Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 12711–12716, November 1997
Plant Biology

Isolation and characterization of neutral-lipid-containing organelles and globuli-filled plastids from *Brassica napus* tapetum

SHERRY S. H. WU, KATHRYN A. PLATT, CHANDRA RATNAYAKE, TZANN-WEI WANG, JULIE T. L. TING, AND ANTHONY H. C. HUANG*

Department of Botany and Plant Sciences, University of California, Riverside, CA 92521-0124

Edited by Paul K. Stumpf, University of California, Davis, CA, and approved September 10, 1997 (received for review July 2, 1997)

ABSTRACT **The monolayer tapetum cells of the maturing flowers of *Brassica napus* contain abundant subcellular globuli-filled plastids and special lipid particles, both enriched with lipids that are supposed to be discharged and deposited onto the surface of adjacent maturing pollen. We separated the two organelles by flotation density gradient centrifugation and identified them by electron microscopy. The globuli-filled plastids had a morphology similar to those described in other plant species and tissues. They had an equilibrium density of 1.02 g/cm^3 and contained neutral esters and unique polypeptides. The lipid particles contained patches of osmiophilic materials situated among densely packed vesicles and did not have an enclosing membrane. They exhibited osmotic properties, presumably exerted by the individual vesicles. They had an equilibrium density of 1.05 g/cm^3 and possessed triacylglycerols and unique polypeptides. Several of these polypeptides were identified, by their N-terminal sequences or antibody cross-reactivity, as oleosins, proteins known to be associated with seed storage oil bodies. The morphological and biochemical characteristics of the lipid particles indicate that they are novel organelles in eukaryotes that have not been previously isolated and studied. After lysis of the tapetum cells at a late stage of floral development, only the major plastid neutral ester was recovered, whereas the other abundant lipids and proteins of the two tapetum organelles were present in fragmented forms or absent on the pollen surface.**

Eukaryotic cells possess intracellular particles that contain high amounts of neutral lipids, usually triacylglycerols (TAGs). These particles, termed oil/fat bodies/globules, etc., are present in plant seeds and pollens (1), algae (2), yeast (3), nematode eggs (4), mammalian brown adipose tissue (5), and mammalian glands (6). Most of them act as food reserves for an upcoming period of active growth, whereas the fat globules in mammalian glands are exported as milk fat droplets. All of the above-mentioned lipid particles are relatively small, of 0.2 to several micrometers in diameter. Much larger intracellular particles (several hundred micrometers) are present in plant fruit mesocarps, in which the TAGs may be used to attract or reward animals for seed dispersal (7), and in mammalian white adipose tissues, where the TAGs are for long-term storage and heat insulation (8). Much less common are intracellular particles containing wax esters instead of TAGs in seeds of the plant jojoba (1) and *Euglena* (9). Regardless of their size, function, and lipid constituents, all of the above-mentioned particles have a very simple morphology. They have an amorphous matrix of neutral lipids enclosed by a layer of amphipathic molecules, which may include phospholipids (PLs) and unique proteins. Similar in morphology are the intercellular lipoproteins in the mammalian circulatory system (10).

Among intracellular lipid particles, those from plant seeds have been studied most intensively (1). Seeds store TAGs in spherical organelles called oil bodies (lipid bodies) that have diameters of about 0.6–2.0 μm. Each oil body contains a TAG matrix surrounded by a layer of phospholipids embedded with unique and abundant proteins termed oleosins. Oleosins have molecular masses between 15 and 26 kDa, depending on the isoforms and plant species in which they occur. Each oleosin molecule has a highly conserved central domain of 72 uninterrupted hydrophobic residues flanked by amphipathic stretches. The structures enable the oleosins to interact with the TAGs and PLs on the surface of oil body. Oleosins form a steric barrier and maintain the oil bodies as small entities, which would facilitate lipase binding and lipolysis during germination.

Aside from the above-mentioned neutral-lipid containing intracellular particles, some nonphotosynthetic plastids in plants also contain a high proportion of neutral lipids (11, 12). These plastids are enclosed by double membranes, and the interior has few membranes (thylakoids) but numerous small lipid globuli. The globuli isolated from lysed plastids were reported to contain a great variety of lipids (13, 14).

During the formation of pollen in the flowers of many plants, the monolayer tapetum cells surrounding the maturing pollen in the anther possess two dominant organelles, which apparently contain abundant lipids (15–17). These organelles are the plastids, which are packed with globuli, and the lipid particles, whose characteristics have not been previously elucidated. Both organelles are spherical, of about 3 μm in diameter. They are supposed to be released from the ruptured tapetum cells and deposited onto the surface of the maturing pollen (15–17). The pollen surface materials, termed pollenkitt or tryphine, could serve one or many functions, which include adhering the pollen together and to the pollinating insect bodies and the carpel surface, preventing the pollen from water loss, aiding hydration and germination of the pollen, protecting the pollen from solar radiation, giving the pollen color, attracting insect pollinators, and exerting self-compatibility and incompatibility (18).

Recently, we isolated the plastids and lipid particles as a mixture from the tapetum of *Brassica napus* and found that they contained two oleosins (19), whose genes had been cloned by random sequencing earlier (20, 21). We were unable to separate these two organelles from each other for biochemical characterization. Here, we provide experimental results on the separation and characterization of the two organelles. The lipid particles are unique organelles that, to our knowledge,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: TAG, triacylglycerol; PL, phospholipid.
*To whom reprint requests should be addressed. e-mail: ahuang@ucrac1.ucr.edu.

have not been described previously in eukaryotic cells. During a late stage of floral development, most of the constituents of the two organelles either disappear or are deposited in fragmented form onto the surface of the mature pollen.

MATERIALS AND METHODS

Plant Materials. *B. napus* L. cv. Westar was grown in a greenhouse (19). Flower buds containing anthers of maturation stage 3, based on the developmental stages we set earlier (19), were used. At this stage, the flower buds and anthers were 6 and 3–4 mm long, respectively, and their tapetum cells were unlysed and filled with organelles. Mature pollen was collected from flowers that had opened on the same day.

Isolation of Organelles from the Sporophytic Flower Buds. All procedures were performed in 4°C. The flower buds were minced with a razor blade in a Petri dish containing a grinding medium of 0.05 M Hepes-NaOH, pH 7.5/0.8 M sucrose (125 buds of 3.1 g/8 ml). Light microscopy revealed that the chopping broke most of the sporophytic floral tissues, especially the tapetum, but left the gametophytic microspores intact. These microspores were stained positively with the vital dye fluorescein diacetate. The homogenate was filtered through Nitex cloth (20×20 μm pore size), which removed the microspores.

The filtrate (4 ml) was placed in a 17-ml centrifuge tube. All the solutions described in this paragraph contained 0.05 M Hepes-NaOH (pH 7.5). For the preparation of a mixture of the two organelles in a one-step density gradient, 12 ml of 0.4 M sucrose solution was layered on top of the filtrate. For the separation of the two organelles in a three-step density gradient, 4-ml successive layers of 0.4, 0.2, and 0 M sucrose solutions were placed on top of the filtrate. For the separation of the two organelles in a linear density gradient, a 12-ml gradient of 0.4 to 0 M sucrose solutions was placed on top of the filtrate. The tube was centrifuged at 9,000 rpm in a Beckman SW 28.1 rotor for 2 h. In the one-step density gradient, the floated two-organelle fraction was collected with a pipette. In the three-step density gradient, the two visible organelle fractions at the interfaces between 0.4–0.2 M, and 0.2–0 M sucrose solutions were collected from the bottom of the tube after puncturing a hole with a needle. The whole linear density gradient was collected into 0.8-ml fractions from the bottom of the tube.

Preparation of Fractions from Pollen. Mature pollen was shaken with diethyl ether or cyclohexane for 1 min. The organic solvent was removed as the surface fraction. The leftover pollen could be stained positively with the vital dye fluorescein diacetate (observed by light microscopy). It was ground in chloroform methanol (2:1, vol/vol) with glass beads (425–600 μm diameter) by using a mortar and pestle. The whole homogenate was used as the leftover pollen fraction.

SDS/PAGE and Immunoblotting. Acrylamide (12.5%) SDS/PAGE was performed as described (22). The gel was stained with Coomassie blue.

The proteins in various fractions were resolved by SDS/PAGE and subjected to immunoblotting as described (22). Rabbit antibodies were raised against specific proteins eluted from SDS/PAGE gels (22), and the IgG were purified from antisera by DEAE Affi-Gel Blue chromatography (Bio-Rad).

Protein Quantitation, N-Terminal Sequencing, and Enzyme Assay. Total proteins were quantified by the Bradford method (23). The proteins in the isolated organelle fractions were resolved by SDS/PAGE, blotted onto a nitrocellulose membrane (22), and N-terminal sequenced (19). UDP-gal-diacylglycerol galactosyltransferase was assayed (24) by using internal diacylglycerols and 64 μM UDP-gal.

Lipid Extraction and TLC. Total lipids were extracted with chloroform methanol (2:1, vol/vol). Their amounts in the organelle fractions were estimated by the following method. Standards of oleoyl oleate, triolein, and oleic acids in increasing amounts and the neutral and polar lipids in the two organelle fractions were resolved by TLC. After the plates were charred with sulfuric acid, the amount of lipid in each visible spot resolved from the organelles was determined by visual comparison with the standards. The darkness and area of the spots of the standards were directly proportional to the amounts of lipids applied to the plate.

TLC plates (silica gel 60A; Whatmann) were developed in hexane/diethyl-ether/acetic-acid (80:20:2, vol/vol) for the separation of neutral lipids, and in chloroform/acetic acid/methanol/water (70:25:5:2, vol/vol) for the separation of polar lipids. After development, the plates were charred with sulfuric acid.

Electron Microscopy. Whole anthers (maturation stage 3) were fixed in Karnovsky's (25) solution (4% paraformaldehyde/5% glutaraldehyde/0.2 M potassium phosphate, pH 7.0) for 8 h at 4°C. The samples were washed twice in 0.1 M K-phosphate (pH 7.0), and postfixed in 1% OsO_4 in 50 mM Na-cacodylate (pH 7.0) for 1.5 h at 20°C.

Isolated plastids (in 0.1 M sucrose/0.05 M Hepes-NaOH, pH 7.5) and lipid particles (in 0.3 M sucrose/0.05 M Hepes-NaOH, pH 7.5) were fixed in a 1/3 volume of Karnovsky's solution for 2 h at 4°C. The isolated organelles were also fixed in solutions containing a higher concentration of osmotica. The organelle fractions were mixed with 1.2 M sucrose/0.05 M Hepes-NaOH, pH 7.5, to bring the sucrose concentration to 0.8 M. They were then mixed with an equal volume of Karnovsky's solution for 2 h at 4°C. Karnovsky's solution had an osmotica concentration of 1.2 OsM, and the equal-volume mixture had about 1.0 OsM. After aldehyde fixation, the organelles were postfixed in 1% OsO_4/0.4 M sucrose/0.05 M Hepes-NaOH, pH 7.5, for 2 h, then in 1% OsO_4/0.05 M K-phosphate, pH 7.0, for 16 h.

The samples were dehydrated, embedded in resin, sectioned, poststained with uranyl acetate and lead citrate, and photographed by using an electron microscope (19).

RESULTS

Plastids and Lipid Particles of the Tapetum Cells Were Isolated and Separated from Each Other. Earlier, we isolated a floated pad of a mixture of plastids and lipid particles from the tapetum cells of *B. napus* by centrifuging the crude extract of the anthers in a 0.8 M sucrose solution in an overlying layer of a 0.4 M sucrose solution (19). In the current study, we separated the two organelles from each other. To reduce efforts for preparing the anthers, we used whole flower buds. The floated pads obtained from the whole flower buds and the separated anthers had an identical protein pattern on SDS/PAGE gel (data not shown). There were no floated materials after the crude extract of the anther-removed flower buds in 0.8 M sucrose solution was centrifuged in an overlying 0.4 M sucrose solution (visual observation).

The crude extract of the flower buds in an 0.8 M sucrose solution was centrifuged in an overlying linear gradient of 0.4–0 M sucrose solutions (Fig. 1). After centrifugation, two white bands among a smear of cloudy white materials were observed at the region of 0.1–0.3 M sucrose. After fractionation, the gradient fractions were subjected to SDS/PAGE. The protein pattern along the gradient (Fig. 1) revealed two types of organelles, which migrated to different density regions. One type of organelle, having migrated to a region of 1.02 g/cm^3 density, had several major polypeptides of 36–31 kDa. The other type of organelle, having migrated to a region of 1.05 g/cm^3 density, possessed major polypeptides of 48–45 kDa and minor ones of 35 and 27–14 kDa. These densities are the equilibrium densities because the two organelles did not migrate further upon longer (4 h instead of 2 h) centrifugation (data not shown).

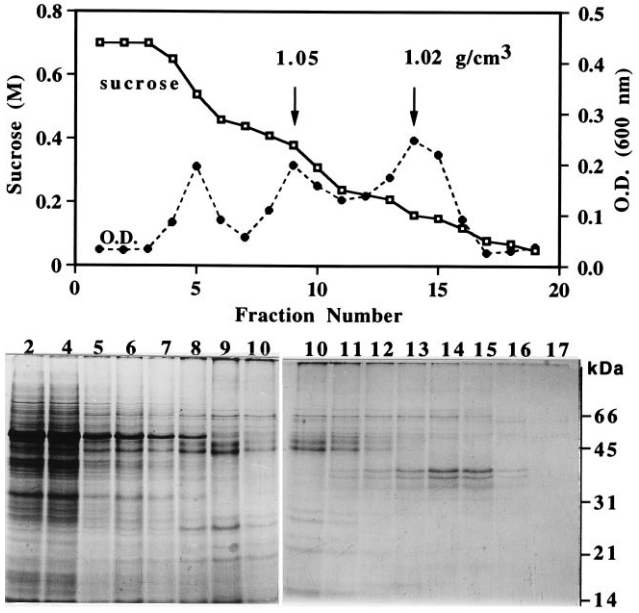

FIG. 1. Separation of organelles from the flower buds of *B. napus* by flotation sucrose density gradient centrifugation and analysis of the gradient fractions by SDS/PAGE. The total extract of flower buds in a 0.8 M sucrose solution was centrifuged with an overlying gradient of 0.4–0 M sucrose solution. After centrifugation and fractionation, the fractions were analyzed for sucrose concentrations with a refractometer and turbidity at 600 nm with a spectrophotometer (*Upper*). The two vertical arrows indicate the locations and equilibrium densities of the two separated organelles. The turbidity peak at fraction 5 represented the junction between the original total extract and the gradient above it. Proteins in the PAGE gel were stained with Coomassie blue (*Lower*). Fractions 2, 4, and 5 used for SDS/PAGE were one-third the amount of the other fractions. Positions of the molecular mass markers are shown on the right.

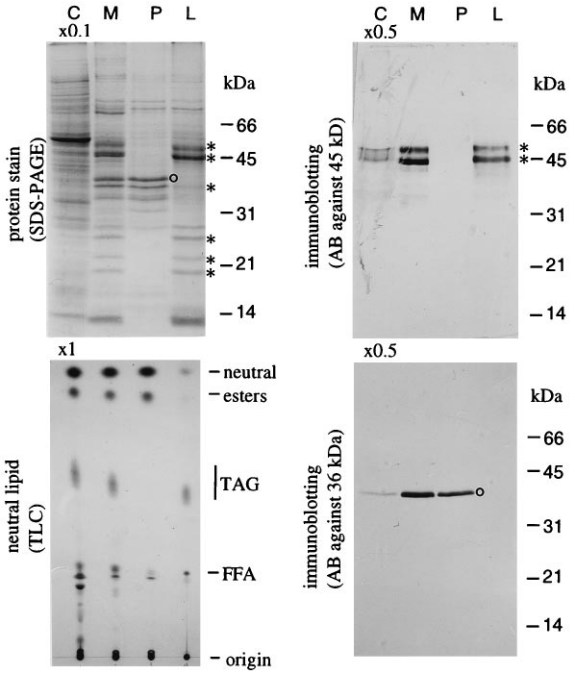

FIG. 2. SDS/PAGE and immunoblotting of proteins and TLC of neutral lipids of the crude extract (lane C), the two-organelle mixture (lane M), the plastid fraction (lane P), and the lipid particle fraction (lane L) from *B. napus* flower buds. Proteins on the PAGE gel were stained with Coomassie blue (*Upper Left*) or treated for immunoblotting using antibodies against the 45-kDa oleosin (*Upper Right*) or the 36-kDa polypeptide (*Lower Right*). Neutral lipids on the TLC plate were allowed to react with sulfuric acid (*Lower Left*). In each of the four panels, the same proportional amounts of the four samples were used, with the exception of the crude extract which varied ×0.1 or ×0.5 to reveal their contents clearly. Positions of markers for protein molecular masses and standard lipids are shown on the right. Asterisks indicate oleosins of 48, 45, 35, 25, 23, and 21 kDa, and circles denote the plastid 36-kDa polypeptide.

On the basis of the above findings, we constructed a three-step sucrose density gradient to separate the two types of organelles for detailed study. The crude extract of flower buds in a 0.8 M sucrose solution was centrifuged in overlying steps of 0.4, 0.2, and 0 M sucrose solutions. After centrifugation, the two cloudy fractions at the interfaces between 0–0.2 and 0.2–0.4 M sucrose solutions were collected. An analysis of the two fractions by SDS/PAGE (Fig. 2) showed unique protein constituents of the two types of organelles predicted from the results of linear gradient centrifugation (Fig. 1).

The organelles at the interfaces between 0 and 0.2 M and between 0.2 and 0.4 M sucrose were identified as the plastids and the lipid particles, respectively, by electron microscopy (Fig. 3). The plastid fraction and lipid particle fraction were each about 90% pure, as judged from the degrees of cross-contamination of the two organelles by electron microscopy (Fig. 3), their unique proteins by SDS/PAGE (Fig. 2), and their unique neutral lipids by TLC (to be described). The two organelles were present in about equal numbers in the tapetum cells (Fig. 3, and other electron micrographs not shown), and this equality was reflected in the roughly equal amounts of their respective unique proteins and neutral lipids in the isolated mixture of the two organelles (Fig. 2, to be described).

The Lipid Particles Did Not Have an Apparent Enclosing Membrane But Had Internal Vesicles and Exhibited Osmotic Properties. The isolated plastids maintained the same morphology, in terms of size and content of numerous globuli (Fig. 3 *B* and *C*), as those *in situ* (Fig. 3*A*). Most of the globuli were retained within the plastid-enclosing membranes, which were barely visible. There were few internal membranes (thylakoids). The plastid fraction contained 0.52 nmol/min per mg protein of UDP-gal-diacylglycerol galactosyltransferase, a marker of the plastid-enclosing membrane (24), whereas the lipid particle fraction contained 0.051 nmol/min per mg protein (see below).

The isolated lipid particles also maintained a morphology similar to those *in situ* (Fig. 3). Depending on the osmotic concentration in the fixation medium for electron microscopy, the organelles appeared in a dilated or dense form. When the isolated organelles in a 0.3 M sucrose solution were fixed in an aldehyde solution containing about 0.4 OsM, they appeared in a dilated form (Fig. 3*D*). When the same organelle fraction was first mixed with a 1.2 M sucrose solution to make a suspension in a 0.8 M sucrose solution and then fixed in an equal volume of an aldehyde solution containing 1.2 OsM, a substantial proportion of the organelles were present in a dense form (Fig. 3*E*). Within each, and among both, of the two organelle preparations fixed in aldehyde solutions of low and high osmotic concentrations the organelles in the dense form were in general smaller than those in the dilated form. A striking feature is that the lipid particles were packed with vesicles, which could be seen clearly only when the organelles were in the dilated form (Fig. 3 *D* and *F*). When the organelles were in the dense form, the internal vesicles were not clearly visible (Fig. 3 *E* and *G*), and they closely resembled those *in situ* (Fig. 3*A*). Patches of electron-dense materials, presumably neutral lipids, were present. It is unknown whether they were present outside of the vesicles or within specific vesicles or enclosed by a layer of PLs and oleosins as are seed oil bodies. The lipid particles *in situ* (Fig. 3*A*, and electron micrographs not shown) or in isolated preparations (Fig. 3 *F* and *G*) did not have a clearly defined boundary membrane, and the outer boundary

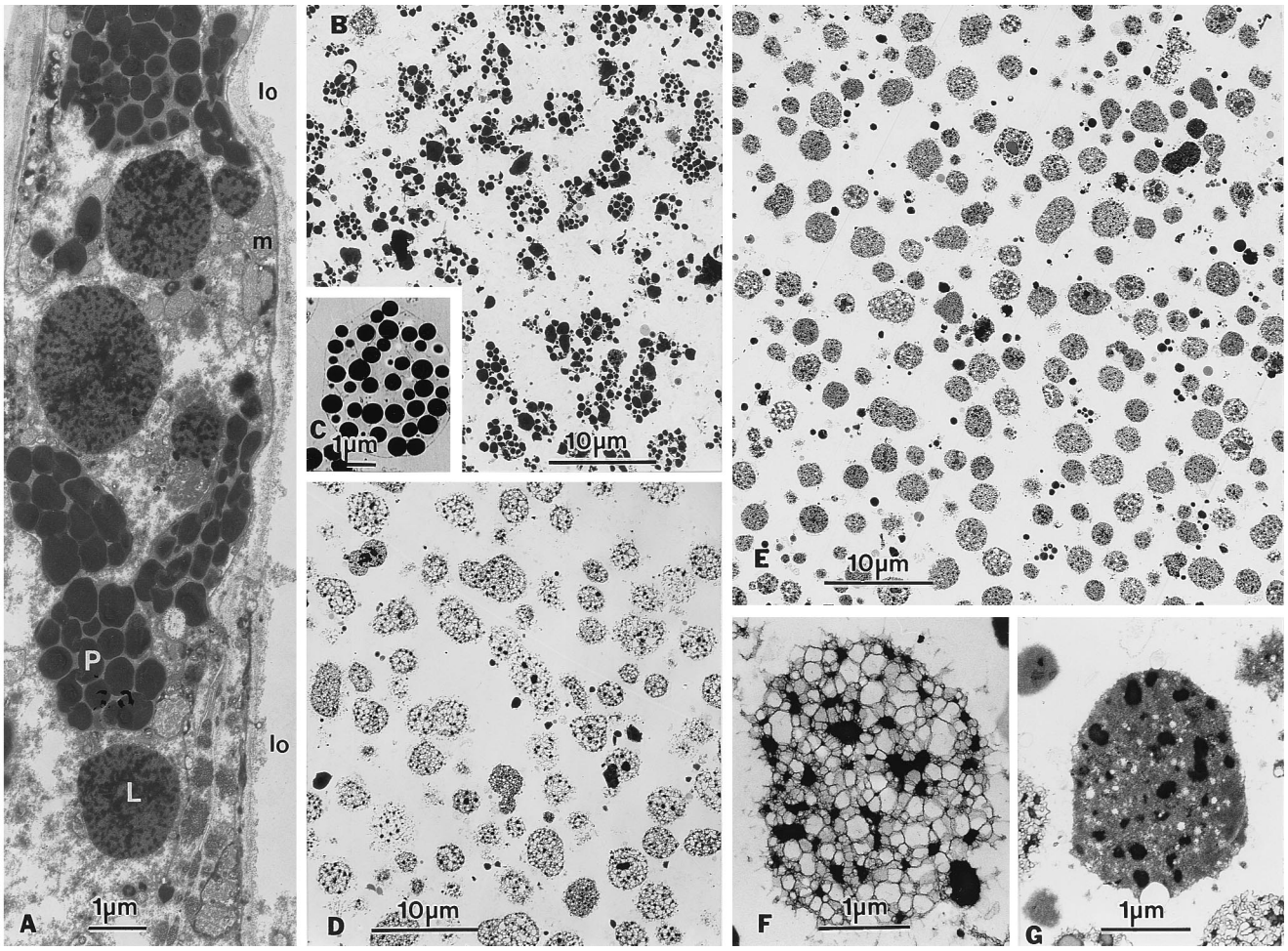

FIG. 3. Electron micrographs of a *B. napus* tapetum cell and isolated fractions of the plastids and lipid particles. (*A*) Portion of a tapetum cell, with its long axis facing the locule (lo). The cell contained abundant and conspicuous globuli-filled plastids (P) and lipid particles (L), as well as several mitochondria (m). (*B*) Fraction of isolated plastids, each of which was filled with globuli. (*C*) Enlarged isolated plastid, with a barely visible enclosing membrane. (*D*) Fraction of isolated lipid particles, aldehyde-fixed in a diluted osmotic solution. Most of the lipid particles were in a dilated form. (*E*) Fraction of isolated lipid particles, aldehyde-fixed in a higher osmotic solution. The lipid particles were in a dilated or dense form. In both *D* and *E*, the lipid particles in the dilated form were, in general, larger than those in the dense form. (*F*) Enlarged view of a lipid particle in the dilated form. Patches of osmiophilic materials, presumably neutral lipids, were scattered among vesicles. (*G*) Enlarged view of a lipid particle in the dense form.

appeared to be made up of that of the outermost vesicles. It is unknown whether the internal vesicles were individually spherical or represented highly coiled network of tubular or lamellar structure.

The isolated lipid particles exhibited osmotic properties, as revealed by their existence in dense and dilated forms, depending on the osmotica concentration of the fixation medium for electron microscopy. Because a clearly defined boundary membrane was absent, the osmotic properties of the organelles presumably were exerted by the internal vesicles, each of which contracted or dilated depending on the osmotica concentration of the medium. Seed oil bodies, which have an amorphous TAG matrix surrounded by a layer of PLs and oleosins, do not change their sizes in solution of different osmotica concentrations (unpublished observation).

The Proportions of Neutral Lipids, Polar Lipids, and Proteins in the Two Organelles Were Consistent with the Organelle Densities and Morphology. Both the plastids and lipid particles of the tapetum had substantially lower proportions of neutral lipids and higher proportions of polar lipids and proteins than seed oil bodies and human chylomicrons (Table 1). These values are consistent with the comparatively higher equilibrium densities of the tapetum organelles. They are also compatible with the morphology of the two organelles, which, especially the lipid particles, have internal membranes.

The two tapetum organelles were isolated by an aqueous procedure of sucrose gradient centrifugation during which the organelles migrated to and dilated in solutions of osmotic concentrations (about 0.1 OsM for plastids and 0.3 OsM for lipid particles) less than the physiological value of about 0.4

Table 1. Parameters of the neutral-lipid containing organelles from *B. napus* tapetum and seed, and lipoprotein particles from human

	Brassica			Human
	Tapetum lipid particles	Tapetum plastids	Seed oil bodies	Intestine chylomicrons
Density, g/cm^3	1.05	1.02	0.92	<0.96
Diameter, μm	≈3	≈3	0.65	0.1–1.0
Constituents, % (wt/wt)				
Neutral lipids	47	69	94	91
Polar lipids	24	18	2	9
Proteins	28	11	3	1–2

Data on seed oil bodies (1) and human extracellular intestine chylomicrons (30) are those reported earlier. Chylomicrons are the only extracellular lipoprotein particles resembling the intracellular lipid organelles in size; all other lipoprotein particles are too small (<0.1 μm) to make a meaningful comparison.

OsM (likely higher in the tapetum cells). We do not know if the isolated organelles had lost some water-soluble constituents. Regardless, there was no appreciable loss of proteins and lipids. This interpretation is based on the findings by SDS/PAGE (Fig. 2) and TLC (to be described) that the two separated organelles in 0.1 and 0.3 M sucrose together contained the same proteins and lipids as the two-organelle mixture isolated in 0.4 M sucrose (Fig. 2).

The Plastids Possessed Neutral Esters, Whereas the Lipid Particles Contained TAGs. TLC analyses revealed that the plastids contained two neutral esters, whereas the lipid particles possessed TAGs as their respective unique and major neutral lipids (Fig. 2). The two plastid neutral esters were so termed because upon alkaline hydrolysis, they released free fatty acids (data not shown). By TLC, the more-hydrophobic neutral ester, which was more abundant, comigrated with palmityl oleate and cholesteryl oleate but migrated slower than tetradecane and hexadecane. A small proportion of the neutral lipids in each of the two organelle fractions was represented by several less-hydrophobic components (Fig. 2), which comigrated with diolein, oleic acid, nervonyl alcohol, and cholesterol.

By TLC analyses, using different developing solvents, the major PLs in each of the two organelles were found to be phosphatidylcholine and phosphatidylethanolamine (data not shown). The plastids also contained some lysophosphatidylcholine, lysophosphatidylethanolamine, and monogalactoacylglycerol.

The Plastids Contained Unique Proteins of Unknown Identities, Whereas the Lipid Particles Possessed Several Oleosins. The plastids and lipid particles each had unique proteins, as revealed by SDS/PAGE (Fig. 2). The plastids possessed several major polypeptides of 36–31 kDa. Antibodies raised against the most abundant polypeptide of 36 kDa recognized the original 36-kDa antigen but did not recognize any other polypeptide in the plastids or any polypeptide in the lipid particles. The identities of the unique proteins in the plastids are unknown.

The lipid particles contained two major polypeptides of 48 and 45 kDa and less-abundant polypeptides of 35 and 27–14 kDa (Fig. 2). Earlier (19), we identified, by recognition with antibodies raised against a synthetic peptide and N-terminal amino acid sequence comparison, the 48- and 45-kDa polypeptides to be tapetum oleosins encoded by two cloned genes, which share a very high degree of amino acid sequence similarity (20). In the current study, we raised antibodies against the whole 45-kDa polypeptide and found that the antibodies reacted with the 45-kDa and 48-kDa polypeptides (Fig. 2). If the immunoblotting reaction was allowed to react for a longer time, the antibodies also recognized the minor 35-kDa polypeptide (data not shown). This 35-kDa polypeptide had a N-terminal amino acid sequence of LGIPESIKPSNIIPE, which was an apparent fragment of the 45-kDa oleosin (21) based on sequence similarities. In addition, we identified the polypeptides of 25 kDa (N-terminal sequence being MKEEIQNETAQTLSQREGRMF, encoded by *Bnolnb*-6), 23 kDa (XILR/LKKKHER, possibly encoded by *I3*), and 21 kDa (GILRKKKHERKPSFKSVLT, encoded by *Pol3*) being oleosins encoded by cloned genes (20, 21) via similarities at their N-terminal sequences (Fig. 2).

The experimental results (Fig. 2) clearly show that the 48- and 45-kDa oleosins, and a related oleosin of 35 kDa, are present in the lipid particles and not in the plastids. The evidence is overwhelming for us to correct our earlier erroneous suggestion that the oleosins were localized in the plastids (19); less-reliable immunocytochemical results using antibodies against a synthetic peptide of the 45-kDa oleosin showed more immunogold particles in the plastids than in the lipid particles (19). We conclude that most if not all of the oleosins in the tapetum are present in the lipid particles.

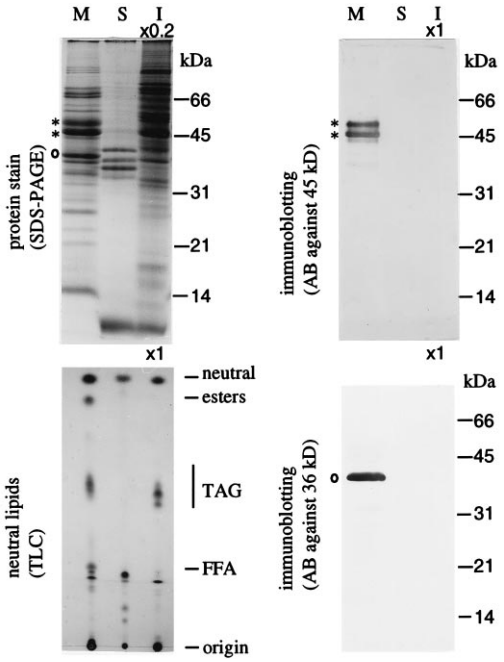

FIG. 4. SDS/PAGE and immunoblotting of proteins and TLC of neutral lipids of the two-organelle mixture (lane M), the ether-washed pollen surface materials (pollenkitt) (lane S), and the extract of the pollen interior after the ether wash (lane I) from *B. napus*. Proteins on the PAGE gel were stained with Coomassie blue (*Upper Left*) or treated for immunoblotting using antibodies against the 45-kDa polypeptide (*Upper Right*) or the 36-kDa polypeptide (*Lower Right*). Neutral lipids on the TLC plate were allowed to react with sulfuric acid (*Lower Left*). Positions of markers for protein molecular masses and standard lipids were shown on the right. Asterisks indicate the 48- and 45-kDa oleosins, and circles denote the plastid 36-kDa polypeptide.

Of the Major Lipids and Proteins in the Two Tapetum Organelles, Only the Abundant Plastid Neutral Ester Was Recovered Intact on the Surface of the Mature Pollen. At a late maturation stage of the *Brassica* anther, the tapetum cells lyse, releasing their organelles, whose constituents are supposed to be deposited onto the maturing pollens (15–17). We followed the relocation process by comparing the constituents in the two-organelle fraction of the tapetum and the surface materials (pollenkitt) of the pollen. The pollenkitt was extracted from the mature pollen by ether (19), and its proteins and lipids were analyzed (Fig. 4). Using the more nonpolar cyclohexane (21, 26) instead of ether for the pollenkitt extraction generated essentially the same results (data not shown).

The more-hydrophobic neutral ester of the plastids apparently was recovered in the pollenkitt, but the less-hydrophobic neutral ester was absent (Fig. 4). The lipid-particle TAGs also disappeared. An earlier report also found little TAGs in the pollenkitt (27). There was insufficient free fatty acid in the pollenkitt (relative to the amount of the more hydrophobic neutral ester) that could possibly account for the loss of the less-hydrophobic neutral ester or TAGs in the two-organelle fraction (Fig. 4). The neutral lipids of the pollenkitt differed from those of the pollen interior (Fig. 4). The pollen interior had two major neutral lipids, one of which was TAGs, which presumably represented that in the storage oil bodies. The other lipid had a migration pattern by TLC similar to that of the tapetum neutral ester (Fig. 4). This pollen-interior lipid gave a pink color whereas the tapetum neutral ester yielded a gray color after the TLC plate had been sprayed with sulfuric acid–ferric chloride (data not shown); so the two lipids were different.

The pollenkitt possessed two distinct molecular mass groups of polypeptides of roughly equal amounts (Fig. 4). One group

consisted of several polypeptides of 37–32 kDa, and the other group was composed of polypeptides of less than 10 kDa. The presence of these two groups of proteins was reported earlier (19, 21, 26), and their relative amounts varied. This variation was due to the use of different organic solvents to remove the lipids in the pollenkitt before SDS/PAGE; the solvents could also remove some of the relatively hydrophobic proteins of higher molecular masses. In the current study, we did not do the solvent extraction and used the pollenkitt in its entirety for SDS/PAGE and confirmed the report (26) that there were roughly equal amounts of the two groups of high and low molecular mass proteins (Fig. 4). Some of the polypeptides in both groups were shown to be fragments of oleosins on the basis of their N-terminal amino acid sequences being identical to those predicted with cloned cDNAs (21).

Using antibodies against the 45-kDa oleosin of the tapetum lipid particles, we could not detect fragments of the two most abundant 48- and 45-kDa oleosins in the pollenkitt (Fig. 4). Similarly, we failed to detect the most abundant plastid 36-kDa polypeptide or its fragments in the pollenkitt by using antibodies against this polypeptide (Fig. 4). In our immunoblotting, the sensitivity allowed us to detect on the same blot one-twentieth the amount of the antigen in the two-organelle fraction. Our failure to detect the 45- and 48-kDa oleosins and the plastid 36-kDa polypeptide, as well as their fragments in the pollenkitt, indicated that these abundant polypeptides of the two tapetum organelles were degraded completely or to smaller fragments (21) with much less antigenicity (per amount of protein) than the original antigens.

DISCUSSION

The tapetum lipid particles are novel organelles whose chemical and structural properties, to our knowledge, have not been described previously in any eukaryote. They differ from the well-studied seed oil bodies, although both organelles contain TAGs and oleosins. Lipid particles identified by their simple *in situ* morphology are present in the tapetum of diverse species including dicots and monocots (28), and whether they all have properties similar to those of the *Brassica* organelles remains to be revealed. If the lipid particles are unique to the tapetum of plants, they could be called "tapetosomes" to distinguish them from all the other closely related fat/oil bodies/particles in plants and non-plant organisms. The unusual morphology of the tapetum lipid particles raises an interesting question of how the organelles and their constituents are synthesized. During the active stage of lipid-particle formation, massive endoplasmic reticulum attached and penetrated into the organelles (19), and therefore the biogenesis of the organelles should be closely related to the endoplasmic reticulum. The role of the lipid particles could be speculated. The TAGs or their fatty acids are not conserved on the mature pollen, and thus likely perform a role within the tapetum. They could provide energy for cellular metabolism at the final development stage of the tapetum cell which contains abundant mitochondria (Fig. 3*A*). They could be the source of polyunsaturated fatty acids which are precursors of essential hormones for the final stage of anther maturation (29). Fragments of oleosins on the pollen surface could perform one or more of the proposed functions of the pollenkitt (see Introduction) or represent remnants that serve no further function. If only TAGs play a direct physiological role, oleosins may be present just to maintain the stability of the TAGs, and the same possibility holds if only oleosins play a direct role. Other than the TAGs and oleosins, the internal vesicles of the lipid particles, through their membrane constituents or the luminal contents or both, may become part of the pollenkitt and play a specific role on the pollen.

Globuli fractions obtained from lysed plastids or crude extracts of a few plant species and organs were found to contain different lipid pigments and TAGs (13, 14). The globuli-filled plastids isolated from *Brassica* tapetum do not contain pigments (no visible color other than cloudiness) or TAGs. In view of the absence of TAGs in the globuli-filled plastids and their presence in other organelles (lipid particles) within the same tapetum cells, the reported existence of TAGs in the globuli of plastids from other plant organs needs to be re-examined to ensure that the TAGs were not contaminants. The tapetum plastids accumulate and probably synthesize neutral (sterol or wax) esters, a role that has not been documented. Within the tapetum plastids, the globuli, although packed, do not fuse among themselves and remain fairly uniform in size. Their surface must contain amphipathic molecules that prevent fusion. The role of the plastids, which are present in the tapetum of both dicots and monocots, appears to synthesize and temporarily store the neutral ester in the tapetum cells before the neutral ester is discharged onto the surface of mature pollen.

This work was supported by U.S. Department of Agriculture Grant 96-00831.

1. Huang, A. H. C. (1992) *Annu. Rev. Plant Physiol. Mol. Biol.* **43,** 177–200.
2. Rossler, P. G. (1988) *J. Physiol. (London)* **24,** 394–400.
3. Clausen, M. K., Christiansen, K., Jensen, P. K. & Behnke, O. (1974) *FEBS Lett.* **43,** 176–179.
4. Rubin, H. & Trelease, R. N. (1976) *J. Cell Biol.* **70,** 374–383.
5. Bukowiecki, L. J. (1986) in *Brown Adipose Tissue*, ed. Trayhurn, P. & Nicholls, D. (Arnold, Baltimore), pp. 105–121.
6. Welsch, U., Schumacherm U., Buchheim, W., Schinko, I., Jenness, P. & Patton, S. (1990) *Acta Histochem.* **40,** 59–64.
7. Platt-Aloia, K. A. & Thomson. W. W. (1981) *Ann. Bot.* **48,** 451–465.
8. Jeanrenaud, B. & Hepp, G. D. (1990) *Adipose Tissue: Regulation and Metabolic Function* (Academic, New York).
9. Osafune, T., Sumida, S., Ehara, T., Ueno, N., Hase, E. & Schiff, J. A. (1980) *J. Electron Microsc.* **39,** 372–381.
10. Rosseneu, M. (1992) *Structure and Function of Apolipoproteins* (CRC, Boca Raton, FL).
11. Tuquet, C. & Newman, D. W. (1980) *Cytobios* **29,** 43–59.
12. Greenwood, A. D., Leech, R. M. & Williams, J. P. (1963) *Biochim. Biophys. Acta* **78,** 148–162.
13. Lichtenthaler, H. K. (1969) *Protoplasma* **68,** 65–77.
14. Steinmüller, D. & Tevini, M. (1985) *Planta* **163,** 201–207.
15. Murgia, M., Charzynska, M., Rougier, M. & Cresti, M. (1991) *Sex. Plant Reprod.* **4,** 28–35.
16. Owen, H. A. & Makaroff, C. A. (1995) *Protoplasma* **185,** 7–21.
17. Polowich, P. L. & Sawhnew, V. K. (1990) *Sex. Plant Reprod.* **3,** 263–276.
18. Pacini, E. & Franchi, G. G. (1993) *Plant Syst. Evol.* **7** (Suppl), 1–11.
19. Wang, T. W., Balsamo, R. A., Ratnayake, C., Platt, K. A., Ting, J. T. L. & Huang, A. H. C. (1997) *Plant J.* **11,** 475–487.
20. Robert, L. S., Gerster, J., Allard, S., Cass, L. & Simmonds, J. (1994) *Plant J.* **6,** 927–933.
21. Ross, J. H. E. & Murphy, D. J. (1996) *Plant J.* **9,** 625–637.
22. Tzen, J. T. C., Cao, Y. Z., Laurent, P., Ratnayake, C. & Huang, A. H. C. (1993) *Plant Physiol.* **101,** 267–276.
23. Smith, P. K., Krohn, R. I., Hermanson, G. T., Malia, A. K., Gartner, F. H., Proverzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85.
24. Douce, R. & Joyard, J. (1980) *Methods Enzymol.* **69,** 290–301.
25. Karnovsky, M. J. (1965) *J. Cell Biol.* **27,** 137A.
26. Doughty, J., Hedderson, F., McCubbin, A. & Dickinson, H. (1993) *Proc. Natl. Acad. Sci., USA* **90,** 467–471.
27. Piffanelli, P., Ross, J. H. E. & Murphy, D. J. (1997) *Plant J.* **11,** 549–562.
28. Hesse, M., Pacini, E. & Willemse, M. (1993) *The Tapetum: Cytology, Function, Biochemistry and Evolution* (Springer, Vienna).
29. McConn, M. & Browse, J. (1996) *Plant Cell* **8,** 403–416.
30. Olson, R. E. & Vester, J. W. (1960) *Physiol. Rev.* **40,** 677–733.