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Coexistence of Both Oleosin Isoforms on the Surface of Seed Oil Bodies and Their Individual Stabilization to the Organelles[1]

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The oil bodies of plant seeds contain a triacylglycerol matrix surrounded by a monolayer of phospholipids embedded with alkaline proteins termed oleosins. Two distinct oleosin isoforms with molecular masses of 18 and 16 kDa are present in rice oil bodies. Chicken antibodies raised against oleosin 18 kDa and rabbit antibodies raised against oleosin 16 kDa did not cross-recognize these two homologous isoforms. This peculiar non-cross recognition was used to locate the two oleosin isoforms on the surface of oil bodies *via* immunofluorescence detection using anti-chicken IgG conjugated with FITC (fluorescein isothiocyanate) and anti-rabbit IgG conjugated with Texas-Red. The results revealed that both oleosin isoforms resided on each oil body *in vivo* and *in vitro*. Artificial oil bodies were reconstituted *via* sonication using triacylglycerol, phospholipid, and oleosins. The results indicated that the two rice oleosin isoforms could stabilize artificial oil bodies individually whereas oleosin 16 kDa provided better stability to the organelles than oleosin 18 kDa.

Key words: immunofluorescence, isoform, oil body, oleosin, organelle stability.

Vegetable cooking oils used for human consumption comprise triacylglycerols (TAGs) extracted from various plant seeds. Plant seeds store TAGs as energy sources for germination and the postgerminative growth of seedlings. The storage TAGs are confined to discrete spherical organelles called oil bodies, lipid bodies, oleosomes, or spherosomes (*1-3*). An oil body is 0.5 to 2.5 μm in diameter and contains a TAG matrix surrounded by a monolayer of phospholipids (PLs) embedded with abundant proteins termed oleosins (*4*).

Oil bodies are maintained as individual small organelles even after a long period of storage in plant seeds (*5*). This stability is a consequence of the steric hindrance and electronegative repulsion provided by oleosins on the surface of the oil bodies (*6*). It has been suggested that the entire surface of an oil body is covered by oleosins (*7*). Therefore, the compressed oil bodies in the cells of a mature seed would never coalesce or aggregate. An oleosin molecule is proposed to comprise three distinct structural domains: an N-terminal amphipathic domain, a central hydrophobic antiparallel β-strand domain, and a C-terminal amphipathic α-helical domain (*6, 8*). The proposed secondary structures of oleosins are in agreement with the results of spectrum analyses (*9*). The central hydrophobic domain of oleosin is highly conserved among diverse species. The possible structural role of the central hydrophobic domain is to anchor the oleosins on the surface of oil bodies by penetrating the surface PL layer and residing in the hydrophobic TAG matrix. As a consequence of the insolubility of this hydrophobic domain, no three-dimensional structure of an oleosin, determined by X-raying or NMR, is available at the present time.

Oleosins are alkaline proteins with molecular masses of 15 to 26 kDa, depending on the isoforms and plant species in which they occur (*10, 11*). To date, the sequences of more than thirty oleosins in diverse plant species have been determined on corresponding cDNA, genomic DNA and direct amino acid sequencing. There are at least two isoform classes of oleosins present in seed oil bodies (*12*). The biological meaning of the presence of these two isoforms remains unknown. Whether the two oleosin isoforms coexist in each oil body or constitute two types of oil bodies has not been clarified. Realization of the above organization of oleosin isoforms in oil bodies will have a direct impact on further investigation of the structure-function relationship of these two oleosin isoforms on the surface of the organelles. Meanwhile, the individual and combined contributions of these two oleosin isoforms to the stability of oil bodies have not been elucidated.

In this report, we took advantage of that antibodies raised against two oleosins isoforms did not cross-recognize each other and selected rice oil bodies as a model system for examining the distribution of the two oleosin isoforms on the surface of oil bodies *via* immunofluorescence detection. The results indicated that both oleosin isoforms coexisted on each oil body. We also examined the individual stabilization by the two oleosin isoforms of the organelles *via* reconstitution of artificial oil bodies. It seemed that oleosin 16 kDa provided better stability of oil bodies than oleosin 18 kDa.

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Abbreviations: TAG(s), triacylglycerol(s); PL(s), phospholipid(s).

MATERIALS AND METHODS

Plant Materials—Mature rice (*Oryza sativa* L., Japonica TNG67) was a gift from the Taiwan Agricultural Research Institute. The mature seeds were soaked in water for 10 min prior to the purification of oil bodies.

Purification of Oil Bodies—Oil bodies were extracted from mature rice, and then subjected to further purification, including two-layer flotation by centrifugation, detergent washing, ionic elution, treatment with a chaotropic agent, and an integrity test with hexane (*13*).

Production of Antibodies in Chickens and Rabbits—Proteins extracted from rice oil bodies were resolved by SDS-PAGE on 12.5 and 4.75% polyacrylamide gels, as the separating and stacking gels, respectively (*14*). After electrophoresis, the gel was stained with Coomassie Blue R-250, and then destained. The stained oleosin bands were cut out and extracted according to the modified gel-extraction procedure reported by Tzen *et al.* (*12*). The purified oleosins were used for the production of antibodies in chickens or rabbits. Antibodies against oleosin 18 kDa were raised in two chickens and antibodies against oleosin 16 kDa were raised in two rabbits. Pre-immune antibodies were obtained from the chickens or rabbits a week before antigen injections, and were used as pre-immune blotting controls. The procedure was the modified one reported by Chuang *et al.* (*15*).

Purification of Monospecific Antibodies on Oleosin Affinity Columns—To prepare oleosin affinity columns, approximately 1 mg of rice oleosin 18 or 16 kDa was incorporated onto 1 g CNBr-activated Sepharose 6 MB gel (from Pharmacia) following the supplier's protocol. A preparation of 10 mg chicken antibodies against rice oleosin 18 kDa or rabbit antibodies against rice oleosin 16 kDa was applied to the oleosin (18 or 16 kDa) affinity column in a buffer of 20 mM Tris-HCl, pH 7.5. The bound antibodies were eluted with 3 ml of an eluting solution containing 500 mM NaCl, 0.5% Tween-20, and 5 mM glycine-HCl, pH 2.4. The eluate was neutralized with 2 ml of 500 mM sodium phosphate, pH 7.5, contiguously. The column was further eluted with 3 ml of a neutralization solution containing 140 mM NaCl, 0.5% Tween-20, and 10 mM Tris-HCl, pH 7.5. The two eluates were combined and precipitated with an equal volume of acetone pre-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 obtain a protein pellet of monospecific antibodies. The pellet was resuspended in 20 mM Tris-HCl, pH 7.5, to a concentration of 1 mg/ml.

Western Blotting—The proteins in the SDS-PAGE gel were transferred to a nitrocellulose membrane for 2 h at 0.25 A in a Bio-Rad Trans-Blot system. The transfer buffer contained 25 mM Tris, 192 mM glycine (pH 8.3), and 20% methanol. The membrane was blocked with 3% gelatin in Tris-buffered saline (TBS) containing 10 mM Tris-HCl, pH 7.5, and 150 mM NaCl for 30 min. It was then incubated with the antibodies or pre-immune antibodies (control) diluted in TBS containing 1% gelatin at room temperature for 2 h. The membrane was rinsed with distilled water and then washed twice (10 min each) in TBS containing 0.05% Tween-20 before the addition of the peroxidase-conjugated goat anti-chicken IgG in TBS containing 1% gelatin. After 1 h incubation, the membrane was briefly rinsed in a large volume of water, and then washed twice (10 min each) in TBS containing 0.05% Tween-20. It was then incubated with 4-chloro-1-naphthol containing H_2O_2 for color development (*16*).

Paraffin Sections of Rice Embryos—Rice embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, overnight. After several rinses with the phosphate buffer, the fixed sample was dehydrated with a graded ethanol series, from 50 to 100%. After dehydration, the sample was rinsed with xylene, soaked in wax at 55°C overnight, and finally embedded in wax at room temperature. Sections of 2 mm were obtained in warm water and layered on slide glasses coated with 2% polyvinyl alcohol.

Immunofluorescence Detection—Paraffin sections of rice embryos fixed on slide glasses were wax-deprived with xylene, rinsed with a graded ethanol series, to wash away the xylene, and the soaked in TBS buffer for 10 min. Paraffin sections and purified oil bodies in Eppendorf tubes were separately blocked in 3% gelatin in TBS buffer for 1 h at room temperature. The samples were incubated with chicken antibodies against oleosin 18 kDa and rabbit antibodies against oleosin 16 kDa or the respective pre-immune antibodies in 1% gelatin in TBS buffer for 1.5 h at room temperature. After washing twice (10 min each) in TBS containing 0.05% Tween-20, the samples were incubated with FITC (fluorescein isothiocyanate)-conjugated anti-chicken IgG from mouse (Sigma) and Texas-Red-conjugated anti-rabbit IgG from goat (Jackson Immuno-Research Laboratories) in 1% gelatin in TBS buffer for 1.5 h at room temperature. After washing twice (10 min each) in TBS containing 0.05% Tween-20, paraffin sections fixed on slide glasses were covered with glycerol. The paraffin sections and purified oil bodies layered on slide glasses were observed under a Nikon type 104 light microscope with different radiation to detect green or red fluorescence.

Reconstitution of Artificial Oil Bodies—Three essential constituents (TAG, PL, and oleosin) of oil bodies were used to reconstitute artificial oil bodies. Commercially prepared trilinolein and dioleoyl phosphatidylcholine were purchased from Sigma. Oleosins eluted on SDS-PAGE were precipitated with an equal amount of acetone pre-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 obtain an oleosin pellet. The pellet was resuspended in 500 μl of 0.1 M sodium phosphate buffer, pH 7.5. The acetone precipitation was repeated two more times to remove SDS in the solution. After the removal of SDS, the insoluble oleosin pellets were sonicated in 500 μl of the sodium phosphate buffer prior to use for the reconstitution of artificial oil bodies.

For the reconstitution of oil bodies, a 1-ml suspension containing 15 mg TAG, 150 μg PL, and 225 μg oleosins was prepared in a 1.5-ml Eppendorf tube. PL dissolved in chloroform was placed at the bottom of the Eppendorf tube, and then the chloroform was allowed to evaporate under nitrogen. After evaporation, the TAG and oleosins were incorporated, followed by sonication with a 3-mm-diameter probe in a Sonics & Materials VCX-400 ultrasonic generator at an amplitude of 30% for 15 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 floated oil-body layer showed a decrease in turbidity. The turbidity below the oil-body layer was measured at time intervals by the following method. A mixture of 1.0 ml was placed in a disposable cuvette of 1.8 ml capacity (from Fisher). The cuvette was covered with parafilm and then subjected to minimal disturbance. The absorbance of the suspension in the lower portion of the cuvette was read at 600 nm with a Jasco V-500 spectrophotometer at intervals. At the start of the measurement, the absorbance (A) was 2.0 (A_o). The turbidity (T) of the suspension was proportional to 10^A, and relative turbidity was expressed as $T/T_o = 10^A/10^{Ao} = 10^A/10^{2.0}$.

RESULTS

Rice Oil Bodies as a Model System for Studies on Oleosin Isoforms—Oil body proteins of seven different species were resolved by SDS-PAGE (Fig. 1). Among the oil bodies purified from these diverse species, rice oil bodies represented a good model system for investigation of the oleosin isoforms because of the comparable abundance of the two oleosin isoforms, the single polypeptide for each oleosin isoform, and less contamination or other minor proteins. Similar oleosin patterns were observed for rice oil bodies extracted from five different varieties from local farms in Taiwan (data not shown). In the following experiments, we selected Japonica TNG67 for further studies.

Non-Cross Recognition of Monospecific Antibodies against Two Rice Oleosins—Judging from the results of analysis of the two rice oleosin sequences deduced from cDNA clones, rice oleosin 16 and 18 kDa are two homologous proteins (*17*). The homology is primarily located in the central hydrophobic domain, *i.e.* not in the amphipathic N- and C-terminal domains. We expected no or weak cross recognition by the antibodies raised against each of the two oleosin isoforms. In order to verify this prediction, both rice oleosin isoforms (18 and 16 kDa) were purified to homogeneity (Fig. 2), and then used for the production of antibodies in chickens and rabbits, respectively. These polyclone antibodies (from chickens or rabbits) were further purified on an oleosin (18 or 16 kDa) affinity column to generate monospecific antibodies. Under the conditions in our experiments, monospecific chicken antibodies against rice oleosin 18 kDa could recognize the original antigen as well as the corresponding protein band (oleosin 18 kDa) in the oil body preparation, but not rice oleosin 16 kDa (Fig. 2). In a similar manner, monospecific rabbit antibodies against rice oleosin 16 kDa could only recognize their antigen, *i.e.* not oleosin 18 kDa. The results were in accord with the results of our previous work involving the respective polyclone chicken antibodies against these two oleosin isoforms (*15*).

Coexistence of Both Oleosin Isoforms in Oil Bodies—In order to determine the distribution of the two oleosin isoforms, we took advantage of the non-cross recognition by the chicken and rabbit antibodies raised against these two isoforms, respectively. Rice oleosin 18 kDa was tracked with chicken antibodies and visualized *via* green fluorescence using anti-chicken IgG conjugated with FITC (fluorescein isothiocyanate), while rice oleosin 16 kDa was tracked with rabbit antibodies and visualized *via* red fluorescence using anti-rabbit IgG conjugated with Texas-Red. Under the conditions in our experiments, the surface of each oil body in a paraffin section exhibited green and red fluorescence after different radiation (Fig. 3). No fluorescence was observed with pre-immune chicken and rabbit antibodies (data not shown). The same results were obtained using purified oil bodies treated under the same conditions (Fig. 4). It was concluded that both oleosin isoforms resided on the surface of rice oil bodies.

Stability of Reconstituted Oil Bodies Provided by Rice Oleosin 16 and 18 kDa—Sonicated TAG droplets without

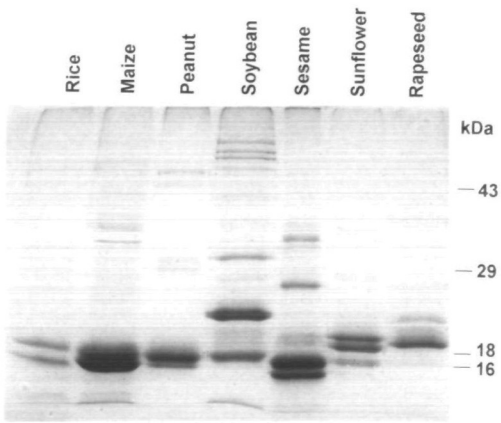

Fig. 1. **SDS-PAGE of proteins of oil bodies purified from the seeds of various species.** The proteins were stained with Coomassie Blue. The molecular masses of the proteins are indicated on the right.

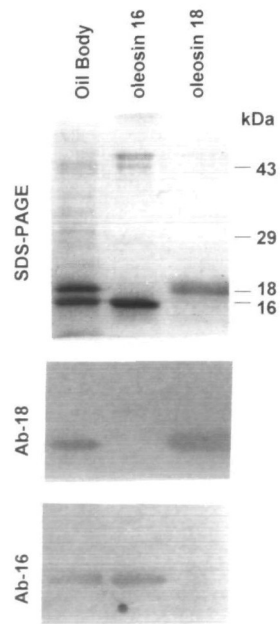

Fig. 2. **SDS-PAGE and Western blotting of rice oil body proteins and purified oleosins.** The proteins of rice oil bodies were extracted with SDS-PAGE sample buffer according to the Bio-Rad instruction manual. The molecular masses of the proteins are indicated on the right. Duplicate SDS-PAGE gels were transferred to two pieces of nitrocellulose membrane, and then subjected to immunoassaying using chicken antibodies against rice oleosin 18 kDa and rabbit antibodies against rice oleosin 16 kDa, respectively.

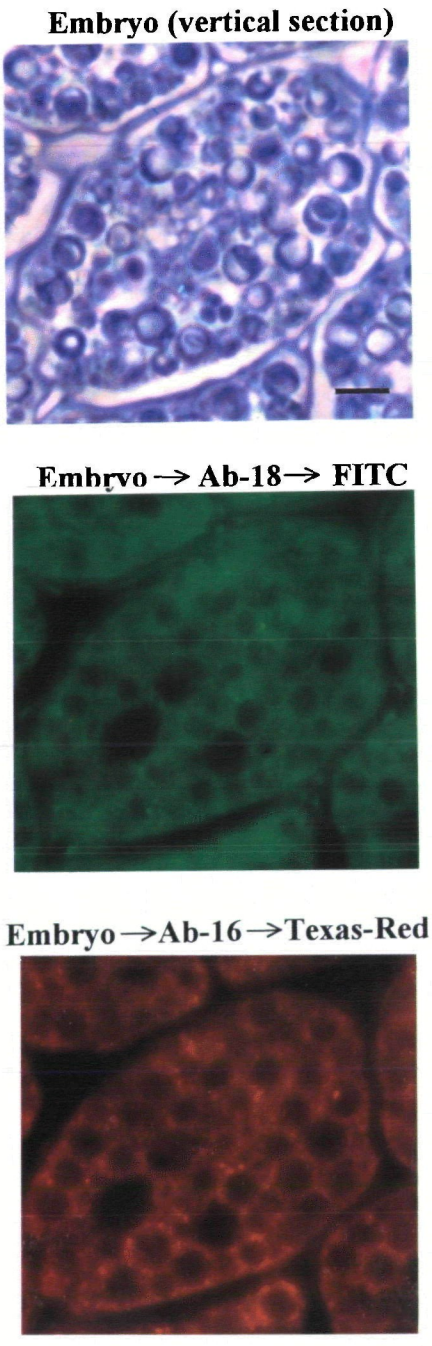

Fig. 3. **Fluorescence microscopy of rice embryos.** Paraffin sections of rice embryos were examined under a light microscope (top panel) or incubated with chicken antibodies against oleosin 18 kDa and rabbit antibodies against oleosin 16 kDa, and then with anti-chicken IgG conjugated with FITC and anti-rabbit IgG conjugated with Texas-Red. After incubation, the sections were observed under a microscope with different radiation to detect green (middle panel) or red (bottom panel) fluorescence. The most abundant spherical particles are oil bodies. The bar represents 1 μm.

oleosins were extremely unstable and fused into large oil drops, which floated to the top of the solution in a few minutes and decreased the relative turbidity rapidly (Figs. 5 and 6). PL slightly increased the stability of the sonicated TAG droplets but did not prevent the formation of large oil drops. Stable artificial oil bodies could be reconstituted *via*

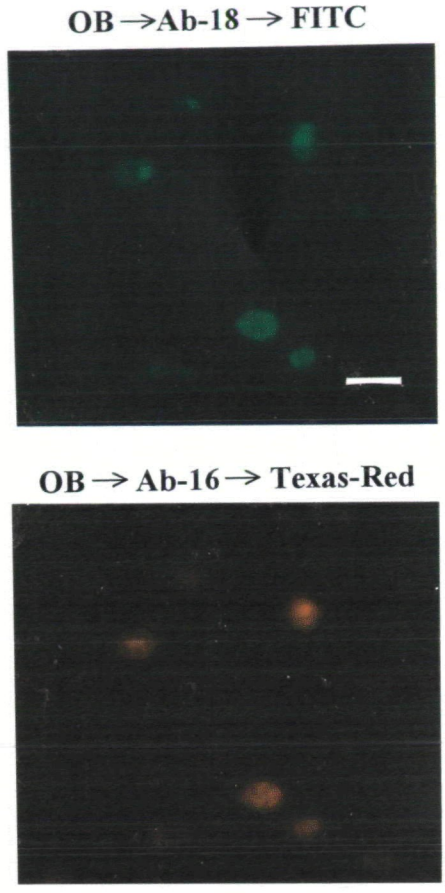

Fig. 4. **Fluorescence microscopy of purified rice oil bodies.** Purified rice oil bodies were treated as described in Fig. 3, and then examined under a microscope with different radiation to detect green or red fluorescence. The bar represents 1 μm.

sonication using TAG, PL, and rice oleosins. Artificial oil bodies reconstituted with oleosin 16 kDa exhibited substantially or slightly higher stability than ones reconstituted with oleosin 18 kDa or with a mixture of oleosin 16 and 18 kDa (1 : 1 ratio by weight). However, none of the reconstituted oil bodies under the conditions of sonication used in our experiments maintained their integrity as well as the natural seed oil bodies purified from mature rice.

DISCUSSION

It is clear that the two oleosin isoforms in rice do not constitute two types of oil bodies but coexist in each oil body. However, there has been no experimental evidence indicating whether the two oleosin isoforms are present individually or as homo- and/or heterodimers or multimers on the surface of oil bodies. Recently, based on the similar abundance of the two maize oleosin isoforms extracted from purified oil bodies, it was proposed that the two oleosin isoforms may form a heterodimer in these organelles (*18*). This speculation may not be correct or applicable, at least, to many other species which do not possess comparable amounts of the two oleosin isoforms (Fig. 1). Indeed, the relative contents of the two oleosin isoforms in oil bodies fluctuate during seed formation and germination (Peng and Tzen, unpublished data).

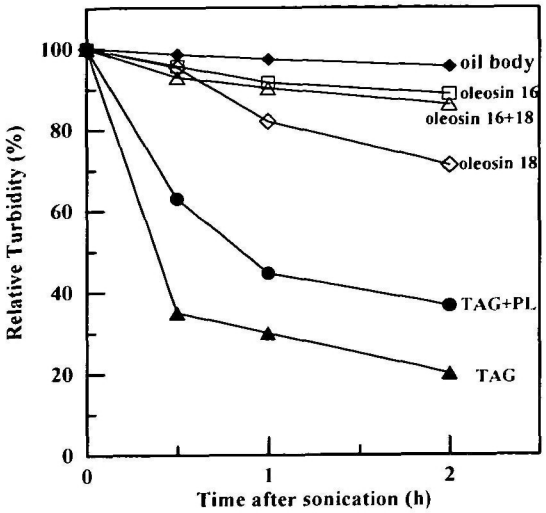

Fig. 5. **Stability of oil bodies reconstituted from TAG, PL, and rice oleosins.** A suspension (1 ml) of rice oil bodies or different artificial oil bodies reconstituted from various combinations of TAG, PL, and oleosins was placed in a 1.8-ml cuvette, and the relatively turbidity (T/T_0) at 600 nm of the approximately bottom 0.5 ml of the suspension was measured at intervals. For the reconstitution of oil bodies, a 1-ml suspension containing 15 mg TAG (trilinolein), 150 μg PL (dioleoyl phosphatidylcholine), and 225 μg oleosins (rice oleosin 16 or 18 kDa, or a mixture of them) was sonicated in a 1.5-ml Eppendorf tube. The data lines designated as oleosin 16, 18, and 16+18 represent artificial oil bodies reconstituted from TAG, PL, and the indicated oleosins.

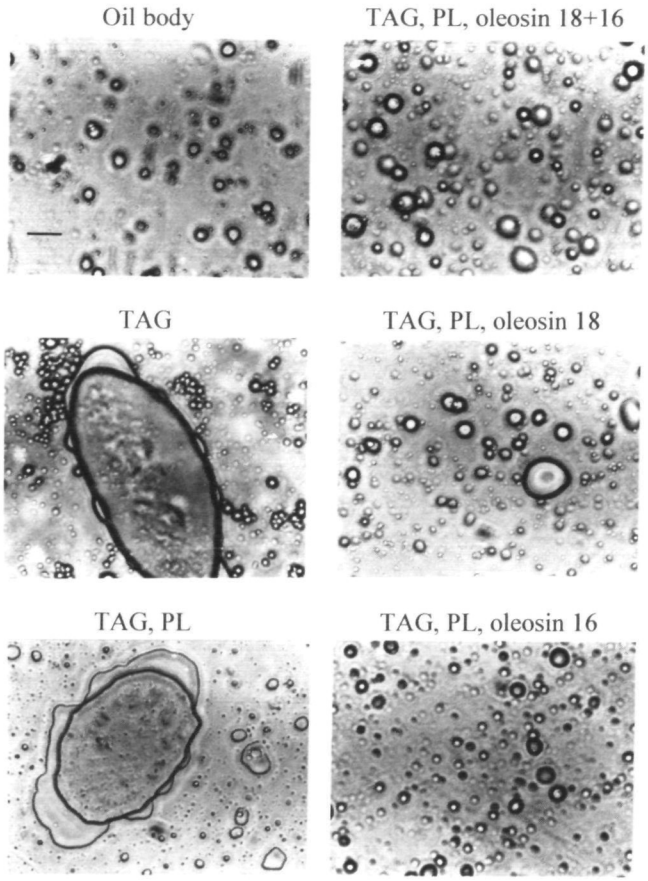

Fig. 6. **Light microscopy of rice oil bodies and reconstituted oil bodies.** Purified rice oil bodies and different artificial oil bodies reconstituted from various combinations of TAG, PL, and oleosins (see Fig. 5) were left at room temperature for 2 h before taking photos. The bar represents 1 μm.

Currently, the two homologous oleosin isoforms are classified as high and low M_r oleosins according to their relative molecular masses in each species. So far, all of the known oleosins can be placed in these two isoform classes. To date, all the examined species of angiosperms have both oleosin isoforms in their seed oil bodies. The physiological importance of the presence of these two isoforms is unknown. According to the results of analysis of the secondary structures of the two oleosin isoforms, the major difference between the two oleosin isoforms is the presence of a unique bent amphipathic α-helix connected to the central hydrophobic domain in the high M_r but not in the low M_r oleosin isoform (*5*). Whether or not this unique amphipathic α-helix implies any specific biological function the high M_r oleosin isoform remains to be seen.

It remains unknown when the splitting of these two homologous oleosin genes occurred in evolution. However, one major oleosin was identified in oil bodies of pine and classified as a low M_r oleosin based on its amino acid sequence deduced from the corresponding gene (*19*). No detectable high M_r oleosin was observed among the proteins extracted from pine oil bodies resolved on SDS-PAGE. Therefore, we speculate that the low M_r oleosin is the original isoform, and that the high M_r oleosin is a duplicated and modified form the low M_r oleosin. The modification may allow the high M_r oleosin to execute a biological function better than the low M_r oleosin at the expense of a slight reduction in its contribution to the stability of the organelles. The potential biological function is probably related to the mobilization of oil bodies during seed germination and the postgerminative growth of seedlings.

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