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Author for correspondence:

Dr. Anthony Huang  
Department of Botany and Plant Sciences, University of California, Riverside, CA 92521  
Email address: anthony.huang@ucr.edu  
Phone: 1-951-827-4783  
Fax: 1-951-827-4437

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Oil bodies and oleosins in *Physcomitrella* possess characteristics representative of early trends in evolution

Chien-Yu Huang\(^2\), Chung Chun-I\(^2\), Yao-Cheng Lin\(^3\), Yue-Ie Caroline Hsing\(^3\), and Anthony H.C. Huang\(^2\)\(^*\)

\(^2\) Center for Plant Cell Biology, Department of Botany and Plant Sciences, University of California, Riverside, CA 92521

\(^3\) Institute of Plant and Microbial Biology, Academia Sinica 11529, Taipei, Taiwan
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*corresponding author, Anthony Huang, anthony.huang@ucr.edu
Searches of sequenced genomes of diverse organisms revealed that the moss *Physcomitrella patens* is the most primitive organism possessing oleosin genes. Microscopy examination of *Physcomitrella* revealed that oil bodies (OBs) were abundant in the photosynthetic vegetative gametophyte and the reproductive spore. Chromatography illustrated the neutral lipids in OBs isolated from the gametophyte to be largely steryl esters and triacylglycerols, and SDS-PAGE showed the major proteins to be oleosins. RT-PCR revealed the expression of all 3 oleosin genes to be tissue specific. This tissue specificity was greatly altered via alternative splicing, a control mechanism of oleosin gene expression unknown in higher plants. During the production of sex organs at the tips of gametophyte branches, the number of OBs in the top gametophyte tissue decreased concomitant with increases in the number of peroxisomes and level of transcripts encoding the glyoxylate cycle enzymes; thus, the OBs are food reserves for gluconeogenesis. In spore during germination, peroxisomes adjacent to OBs, along with transcripts encoding the glyoxylate cycle enzymes, appeared; thus, the spore OBs are food reserves for gluconeogenesis and equivalent to seed OBs. The one-cell-layer gametophyte could be observed easily with confocal microscopy for the subcellular OBs and other structures. Transient expression of various gene constructs transformed into gametophyte cells revealed that all OBs were linked to the endoplasmic reticulum (ER), that oleosins were synthesized in extended regions of the ER and that two different oleosins were co-located in all OBs.
Eukaryotes and prokaryotes contain neutral lipids in subcellular droplets as food reserves and/or for other purposes (Hsieh and Huang, 2004; Martin and Parton, 2006; Goodman, 2008; Rajakumari et al., 2008). These lipid droplets are present in seeds, pollens, fruits and flowers 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. Among all these lipid droplets, oil bodies (OBs) in seeds are the most prominent and have been extensively studied.

Seeds of diverse plant species store oils (triacylglycerols [TAGs]) as food reserves for germination and postgermination growth (Napier et al, 1996; Frandsen et al, 2001; Murphy, 2001; Hsieh and Huang, 2004). The TAGs are present in small subcellular, spherical OBs of approximately 0.5-2 μm in diameter. Each OB has a matrix of TAGs surrounded by a layer of phospholipids (PLs) and the structural protein oleosins. The massive oleosins completely cover the surface of the OBs and prevent them from coalescence; so, a large surface area per unit TAG is available for lipase binding and catalysis during germination. Each oleosin molecule has a characteristic long central hydrophobic stretch, which forms a hairpin penetrating into the matrix TAGs for stable anchorage.

Other than being present in the seeds of plants, oleosin-coated OBs are also present in pollen (probably for storage of acyl moieties for tube elongation [Kim et al., 2002]) and the tapeta of Brassica and Arabidopsis (Hsieh and Huang, 2005, 2007). Inside each tapetum cell, many oleosin-coated OBs associate with numerous flavonoid-containing vesicles to form large subcellular particles termed tapetosomes, each 2-3 μm in diameter. Tapetosomes temporarily store lipids and flavonoids, which are deposited onto the maturing pollen as a pollen coat for water-proofing and UV protection, respectively. In fruits of some species, such as olive, avocado and oil palm, the fleshy mesocarp possesses much larger (10-50 μm diameter) subcellular lipid particles of TAGs, which are devoid of surface oleosins and apparently are for attracting animals for seed dispersal (Murphy 2001; Hsieh and Huang, 2004). OBs are also present, although generally in low abundance, in leaves of diverse plant species, and their structures and functions are unknown (Lersten et al., 2006).

Oleosins of all plant species contain a conserved central hydrophobic hairpin of ~72 residues flanked by less conserved amphipathic N and C termini of highly variable length (Hsieh and Huang, 2004). Within the hairpin, the turn consists of 12 most-conserved residues
(PX₃SPX₃P), of which the 3 proline and 1 serine residues (termed the proline knot) are completely conserved without a single exception among hundreds of examined oleosins of various species. Paralogs of oleosin genes are present within each species and individuals and are expressed in a tissue-specific manner. For example, *Arabidopsis* has 17 oleosin genes, which are selectively expressed in seed, pollen and the tapetum (Kim et al., 2002).

Oleosins and TAGs are generally believed to be synthesized on the ER inside a seed cell. Whereas the nascent oleosins are attached to the ER surface via the long hydrophobic hairpin stretch, TAGs are sequestered between the 2 PL layers of the ER membrane. These oleosins and TAGs migrate to and are eventually concentrated in confined ER regions, which are detached to form mature OBs (Napier et al., 1996; Murphy, 2001; Abell et al., 2004; Hsieh and Huang, 2004). What is uncertain is the location of the ER on which oleosins and TAGs are synthesized. These major OB components could be synthesized in specific ER subdomains, as interpreted from results of immunodetection of oleosins with transmission electron microscopy (TEM) (Herman, 1987), biochemical isolation and enzymatic analysis of ER subfractions (Lacey et al., 1999) and fluorescence detection of TAG-synthesizing enzymes with confocal laser scanning microscopy (CLSM) (Shockey et al., 2006). Existence of OB-synthesizing ER subdomains would raise the possibility that each subdomain produces an OB with only 1 of the several oleosin isoforms in the cell. Alternatively, oleosin and TAG synthesis could occur in non-specific, extended regions of the ER (Hsieh and Huang, 2004).

Lipid droplets in cells of non-plant organisms, such as mammals and yeast, also possess surface proteins with structural and/or metabolic functions (Martin and Parton, 2006; Goodman, 2008; Rajakumari et al., 2008). These surface proteins are not related to oleosins (Ting et al., 1997) and do not possess a long hydrophobic stretch. Their polypeptides wrap around rather than penetrate into the lipid droplets. Whereas seed OBs possess only TAGs as the matrix lipids, the lipid droplets in mammals and yeast contain both TAGs and steryl esters (SEs). The evolutionary relationship of plant OBs and non-plant lipid droplets is unknown.

To explore the evolutionary trends of OBs and oleosins in primitive plants, algae and fungi, we searched for genes encoding oleosins in these organisms having completely sequenced genomes. Only the moss *Physcomitrella* (3 paralogs) and the primitive fern (fern ally) *Selaginella* (8 paralogs) possess genes encoding oleosins. Primitive plants, including bryophytes (mosses) and ferns, contain neutral lipids and oil bodies (Swanson et al., 1976; Pihakaski et al.,
We chose the more primitive *Physcomitrella* for more intensive study. The 3 oleosin genes are expressed in a tissue-specific manner, which is further regulated via alternative splicing, a process unknown with oleosin genes in higher plants. OBs in the dehydrated spore resemble those in seeds in being food reserves for germination. OBs in the non-dehydrated, photosynthetic gametophyte, although harboring surface oleosins, possess both TAGs and SEs esters and thus resemble more the lipid droplets in mammals and yeasts. The gametophyte OBs are for gluconeogenesis when food reserves are needed, such as during sex organ production. The 1-cell-layer gametophyte can be used for transient expression of oleosin genes for convenient microscopy exploration. The approach shows that all OBs are linked to extended regions of the ER, on which different oleosins are synthesized concurrently. Here we report our findings.
RESULTS

Abundant OBs are present in the photosynthetic gametophyte and dehydrated spore

We used light microscopy and TEM to observe OBs in Physcomitrella cells throughout the life cycle (Fig. 1). Cells of the protonema, which were young tissues grown from spore after germination for 10 d, contained no or few OBs. The cells had conspicuous plastids with large starch grains. Cells of the predominate mature gametophyte, which was the conspicuous photosynthetic branches, contained numerous OBs. These OBs could be observed after Sudan Black staining and were as numerous as the larger chloroplasts (~150 per 100 μm x 100 μm). The spherical OBs had heterogeneous sizes, of <1 μm to several μm in diameter. Cells of the antheridium, the male reproductive structure, had 1-2 OBs per cell; they were strongly electron dense after osmium fixation, presumably possessing highly unsaturated lipids. Early cells of the archegonium, the female sex structure, contained no or few OBs. Some internal cells of the archegonium differentiated into spore mother cells and began to accumulate OBs, even before meiosis. After meiosis, the spore continued to accumulate OBs, and the mature, dehydrated spore were packed with OBs of various sizes, from 0.2 to 3 μm in diameter. Upon germination, the spore became less hydrated, and peroxisomes (glyoxysomes) appeared.

OBs isolated from the photosynthetic gametophyte contain oleosins, SEs and TAGs

We could not collect enough spore, which were of minute sizes (20 μm in diameter), and then crack their hard shell gently for isolation of the internal OBs. Thus, we isolated the OBs from the photosynthetic gametophyte after gentle homogenization of the cells and floatation centrifugation. TEM of the floated OB fraction revealed OBs of heterogeneous sizes, ranging from 0.5 to 5 μm (Fig. 2A). SDS-PAGE showed that the OB fraction was highly enriched with protein(s) of ~17 kD (Fig. 2B). The protein was extracted from the gel and subjected to trypsin digestion. The resulting fragments were analyzed with Matrix-assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry and identified with use of the predicted protein database derived from the Physcomitrella genome (http://moss.nibb.ac.jp/). They corresponded to the N-terminal fragments of OLE1 and OLE2 (whole proteins predicted to be 13-21 kD; to be described). Thus, the ~17-kD proteins highly enriched in the OB fraction were OLE1 and OLE2.
The neutral lipids of the OB fraction were analyzed with thin layer chromatography (TLC) and visualized after sulfuric acid spraying (Fig. 2C). The major lipids were SEs and TAGs, and diacylglycerols (DAGs) were in lesser amounts. The SEs were identified with HPLC-mass spectrometry (HPLC-MS) to be lanosterol esters (42.7% of all SEs), stigmasterol esters (30.32%), β-sitosterol esters (19.97%) and campesterol esters (8.89%). The TAGs contained the common acyl moieties of oleic (O), linoleic (L), linolenic (Ln) and palmitic (P) acids and were O/L/Ln (28.68% of all TAGs), L/L/O and O/O/L (not separated, 20.73%), P/L/L (20.73%), L/L/L (16.90%), L/L/Ln (7.76%) and O/O/O (5.21%).

**Physcomitrella is the most primitive organism possessing oleosins**

We used (A) the conserved proline knot sequence (PX$_5$SPX$_3$P) and (B) the complete hairpin sequence of oleosins from different plant species as queries to search for oleosins in the JGI Genomic database. Attention was paid to organisms whose genomes have been completely sequenced. The searched primitive species included lycophytes (*Selaginella moellendorffii*), bryophytes (*Physcomitrella patens*), algae and related organisms (*Aureococcus anophagefferens*, *Chlamydomonas reinhardtii*, *Chlorella* sp., *Emiliania huxleyi*, *Micromonas pusilla*, *Ostreococcus lucimarinus*, *Phaeodactylum tricornutum* and *Volvox carteri*), fungi and related organisms (*Aspergillus niger*, *Batrachochytrium dendrobatidis*, *Cochliobolus heterostrophus*, *Laccaria bicolor*, *Mycosphaerella fijiensis*, *Nectria haematococca*, *Phanerochaete chrysosporium*, *Phycomyces blakesleeanus*, *Saccharomyces cerevisiae*, *Pichia stipitis*, *Postia placenta* and *Sporobolomyces roseus*) and oomycetes (*Phytophthora ramorum*). Among these species, only the primitive fern (ferm ally) *Selaginella moellendorffii* (8 genes) and the moss *Physcomitrella patens* (3 genes) had oleosin genes. Clearly, no oleosin gene was present in algae, fungi and oomycetes. Thus, the moss *Physcomitrella* was the most primitive organism found to possess oleosin genes.

An unrooted phylogenetic tree of oleosins in species with completely sequenced genomes was constructed on the basis of their conserved hairpin sequences plus the moderately conserved sequences immediately flanking the hairpin (Fig. 3). The tree includes 17 oleosins from *Arabidopsis*, 6 from rice, 8 from *Populus*, 8 from *Selaginella* and 3 from *Physcomitrella*. The oleosin genes in the higher plants *Arabidopsis*, rice and *Populus* have had more variations.
Physcomitrella has the least variations and the fewest oleosin genes. A pileup of the amino acid sequences of these oleosins revealing the conserved and non-conserved residues is in Table S1.

The 3 oleosin genes are expressed in a tissue-specific manner, which can be altered via alternative splicing

RT-PCR with use of gene-specific primers was performed to examine the levels of transcripts encoding oleosins and related proteins in various tissues throughout the life cycle of Physcomitrella (Fig. 4). For each of the 3 oleosin transcripts, the RT-PCR primers detected the sequence encoding the oleosin hairpin region and thus would detect both oleosin isoforms generated via alternative splicing (see next paragraph). OLE1 and OLE2 transcripts were present in all tissues, and their levels in zygotes and spore increased during spore maturation. The OLE3 transcript was present only in the spore samples. Transcripts of genes encoding malate synthase and isocitrate lyase (1 gene each per haploid genome) were also present in all tissues but at higher levels in mature and germinated spore. Physcomitrella has 4 genes encoding putative DAG acyltransferase with use of acyl-CoA as the acyl donor (DAGAT1a, b, c and d), 2 genes encoding putative DAG acyltransferase with use of PLs as the acyl donor (DAGAT2a and b) and 1 gene encoding a putative steryl acyltransferase (SEAT). These genes were annotated on the basis of their sequence similarities with the annotated genes in yeast (Rajakumari et al., 2008). The transcripts of these acyltransferases were present at different levels in diverse tissues. Only the expression of DAGAT1d and SEAT had a clear pattern, similar to that of OLE1 and OLE2, of increasing in level from zygotes to maturing spore. Thus, the data strongly suggest that DAGAT1d and SEAT encode the acyltransferases for synthesis of the storage TAGs and SEs, respectively, in maturing spore.

OLE1 and OLE2 could each produce 2 different transcripts via alternative splicing, which would result in 2 oleosin isoforms of different sizes (Fig. 5). The alternative splicing sites occurred downstream of the sequence encoding the hydrophobic hairpin stretch, and thus the resulting 2 oleosin isoforms still possessed the hairpin stretch and the structural characteristics of an oleosin. Alternative splicing of OLE1 gave OLE1a of 15.3 kD (predicted) and OLE1b of 16.1 kD, and that of OLE2 gave OLE2a of 12.5 kD and OLE2b of 21.7 kD. Importantly, transcripts encoding OLE1a and OLE2a were restricted to spore, whereas those encoding OLE1b and OLE2b were ubiquitous. Thus, the tissue-specific presence of the machineries of alternative
splicing (Barbazuk et al., 2008) allows for substantial changes in expression and thus, presumably, differentiation of the oleosin genes and oleosin functions. Preliminary testing of OLE3 for alternative splicing generated negative results, and the predicted OLE3 had 13.8 kD.

**OBs in spore are equivalent metabolically to those in seeds**

During the life cycle of *Physcomitrella*, haploid spore were produced via meiosis. Each spore became dehydrated and packed with OBs (Fig. 1C) and could stay dormant or germinate in favorable conditions. Many of these physiological aspects are similar to those of seeds. In spore that had just germinated, peroxisomes appeared adjacent to the OBs (Fig. 1). These peroxisomes were most likely the glyoxysomes, in reference to those in germinated seeds (Pracharoenwattana and Smith, 2008). Attempts to use antibodies against cotton malate synthase to detect the enzyme in *Physcomitrella* spore peroxisomes (glyoxysomes) via immunofluorescence microscopy were unsuccessful, presumably because the antibody-antigen reaction was not strong enough. Nevertheless, the levels of transcripts encoding malate synthase and isocitrate lyase, 2 marker enzymes of the glyoxysomes, in spore increased substantially during germination (Fig. 4). Thus, the spore OBs are present as food reserves for future gluconeogenesis via the glyoxysomes and other metabolic machineries.

**OBs in the photosynthetic gametophyte serve as food reserves and are mobilized via the glyoxysomes, as exemplified in the production of sex organs**

The vegetative gametophyte was induced to produce sex organs by switching the culture temperature from 25°C to 15°C. Within a 7-d period, brown antheridia (to produce sperms) and greenish archegonia (not easily visible) were produced (Fig. 6). OBs and peroxisomes in the uppermost leafy tissue were observed during this period of induction with BODIPY dye (for OBs) and antibodies against cotton catalase (for peroxisomes). During the 7-d period, the OB number rapidly deceased, concomitant with increased number of peroxisomes (glyoxysomes) (Fig. 6).

The above changes in OBs and peroxisomes occurred only in the uppermost leafy tissues but not in the middle leafy tissues of a standup branch (Fig. 7A). During the 7-d period, the OB number in the uppermost leafy tissue decreased by 80%, concomitant with a marked increase in peroxisome number. No such changes of the 2 organelles occurred in the mid leafy tissues of a
standup branch. In both the uppermost and mid leafy tissues, the number of chloroplasts remained unchanged. Thus, mobilization of lipid reserves to initiate production of sex organs in a standup branch required only OBs in the uppermost leafy tissue. Presumably, a longer sustained sexual reproduction process would require mobilization of the lipid reserves in the lower leafy tissues of a branch. The plant was cultivated in a sugar-rich medium, and thus there was a lesser need for mobilizing all lipid reserves in a branch for sexual reproduction.

During the 7-d period, transcripts encoding malate synthase and isocitrate lyase, markers of glyoxysomes, in the uppermost leafy tissue rapidly increased in level, concomitant with a decrease in levels of transcripts encoding OLE1 and OLE2 (Fig. 7B). These changes in transcript levels did not occur in the mid leafy tissue.

All of the OBs in a gametophyte cell are linked to extended regions of the ER, on which different oleosins are synthesized concurrently

The leafy tissue of the gametophyte consists of only 1 cell layer. We tried to establish the leafy gametophyte as a transient expression system for cells that contain abundant OBs, that can be transformed easily with bombardment and that can be observed clearly with CLSM. Such a plant system has not been previously established (Miao and Jiang, 2007).

When cells were transformed with GFP or RFP driven by a 35S promoter, GFP or RFP was observed in the cytosol and was not associated with specific subcellular structures (Fig. 8A). When GFP was attached to the 3’-terminus of a complete OLE1a open reading frame, OLE1a-GFP initially appeared in a network and the associated droplets (Fig. 8B). Gradually, from 7, 10 to 12 h, proportionally less OLE1a-GFP was present in the network and more in the associated droplets. The network and the associated droplets were the ER and OBs, respectively, because after co-transformation with OLE1a-GFP and BiP-RFP (chaperone binding protein [BiP], an ER marker, from Arabidopsis [Kim et al., 2001]), OLE1a-GFP overlapped with BiP-RFP in the network and was highly enriched in the droplets (Fig. 8C). When OLE1a-GFP was used, OLE1a-GFP and the lipid dye Nile Red superimposed in all the droplets (Fig. 8D). When OLE1a-RFP and OLE2a-GFP were co-transformed, their encoded proteins appeared in all the droplets (Fig. 8E). The overall findings indicate that different oleosins are synthesized in extended regions of the ER and move to the associated OBs.
DISCUSSION

The OBs in both *Physcomitrella* and seeds apparently are similar in having a matrix of oils enclosed by a layer of oleosins and presumably also PLs. However, *Physcomitrella* OBs have the following early evolutionary trends. (A) The sizes of OBs in both the photosynthetic gametophyte and mature spore vary substantially, from 0.2 to 5 μm in diameter. Seed OBs have a narrow size range within a species (Tzen et al., 1993). *Physcomitrella* might not have evolved a mechanism to control the sizes of OBs. OBs in the photosynthetic gametophyte may resemble the lipid droplets in yeast and mammal cells in that the droplet sizes are related to the metabolic conditions of the cells. (B) Among all examined plant species, *Physcomitrella* has the fewest number of oleosin genes with minimal diversification. Alternative splicing that alters the tissue-specific expression of the oleosin genes in *Physcometrella* may represent a mechanism for gene diversification. (C) Whereas seed OBs contain mostly TAGs, *Physcomitrella* OBs possess largely SEs and TAGs and some DAGs. This lipid composition of *Physcomitrella* OBs is similar to that of the intracellular and/or extracellular lipid droplets in yeast and mammals (with SEs and TAGs) and the extracellular lipid droplets in insects (largely DAGs; Ryan, 1994). (D) All the OBs within a *Physcomitrella* gametophyte cell apparently are physically linked to the ER, a trend suspected to occur in yeast, mammals and other non-plant organisms (Martin and Parton, 2006; Goodman, 2008; Rajakumari et al., 2008). Actually, this characteristic may be normal for lipid droplets in all non-dehydrated vegetative cells, in which metabolic dynamics is expected. No such information is available for the OBs in not-yet-dehydrated, maturing seed cells, and the OBs in mature seed cells are apparently not linked to the ER, which disappears during dehydration. (E) Unlike mature seeds, the *Physcomitrella* gametophyte possesses oleosin-coated OBs in highly hydrated vegetative cells; this situation is similar to the lipid droplets in yeast and mammalian cells. Oleosins on seed OBs may protect the OBs from dehydration (Napier et al, 1996; Murphy, 2001; Hsieh and Huang, 2004). This idea could be applied to the oleosins on OBs in the vegetative non-dehydrated *Physcomitrella* gametophyte, because many moss tissues can undergo extreme and prolonged dehydration and still resuscitate upon water uptake. The evolutionary acquisition of oleosins, which are absent in algae, would represent one of the desiccation and stress tolerance features adapted by *Physcomitrella* (Rensing et al., 2008). Whether during early evolution, OBs coated with oleosins appeared first in vegetative cells
(photosynthetic gametophyte and then leaves) or in desiccated sexual organs (spore and then seed) is unknown.

Lipid droplets are present in green leaves of diverse species, although they are less obvious and abundant (Lernsten et al., 2006). Whether these lipid droplets possess oleosins and other characteristics and play a similar physiological role of food storage as do Physcomitrella gametophyte OBs remains to be elucidated. No or few (authentic or simply background) oleosin transcripts are present in high-quality MPSS or SBS leaf transcriptomes of Arabidopsis (Meyers et al., 2004) and rice (Nobuta et al., 2007). Regardless, oleosin encoded by an Arabidopsis gene transformed into tobacco leaf cells targets to lipid droplets via the ER (Wahlroos et al., 2003). In some species, the leaf lipid droplets may contain hydrophobic secondary metabolites (e.g., rubber droplets in guayule) instead of TAGs. In some other species, the leaf lipid droplets may be remnants of OBs in primitive plants and may be induced to proliferate under special situations, such as starvation and senescence. Under the latter situations, glyoxysomes and other machineries appear and convert degraded lipids into sugar for internal use or for export to nonsenescing tissues (Pracharoenwattana and Smith, 2008). Overall, leaf lipid droplets in diverse species, unlike those in seeds, are heterogeneous in structures and lipid contents and have diverse functions under different developmental, physiological and environmental conditions.

The haploid Physcomitrella spore is genetically and physiologically similar to the haploid pollen in higher plants. Both the spore and pollen also contain storage OBs coated with oleosins. However, the function of Physcomitrella spore OBs is for gluconeogenesis via the glyoxysomes. Pollen OBs are not metabolized via the glyoxysomes and likely act as reserves of acyl moieties for synthesis of new plasma membrane during pollen tube elongation.

Physcomitrella can be easily transformed and examined with CLSM for transient expression of genes that are involved in storage TAG and SE metabolism and contain abundant OBs. Such a plant system was not previously available (Miao and Jiang, 2007), and transient expression of genes in Physcomitrella has been performed only with juvenile protonema (e.g., Marella et al., 2006), which contain few or no OBs (Fig. 1A). Earlier, maturing embryos of flax and microspore cultures of Brassica were used for transient expression of modified oleosin genes, and the transformed plant materials were examined with in vitro biochemical analyses (Abell et al., 2004). The OB-containing internal cells in an embryo may not be transformed easily with bombardment or observed clearly with CLSM, and the microspore culture is highly artificial, and
the microspore (pollen) OBs are not for gluconeogenesis. Another major advantage of the *Physcomitrella* transient expression system is that the growth condition of the plant can be altered easily for study of storage lipid metabolism and packaging.

The sizes of OBs in the *Physcomitrella* gametophyte and spore are highly variable, whereas those of OBs in seeds of individual species are more confined. Presumably, *Physcomitrella* has not evolved a mechanism to control the coordinate synthesis of TAGs and oleosins, and thus the sizes of OBs, within the same cell. In seeds, OB sizes are directly related to the ratio of TAGs to oleosins, as in kernels of maize lines that were bred for high or low oils (Ting et al., 1996) and seeds of *Arabidopsis* mutants whose oleosin genes were knocked out or down (Siloto et al., 2006; Shimada et al., 2008). In maize lines bred for low oils (resulting in a low ratio of oils to oleosins), the OBs are not only smaller but also have irregularly shaped surface that could accommodate more surface oleosins per unit of matrix TAGs. In high-oil maize lines, the OBs are spherical and substantially larger. Similarly, in mutant Arabidopsis seeds with lesser amounts of oleosins, the OBs are considerably bigger. Occasional dumbbell-shaped OBs are present and looked upon as fusing detached OBs that do not have sufficient surface oleosins. An alternative explanation is that the apparent fusion occurred among budding OBs that were still attached to the ER.

In maturing seeds, whether oleosins and TAGs are synthesized in extended regions or restricted subdomains of the ER is uncertain. Earlier, OB synthesis in restricted ER subdomains has been suggested on the basis that immuno-TEM reveals more oleosins in the ER near ER-OB structures (Herman, 1987) and that a low-density, isolated subfraction (ER-OB structures) could synthesize more TAGs in vitro (Lacey et al., 1999). However, these results could also be interpreted as OBs being synthesized in extended ER regions, such that there is a concentration gradient in the ER, with more oleosins near the budding OBs; the abundant oleosins in these ER-OB structures, upon isolation, would facilitate in vitro TAG synthesis. More recently, fluorescence microscopy revealed specific DAGAT for TAG synthesis in highly defined ER subdomains in transformed BY2 cells (Shockey et al., 2006); uncertainty exists because BY2 cells do not contain OBs. On the contrary, in cells of both *Physcomitrella* gametophyte (current report) and *Brassica* tapetum (Hsieh and Huang, 2005), oleosins are synthesized in extended regions of the ER. Nevertheless, neither of these systems is directly related to maturing seeds, which is specialized to produce massive OBs. It is also possible that oleosins are synthesized in
extended ER regions and diffuse to restricted TAG-synthesizing ER subdomains from which nascent OBs detach to become solitary entities.
MATERIALS AND METHODS

Plant Materials

Spore of Physcomitrella patens subsp. patens was kindly provided by Dr. Eugene Nothnagel of the University of California, Riverside. Gametophytes were grown axenically on a solid Knop’s medium containing 125 mg.L⁻¹ KNO₃, 125 mg.L⁻¹ KH₂PO₄, 125 mg.L⁻¹ MgSO₄.7H₂O, 500 mg.L⁻¹ Ca(NO₃)₂.4H₂O and 10 g.L⁻¹ glucose supplemented with 1 ml.L⁻¹ 1000X Hunter’s “metal 49” micronutrients (76 mg.L⁻¹ 5-sulfosalicylic acid dihydrate, 7 g.L⁻¹ Fe(NH₄)₂(SO₄)₂.6H₂O, 3.04 g.L⁻¹ MnSO₄.7H₂O, 2.2g.L⁻¹ ZnSO₄.7H₂O, 0.025 mg.L⁻¹ (NH₄)₆Mo₇O₂₄.4H₂O, 616 mg.L⁻¹ CuSO₄.5H₂O, 238 mg.L⁻¹ CoSO₄.7H₂O, 57.2 mg.L⁻¹ H₃BO₃, 18 mg.L⁻¹ Na₃VO₄; Basile, 1978) and 1.2% (w/v) agar, pH 4.6. Plants were cultured at 25±1°C under a 16-h light (60~100 μE m⁻²s⁻¹)/8-h dark cycle. Sexual development was carried out with cold stimulation. Cultures of 45 d were half-submerged in water and cultured at 15°C. After the cold treatment for 60 d, mature sporophytes were harvested from the apex. From these sporophytes, spore was collected.

Tissues for RT-PCR analysis were protonema (tissue grown from spore after germination for 10 d); mature gametophytes (60-d-old culture) subdivided into top, middle, and bottom leafy tissues; antheridia; antheridia and archegonia; zygote; young, maturing and old sporophytes (S₁, S₂ and S₃ obtained after 32, 45 and 56 d of cold stimulation, respectively), mature spore and germinating spore (on a solid Knops’ medium covered with a layer of cellophane at 25°C and with continuous light for 2 d).

Staining of OBs in situ

OBs in situ were stained with Sudan Black B, Nile Red (Greenspan et al., 1985) or 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY® 493/503, D-3922 from Invitrogen Corp., Carlsbad, CA). For Sudan Black staining, fresh tissues were placed in 70 % (v/v) propylene glycol for 5 min, transferred to a saturated Sudan Black B solution (in 70 % propylene glycol) for 10 min, washed with 50 % propylene glycol twice and observed with light microscopy. For Nile-Red or BODIPY staining, fresh tissue or fixed tissue (after immunofluorescence treatment, to be described) were placed in a solution consisting of Nile Red stock (100 μg/ml acetone) or BODIPY® 493/503 stock (10 mg/ml DMSO) diluted 100x with 1x...
phosphate buffered saline (PBS, [10 mM K phosphate, pH 7.4, 138 mM NaCl and 2.7 mM KCl]) for 10 min, washed with PBS twice, and observed with a Zeiss LSM 510 META NLO confocal microscope. Nile Red and BODIPY® 493/503 were excited with the 543 and 488 nm lines, and its emission was detected with filter BP 565-615 and BP 500-530, respectively.

**Isolation of OBs from the Gametophyte**

All procedures were performed at 4°C. Fresh, 60-d-old gametophytes were soaked in a grinding medium (0.6 M sucrose, 0.1 M HEPES-NaOH, 4 mM dithiothretol, pH 7.5) for 20 min and chopped with a razor blade and then ground with a mortar and pestle. The ground sample was filtered through a layer of Nitex cloth (50 μm x 50 μm) to yield a total extract. The total extract was placed at the bottom of a centrifuge tube, and a lighter solution (0.4 M sucrose, 0.1 M HEPES-NaOH, pH 7.5) was loaded above the extract. The tube was centrifuged at 18,000 rpm for 45 min in a Beckman SW28 rotor. Floated OBs at the top of the gradient were collected with use of a spatula.

**Analysis of Lipids**

Lipids in the total gametophyte extract and isolated OB fraction were extracted with 1.2x volume of lipid extraction buffer (chloroform/heptanes/methanol, 4/3/2, v/v/v) 3x. The extract was evaporated to dryness with a stream of nitrogen gas and re-dissolved in ether or acetone for TLC or HPLC, respectively.

Lipid samples were applied to TLC plates (silica gel 60A; Whatman), which were developed in hexane:diethyl ether:acetic acid (80:20:2, v/v/v). Lipids on the plates were visualized after sulfuric acid spray. HPLC-APCI/MS was performed with Agilent 1100 series liquid chromatography coupled with ThermoFinnigan LCQ Advantage ion trap mass spectrometer (San Jose, CA) with an APCI interface. HPLC was carried out with a 5-μl sample (20 mg/ml) and a RP-18 column (Phenomenex Luna 3μ C18, 150 mm×2.0 mm) at 30 °C. Elution was performed with isocratic acetone-acetonitrile (1:1, v/v) at a flow-rate of 0.2 ml/min. The peaks were analyzed with DAD detection at 205 nm and then MS detection. Ionization was performed in the positive ion mode for all analyses.

**Electron Microscopy**
Tissues (cut into ~2 mm x 2 mm pieces) and the isolated OB fraction (in 0.4 M sucrose and 0.01M K-phosphate buffer [pH 7.4]) were fixed with 2.5% glutaraldehyde, 4% paraformaldehyde and 0.1 M K-phosphate (pH 7.0) at 4°C for 24 h. The samples were washed with 0.1 M K-phosphate buffer for 10 min 2x and then treated with 1% OsO₄, 0.1 M K-phosphate (pH 7.0) at room temperature for 4 h. The fixed samples were rinsed with 0.1 M K-phosphate buffer and dehydrated through an acetone series and embedded in Spurr medium. Ultrathin sections (70 to 90 nm) were obtained with a Leica Reichert Ultracut S or Leica EM UC6 ultramicrotome (Wetzlar, Germany). Sections were stained with uranyl acetate and lead citrate and examined with a Philips CM 100 transmission electron microscope at 80 KV.

**Immunofluorescence CLSM**

All antibody treatments were performed with 1:50 dilution of the IgG fraction (isolated and resuspended into the same original anti-catalase serum volume), 1% (w/v) milk powder and 1x PBS. Each wash was performed with PBST (1x PBS and 0.05% [w/w] Tween-20) for 10 min. Tissues were fixed in 4% paraformaldehyde, 1x PBS and 0.15 M sucrose at 4°C for 16 h. After 2 washes, the tissues were treated with 1% cellulase R10 (Yakult in Honsa, Japan) in 1 x PBS for 20 min at 25°C. After 2 washes, the tissues were incubated with 1x PBS with 0.1 % Tween-20 for 20 min at 25°C. After 2 washes, the tissues were treated with a blocking solution (3% milk, 1x PBS) at 25°C for 1 h and then rabbit antibodies against cotton seed catalase at 4°C for 16 h. After 3 washes, the tissues were treated with cyanine 3-conjugated donkey antibodies against rabbit IgG (Jackson Immuno Research Laboratories, West Grove, PA) for 1 h at 25°C. After 3 washes, the tissues were stained with BODIPY® 493/503 for OB staining. The tissues were placed on a slide and observed with a LSM 510 META confocal microscope (Carl Ziess in Jena, Germany). BODIPY® 493/503, cyanines 3 and chloroplast were excited with the Argon 488-, HeNe 543- and Argon 488-nm lines, respectively, and the emissions were detected with emission filters of BP 500–530, BP 565–615 and BP 650–710 nm, respectively.

**SDS-PAGE and Identification of Oleosins with MALDI-TOF**

Proteins in the total cellular extract and the isolated OB fraction were separated with 12 % (w/v) SDS-PAGE (Wu et al., 1997). The gel was stained with Coomassie Blue. The gel containing the visible ~17-kD proteins of the OB fraction was cut. The proteins were extracted
and subjected to trypsin digestion and mass spectrometry (MALDI-TOF) analysis with Voyager DE-STR (PerSeptive Biosystems, Framingham, MA).

**Searches for Oleosin Genes of Physcomitrella and Other Organisms**

Sequences of the conserved hairpin domain of oleosins and several complete oleosins of *Arabidopsis* (Kim et al., 2002) were used as query sequences for the BLAST program (tblastn) against genome and transcriptome databases of *Physcomitrella patens* (http://www.cosmoss.org/). Three oleosin genes, *PpOLE1*, *PpOLE2* and *PpOLE3*, were found on scaffold 84, scaffold 21 and scaffold 180, respectively. Similar searches yielded oleosin genes of *Oryza sativa* (from http://rice.plantbiology.msu.edu/), *Arabidopsis thaliana*, *Selaginella mutica* and *Populus trichocarpa* (from JGI Eukaryotic Genomics database, http://www.jgi.doe.gov/). An oleosin of *Pinus ponderosa*, *Pinus-OLE*, was obtained from an earlier study (Lee et al., 1994). A phylogenetic tree of the above oleosins was constructed on the basis of protein sequence similarities (of the conserved hairpin sequence plus the moderately conserved sequences immediate flanking the hairpin) and constrictions with the Clustal method and a distance method (Neighbor-Joining) and PHYLIP with 1,000 bootstrap replicates.

**Reverse Transcription-PCR analyses**

RNA was extracted from tissues with use of an RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA (2 μg) was first treated with DNaseI (Invitrogen) for 35 min at 37°C. The proteins were removed with PCI (Phenol/chloroform/isoamyl alcohol [25:24:1], pH 4.5), and the phenol with chloroform-isoamyl alcohol (24/1, v/v). The RNA was precipitated with 0.1x volume of 3 M NaOAc (pH 5.2) and 2.5x volume of absolute ethanol at -20°C for 16 h. The RNA (1μg) was used to make cDNA with the SuperScriptIII RT-PCR system (Invitrogen). The RNA was pre-incubated with oligo(dT)_{12,18} and dNTPs at 65°C for 5 min and then placed on ice. cDNA Synthesis Mix was added to the RNA, and the mixture was incubated at 50°C for 1 h. The reaction was terminated by heating at 75°C for 5 min. PCR was carried out with use of 0.2 μl of the cDNA as template and DyNazyme DNA polymerase with dNTP and primers. From the sequence information of the genes and their transcripts, primers were selected and synthesized. Primer pairs for amplifying full-length cDNA of *PpOLE1a*, *PpOLE2a* and *PpOLE3* are shown in Supplemental Table S1. Amplified DNA fragments were subcloned into pGEM-T Easy
(Promega, Madison, WI) and subjected to DNA sequencing with use of M13 forward and reverse primers. Primer pairs for amplification of specific gene fragments in the study of gene expression patterns are shown in Supplemental Table S1. Amplified DNA fragments of ~200bp were analyzed on a 1.8% agarose gel.

**Transient Expression assays**

DNA sequences encoding the complete coding region of *PpOLE1a* and *PpOLE2a* were amplified by PCR with use of primers shown in Supplemental Table S1. The resulting coding fragments were digested with BamH I and cloned into the expression site of a GFP expression vector (Chiu et al. 1996) or an RFP expression vector (Lee et al., 2001) to be driven by a CaMV 35S promoter. A BIP-RFP expression vector of a similar construct (Kim et al., 2001) was obtained from Dr. David Ho, Institute of Plant and Microbial Biology, Taipei. Transformation of the gametophyte was carried out with particle bombardment. Sixty-day-old gametophyte tissues were placed on solid Knop’s medium. Plasmid DNA (5 μg) was coated onto the surface of 1.25 mg 1.6-nm gold particles, which would be used for 6 different shootings. The gold particles were bombarded with 900 psi under 28-in Hg vacuum onto the gametophyte from a distance of 6 cm in PDS-1000 (BIO-RAD, Hercules, CA). After bombardment, the tissues were left on the culture medium and observed with CLSM at time intervals. GFP and RFP were excited with the Argon 488- and HeNe 543-nm lines, and their emissions were detected by emission filters of BP 500–530 and BP 565–615, respectively.

**SUPPLEMENTAL MATERIALS**

The following materials are available in the online version of this article:

**Supplemental Figure S1.** A pile-up of 45 oleosins from *Physcomtrella, Arabidopsis, Populus, Oryza, Selaginella* and pine.

**Supplemental Table S1.** Primers for PCR and RT-PCR.

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**FIGURE LEGENDS**

**Figure 1.** Microscopy images of different tissues of *Physcomitrella* during its life cycle, showing the presence or absence of subcellular OBs. Samples were photographed with light microscopy (all in color) or transmission electron microscopy (black and white).

A. Haploid cells (from top to bottom) of protonema (immature tissue grown from spore after germination for 10 d), mature gametophytes (dark OBs [stained with Sudan Black B] among green chloroplasts), antheridium (dark OBs) and archegonia (no OBs).

B. Sporophytes during maturation (from top to bottom). Left column shows the whole sporophyte, and right column reveals diploid cells enclosing the locule in which spore-mother cells (smc) became spore.

C. Mature spore packed with OBs but no peroxisomes (left 2 images) and germinating spore with numerous peroxisomes (p) (right 2 images).

**Figure 2.** Analyses of an OB fraction isolated from mature, green gametophyte branches.

A. TEM of the OB fraction, showing spherical OBs of heterogeneous sizes.

B. SDS-PAGE gel of proteins in total extract and OB fraction. Molecular marker positions are indicated.

C. TLC plate of neutral lipids in total extract and OB fraction. Approximate locations of lipid groups, steryl esters (cholesteryl palmitate as marker), TAGs (triolein), FFA (free fatty acids, oleic acid), sterols (cholesterol), DAGs (1,3- and 1,2-diolein) and MAGs (1-monoolein) are indicated.

**Figure 3.** An un-rooted phylogenetic tree of oleosins from *Physcomitrella* and several other representative species constructed on the basis of their predicted amino acid sequences. *Arabidopsis thaliana* (At, a non-woody dicot), *Populus trichocarpa* (Pt, a woody dicot), *Oryza sativa* (Os, a monocot), *Selaginella mutica* (Sm, a primitive fern) and *Physcomitrella patens* (Pp) genomes have been completely sequenced, and all their oleosins are included. One pine (*Pinus ponderosa*) oleosin is used to represent gymnosperm proteins. Nomenclature of the *Arabidopsis* oleosins follows that reported (Kim et al., 2002); S, T and SM denote oleosins present specifically in seed, tapetum, and seed-and-microspore, respectively. The phylogenetic tree was
constructed from aligned sequences of oleosins (the conserved hairpin sequence plus its immediately flanking semi-conserved sequences) by a distance method (Neighbor-Joining) with use of PHYLIP and 1,000 bootstrap replicates. Bootstrap values higher than 50 are indicated.

**Figure 4.** RT-PCR of transcripts encoding oleosins and related proteins in various tissues. Tissues shown from left to right are protonema (P); top, middle and bottom leafy tissues of gametophyte branches (T, M, and B, respectively); antheridia (A); antheridia and archegonia (AA); zygotes (Z), sporophytes of early, middle and late developmental stages (S1, S2 and S3, respectively); and mature spore (MS) and germinated spore (GS). Transcripts are those encoding oleosins (OLE1, 2 and 3), malate synthase (MS), isocitrate lyase (ICL), DAG acyltransferases (DAGAT1 [4 paralogs] and DAGAT2 [2 paralogs] utilizing acyl CoA and PLs as the acyl donors, respectively) and steryl acyltransferase (SEAT). Approximately equal amounts of the transcript encoding actin (ACT2) were present in the samples.

**Figure 5.** Gene structures of OLE1 and OLE2 and alternative splicing of each gene resulting in 2 tissue-specific transcripts.

A. Arrangement of OLE1 and OLE2 in 2 scaffolds. Occurrence of 2 open reading frames (shaded boxes) in each of the 2 genes via alternative splicing is indicated. The dotted lines represent the sequences encoding the hairpin region. Primers for RT-PCR are shown.

B. A pile-up of OLE1a, OLE1b, OLE2a, OLE2b and OLE3. The hairpin sequences in the second row are dotted, and the 3P and 1S in the central hairpin turn, PX$_3$SPX$_3$P, are highlighted with asterisks.

C. RT-PCR of transcripts of OLE1a and OLE1b, OLE2a and OLE2b, as well as ACT2 (a loading control) in various tissues (see Fig. 4 legend for labels). Primers were 1HF and 1aR for OLE1a and 1HF and 1bR for OLE1b (see A); and 2HF and 2aR for OLE2a and 2HF and 2bR for OLE2b.

**Figure 6.** Images of the gametophyte after induction of sporophyte development. The gametophyte was examined after induction of sporophyte development on cold treatment for 0, 5 and 7 d.

A. The uppermost row shows images of the tip of gametophyte, which was producing antheridia (brown color) and archegonia (not visible). The subsequent rows are fluorescence CLSM images
of several cells in an uppermost leafy tissue of a branch. The cells were examined for OBs with use of the lipid probe BODIPY 493/503 (green), peroxisomes with rabbit anti-catalase antibodies and then anti-rabbit IgG antibodies conjugated to Cy3 (red) and chloroplasts with autofluorescence (blue). Each column shows identical cells after the indicated days of cold treatment. Photos were taken to reveal OBs or peroxisomes alone, or in combination (merge-1 for OBs and peroxisomes, and merge-2 for chloroplasts also and with dotted lines to outline the cell circumference).

B. TEM pictures of portions of cells in an uppermost leafy tissue of a gametophyte branch showing the presence of OBs (OB) at d 0 and a peroxisome (p) at d 7.

Figure 7. Changes in the number of organelles and levels of gene transcripts in the uppermost and mid leafy tissues of gametophyte branches upon induction of sporophyte development for 0, 3, 5 and 7 d.

A. Number of OBs, peroxisomes (PEX) and chloroplasts (CLP) per cell area.

B. RT-PCR results of transcripts encoding malate synthase (MS), isocitrate lyase (ICL), OLE1 (OLE1), OLE2 (OLE2) and actin (ACT2, as a loading control).

Figure 8. Transient expression of various GFP and RFP constructs in individual gametophyte cells. Chloroplast autofluorescence is shown in red or blue in merged pictures. The speed of transient expression varied among experiments, and CLSM images were obtained at 6-8 (early), 8-10 (mid) and 10-12 (late time point) h after bombardment.

A. Expression of control GFP or RFP not attached to OLE1a at a late time point.

B. Expression of OLE1a-GFP at time intervals. GFP (green) was present largely in a cellular network at an early time point but was associated more with subcellular droplets at a late time point.

C. Co-expression of OLE1a-GFP and BiP-RFP. GFP (green) and RFP (red) at a mid time point are shown.

D. Expression of OLE1a-GFP. GFP (green) and OBs (red, stained with Nile Red) at a late time point are shown.

E. Co-expression of OLE1a-GFP and OLE2a-RFP. GFP (green) and RFP (red) at a late time point are shown.
