method. A 1-ml portion of the mixture was placed in a disposable cuvette of 2-ml capacity. The cuvette was covered with parafilm and kept undisturbed. The absorbance of the suspension in the lower portion of the cuvette was read at 600 nm in a Beckman DU 530 spectrophotometer at intervals. At the start, the absorbance A was 2.0 (A_0). The turbidity (T) of the suspension was proportional to 10^A , and the relative turbidity is expressed as

$$T/T_0 = 10^A/10^{A_0} = 10^A/10^{2.0}.$$

Results and Discussion

Cloning and sequencing of oleosin genes from maturing sesame seeds

Three oleosins of the molecular masses 17, 15.5, and 15 kDa have been identified in sesame seed oil bodies, and a full-length cDNA clone encoding the 15.5-kDa oleosin has been sequenced and classified as an H-oleosin.¹⁸⁾ We set out to clone the other two sesame oleosin genes. With the sesame 15.5-kDa H-oleosin probe, a full-length cDNA clone (accession no. AF302807) with an open reading frame encoding a putative 17-kDa oleosin was obtained. The cDNA fragment comprised 808 nucleotides, consisting of a 23-nucleotide 5' untranslated region, an open reading frame of 501 nucleotides, and a 284-nucleotide 3' untranslated region. The deduced polypeptide had 166 amino acid residues with a molecular mass of 17,374 Da.

With the rice L-oleosin probe, a full-length cDNA clone (accession no. AF091840) with an open reading frame encoding a putative 15-kDa oleosin was obtained. The cDNA fragment comprised 708 nucleotides, consisting of a 34-nucleotide 5' untranslated region, an open reading frame of 438 nucleotides, and a 236-nucleotide 3' untranslated region. The deduced polypeptide had 145 amino acid residues with a molecular mass of 15,194 Da.

Identification of sesame oleosin clones by immunological recognition of their overexpressed oleosins in E. coli

The overexpressed fusion oleosins of the cDNA clones screened by the sesame 15.5-kDa oleosin clone and rice 16-kDa oleosin clone were recognized as strongly as their original antigens by antibodies against 17- and 15-kDa oleosin, respectively (Fig. 1). The results confirmed that the two cDNA clones encoded sesame 17-kDa or 15-kDa oleosin.

Classification of sesame oleosin isoforms

Sequence comparison with known oleosin sequences showed that sesame 17-kDa oleosin and 15.5-kDa oleosin were H-oleosins and 15-kDa oleosin was an L-oleosin. Sequence alignment of three structural domains of oleosin isoforms from one gymnosperm (pine), two dicot species (sesame and *Arabidopsis thaliana*), and two monocot species (maize and rice) is shown in Fig. 2. There were two H-oleosins and one L-oleosin in seed oil bodies of sesame and maize, one H-oleosin and one L-oleosin in oil bodies of rice, and three H-oleosins and three L-oleosins encoded by *Arabidopsis* genome. The N-terminal domains and the C-terminal domains of H-oleosin and L-oleosin were different, whereas the central hydrophobic domain was highly conserved. Even

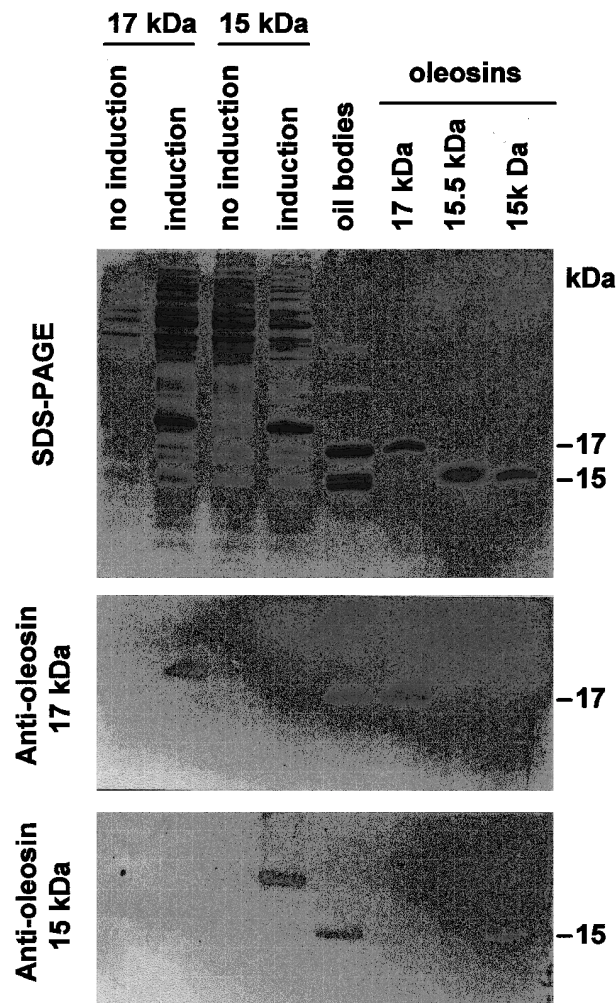


Fig. 1. SDS-PAGE (above) and Western Blotting (below) of Two Overexpressed Sesame Oleosin Clones as Fusion Polypeptides in *E. coli*.

Putative sesame 17- and 15-kDa oleosins were overexpressed in a fusion vector without ("no induction") or with ("induction") IPTG induction in *E. coli*. In addition, oil bodies and the three oleosins (17, 15.5, and 15 kDa) in sesame oil bodies were resolved by SDS-PAGE. A duplicate gel with one third of the protein content of the PAGE was transferred to nitrocellulose paper and then immunoassayed with antibodies against sesame 17- and 15-kDa oleosins.

within the same isoform class, the N-terminal domains and the C-terminal domains were only slightly conserved. Not counting the last 10 to 30 residues (depending on the species), oleosins were conserved but the two isoforms were distinguishable in the C-terminal domain. In particular, there was an additional 18-residue fragment in H-oleosins inserted near the end of the conserved region in the C-terminal domain (boxed in Fig. 2). On the basis of this difference, the single oleosin in pine was confirmed to be an L-oleosin. Moreover, all known oleosins can be grouped into these two isoform classes, including those species with at least three oleosins (data not shown).

In several species, the difference in molecular mass

between H-oleosin and L-oleosin is 2 kDa: maize has 18- and 16-kDa oleosins;⁸⁾ *Arabidopsis thaliana* has 21- and 19-kDa oleosins;^{24,25)} barley has 18- and 16-kDa oleosins;²⁶⁾ rice has 18- and 16-kDa oleosins;²⁷⁾ and sesame has 17- and 15-kDa oleosins (this report). Presumably, the difference is constant because of the insertion of 18 residues (~2 kDa) in the C-terminal domain of the H-oleosins. This insertion seems to occur at the time of the splitting of H-oleosin and L-oleosin during evolution, or soon after, accounting for its being found in H-oleosins of all angiosperm species.

On the basis of immunological cross-recognition¹¹⁾ and sequence comparison, it seems likely that H-oleosin was derived from L-oleosin before the divergence of monocot and dicot species. Duplicate H-oleosins have been found in six species including monocots and dicots (Table 1). However, the duplication was not ubiquitously in monocots and dicots, and the similarity of the two H-oleosins in the same species, ranged from 52.8% in sesame to 98.3% in perilla. Probably, the occurrence of duplicate H-oleosins in several species was not the result of a single duplication. Interestingly, sesame 15.5-kDa oleosin was shorter than the other H-oleosin, at 17 kDa. The difference of molecular mass arose from the loss of 22 amino acids in both N-terminal and C-terminal regions, including a shortening of the 18-residue insertion of H-oleosins to 12 residues.

Single copy or a low copy number of oleosin genes in the sesame genome

Results of copy number of 17 kDa, 15.5 kDa and 15 kDa oleosin genes in sesame genome are shown in Fig. 3. In each enzymatic digest of each of the three oleosin genes, one fragment was abundant, suggesting that there was a single copy or a low copy number of each oleosin gene in the sesame genome.

Expression of oleosin isoforms during seed maturation

Results of northern hybridization are shown in Fig. 4. Accumulation of 17-kDa oleosin mRNA was started in maturing seeds some 2 weeks after flowering and continued steadily thereafter until the amount decreased in the seed-drying stage, reaching undetectable levels in mature seeds. Similar expression patterns were found for mRNAs of 15.5- and 15-kDa oleosin. The 15.5-kDa oleosin mRNA appeared a few days later and 15-kDa oleosin mRNA a few days earlier than 17-kDa oleosin mRNA in the maturing seeds. The difference times when the mRNAs were expressed matched the accumulation of oleosin in maturing sesame seed oil bodies.²¹⁾ It is likely that these three oleosin genes are regulated as seed maturation proceeds to be expressed in varying intensities. Presumably, 15.5-kDa H-oleosin was duplicated from 17-kDa H-oleosin after the diver-

Sesame H1	MADRDR-----PHPHQIQVHPQHPRHYEGGV-----KSLLPQKGPSTTQILA	42
Sesame H2	MAD-----EPHD-----QRPTDVI-----KSYLPEKGPSTSOVLA	30
Arab. H1	MADTHR-----VDRTDRHFQFQSP---YEGGRGQGQYEGDRGYGGGG---YKSMPPECSGPSSTQVLS	56
Arab. H2	MANVDRDRRVHVDRTDKRVH-QPN---YEDDVGFGGYG--GYAGSD----YKSR----GPSTNQILA	54
Arab. H3	MAD-----VRTHSHQLQVHPQRQ---HEGGI-----KVLYPQSGPSSTQVLA	39
Maize H1	MADRDR-----SGIYGG---AHATYGGQQQQGGGGRPMGEQVKKGMLHDKGPTASQAALT	51
Maize H2	MADRDR-----SGIYGG---G---AYGQQQ---GRPPMGEQVK-GMIHDKGPTASQAALT	44
Rice H	MADRDR-----AGQY-----YQQQRGQ---VGETVK-GILPEKAPSASAQAALT	38
Sesame L	MAEH-----YGQQQQTRAPHLQLQPRAQR-----VVK	27
Arab. L1	MVSLLK-----LQKHRTLNPYSLRKRKKEMADHQHQHQQQQP---IMRS-LHESSPSTROIWR	55
Arab. L2	MADTARG-----THHDIIGRDQYPMMGRDRDQYQMSEGRGS DY-----SKSRQIAK	45
Arab. L3	MADQTR-----THHEMISR-----STQE AH-----PKARQMVK	42
Maize L	MADHHRG-----A---TG GGGGYGD LQRGGGMHGEAQQQQKQ-----GAMMTALK	29
Rice L	MADQHRG-----V---IGGGG-YGD-RGG---OE QQEKQ-----PFMMTALK	34
Pine L	M-----OKIQENA-----PSAQQVMK	16

proline knot

Sesame H1	IITLLPISGTTLLCLAGITTLVGTLLIGLAVATPVLVIFSFVLVPAALIIAGAVTAFLTSGAFLGTGLSSLSWV	113
Sesame H2	VVTLPPLGAVLLCLAGLIILTGTIIIGLAVATPLVVFISFVLVPAALTIALAVTGFLTSGAFLTALSSISWL	101
Arab. H1	LLIGVPVVGSLLALAGLLLAGSVIGLMLAFPLFLFSEVIVPAALTIGLAMTGFLASGMFGLTGLSSISWV	127
Arab. H2	LIAGVPIGGTLLTLTLAGTLTAGSVIGLLVSIPLFLFSEVIVPAALTIGLAVTGILASGLFGLTGLSSVSWV	125
Arab. H3	VFVGVPPIGGTLLTIAGTLTAGSVIGLMLAFPLFLFSEVIVPAAEVIGLAMTGFLASCAIGLTGLSSMSWV	110
Maize H1	VATLPPLGGLLLVLSGLALTASVVGLAVATPVLVIFSFVLVPAALLIGTAVMGFLTSGALGGLGSSITCL	122
Maize H2	VATLPPLGGLLLVLSGLAALASTVGLAVATPVLVIFSFVLVPAALLIGTAVAGFLTSGALGGLGSSITCL	115
Rice H	VATLPPLGGLLLVLSGLAALASVVGLAVDTPVLVIFSFVLVPAALLIGLAVAGFLTSGALGGLGSSITFL	109
Sesame L	AATAVTAGGSLLVLVSGTLTLAGTVIALTIATPVLVIFSFVLVPAVITIFLLGAGFLASGGFGVAALSVLSWI	98
Arab. L1	FVTAATIGLSLLVLVSGTLTGTGTIGLIVATPLMVLFSEVLPFAVITIGLLTMGFLFSGGCGVAATALTWI	126
Arab. L2	AATAVTAGGSLLVLVSSLTLVGTVIALTVATELLVIFSFVLVPAALITVALLITGFLSSGGFGIAAITVFSWI	116
Arab. L3	AATAVTAGGSLLVLVSGTLTLAGTVIALTVATELLVIFSFVLVPAVVTALAIITGFLSAGGFGIAAITAFSWL	100
Maize L	AATAATFGGSMVLVSGLLIAGTVIALTVATPVLVIFSFVLVPAALIALMALMAAGFVTSGGLGVAALSVFSWM	113
Rice L	TVTAATAGGSMLVLVSGLLIAGTVIALTVATPVLVIFSFVLVPAALIALMALMAAGFVTSGGLGVAALSVFSWM	105
Pine L	MMAAITVGGALLLLLAGITTLTGTVTLTVATEPVLVIFSFVLVPGVTVLLTTIGFLSAGGFGIAALSSLSWI	87

H-form insertion

Sesame H1	LNSFRATGQGP--LEYAKRGVQEGTLYVGEKTKQAGEAIKSTAKEG-----GREGTART	166
Sesame H2	LNIVRRMRGSLPEQLDHARRRVQET---VGQKTREAGQR-----SQDVIRP	144
Arab. H1	MNYLRGTRRTVPEQLEYAKRRMADAVGYAGQKGKMGQHVQNKAQDVQKQ-YD-ISKPHD'TTKGHETQGRITAA	199
Arab. H2	LNLYRGTSDTVPEQLDYAKRRMADAVGYAGMKGKMGQYVQDKAHEARE-TE-F-----MTETHEPGKARRGS	191
Arab. H3	LNIIIRAGQHIPEELEAKHRLADMAEYVGQRTKDAQTIEDKAHDVRE-AKTFDVRDRD'TTKGTHNVRD'TKTT	183
Maize H1	ANTARQAFQRTPDYVEEARRRMAFAAAQAGHKTAQAGQAIQGRAQEA-----GTGGAGAGAGGGGGRASS	187
Maize H2	ANTARQAFQRTPDYVEEARRRMAFAAAHAGHKTAQAGHGIQSKAQEA-----GACTGAG-----GGRTSS	175
Rice H	ANTARQAFQRTPDYVEQARRRMAFAAAHAGHKTAQAAHAIQGRADQA-----GTG--AGAGGGAGTKTSS	172
Sesame L	YRYLTGKHPPGADQLESAKTKLAS-----KAREMKDRAEQFSQQPVA-----GSQTS	145
Arab. L1	YKYVTGKHPPMGADKVDYARMRIAE-----KAKEL--GHYTHSQ-P-QQTHQ-----TTTTTH	174
Arab. L2	YKYATGEHPQGSCLKDSARMKLGs-----KAQDLKDRAQYYGQOHTTGGEHDRDRTRGGQHTT	173
Arab. L3	YRHMTGS--GSDKIENARMKVGs-----RVQDTK-----YGOHNIGVQH-----QQVS	141
Maize L	YKYLTKGKHPPAADQLDHAKARLAS-----KARDVKDAA-----QHRID-----QAQG-S	156
Rice L	YKYLTKGKHPPGADQLDHAKARLAS-----KARDIKEAA-----QHRID-----QAQA-S	148
Pine L	VNYIRGKHPPGADKVDYARORIAS-----KAKDKDRAGGYMOKKGDOATOG-----LPEHTS	140

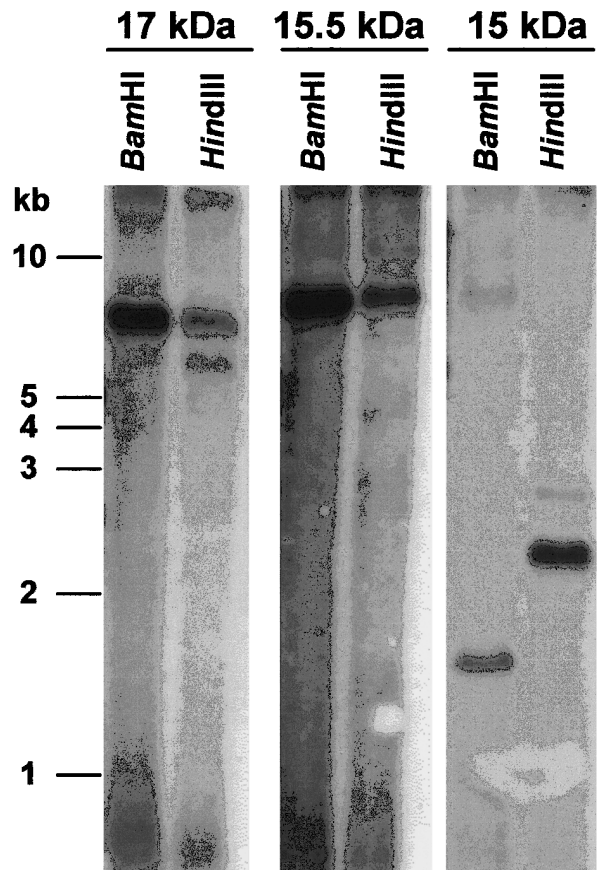
Fig. 2. Sequence Alignment of H- and L-Oleosin Isoforms from One Gymnosperm,³⁰⁾ Two Dicot Species and Two Monocot Species.

The sequences are aligned according to the three structural domains (N-terminal, central hydrophobic, and C-terminal domains) of oleosins. The amino acid number of the last residue of each domain is listed on the right for each species. Broken lines in the sequences show gaps introduced for best alignment. Residues identical in different species are shaded. The location of a putative insert of an 18-residue fragment in the C-terminal domain of H-oleosins is boxed. The four invariable residues in the proline knot motif are enclosed. The accession numbers of the aligned oleosin sequences are: AF302807, U97700, and AF091840 for sesame H1, H2, and L; S71180, Z54164, AAF01542, AAC42242, S22538, and Z54165 for *Arabidopsis* (Arab.) H1, H2, H3, L1, L2, and L3; P21641, S52030, and S52029 for maize H1, H2, and L; U 43931 and U43930 for rice H and L.

Table 1. Sequence Similarity of Duplicate H-Oleosins in Six Species

The accession numbers of the two H-oleosins H1 and H2 are: sesame, AF302807 and U97700; sunflower, S60482 and P29529; cotton, P29527 and P29528;²⁸⁾ maize, P21641 and S52030;²⁹⁾ soybean, P29530 and P29531; and perilla, AAG24455 and AAG09751.

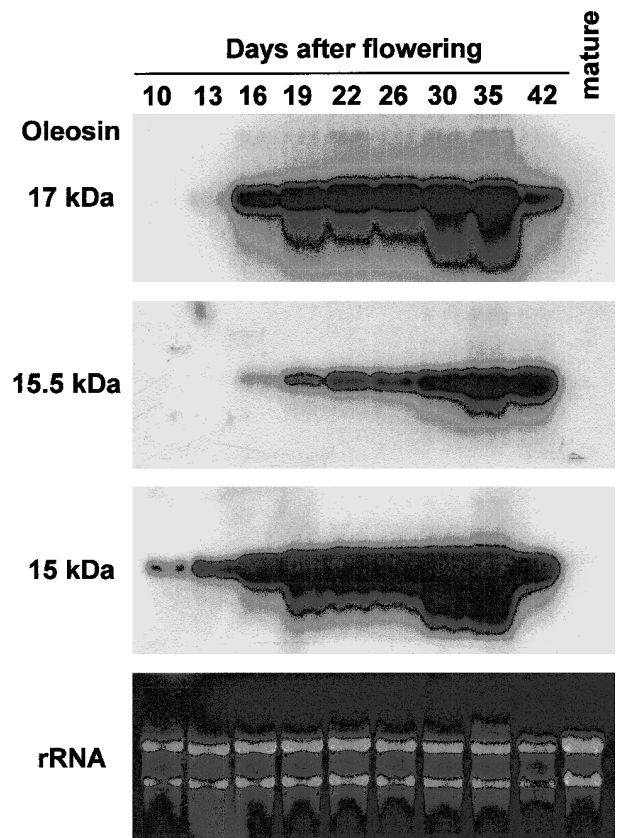
	Sesame		Sunflower		Cotton		Maize		Soybean		Perilla	
	H1	H2	H1	H2	H1	H2	H1	H2	H1	H2	H1	H2
Residues	166	145	184	181	168	154	187	175	226	223	177	175
Sequence similarity of two H-oleosins (%)	52.8		63.5		73.4		83.4		85.2		98.3	

**Fig. 3.** Southern Blotting of Genomic DNA Extracted from Leaves of Sesame Plants.

Each lane had 10 μ g of genomic DNA completely digested with *Bam*HI and *Hind*III. After being blotted, the membrane was hybridized with a ³²P-labeled probe containing the clone of sesame 17- or 15-kDa oleosin.

gence of H-oleosin from L-oleosin in evolution. Surprisingly, the mRNA expression pattern of 17-kDa H-oleosin resembled that of 15-kDa L-oleosin more than that of 15.5-kDa H-oleosin. Compared with the other duplicate H-oleosins in various species, sesame 15.5-kDa H-oleosin seemed to be derived from 17-kDa H-oleosin earlier or to change faster after duplication.

Of the three oleosins in sesame oil bodies, 15.5-kDa oleosin is least abundant (Fig. 1). The 3' untranslated region (109 nucleotides) of the 15.5-kDa oleosin clone was shorter than that (284 or 236

**Fig. 4.** Northern Blotting of Total RNA Extracted from Various Stages of Maturing Sesame Seeds.

Each lane had 5 μ g of total RNA extracted from maturing seeds at various days after flowering. After being blotted, the membrane was hybridized with a ³²P-labeled probe containing the coding sequence of sesame 17-, 15.5-, or 15-kDa oleosin. Only the portion of the membrane corresponding to the position of visible hybridized RNA is shown.

nucleotides) of the two other clones. Whether the 3' untranslated regions of oleosin genes were related to their changes in mRNA expression, and relative protein abundance in seed oil bodies is not known.

Stability of reconstituted oil bodies as affected by sesame oleosin isoforms

Sonicated TAG droplets without oleosins were extremely unstable, and floated to the top of the solution in a few minutes, decreasing the turbidity rapidly (Fig. 5). Inclusion of amphipathic proteins or surfactants may increase the stability of the sonicated

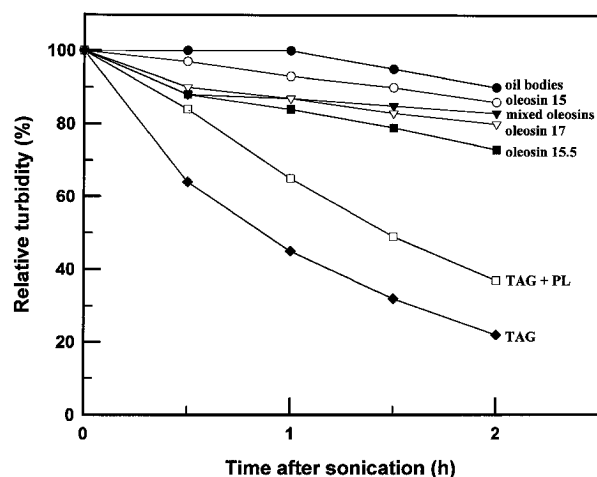


Fig. 5. Stability of Artificial Oil Bodies Reconstituted with TAG, PL, and Sesame Oleosin(s).

A suspension (1 ml) of sesame oil bodies or artificial oil bodies reconstituted with TAG, PL, and various sesame oleosins was placed in a 2-ml cuvette. The relative turbidity (T/T_0) at 600 nm of the lowest 0.5 ml of the suspension was measured at intervals. For reconstitution of oil bodies, a 1-ml suspension containing 15 mg of TAG, 150 μ g of PL, and 225 μ g of oleosin(s) (one or a mixture of the three sesame oleosins) was sonicated in a 1.5-ml Eppendorf tube. The curved labelled oleosin 17, 15.5, 15, and mixed oleosins are for artificial oil bodies reconstituted with TAG, PL and the oleosin(s) indicated.

droplets by forming oil emulsions. In our experiments, stable artificial oil bodies could be reconstituted through sonication with TAG, PL, and oleosin(s). Artificial oil bodies reconstituted with 15-kDa L-oleosin were more stable than those reconstituted with 17- or 15.5-kDa H-oleosin or with a mixture of natural oleosin isoforms. The different stability was reproducible. The result is in accord with that obtained with reconstituted oil bodies and rice oleosin isoforms.¹⁰ Nevertheless, none of the oil bodies reconstituted under our experimental conditions maintained their integrity as natural seed oil bodies purified from mature sesame seeds did, presumably because of imperfect reconstitution.

H-oleosin in angiosperm species is not essential for seed oil bodies; only L-oleosin is present in seed oil bodies of gymnosperm species.¹¹ Though H-oleosin was less stabilizing than L-oleosin, some unidentified evolutionary benefit may account for its occurrence in angiosperms and its duplication in several species. We speculate that H-oleosin was derived from L-oleosin and modified, mostly in the insertion of an 18-residue fragment in the C-terminal domain, executing some biological function better than L-oleosin at the expense of slightly reduced stability of the organelles. This biological function may be related to the mobilization of oil bodies during seed germination and postgerminative growth of seedlings. H-oleosin may be a better receptor than L-oleosin for lipase attachment or for organelle interaction with

glyoxysomes.

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