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Oleosins and Endoplasmic Reticulum in Seeds and Anthers

Anthony H. C. Huang

Center for Plant Cell Biology, Department of Botany and Plant Sciences,
University of California, Riverside, CA 92521, USA
Anthony.Huang@UCR.edu

Abstract Three types of related subcellular oil-rich particles are present in plants: storage oil bodies in seeds for gluconeogenesis during germination, storage oil bodies in pollen providing acyl moieties for membrane synthesis in the pollen tube, and tapetosomes in the anther tapetum for delivering lipids and proteins to the maturing pollen surface. Each of these oil-rich particles has a basic structure of an oil body, which consists of a triacylglycerol matrix enclosed by a layer of phospholipids and the structural protein oleosins. All components of an oil body are synthesized and assembled in endoplasmic reticulum (ER), from which a budding oil body is released. An oleosin molecule has a highly conserved central domain of 72 uninterrupted hydrophobic residues flanked by variable amphipathic *N*- and *C*-terminal segments. Its unique central domain is presumed to have evolved from a transmembrane segment of an ER protein. An oleosin molecule does not have an *N*-terminal ER-targeting signal and is targeted to the signal recognition particle and then ER via its central hydrophobic domain. Targeting studies of oleosin molecules that have been modified by adding a *N*-terminal ER-targeting signal, shortening the central hydrophobic stretch and eliminating the *N*- or *C*-terminal amphipathic stretch, have provided a model delineating the mechanism of oleosin targeting to ER and oil bodies. A tapetosome possesses numerous oleosin-coated oil bodies associated ionically with abundant membranous vesicles, both of which are assembled in and then detached from ER.

1 Introduction

Eukaryotes and prokaryotes contain neutral lipids in subcellular structures for food reserves and other purposes. These lipid particles are present in the seeds, flowers, pollen, and fruits of higher plants; the vegetative and reproductive organs of lower plants, algae, fungi, and nematodes; mammalian organs/tissues such as mammalian glands and adipose tissues; and bacteria. Oil bodies (OBs) in seeds are the most prominent and best studied of all of these lipid particles.

Seeds of most plant species store oils (triacylglycerols [TAGs]) as a food reserve for germination and postgerminative growth. The TAGs are present in small spherical OBs of approximately 0.5–1 μm in diameter. Each OB has a matrix of TAGs surrounded by a layer of phospholipids (PLs) and structural proteins termed oleosins. The small size of OBs provides a large surface

area per unit TAG, which facilitates lipase binding and lipolysis during germination. OBs inside the cells of mature seeds or in isolated preparations are highly stable and do not aggregate or coalesce. This stability is in contrast to the instability of artificial liposomes made from amphipathic and neutral lipids; the liposomes gradually coalesce after formation. Also, lipid particles in yeast and special mammalian cells, as well as extracellular lipoproteins in mammals and insects, are unstable because they undergo dynamic metabolic fluxes of their surface and matrix constituents. Seed OBs are stable because their surface is shielded by a layer of oleosins, which, for firm anchorage, has a long hydrophobic stretch that is absent in proteins on lipid particles of other organisms. In maturing seeds, TAGs, PLs and oleosins are synthesized in endoplasmic reticulum (ER), from which budding OBs are released.

Research into seed OBs and oleosins has been reviewed in the past by Huang (1992; which reviewed earlier literature), Frandsen et al. (2001), Galili et al. (1998), Hsieh and Huang (2004), Murphy (2001) and Napier et al. (1996). This article emphasizes recent studies, including the findings of a novel organelle, the tapetosome, in the tapetum cells of floral anthers.

2
Distribution and Structure of Oleosins

Oleosins in seeds are small proteins of about 15–26 kDa. They completely cover the surface of a subcellular OB (Fig. 1). They can be abundant in seeds with a high proportion of oils and small OBs (therefore more OB surface area). For example, *Arabidopsis* seeds have more than 40% (wt/wt) oils and small OBs of $\sim 0.5\ \mu m$ diameter, and 10% of the seed proteins are oleosins.

More than 200 genes encoding oleosins have been identified, and oleosins are restricted to plants alone. Recently, genes encoding oleosins on the storage OBs in Arabidopsis pollen (Kim et al. 2002) and tropical cacao seeds (Guilloteau et al. 2003) have been described. These findings negate a proposal of an alternative subcellular mechanism for stabilizing the OBs in pollen and another proposal explaining the short lifespan of tropical seeds such as cacao on the basis of their having unstable OBs. The transcript of a gene encoding an oleosin in the moss *Physcomitrella* can be found in an EST database; this is the most primitive plant known to contain oleosins. Whether algae contain oleosins is not known. Arabidopsis has 17 genes encoding oleosins: nine (eight in tandem) on chromosome 5 that are active in the tapetum cells, five active in seeds, and three active in both seeds and pollen (Kim et al. 2002). Minor proteins present in isolated OB fractions of some seeds have been termed caleosin and steroleosin (Frandsen et al. 2001). They do not have a long hydrophobic sequence, even though they have a short sequence similar to, but much less conserved than, the proline knot sequence in oleosins (to be described). Their mode of association with OBs should not be similar to that

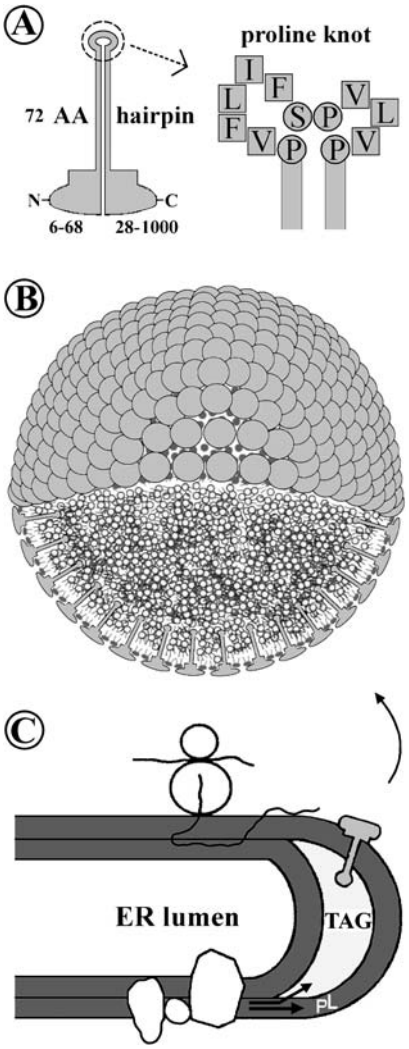

Fig. 1 Models of an oleosin molecule, a seed oil body, and the synthesis of an oil body on endoplasmic reticulum. **a** The three portions of an oleosin molecule, showing the *N*-terminal hydrophilic/amphipathic portion, the central hydrophobic hairpin (and residues at the turn, including the proline knot of three proline residues and one serine residue), and the *C*-terminal hydrophilic/amphipathic portion. The number of residues and their ranges in the three portions in all 17 Arabidopsis oleosins are shown. **b** An OB having oleosins (*medium grey*) and PLs (*dark*) enclosing the matrix TAGs (*light grey*). All molecules are drawn approximately to scale, whereas the diameter of the OB has been reduced 24 times to magnify the surface structure. **c** A budding OB being produced on RER. The ER lumen, the two PL layers (*dark*), the sequestered TAGs (*light grey*) in a budding OB, a ribosome with an mRNA synthesizing an oleosin polypeptide (*dark line*, of an unknown configuration), and enzymes (*irregular circles*) for the synthesis of TAGs and PLs are shown (modified from Hsieh and Huang 2004)

of oleosins, and the possibility of their being contaminants or remnants of ER after biogenesis needs to be explored.

An oleosin molecule can be divided into three portions according to its amino acid sequence (Fig. 1a). The *N*-terminal portion can be short or long (e.g., 6–68 residues in Arabidopsis) and hydrophilic or amphipathic. The central portion is a long hydrophobic stretch of 72 residues. The *C*-terminal portion can be short or very long (e.g., 28–1000 residues in Arabidopsis oleosins), and its ~ 30 residues adjacent to the central hydrophobic stretch can form an amphipathic α-helical structure that interacts horizontally with the charged phosphate and choline groups of the PL layer on the OB surface. The *C*-terminal portions of most Arabidopsis oleosins consist of fewer than 100 residues; a few have 100–150 residues; one has 403; and another has 1000 residues. Each of the longer *C*-terminal portions contains many repeats of short peptides, which are not conserved among oleosins and may not have any functional significance. Some of the repeated short peptides have several glycine residues. Oleosins in the tapetum are sometimes called glycine-rich proteins, although such a terminology does not describe the important characteristics of the proteins.

The central hydrophobic stretch of 72 uninterrupted hydrophobic residues is the hallmark of an oleosin. No other protein in any organism has such a long, or even half as long, hydrophobic stretch. Proteins on the surface of extracellular or intracellular lipid droplets, such as apolipoproteins, perilipin, adipophilin, and caveolin in mammals, phasin in bacteria and lipid-associated protein (PAP) in plastids, do not have such a long hydrophobic stretch; their polypeptides run parallel to the surface of the lipid droplets rather than penetrate into them. The 72-residue hydrophobic stretch of an oleosin is long enough (a transmembrane [PL bilayer] peptide has 20–25 residues) to form a hairpin that penetrates the surface PL monolayer of an OB into the matrix (Fig. 1b).

The center of the hydrophobic stretch has three proline residues and one serine residue that could interact to form a "proline knot" (Fig. 1a). This proposal is based on the presence of the less hydrophobic proline and serine residues among the other more hydrophobic residues and TAGs in the matrix of an OB, as well as on the fact that proline residues on polypeptides are breakers or turners of α-helical and β-structures. The formation of the proline knot could permit creation of a hairpin structure of the whole hydrophobic stretch, with two arms of 30 residues joined by a turn of 12 residues. The 72 residues of the hydrophobic stretch are conserved in terms of hydrophobicity among oleosins of diverse species, and the conservation is higher at the proline knot and its immediate vicinity ($-\,PX_5SPX_3P\,-$). All oleosins contain the three proline and one serine residues at identical locations in the center of the hydrophobic stretch.

All researchers agree that the central hydrophobic stretch forms a hairpin structure with a proline knot at the turn, but disagree on the secondary struc-

tures of the two hairpin arms. Earlier, it was proposed that the two arms have an antiparallel α-helical structure on the basis of an algorithm prediction (actually no database for predicting secondary structures of polypeptides in a hydrophobic environment exists) or an antiparallel β-structure on the basis of symmetry of residues between the two arms (Huang 1992). If the two arms had an antiparallel β-structure, they could bend at several locations where pairing of small glycine residues occurs; the bending would create more interactions among residues and thus offer higher stability. Two laboratories used circular dichroism and Fourier transform infrared spectroscopy to determine the secondary structures of the oleosin hairpin in its imitated native conditions. They arrived at the opposite conclusions of either an α-helical structure (Alexander et al. 2002) or β-structure (Li et al. 2002). The controversy underlines the difficulties in measuring the uniquely long hydrophobic polypeptide in a neutral-lipid environment. In addition, the arms or even the turn could interact with those in adjacent oleosins in the OB matrix. Such interactions could provide higher stability to the oleosin hairpin, in which the peptide bonds are relatively hydrophilic, in the hydrophobic environment. In maize, oleosins of two isoforms coexist in a 1 : 1 ratio, and an interaction between the pair is likely (Lee and Huang 1996). Furthermore, the *N*- and *C*-terminal portions of an oleosin, even though on the OB surface, may play a role in maintaining the hairpin in a special configuration. The *N*- and *C*-terminal portions of an oleosin on the surface of a seed OB may act as receptors for the binding of lipase during germination. This possibility can be tested by using a seed lipase protein derived from a cloned lipase gene, the cloning being facilitated by currently available genomics and proteomics technologies.

3
Synthesis of Oils, Oleosins, and Oil Bodies in Endoplasmic Reticulum

Oil bodies, including their constituent TAGs, PLs, and oleosins, are synthesized on ER. Diacylglycerol acyltransferase (DAG AT), the last enzyme and the only one unique to the synthesis of TAGs, as well as enzymes for the synthesis of precursor DAGs and PLs, are associated with rough ER (RER). An alternative TAG-synthesizing enzyme, which can transfer the acyl moiety from PLs instead of acyl-CoA to DAG, is also located in ER. The presence of these enzymes in ER is not surprising in view of the hydrophobicity of TAG and its metabolic precursors. TAGs synthesized in ER are sequestered in the hydrophobic region (i.e., the acyl region of the PL bilayer). Continuation of TAG accumulation at a domain of ER forms a budding OB, which is enclosed by a single layer of PLs (Fig. 1c). This budding OB, covered with a PL monolayer, is stabilized by inclusion of oleosins to its surface.

Ribosome-mRNA with a nascent oleosin peptide can be guided to ER via the signal-recognition particle (SRP) pathway. mRNA for synthesis of oleosin

is associated with RER. Translation of oleosin mRNA in an in vitro synthesis system is retarded or enhanced, respectively, when SRP or microsomes are added (Abell et al. 2002; Beaudoin and Napier 2002; Loer and Herman 1993; Thoyts et al. 1995). The findings suggest that the translation of oleosin mRNA pauses after binding of SRP to the nascent peptide and accelerates when the newly synthesized oleosins incorporate into ER. In addition, stable incorporation of in vitro-synthesized oleosin (commercial in vitro synthesis systems usually contain SRP) into microsomes is inhibited when the SRP receptor (SRP-60) on microsomes is removed beforehand through proteolysis. This inhibition can be restored with reconstituted SRP receptor. Yeast transformed with an oleosin gene synthesizes and targets oleosin to OBs (Ting et al. 1997). When the transformed yeast strains are mutants defective in SRP components, oleosin is not targeted to OBs, and the nontargeted oleosin is proteolytically degraded (Beaudoin et al. 2000).

Targeting of oleosin to ER in vitro can occur with the use of SRP components and microsomes from yeast, mammals, or plants. Thus the unique aspect of the targeting mechanism is the targeting signal(s) in the oleosin molecule. Modified oleosins produced via gene recombination can be tested for their stable insertion into microsomes in vitro or into ER in vivo (Abell et al. 1997, 2002; Beaudoin and Napier 2002). The *N*- and *C*-terminal portions are relatively unimportant in targeting oleosin to ER. Rather, the long hydrophobic stretch of oleosin is the predominant factor for targeting. No specific signal sequence in the hydrophobic stretch is required. Instead, any of the multiple and probably overlapping sequences along the hydrophobic stretch can target the protein to ER. Significantly, the highly conserved proline knot is not important for the in vitro targeting of oleosin to microsomes, because replacement of the three proline residues with leucine residues does not affect the targeting. The finding that multiple peptides along the hydrophobic stretch can be the targeting signals is consistent with the knowledge that the hydrophobic pocket of an SRP can recognize a diverse array of hydrophobic ER-targeting peptides at the *N* termini or interior of many proteins.

The nascent oleosin polypeptide synthesized or being synthesized on ER assumes a topology on the basis of its hydrophobic and hydrophilic interactions with the PL bilayer. The hydrophilic/amphipathic *N*- and *C*-terminal portions interact with the PL layers on the cytosolic side of ER (Fig. 1c), whereby the central hydrophobic stretch buries itself in the hydrophobic acyl portion of the PL bilayer. Much evidence from in vivo and in vitro experiments exists for such a topology for the nascent oleosin (Abell et al. 1997, 2002; Beaudoin et al. 2002). The *N*- and *C*-terminal portions, but not the hydrophobic stretch, of oleosin in isolated microsomes are susceptible to proteolysis by exogenously added proteases; this observation is similar to that of oleosins on mature OBs. The secondary structure of the 72-residue hydrophobic stretch in the hydrophobic portion of the PL bilayer is unknown but likely differs from that in a mature OB. The matrix of a mature OB, but not the hy-

drophobic region of ER, provides an excess of hydrophobic volume for the hydrophobic stretch to assume its presumably most stable hairpin configuration. The hydrophobic stretch of oleosin within the hydrophobic region of ER could assume a bended hairpin structure or an extended structure with or without coiling, running parallel to the PL bilayer (Fig. 1c). An additional consideration is the actual thickness of the hydrophobic region of the PL bilayer. While ER is synthesizing oleosins, it also produces massive amounts of TAGs, which will be temporarily sequestered in, and thus enlarge, the hydrophobic region of the PL bilayer. Thus, the hydrophobic region of the PL bilayer may have more room for the hydrophobic stretch of a nascent oleosin than that defined by the length of the two acyl chains.

Both the newly synthesized oleosins and the temporarily located TAGs on ER diffuse to budding OBs. This movement is made possible in accordance with the fluid mosaic model of membrane action and thermodynamic considerations. TAGs and the oleosins will be more stable in the hydrophobic environment of a budding OB. A native oleosin stably inserted into ER diffuses to the budding OB, but a stably inserted, artificially modified oleosin may not. The mechanism of this oleosin movement has been studied in vivo through using modified oleosins and measurements of oleosins recovered in ER and OB fractions (Abell et al. 1997, 2002, 2004; Beaudoin and Napier 2002). Strictly speaking, this approach measures not just targeting success per se, but also the stability of the modified oleosins in OBs. Modified oleosins that can diffuse to the budding OBs may be unstable there and removed by endogenous proteolysis. The molecular requirements for oleosin to diffuse successfully to, and incorporate stably into, OBs are similar to those for targeting the protein to ER; however, more are required. The proline knot in the hydrophobic stretch is also essential, presumably for stable anchoring of oleosin on OBs. A modified oleosin without the proline knot (e.g., having the three proline residues replaced with leucine residues) can probably insert into ER and also diffuse to the budding OB but would be unstable there and thus eliminated by endogenous proteolysis. In addition to the need for the proline knot, decreased length or elimination of the *N*- or *C*-terminal portions or decreased length of the hydrophobic stretch all lead to a reduced recovery of the modified oleosin in OBs.

Oleosins must be only on the cytosolic side of ER to be able to diffuse to the budding OB. Attempts to insert the whole oleosin molecule into the luminal side of ER have been unsuccessful. An *N*-terminal ER targeting peptide from a nonoleosin protein attached to the *N* terminus of an oleosin, produced via gene recombination, can pull the *N*-terminal portion of the oleosin but not the hydrophobic stretch (with or without the *C*-terminal portion) into the ER lumen (Abell et al. 2002, 2004). Apparently, the hydrophobic interaction between the long hydrophobic stretch and the acyl moieties of the PL bilayer (with or without the added hydrophilic interaction between the *C*-terminal portion and the PL layer on the cytosolic side) is too strong for the oleosin

to leave the PL bilayer and insert into the lumen. This modified oleosin can incorporate into ER but cannot insert into the budding OB. Obviously, its polypeptide spanning across the whole PL bilayer of ER cannot diffuse to the PL monolayer of a budding OB (Fig. 1c). Even if it could, it would be unstable there.

It is uncertain whether a ribosome-mRNA-oleosin complex can target to ER or the budding OB directly without involvement of the SRP pathway. All the evidence from in vitro experiments shows that the SRP system is involved. A ribosome-mRNA-oleosin complex with the hydrophobic stretch dangling outward in vitro could bind to the hydrophobic pocket of an added SRP, regardless of whether SRP is actually involved in vivo. Certainly, it has been shown that oleosin synthesized in vitro cannot insert into mature OBs co- or posttranslationally (Hills et al. 1993). However, a mature OB is packed with oleosins on its surface and has no extra room for new oleosins. Oleosin synthesized in vitro can insert into artificial OBs whose surface has not been filled completely with oleosins (Chen and Tzen 2001). A ribosome-mRNA-oleosin complex with the hydrophobic stretch dangling outward could theoretically bind to the hydrophobic region of ER or a budding OB whose surface had not been filled completely with oleosins. Nevertheless, the strongest evidence for the need of SRP to guide oleosin to ER has come from in vivo studies with yeast mutants defective in SRP components (Beaudoin et al. 2000). This finding with yeast should be tested with plants. Further, whether oleosin synthesis employs both the SRP system and a direct insertion mechanism has not been evaluated.

As newly synthesized TAGs and oleosins on ER diffuse to and converge at the budding OB, a gradient of enrichment of these two components should exist from the point of synthesis to the budding OB. This concentration gradient can explain the immunocytochemical observation that more oleosins are present in the ER near the budding OBs (Herman 1987). Whether subdomains of ER for TAG and oleosin synthesis are present remains to be documented. In an in vitro study, sunflower seed microsomes supplied with precursors synthesized TAGs and, after this synthesis, were subfractionated by density gradient centrifugation (Lacey et al. 1999). The fraction with the lowest buoyant density contained more TAG, oleosin, and lipid synthesis activity on a per fraction basis. This fraction may represent ER subdomains specialized for TAG and oleosin synthesis, or simply fragments of ER regions originally closest to the budding OBs and thus having more TAGs and a lower buoyant density. In an earlier experiment, when an extract of maturing maize kernel was subfractionated by density gradient centrifugation, DAG AT, the last and unique enzyme for TAG synthesis, was found with cytochrome reductase in RER fragments of diverse buoyant densities (Cao and Huang 1986). The DAG AT was not concentrated in ER fragments with the lowest buoyant densities and therefore most TAGs (or fewest polysomes). Thus, in the maize cells, TAGs are probably synthesized in diverse regions of ER and diffuse to the budding OBs. In the tapetum in

Brassica anthers, oleosin-coated oil droplets are structural analogs of seed oil bodies (see Sect. 4). During synthesis of these tapetum oil droplets, oleosin and the ER chaperone calreticulin were colocalized in extensive regions of the ER network, as seen in situ by immunofluorescence microscopy. Thus, the tapetum oleosins, and perhaps TAGs also, are synthesized in diverse regions of ER rather than in highly restricted ER subdomains.

A budding OB is released from ER as a solitary oil body (Fig. 1c). An early release will generate a smaller OB, and vice versa. The size and shape of an OB are determined in part or completely by the relative amount or rate of synthesis of oils and oleosins. High-oil maize kernels (having a high oil-to-oleosin ratio) generated by breeding have large, spherical OBs, whereas low-oil kernels have small OBs with irregularly shaped surface (Ting et al. 1996). In cells that do not synthesize oleosins, such as those in the fatty mesocarp of fruits, the OBs (lipid globules) become very large (see next paragraph). A special mechanism may exist for the physical release of a budding OB from ER. Oleosins accumulated on the bud surface may interact among themselves to produce a physical force of constriction at the neck of the bud, thereby releasing the OB. Or, the physical release may require specific cytosolic proteins (e.g., dynamins). These possibilities can be tested by screening for Arabidopsis mutants whose seeds have larger or smaller OBs or only budding OBs viewed with a light microscope after lipid staining. Some of these mutants may be defective in the mechanism for physical release of OBs from ER.

In the fatty mesocarp of fruits such as avocado, oil palm, and olive, each cell has one to several large lipid globules, which occupy the bulk of the cell volume. Little or no oleosins are present on these lipid globules. Mesocarp lipids are for attracting animals and serve for seed dispersion and thus are not required to be in small entities such as seed OBs. Mostly likely, TAGs are synthesized in ER, as in seeds, but without a cosynthesis of oleosins (Fig. 1c). As a consequence, the budding OB enclosed only by PLs becomes larger (and/or fuses with adjacent budding OBs) before it is released from ER. This is equivalent to the synthesis of larger OBs in maize kernels having a high oil-to-oleosin ratio. It is possible that the mesocarp cells can be modified to synthesize small OBs instead of large lipid globules if oleosin is allowed to be cosynthesized with TAGs via genetic engineering. Although in theory this genetic engineering project can be easily achieved, in practice it is difficult because the better-known avocado, oil palm, and olive that contain fatty mesocarp are tree crops.

4
Oleosins in Tapetum Cells and the Novel Organelle, Tapetosome

The presence of oleosins in tapetum cells of anthers in Arabidopsis and Brassica was discovered a decade ago from unintended gene cloning results

(deOliverira et al. 1993; Roberts et al. 1994). The finding was unexpected because tapetum cells were not known to contain OBs similar to those in seeds. Subsequently, these oleosins were found to be present in a novel, neutral lipid-containing organelle, which has been termed the tapetosome because of its unique presence in the tapetum of plants (Wu et al. 1997). To date, the presence of tapetal oleosins is limited to species of the insect/self-pollinating Brassicaceae family, especially Brassica and Arabidopsis.

In Arabidopsis, nine genes encode the tapetal oleosins, eight of which are in tandem on chromosome 5 (Fiebig et al. 2004; Kim et al. 2002; Schein et al. 2004). One of these genes is highly expressed to produce an oleosin of 53 kDa, which represents about 70% of all tapetal oleosins. Most of the other Arabidopsis tapetal oleosins are smaller (10–23 kDa), but one has 115 kDa. As expected, Brassica has a similar oleosin gene system (Roberts et al. 1994; Ross and Murphy 1996; Ruiter et al. 1997), and the most active gene (ortholog of the Arabidopsis gene encoding the 53-kDa oleosin) produces a major oleosin of 45 or 48 kDa (from the *B. rapa* AA genome or *B. oleracea* CC genome, respectively). Genes encoding the tapetal oleosins have undergone rapid evolution that altered the *N*- and *C*-terminal regions but not the hairpin regions, as the genes encoding seed oleosins do. Findings of these evolutionary changes reiterate that the *N*- and *C*-terminal regions of oleosins have minimal constraints for protein structures, and thus functions.

The tapetum is a one-cell layer enclosing the anther locule, in which microspores mature to become pollen. Tapetum cells are the only anther sporophytic cells that are metabolically very active and control maturation of microspores. At an early stage of anther development, the tapetum cells are specialized for active secretion and contain abundant RER and secretory vesicles. At a late stage of anther development, at least in Brassicaceae species, the cells become a temporary storehouse of ingredients to be deposited onto maturing pollen as pollen coat. The tapetum cells at this late stage of development are packed with two predominant storage organelles, the elaioplasts and tapetosomes (Owen and Makaroff 1995; Platt et al. 1998; Polowick and Sawhney 1990). The elaioplasts, of 3–4 μm in diameter, are specialized plastids largely devoid of thylakoids but filled with small spherical lipid droplets of steryl esters enclosed by the structural protein PAP. Although elaioplasts of similar morphology can be found in nontapetum cells, such as fruit and petal cells, tapetosomes are unique to the tapetum cells. Each spherical tapetosome, of 2–3 μm in diameter, has oleosin-coated TAG droplets associated with vesicles derived from ER. These oleosin-coated TAG droplets are similar in structure and constituents to seed OBs.

The contents of tapetosomes and elaioplasts are selectively retained and discharged to the anther locule after death of the tapetum cells during the final stage of anther development. Oleosins, but not TAGs, of tapetosomes and steryl esters, and not the structural protein PAP of elaioplasts are selectively retained and transferred to the pollen surface, forming the bulk of pollen coat

(Wu et al. 1997, 1999). The rationale and mechanism for the selectivity are unclear. It is intriguing that in seed OBs, TAGs are the prime ingredient for physiological function and oleosins are the accessories, whereas in tapetosomes, oleosins may be the main element for physiological function (to be described) and TAGs are the accessories. The tapetum TAGs disappear after death of the cells, and their function and metabolic fate are unknown. They may be used as an energy source for active metabolism of the tapetum cells. Their fatty acids could also be used to produce jasmonic acid as a floral maturation hormone, or alkanes as one of the two major lipid constituents (the other being the elaioplast steryl esters) for deposition onto maturing pollen. These possibilities are testable with Arabidopsis mutants defective in tapetum TAG synthesis or degradation.

Although the steryl esters and other lipids on pollen form a useful waterproofing layer, the function of the abundant oleosins there is less clear. In Brassica, the predominant 45/48-kDa oleosin on pollen has been cleaved selectively into two fragments, one containing the *N*-terminal portion and the central hydrophobic stretch, and the other the long hydrophilic *C*-terminal portion (Ross and Murphy 1996; Ting et al. 1998). Whether other smaller oleosins on pollen are cleaved is not known. The cleavage may be fortuitous in mutation and have no physiological relevance. The most abundant oleosin on pollen has a large size (53 kDa in Arabidopsis and 45/48 kDa in Brassica) owing to its possession of numerous repeats of short peptides at its *C* terminus. Each of these repeats possesses several glycine residues, which again makes the protein glycine-rich.

Because of its glycine-rich nature, it has been speculated that this pollen oleosin (and extrapolating to other oleosins) might interact the cell walls of the stigma. Such a speculation should be taken with caution. Oleosins have undergone rapid evolutionary changes, and both tapetal and seed oleosins have repeats of short peptides at their *C* termini; some of these repeats have high glycine contents, whereas others do not. The rapidity and extensiveness of changes at the *C* termini may reflect the minimal structural constraints on this part of the protein to perform functions. The high glycine contents at the *C* termini of oleosins may be fortuitous, and certainly the glycine-rich *C* termini in some seed oleosins do not have an apparent function for interaction with cell walls. In fact, the short repeats at *C* termini of the most abundant tapetum oleosins have not only a high glycine content but also high serine and lysine contents, making the oleosin also serine-rich and lysine-rich (the Arabidopsis 53-kDa oleosin has 26, 16, and 14 mol %, and the Brassica 48-kDa oleosin 21, 16, and 11 mol % of glycine, serine, and lysine, respectively).

An oleosin molecule may serve dual functions on pollen and subsequently on the stigma because of its amphipathic property. Its *N*- and *C*-terminal portions are hydrophilic/amphipathic, and its central portion is hydrophobic. The overall amphipathic oleosin can act as an emulsifying agent to uniformly

coat the pollen with steryl esters, alkanes, flavonoids, and other ingredients. It may also aid in water uptake for germination after the pollen grain has landed on the stigma. Brassicaceae species have dry stigmas, and water must be drawn from the stigma interior to the pollen for germination and tube growth. Steryl esters and other neutral lipids are not amphipathic enough to be able to act as a wick. However, the abundant and amphipathic oleosins (and/or flavonoids) could act in this manner. On the basis of these two proposed functions, the mutational addition of repeats of short peptides, which are all fairly hydrophilic, to the *C* termini and fragmentation of the Brassica 45/48-kDa oleosins into two halves do not affect the function of the oleosins. The proposed functions are also in agreement with the observation that the pollen of an Arabidopsis null mutant in the major pollen-coat oleosin does not hydrate efficiently on the stigma (Mayfield and Preuss 2000). This partial loss of function could have been due to the lack of sufficient oleosins on the pollen to serve as a wick and/or the pollen coat not having been properly emulsified. Overall, the major structural constraints on oleosins to perform the proposed functions are a long hydrophobic stretch to interact with the TAG droplets in tapetosomes (not a function per se but for storage in the organelles) and an amphipathic molecule to emulsify the pollen coat materials and take up water from stigma. All the observed mutational changes on tapetal oleosins have not affected these constraints and are thus extensive because of the lack of selective pressure.

Tapetosomes have a unique morphology (Platt et al. 1998; Wu et al. 1997). Transmission electron microscopy has revealed that in situ each tapetosome has a nonhomogeneous interior whose internal structures cannot be recognized. However, these structures can be observed clearly after the tapetosomes has been isolated and subjected to osmotic swelling. A tapetosome consists of oleosin-coated TAG droplets associated via ionic linkage with ER-derived vesicles (Fig. 2). Isolated tapetosomes, after a high- or low-pH treatment, can be subfractionated into TAG droplets (which contain oleosins and TAGs), and membranous vesicles (which possess ER-derived calreticulin and luminal binding protein).

Tapetosomes are synthesized via a special mechanism, as revealed in a recent study with immunofluorescence microscopy and transmission electron microscopy (Hsieh and Huang 2005). During early development of a tapetum cell, the ER luminal protein calreticulin exists as a network, and contains no oleosins. Subsequently, oleosins appear together with calreticulin in the ER network, which possesses centers with a high ratio of oleosin to calreticulin. Transmission electron microscopy shows that at this stage massive ER cisternae interconnect the numerous maturing tapetosomes in a cell. Finally, the ER network largely disappears, and solitary tapetosomes containing oleosins and calreticulin prevail. These and other (Platt et al. 1998) microscopical studies, along with findings from subcellular fractionation, allow for a model depicting the biogenesis of tapetosomes from RER (Fig. 2). Initially,

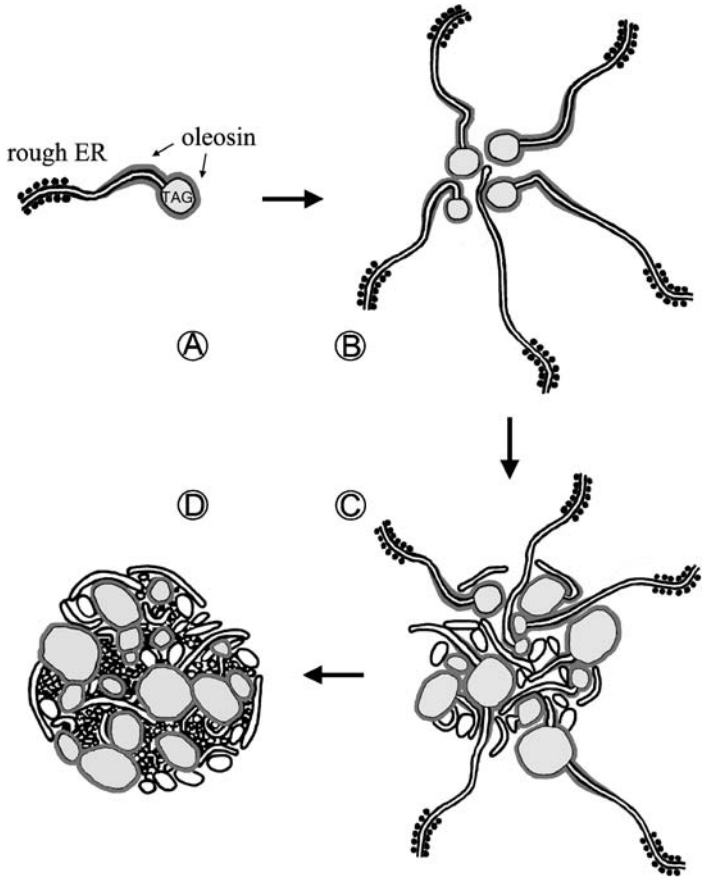

Fig. 2 Model for the synthesis of a tapetosome in Brassica tapetum cells. **a** formation of an oleosin-coated oil droplet from RER by a mechanism similar to that in Fig. 1c. Each oil droplet consists of an oil matrix (*light grey*) enclosed by a layer of PL (*dark*) and oleosins (*medium grey*). **b** Association of several budding oil droplets and ER cisternae. **c** A maturing tapetosome containing detached ER vesicles. **d** A mature tapetosome (modified from Hsieh and Huang 2004)

TAG droplets are produced via an ER-budding mechanism identical to that in maturing seeds. These TAG droplets are covered by oleosins and PLs. As many are produced they converge. More ER cisternae are connected to the droplet clusters and eventually break off as vesicles. As a consequence, a tapetosome is formed. During the peak period of tapetosome formation, all the maturing tapetosomes in the cell are interconnected via ER cisternae.

The function of the abundant ER-derived vesicles in tapetosomes remains to be elucidated. These vesicles possess the same basic constituents of calreticulin and luminal binding protein as the ER cisternae do. The vesicles in tapetosomes may aid in the transfer of oleosins from lysed tapetum cells to

the pollen surface. They may possess proteins that would subsequently exert action on the stigma, such as incompatibility factors and other signaling proteins. They may contain ions such as calcium and boron for the pollen surface; these ions would subsequently modulate the cell wall structures of the stigma. Or, they may contain flavonoids and other secondary metabolites for the pollen surface. Such pollen-surface metabolites are well known, but of uncertain function. Subcellular fractionation and modern microscopy should be used to test the presence of these ingredients in the tapetosome vesicles.

Future studies on the tapetal oleosins and tapetosomes should aim at expanding the existing findings to non-Brassicaceae species, pinpointing the roles of oleosins on pollen, and examining the contents of the ER-derived vesicles in tapetosomes. Working hypotheses exist and are testable. In addition, use of Arabidopsis mutants defective in individual constituents will aid these tests.

5
Evolution of Oleosins, Oil Bodies, and Tapetosomes

Prokaryotes, in general, do not store TAGs as food reserves. A minor exception is *Actinomyces*, which produce TAGs under certain nutritional and other environmental conditions. TAGs were likely to have evolved as efficient food reserves in primitive eukaryotes by the addition of one enzyme, DAG AT, which was evolved from one of the existing acyltransferases. This enzyme diverted DAGs from the ubiquitous PL metabolic pathway to TAGs. Initially, the hydrophobic TAGs were present between the two PL layers of the ER membrane, where DAG AT was. Today, seeds of some species on occasions still have some TAGs present along the hydrophobic region of the PL bilayers in ER (Wanner et al. 1981). The presence of excess TAGs in the ER membrane would interfere with the normal functioning of ER. This problem was overcome by removal of the TAGs from ER via budding to become solitary droplets. The droplets, each containing a TAG matrix enclosed by a layer of PLs originated from ER, would be unstable. In yeasts, the droplets were made more stable through a coat of amphipathic proteins, especially the TAG synthesizing and hydrolyzing enzymes. The semistability would allow the droplets to undergo dynamic metabolic fluxes. In mammals, the droplets were modified to different forms with proteins and membranes, such that they were also semistable and amenable to metabolic fluxes. In plants, the droplets were stabilized by the evolutionary appearance of oleosins, whose long hydrophobic hairpin could stabilize the droplets so effectively that they were amenable to prolonged storage in desiccated seeds. Oleosins and their coated oil droplets have been found in diploid and triploid storage sporophytic cells of seeds of angiosperms and gymnosperms, haploid storage cells of female gametophytes (in seeds) in gymnosperms, haploid cells of male ga-

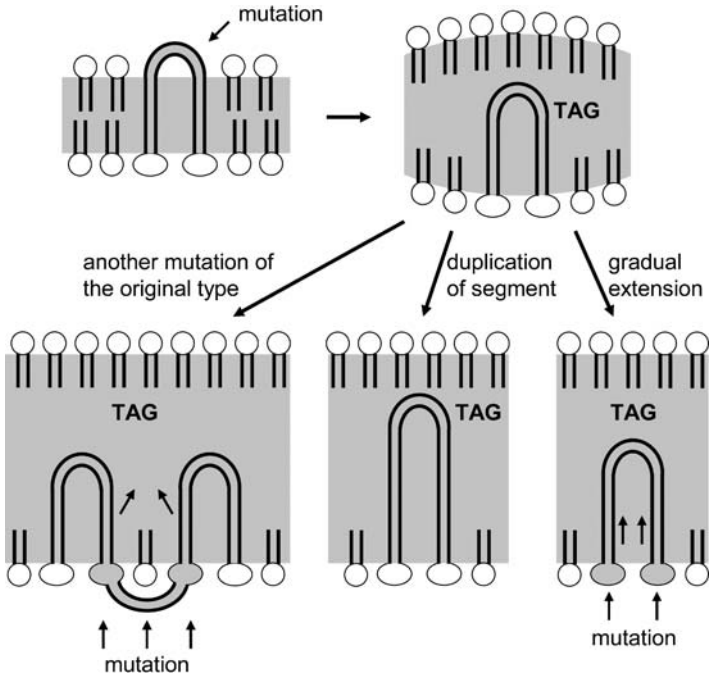

Fig. 3 Model for the evolution of oleosins. The 72-residue hydrophobic segment of an oleosin molecule was viewed as having evolved from the transmembrane segment of an ER protein. The shaded area and *thick lines* represent hydrophobic regions. These include the acyl moieties of PL (*two lines joining a circle*), TAGs, and the transmembrane, hydrophobic portion of an ER protein being evolved to an oleosin hairpin (*enclosed column*). *Unshaded circles* depict hydrophilic portions of PL and proteins (modified from Huang 1996)

metophytes (pollen), the moss *Physcomitrella* (possibly in diploid sporophyte or haploid gametophytes) and the diploid sporophytic cells of floral tapetum.

The hydrophobic stretch of 72 resides in oleosins is the longest, and is actually more than twice as long as any found in any prokaryotic or eukaryotic protein. The mechanism by which it has evolved is intriguing. A hypothesis has been proposed (Fig. 3) on the basis of the following observations (Huang 1996):

1. The length of 72 residues is about four times that of a transmembrane polypeptide ($\sim$ 20 residues)
2. Several relatively hydrophilic residues are present in the middle of both antiparallel stretches
3. A certain degree of residue symmetry exists along the two antiparallel stretches

The hypothesis depicts that the long hydrophobic stretch has evolved from duplications of a transmembrane peptide of an ER protein in a primitive plant or alga. The hypothesis can be tested by comparing the amino acid sequences

of the oleosin hairpins (and the corresponding nucleotide sequences) with those of transmembrane segments of proteins, especially of ER, in the most primitive organisms (currently, the moss *Physcomitrella*). Whereas the hairpin hydrophobic stretch is conserved, the *N*- and *C*-terminal portions have undergone extensive evolutionary changes because of limited structural and functional constraints.

Oleosin-coated oil droplets in diverse plant species can be categorized into two groups according to their structures and functions. The solitary oleosin-coated OBs in seeds and pollen store TAGs for germination and postgerminative growth in the respective organs. The tapetosomes contain clustered oleosin-coated oil droplets associated with ER-derived vesicles and store and deliver materials to the surface of maturing pollen. Whether, during evolution, solitary oleosin-coated oil droplets similar to the modern seed OBs appeared before the complex tapetosomes, or vice versa, is a matter for speculation. The most primitive plant known to contain oleosin is the moss *Physcomitrella*. The moss oleosin is presumably associated with storage OBs in the sporophyte or gametophytes. The moss does not have flowers or tapetum and thus would not have analogs of tapetosomes. Brassicaceae species contain abundant tapetosomes in tapetum, whereas the maize tapetum does not have any. Thus, tapetosomes were likely to have evolved from solitary oleosin-coated TAG droplets similar to the modern OBs in seeds. Initially, these ancestral droplets, solitary or in groups, in tapetum delivered oleosins to the pollen surface. Subsequently, they became associated with vesicles that also contain materials for the pollen surface. Thus, tapetosomes are thought to have evolved to perform the overall function of packaging and storing materials for delivery to the pollen surface.

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