Oleosin of Subcellular Lipid Droplets Evolved in Green Algae

Nan-Lan Huang, Ming-Der Huang, Tung-Ling L. Chen, and Anthony H.C. Huang*

Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan 11529 (N.-L.H., M.-D.H., T.-L.L.C., A.H.C.H.); and Center for Plant Cell Biology, Department of Botany and Plant Sciences, University of California, Riverside, California 92521 (A.H.C.H.)

In primitive and higher plants, intracellular storage lipid droplets (LDs) of triacylglycerols are stabilized with a surface layer of phospholipids and oleosin. In chlorophytes (green algae), a protein termed major lipid-droplet protein (MLDP) rather than oleosin on LDs was recently reported. We explored whether MLDP was present directly on algal LDs and whether algae had oleosin genes and oleosins. Immunofluorescence microscopy revealed that MLDP in the chlorophyte *Chlamydomonas reinhardtii* was associated with endoplasmic reticulum subdomains adjacent to but not directly on LDs. In *C. reinhardtii*, low levels of a transcript encoding an oleosin-like protein (oleolike) in zygotes-tetrads and a transcript encoding oleosin in vegetative cells were associated with endoplasmic reticulum subdomains adjacent to but not directly on LDs. In *C. reinhardtii*, low levels of a transcript encoding an oleosin-like protein (oleolike) in zygotes-tetrads and a transcript encoding oleosin in vegetative cells were associated with endoplasmic reticulum subdomains adjacent to but not directly on LDs.

Eukaryotes and prokaryotes contain neutral lipids in subcellular lipid droplets (LDs) in diverse cell types for food reserves and other purposes (Hsieh and Huang, 2004; Bickel et al., 2009; Murphy, 2012). These LDs are present in seeds, flowers, pollen, and fruits of higher plants; the vegetative and reproductive organs of primitive plants, algae, fungi, and nematodes; mammalian organs/tissues such as mammalian glands and adipose tissues; and bacteria. Of all the LDs, those in seeds, usually called oil bodies, are the most prominent and were studied extensively early on (Huang, 1992; Frandsen et al., 2001).

Seeds of most plant species store oils (triacylglycerols [TAGs]) as a food reserve for germination and postgerminative growth. The TAGs are present in subcellular spherical LDs of approximately 0.5 to 2 μm in diameter (Frandsen et al., 2001; Hsieh and Huang, 2004; Beller et al., 2010; Murphy, 2012). Each LD has a matrix of TAGs surrounded by a layer of phospholipids (PLs) and structural proteins termed oleosins. Oleosins cover the LD surface completely and prevent LDs from coalescence. The small size of LDs provides a large surface area per unit of TAG, which would facilitate lipase binding and lipolysis during germination. The stability of seed LDs in vivo and in vitro is in contrast to the instability of artificial liposomes produced from amphipathic and neutral lipids; liposomes generated by sonication gradually coalesce. LDs in yeast and various mammalian cells, as well as extracellular lipoproteins in mammals and insects, are also unstable and undergo dynamic metabolic fluxes of their surface and matrix constituents (Goodman, 2008; Beller et al., 2010; Murphy, 2012).

Oleosins on LDs are present in diverse plant species, from higher to primitive plants, including the moss *Physcomitrella patens* (Huang et al., 2009). Arabidopsis (*Arabidopsis thaliana*) has 17 genes encoding oleosins: five active in seed, three active in both seed and pollen, and nine (eight in tandem) active in the floral tapetum cells (Kim et al., 2002). Minor proteins termed caleosin and sterolesin are present in isolated seed LD fractions of some species (Frandsen et al., 2001). They do not have a long hydrophobic segment, even though they have a short segment similar to but much less conserved than the Pro-knot motif in oleosins (see

---

1 This work was supported by the Institute of Plant and Microbial Biology, Academia Sinica, the National Science Council, Taiwan (grant no. NSC101-2311-B-001-034), and a U.S. Department of Agriculture-National Research Initiative grant.

2 These authors contributed equally to the article.

* Corresponding author; e-mail ahuang@gate.sinica.edu.tw.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Anthony H.C. Huang (ahuang@gate.sinica.edu.tw or Anthony.Huang@ucr.edu).

[OA] Open Access articles can be viewed online without a subscription.

1862 Plant Physiology®, April 2013, Vol. 161, pp. 1862-1874, www.plantphysiol.org © 2013 American Society of Plant Biologists. All Rights Reserved.
Most oleosins are small proteins of 15 to 26 kD (Hsieh and Huang, 2004; Liu et al., 2012). An oleosin molecule can be divided into three portions according to its amino acid sequence. The N-terminal portion can be short or long (e.g. six to 68 residues in Arabidopsis) and hydrophilic or amphipathic. The central portion is a long uninterrupted hydrophobic stretch of approximately 72 nonpolar residues. The C-terminal portion is a short or long polypeptide (e.g. 28–1,000 residues in Arabidopsis tapeta), and its approximately 33 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 LD surface. The long polypeptide of the C-terminal portion beyond these 33 residues may have little functional significance and may have arisen from fortuitous short polypeptide duplications. The N- and C-terminal portions of an oleosin on the surface of a seed LD may act during germination as a receptor for the binding of lipase for TAG degradation, a site for ubiquitination and then degradation of the whole LDs, and/or a location for interacting with glyoxysomes during germination (Hsieh and Huang, 2004).

The central hydrophobic stretch of approximately 72 uninterrupted nonpolar residues is the hallmark of an oleosin. No other protein in any organism has such a long hydrophobic stretch. Proteins on the surface of extracellular and intracellular LDs, such as apolipoproteins, perilipin, adipophilin, and caveolin in mammals; phasin in bacteria; and lipid-associated protein in plastids, do not have a long hydrophobic stretch (Hsieh and Huang, 2004; Bickel et al., 2009; Murphy, 2012); their polypeptides run parallel to the surface of rather than penetrate into the LD. The LD surface protein of Nannochloropsis oceanica (a photosynthetic heterokont) has a hydrophobic stretch of 60 nonpolar residues; this protein has not been found in other organisms (Vieler et al., 2012). The 72-residue hydrophobic stretch of an oleosin is sufficiently long (a transmembrane [PL bilayer] peptide has approximately 20–25 residues) to form a hairpin penetrating the surface PL monolayer of an LD into the matrix. Importantly, the center of the hydrophobic stretch has three Pro residues and one Ser residue that could interact among themselves to form a “Pro knot,” thus creating a nonpolar hairpin structure, with a turn of 12 residues and two arms each of 30 residues. The 72 residues of the nonpolar stretch, in terms of hydrophobicity, are conserved among oleosins of diverse species, and the conservation is highest at the Pro knot (PX3SPX3P, with X being a highly nonpolar residue).

Oleosins are present in higher and primitive plants, including lycophytes (deduced from the genome sequence of Selaginella moellendorfii) and mosses (P. patens; Huang et al., 2009). In green algae (chlorophytes and the more advanced charophytes), whose ancient members evolved to become plants, the presence of oleosin has not been reported previously. The Chlamydomonas reinhardtii genome has a sequence that could encode an oleosin-like protein (referred to as “oleolike” in this report), which has the characteristic 12-residue Pro-knot sequence of an oleosin but only short nonpolar arms (eight and four residues). In C. reinhardtii, oleolike or its transcript has not been reported previously. Rather, a protein termed major lipid-droplet protein (MLDP) was detected in LD fractions of C. reinhardtii (Moellinger and Benning, 2010) and two other chlorophytes, Haematococcus pluvialis (Peled et al., 2011) and Dunaliella salina (Davidi et al., 2012), but was absent in a LD fraction of C. reinhardtii by a different laboratory (Wang et al., 2009). MLDP does not have a long hydrophobic polypeptide for stable association with the matrix of LDs. The subcellular location of MLDP on LDs and/or other sites of C. reinhardtii and other green algae need to be explored.

We have explored proteins that could be associated with LDs in green algae. First, in view of the contrasting reports on MLDP in C. reinhardtii LD fractions, we examined and found that MLDP was associated with endoplasmic reticulum (ER) subdomains adjacent to but not directly on LDs. Then, we probed the presence of oleolike and/or oleosin transcripts and proteins in chlorophytes and charophytes and found them at minimal levels in vegetative cells and elevated levels in sexual reproductive cells. Although the minimal oleolike/oleosins were difficult to detect in green algae, they behaved like bona fide LD surface proteins in P. patens transformed with the algal genes tagged with a GFP gene. Overall, oleosin genes having weak and cell/development-specific expression were present in green algae. We present a hypothesis for the evolution of oleosins from algae to plants.

RESULTS

Diverse Species of Chlorophytes and Charophytes Possessing LDs in Vegetative Cells Were Selected for Studies

Green algae include the primitive chlorophytes and the advanced charophytes. Their ancient members evolved to become higher (land) plants (Karol et al., 2001). We followed a phylogenetic tree of these algae described earlier (Fig. 1) and selected available species that are representatives in the phylogenetic tree and that contained easily observable LDs in vegetative cells under the growth conditions in our laboratory. Microscopy images of these algae species after staining with Nile Red or BODIPY 505/515 are shown in Figure 2. In general, each cell contained several LDs of approximately 1 µm in diameter. Transmission electron microscopy (TEM) of C. reinhardtii and Spirogyra grevillea cells revealed that the LDs were similar to those in seeds in having a homogeneous matrix surrounded by an electron-dense layer that appeared to...
In *C. reinhardtii*, MLDP Was Associated with ER Subdomains Adjacent to But Not Directly on LDs

We used immunofluorescence confocal laser scanning microscopy (immuno-CLSM) to localize MLDP in nitrogen-starved *C. reinhardtii* cells. We prepared rabbit polyclonal antibodies against a peptide of *C. reinhardtii* MLDP unique to MLDP among all *C. reinhardtii* proteins deduced from the available genome sequence and used them for immunoblotting and immuno-CLSM. In an immunoblot of a SDS-PAGE gel of the total *C. reinhardtii* cell extract, the antibodies recognized a protein of the expected MLDP mass of 28 kD (Fig. 3A).

Immono-CLSM with the antibodies identified MLDP in numerous cup-shaped structures in the cell. These MLDP-containing structures colocated with a portion of ER structures recognized by antibodies against the ER chaperone calreticulin (Fig. 3B). The cup-shaped MLDP structures often partially wrapped around LDs, which were identified by the neutral lipid dye BODIPY 505/515 (Fig. 3B). Although the MLDP structures were often close to LDs, they sometimes were at a distance from the LDs (Fig. 3B, arrows). The cup-shaped MLDP structures did not completely enclose the LDs. An LD could be seen apart from the MLDP structures (Fig. 3B, arrowheads) in a specific optical section but actually had distant contact with a short stretch of the MLDP structures at different focal planes, as revealed in a three-dimensional reconstruction model (Fig. 3B). We interpret these findings to indicate that MLDP is concentrated in subdomains of ER and only intermittently interacts with the LD surface. In time-course studies of *C. reinhardtii* of all examined developmental stages (data not shown), we did not observe MLDP fully wrap the LD surface, contrary to the tight enwrapping configuration of oleosin on LDs reported by us and other laboratories (Huang et al., 2009; see also Fig. 7A in this study).

Oleolike and/or Oleosin Transcripts of Low Levels Were Detected in *C. reinhardtii*, *Volvox globator*, and Several Charophytes

We used Arabidopsis and *P. patens* oleosin and Pro-knot (PX$_5$SPX$_3$P) sequences in BLAST searches for oleosin genes and proteins in genomic and transcriptome databases from the Joint Genome Institute (JGI) or the National Center for Biotechnology Information (NCBI). We applied the criterion that 60% or more of the X in the X$_3$ and X$_5$ should consist of the four highly nonpolar residues F, L, I, and V. We found that candidate proteins with less than 60% of these nonpolar residues all have interrupting charged residues within or adjacent to the Pro knot. We did not find transcripts that could encode oleosin in green algae (chlorophytes and charophytes), stramenopila (brown algae, golden algae, diatoms, and oomycetes), alveolata (dinoflagellates, etc.), or other primitive and advanced organisms of other kingdoms.

The genomes of most green algae have not been sequenced. We expanded the above BLAST search to transcriptomes of *C. reinhardtii* of different developmental stages, *V. globator*, and several charophytes constructed by our laboratory (Table I).

The transcriptomes of *C. reinhardtii* and *V. globator* contained transcripts that could encode oleolike (Table I), which has the characteristic 12-residue Pro-knot sequence of an oleosin but short nonpolar arms having only eight and four nonpolar residues (Fig. 4). The predicted sequences of *C. reinhardtii* and *V. globator* oleolikes are highly similar (Fig. 4), reflecting the close phylogenetic relationship of the two chlorophytes classified on the basis of DNA sequences of several plastid, mitochondrial, and nuclear genes (Fig. 1). The *C. reinhardtii* oleolike polypeptide has 77 residues (8.6 kD) and is rich in Leu (18) and Pro (nine). It has no acidic residues of Glu and Asp and a predicted pI of 11.1. The Pro-knot sequence at the center is flanked, after interruptions by basic residues, on either side with a 19-residue semisymmetric motif. The N-terminal motif has NX$_{17}$K and the C-terminal motif has RX$_{17}$K, and both include a sequence of PX$_x$PX$_x$P (with X representing nonpolar residues). The two PX$_x$PX$_x$P could represent partial repeats of the Pro knot (PX$_x$SPX$_x$P).

The *C. reinhardtii* oleolike is similar to a hypothetical protein encoded by a *C. reinhardtii* gene in the sequenced genome described earlier, although the protein was not found in the cell extract or an LD.
preparation (James et al., 2011). Transcripts encoding similar oleolikes were absent in the transcriptomes of all the examined charophytes (Table I).

Low levels of oleosin transcripts were found in *C. reinhardtii* and several charophytes (Table I). Each of these transcripts encodes oleosin with characteristics of plant oleosins in possessing a 12-residue Pro knot and two approximately 30-residue nonpolar hairpin arms (Fig. 4).

Oleolike and Oleosin Transcripts Were Present in Specific Cells in the Life Cycle of *C. reinhardtii*

In the life cycle of *C. reinhardtii* from vegetative cells, gametes, zygotes, and tetrads, the number of LDs in the cells increased (Fig. 5A). We explored the occurrence of oleosin and oleolike transcripts in these cells.

During the life cycle, oleolike transcript was absent in *C. reinhardtii* vegetative cells and gametes and present in zygotes and tetrads (Table I). This pattern of occurrence in the transcriptomes was confirmed with reverse transcription (RT)-PCR analysis (Fig. 5B). It differs from that for MLDP, which was ubiquitously present in all cells (Table I; Fig. 5B).

Although oleosin transcript was absent in the transcriptomes of *C. reinhardtii* vegetative cells, gametes, zygotes, and tetrads, the four main cell types in our studies (Fig. 5A), it was present at a low level in vegetative cells transferred to an acetate-rich medium (Table I). We used the very sensitive nested RT-PCR procedure, with primers derived from the *C. reinhardtii* oleosin transcript, and observed low levels of the transcript in cells of different developmental stages (Fig. 5B). Sequencing of the RT-PCR fragment validated it to be that of the oleosin (Fig. 4). We searched publicly available *C. reinhardtii* transcriptomes and found two oleosin transcripts from the raw RNA sequences of a transcriptome of pH stress-induced deflagellated vegetative cells (Lechtreck et al., 2009). These two transcripts would encode two oleosins with sequences comparable to the *C. reinhardtii* oleosin encoded by the transcript found by our laboratory (Fig. 4) but with minor residue differences in the variable, nonconserved portion of the polypeptide. The differences presumably reflect the strain varieties of *C. reinhardtii* used in the two studies.

LD Fractions Isolated from *C. reinhardtii* Zygotes Contained Minimal Proteins with No Detectable Oleolike or Oleosin

We obtained rabbit antibodies raised against a synthetic peptide containing a segment of the *C. reinhardtii* oleolike. However, SDS-PAGE immunoblotting and immuno-CLSM revealed that the antibodies were not sufficiently specific for the sole detection of oleolike (data not shown).

We prepared an LD fraction from *C. reinhardtii* zygotes (Fig. 5C) and compared its protein band pattern
on an SDS-PAGE gel with that of the total cell extract. No protein bands were observed in the LD fraction when we loaded an amount of the LD fraction equivalent to that obtained from an equal amount of total cell extract; in such a loading, the proteins in the total cell extract in a gel lane could be resolved and observed clearly. In the study of seed, we used a similar 1:1 loading equivalent of the LD fraction and total cell extract and observed the oleosins in the LD fraction clearly (Lee et al., 1991). In the study of *C. reinhardtii*, we had to load the LD fraction equivalent to 10,000× the total cell extract to observe several faintly visible protein bands (Fig. 5D). None of these protein bands matched closely the predicted sizes of oleolike (8 kDa) and oleosin (more than 12 kDa). We subjected the LD fraction to liquid chromatography (LC)-tandem mass spectrometry (MS/MS) analysis and found no oleolike or oleosin, even though we used a more extensive and sensitive LC gradient (see “Materials and Methods”). Proteins identified with high confidence included aconitate hydratase, eukaryotic translation elongation factor1α2, mitochondrial substrate carrier protein, PSII reaction centers CP47 apoprotein, and rhodanese-like calcium-sensing receptor. We considered these proteins to be contaminants of the LD fraction. We believe that the amounts of oleolike and oleosin were too minimal for detection; the minimal amounts reflect the low reads per kilobase of exon model per million (RPKM) of their transcripts (Table I).

The neutral lipids of the *C. reinhardtii* LDs were analyzed with thin-layer chromatography (TLC). Most of the neutral lipids in the LD fraction were TAGs (Fig. 5D). In TLC, we loaded an amount of LD fraction equivalent to that obtained from the same amount of total cell extract and were able to observe the TAGs clearly.

In *S. grevilleana*, the Level of Oleosin Transcript Increased Greatly in Cells Induced to Produce Conjugation Tubes

Low levels of oleosin transcripts were found in the transcriptomes of several charophytes (Table I). The deduced sequences of these charophyte oleosins are similar to those of *C. reinhardtii*, *P. patens*, and Arabidopsis (Fig. 4). The levels of oleosin transcripts in charophytes in terms of RPKM were low but comparable to those in vegetative cells of *P. patens* and Arabidopsis (Table I). In *P. patens*, sporophytes generated from vegetative (gametophytic) cells contain many more LDs and oleosin transcripts (Huang et al., 2009; Table I), as do Arabidopsis seed cells. This observation indicates a possibility that the levels of charophyte oleosin transcripts were low because we used vegetative cells instead of reproductive cells to generate transcriptomes. We explored this possibility in *S. grevilleana*.

*S. grevilleana* vegetative cells contained numerous LDs (Fig. 6A) located near the spiral ribbon-shaped chloroplasts. They also contained transcripts encoding three oleosins (Table I). When the cells were induced to...
produce conjugation tubes for zygote formation, they accumulated more LDs, as revealed by confocal laser scanning microscopy (CLSM; Fig. 6A) and TEM (Supplemental Fig. S1). During this sexual reproduction process, the levels of the three oleosin transcripts, as detected by RT-PCR, increased greatly (Fig. 6B). The observed increase would in reality be higher, because the induction process converted only about 15% to 35% (depending on the experiment) of the cells to produce conjugation tubes.

**LD Fractions Isolated from *S. grevilleana* Cells with Conjugation Tubes Contained Minimal Proteins, Two of Which Were Identified as Oleosins**

An LD fraction (Fig. 6C) from the total extract of *S. grevilleana* culture containing conjugating cells was analyzed by SDS-PAGE for proteins and by TLC for neutral lipids (Fig. 6D). No proteins of the LD fraction on the gel were observed when the gel loading was a 1:1 equivalent of the LD fraction and total cell extract. When we loaded the gel with the LD fraction 20,000× the total extract to the gel, we observed not-well-defined protein bands (Fig. 6D). We subjected the LD fraction to LC-MS/MS analysis and found two peptides of oleosin3 and one peptide of oleosin2 (Supplemental Table S2). These identified peptides were subjected to a BLAST search in the *S. grevilleana* transcriptome, and the result indicates that they are unique to oleosin3 and oleosin2, respectively. Even though one unique peptide of oleosin2 was identified, it has well-matched ion spectra (data not shown); its identification as that of oleosin2 passes a stringent threshold of a significant expectation value of 0.001, resulting in 99.9% confidence. The barely detectable oleosin3 and oleosin2 in the LD fraction were those, among the three *S. grevilleana* oleosins, with the most abundant transcripts in the transcriptome (Table I) and with levels substantially elevated during conjugation tube formation (Fig. 6B). This finding reiterates the ideas that oleosins were present in minute amounts and that we were reaching the resolution limit of the proteomic analyses.

| Species                      | MLDP (RPKM) | Oleolike (RPKM) | Oleosin1 (RPKM) | Oleosin2 (RPKM) | Oleosin3 (RPKM) | Reference                  |
|------------------------------|-------------|-----------------|-----------------|-----------------|-----------------|---------------------------|
| *Chlamydomonas reinhardtii*  |             |                 |                 |                 |                 | This study                |
| Vegetative                   | 88          | 0               | 0               |                 |                 | This study                |
| Gamete                       | 136         | 0               | 0               |                 |                 | This study                |
| Zygote                       | 141         | 70              | 0               |                 |                 | This study                |
| Tetrad                       | 153         | 81              | 0               |                 |                 | This study                |
| Vegetative (in acetate-rich medium) | 28         | 0               | 7               |                 |                 | This study                |
| *Volvox globator*            | 223         | 0               | 0               |                 |                 | This study                |
| *Cosmarium turpinii*         | ?           | None            | 5               | 4               |                 | This study                |
| *Closterium acerosum*        | ?           | None            | 6               | 3               |                 | This study                |
| *Spirogyra grevilleana*      | ?           | None            | 3               | 4               | 27              | This study                |
| *Coleochaete scutata*        | ?           | None            | None            |                 |                 | This study                |
| *Nilia spp.*                 | ?           | None            | None            |                 |                 | This study                |
| *Physcomitrella patens*      |             |                 |                 |                 |                 | This study, Chen et al. (2012) |
| Protonema                    | None        | None            | 1               | 3               | 3               |                                           |
| Gametophyte                  | None        | None            | 3               | 0               | 6               |                                           |
| Sporophyte                   | None        | None            | 1,374           | 814             | 281             |                                           |
| *Arabidopsis*                |             |                 |                 |                 |                 | SRR068965, SRR388666, SRR515193, SRR331227 |
| Seed                         | None        | None            | 1,478           | 1,409           | 644             |                                           |
| Seedling                     | None        | None            | 2               | 1               | 1               |                                           |
| Leaf                         | None        | None            | 0               | 0               | 0               |                                           |
| Root                         | None        | None            | 0               | 1               | 0               |                                           |

*Transcripts are in vegetative cells, unless otherwise specified. *b* indicates uncertainty. No reliable conserved MLDP sequences other than those in chlorophytes exist for the identification of MLDP transcripts in charophytes and plants. *c* None indicates no transcript detected but also no information about whether the gene exists in the organism. *d* The three oleosin genes in *P. patens* are oleosin1 (Pp1s84_138V6), oleosin2 (Pp1s21_351V6), and oleosin3 (Pp1s180_169V6). *e* Arabidopsis has five seed-specific oleosins, and the three genes with the highest expression are listed: oleosin1 (At4g25140), oleosin2 (At5g51210), and oleosin3 (At3g27660).
sensitive proteomic techniques. Therefore, we explored the subcellular locations of oleolike and oleosin in a heterologous system. We transformed *P. patens* with the respective algal genes tagged with a GFP gene. We used *P. patens* instead of *C. reinhardtii* because of the ease in transformation (although modified strains of *C. reinhardtii* could be transformed efficiently), the stability of GFP in *P. patens*, and our established transient expression of a GFP-Candidate gene in *P. patens* (Huang et al., 2009). In *P. patens*, the prominent structure is the haploid vegetative gametophyte, which has a plant-like photosynthetic leafy frame and a nongreen root-like absorptive frame, and each of the single-layered leafy cells contains numerous LDs (Huang et al., 2009). In transformed *P. patens* leafy cells (Fig. 7A), oleolike-GFP was associated almost exclusively with LDs identified with Nile Red staining. For some of the LDs, oleolike-GFP fluorescence can be seen enclosing the Nile Red-stained neutral lipid cores of LDs (Fig. 7A, arrow).

**DISCUSSION**

**MLDP**

Our findings with in situ immuno-CLSM clarify the subcellular location of MLDP in *C. reinhardtii*. Earlier, several laboratories reported contrasting results of MLDPs in LD fractions from *C. reinhardtii* and two other chlorophytes. A *C. reinhardtii* LD fraction obtained by an osmotic breakage procedure contained minimal proteins, all of which were attributed to minor contaminants (Wang et al., 2009). Another *C. reinhardtii* LD fraction obtained via a sonication procedure possessed numerous proteins with an SDS-PAGE pattern quite similar to those of the total extract, thylakoid, and eye spot fractions, and one protein enriched in the LD fraction was taken as the MLDP (Moellering and Benning, 2010). An *H. pluvialis* LD fraction prepared via drastic physical breakage of the cells contained numerous proteins, one of which was identified as MLDP (Peled et al., 2011). A *D. salina* LD fraction obtained via an osmotic procedure and then centrifugation in the presence of artificial LDs as carriers possessed MLDP as the major protein (Davidi et al., 2012). None of these reports used immuno-CLSM for in situ subcellular localization of MLDP, and in *D. salina*, MLDP was localized on the LD surface with in situ immuno-TEM.

The intracellular distribution of MLDP in *C. reinhardtii* is distinct from that of oleosin in higher plants, in which the oleosin exclusively locates on and fully encloses the LD. Since MLDP lacks a long nonpolar stretch like that in oleosin, we predict that if MLDP directly contacts LDs, the contact is through its polar and/or charged residues with the PLs or other components on the LD surface; such contact would not be as strong as that between oleosin and LDs.

**Figure 4.** Sequence alignment of oleolikes and oleosins in algae and plants. A Pile-Up with emphasis on the nonpolar hairpin is shown. The three conserved Pro residues in the Pro knot (PX5SPX3P) are in yellow, and the nonpolar residues in the Pro knot and the adjacent hairpin are in cyan. The 10th, 20th, and 30th residues in the flanking region on both sides of the Pro knot are marked by ticks for easy visualization. Chlamy_oleo_454-1 and Chlamy_oleo_454-2 are from Lechtreck et al. (2009). Sources of other sequences are shown in Table I.
shaped MLDP-ER subdomains to LDs in *C. reinhardtii*. However, in time-course studies of *C. reinhardtii* of all examined developmental stages (data not shown), we never observed MLDP fully enwrapping the LD surface. This is contrary to oleosins, which tightly wrap around the whole surface of higher plant LDs reported by many laboratories, including ours. At present, we

**Figure 5.** LDs, MLDP, oleolike, and oleosin in *C. reinhardtii*. A, CLSM images of cells stained for LDs with BODIPY 505/515. Cells of different developmental stages were used: vegetative cells, gametes, zygotes, and tetrads. The BODIPY stain was pseudocolored in canary yellow and merged with the autofluorescence of chlorophyll (red). The image of the zygote is a wide field image. All images are of the same magnification, and the bar is in μm. B, RT-PCR of oleosin, MLDP, and oleolike transcripts and PCR of the oleosin gene. The actin transcript was used as an internal control. In the PCR analysis, genomic DNA (gDNA) of vegetative cells was used. Abbreviations are as follows: vegetative cells (v), gametes (g), zygotes (z), and tetrads (t). C, LD fraction from zygotes visualized by DIC and fluorescence microscopy. The LD fraction contained mainly variably sized spherical structures, which were stained by BODIPY 505/515. The bar is in μm. D, LD fraction from zygotes analyzed by SDS-PAGE for proteins (top) and TLC for neutral lipids (bottom). The numbers on top of each lane indicate the relative amount of LDs obtained from 1× total extract. Locations of molecular markers of proteins and a marker of TAGs are indicated on the right.

**Figure 6.** LDs and oleosin in the charophyte *S. grevilleana*. A, CLSM images of vegetative and conjugating cells stained for LDs with BODIPY 505/515 (pseudocolored in yellow). Autofluorescence of chlorophyll is in red. Both images are of the same magnification, and the bar is in μm. B, RT-PCR of oleosin1, oleosin2, oleosin3, and actin transcripts in vegetative and conjugating cells. C, LD fraction from conjugating cells visualized by DIC and fluorescence microscopy. The LD fraction contained mainly variably sized spherical structures, which were stained by BODIPY 505/515. The bar is in μm. D, LD fraction from conjugating cells analyzed by SDS-PAGE for proteins (top) and TLC for neutral lipids (bottom). The numbers on top of each lane indicate the relative amount of LDs obtained from 1× total extract. Locations of molecular markers of proteins and a marker of TAGs are indicated on the right.
have no evidence that MLDP was on the LD surface or would move from the ER to the LDs. Whether, in C. reinhardtii, the MLDP-ER is involved in LD synthesis or degradation remains to be elucidated. Regardless, our observation of the cup-shaped MLDP-ER could explain the positive or negative findings of MLDP in LD fractions by different laboratories. Our findings were obtained with C. reinhardtii. Whether they can be applied to MLDP in D. salina (Davidi et al., 2012) needs further investigation.

Oleolike

The minimal oleolike was difficult to detect in C. reinhardtii. Nevertheless, it behaved like bona fide LD surface proteins in P. patens transformed with the algal gene tagged with a GFP gene. The exclusive location of oleolike-GFP on LDs of transformed P. patens indicates that the association of oleolike to the LD surface is tight, because the larger tagged hydrophilic GFP (approximately 238 residues) did not pull the smaller oleolike (77 residues) to the cytosol. This tight association could reflect a unique structure for the binding of oleolike to the LD surface.

Following the established oleosin hairpin structure (Huang, 1992), we hypothesize that oleolike forms a three-pike fork, with a perfect oleosin-type Pro knot at the center (Fig. 7C). The fork would insert into the hydrophobic region of the surface PL layer and slightly into the matrix TAGs. The basic Lys and Arg residues could form ionic bonds with the acidic carboxylate of the surface amphipathic lipid diacylglyceryl trimethylhomo-Ser. The three-pike fork and the ionic bonds together could anchor the protein tightly on the LD surface.

Oleolike transcript is present in chlorophytes and absent in charophytes, plants, and nonplant organisms. The subcellular location, gene expression pattern, and sequence-derived structural model of oleolike are related to those of oleosins. We suggest that oleolike is evolutionarily related to oleosin, as a precursor of oleosin, a branch-off oleosin, or a degenerated oleosin. If oleolike was a precursor, its short hairpin structure with a Pro knot could become the long hairpin of oleosin on the mutation of several DNA codons for charged residues to codons for non-polar residues (Fig. 7C). This is in accord with the portion of a transformed cell and small portions of three adjacent nontransformed cells on the left and top part of the image. In the DIC image, dotted lines outline the cell boundary, and dark spheres are chloroplasts. C, A possible arrangement of the oleolike polypeptide on the surface of an LD. The polypeptide is depicted as a three-pike fork inserted into the LD surface. The two horizontal lines represent the location of the monolayer of diacylglyceryl trimethylhomo-Ser. The two horizontal lines represent the location of the monolayer of diacylglyceryl trimethylhomo-Ser (indicated with a dark oval attached to two lines of acyl moieties).
hypothesis that the long arms of the oleosin hairpin evolved from the extension of shorter hydrophobic arms (Hsieh and Huang, 2004). Other evolutionary mechanisms, such as the acquisition of the long hairpin arms from other proteins, remain possible.

**Oleosin Gene in C. reinhardtii**

The oleosin transcript was present in the transcriptome of cells transferred to an acetate-rich medium generated by our laboratory and in the raw data of transcriptomes of acid-stressed cells produced by a different laboratory. These cells were grown under similar conditions (acetate-rich and acid-stress media). The common thread may be the acetate in the media, and acetate can induce the production of more LDs (Goodson et al., 2011). We examined the codon usage of three C. reinhardtii oleosin transcripts and found them to be consistent with the codon usage of C. reinhardtii genes (data not shown); as controls, Arabidopsis oleosin genes have different codon usages. Yet, the oleosin transcript sequence is absent in the available C. reinhardtii genome sequence (http://genome.jgi-psf.org/chlamy/chlamy.info.html). We used primers derived from the C. reinhardtii oleosin transcript to obtain a PCR fragment, which had identical length (Fig. 5B) and nucleotide sequence to the oleosin transcript. The identical length indicates that there is no intron in the DNA sequence between the two primers. Apparently, the C. reinhardtii oleosin gene is missing in the available C. reinhardtii genome DNA sequence (version 5), which covers about 98% of the whole genome (http://www.chlamy.org/).

**Oleosin Evolution**

Our findings show that oleosin transcript and protein were at minimal levels in C. reinhardtii and charophytes. The oleosins in these green algae would cover a very small portion of the surface of an LD. Nevertheless, we observed an apparent increase of the levels of oleosin transcript and protein from C. reinhardtii to S. grevilleana and were able to detect the minimal oleosin transcripts and proteins at a defined developmental stage when storing lipids was advantageous. C. reinhardtii produces or maintains minimal amounts of oleolike, and to a lesser extent oleosin, transcript and protein at a defined developmental stage when TAGs accumulate. The more advanced charophytes, as exemplified by S. grevilleana, also produce or maintain minimal amounts of oleosin transcripts and proteins at a defined developmental stage when TAGs accumulate. The minimal amounts of oleosin transcripts and proteins in algae are evident in the low levels of the transcripts in transcriptomes similar to those of Arabidopsis leaves, roots, and seedlings (Table I) and in our difficulties in detecting the proteins biochemically. The moss P. patens apparently has stronger oleosin gene promoters to yield more transcripts in the sporophyte (Table I); yet, the oleosins produced are not excessive, which results in large and nonuniformly sized LDs (Huang et al., 2009). Higher plants, such as maize (Zea mays) and Arabidopsis, produce abundant oleosins and thus small and uniformly sized LDs in seeds, as a consequence of having stronger gene promoters and more gene copies.

During evolution, oleosin genes duplicated. The number of oleosin genes in organisms along the phylegenetic tree increased (Table I): one in C. reinhardtii, two each in Cosmarium turpinii and Closterium
acerosum, three in S. grevilleana, three in P. patens, eight in S. moellendorfii (Huang et al., 2009), and 17 in Arabidopsis (Kim et al., 2002). Before and after duplication, individual oleosin genes via mutation of the promotors became more active and also specialized for cell- or tissue-dependent expression. The three individual oleosin genes in P. patens are specifically expressed in the gametophyte and/or sporophyte, as are the 17 genes in Arabidopsis in seed, pollen, and tapetum. During evolution, the functions of the oleosins diversified. In Arabidopsis, the tapetum cells contain oleosins in tapetosomes, which enable orderly assembly and transfer of pollen coat materials to the pollen (Hsieh and Huang, 2007). Whether, during evolution, other proteins, such as caleosin, would be involved in the assembly and transfer of pollen coat materials to the egg cell- or tissue-dependent expression. The three individual oleosin genes via mutation of the promotors became more active and also specialized for cell- or tissue-dependent expression. The three individual oleosin genes in P. patens are specifically expressed in the gametophyte and/or sporophyte, as are the 17 genes in Arabidopsis in seed, pollen, and tapetum. During evolution, the functions of the oleosins diversified. In Arabidopsis, the tapetum cells contain oleosins in tapetosomes, which enable orderly assembly and transfer of pollen coat materials to the pollen (Hsieh and Huang, 2007). Whether, during evolution, other proteins, such as caleosin, would be involved in the assembly and transfer of pollen coat materials to the egg.
containing 0.15 M NaCl (TN), and blocked in 0.5% blocking reagent (PerkinElmer) in TN. The cells were then incubated sequentially with anti-MLDP (1:250) for 2 h at 37°C, cyamine 3-conjugated anti-rabbit IgG (1:400) for 45 min at 37°C, and 1 µl BODIPY 505/515 for 30 min, with three washes (TN with 0.05% saponin) between incubations, and mounted in ProLong Gold antifade reagent (Molecular Probe). For double immunofluorescence, the procedure for antibody incubations was as follows: anti-MLDP (1:250), Alexa Fluor 488-conjugated Fab fragment anti-rabbit IgG (1:200), anti-rabbit IgG Fab fragment (1:50, for blocking excess rabbit IgG), anti-calreticulin (1:400; against the castor bean [Ricinus communis] enzyme [Coughlan et al., 1997]), and cyamine 3-conjugated anti-rabbit IgG (1:400). Because both the anti-MLDP and anti-calreticulin antibodies were from rabbits, monovalent Fab fragment and anti-rabbit IgG were used in the first immunoreaction (anti-MLDP) to minimize the cross-reactivity with the rabbit IgG from the second immunoreaction (anti-calreticulin). Images were captured with a Zeiss LSM 510Meta confocal microscope with 100X (numerical aperture 1.4) oil-immersion objectives and Zeiss LSM 510 version 3.2 software. Cyamine 3 was excited with the 543-nm helium-neon laser line, and emission was detected at 560 to 615 nm. BODIPY 505/515 and Alexa Fluor 488 were excited with the 488-nm argon laser line, and emission was detected at 500 to 550 nm. Nile Red was excited with the 543-nm helium-neon laser line, and emission was detected at 560 to 615 nm. The wide-field epifluorescence image was captured by a CoolSNAP HQ2 monochrome CCD camera on an Olympus IX71 inverted microscope controlled by the DeltaVision system (Applied Precision). Three-dimensional reconstruction modeling was performed with Imaris 6.4 (Bitplane).

Search for Oleolike and Oleosin Genes in Algae and Other Organisms

Public algae genome sequences and transcript databases were downloaded from JGI (http://genome.jgi.doe.gov/) and NCBI (http://www.ncbi.nlm.nih.gov/) for species whose genomes have been sequenced completely. For the transcriptome database, RNA-Seq raw data were downloaded from JGI and NCBI and generated with the use of an Illumina Genome Analyzer. The RNA-Seq reads were subjected to de novo assembly by use of the programs CLC or OASES to generate transcript databases, and the RPMK values were counted by use of CLC. We used BLAST to search for homologs of oleosins and oleolikes and proteins with Pro-knot motifs. More than 600 annotated oleosin sequences were collected from NCBI, JGI, and other databases, and in turn these were used as queries in additional homologous sequence searches. These oleosins were used for amino acid sequence pattern determination of the Pro-knot sequence. The oleosin candidates from homologous sequence and Pro-knot motif searches were further confirmed with the use of Pfam (http://pfam.sanger.ac.uk/).

RNA Extraction, Transcriptome Preparation, RT-PCR, and PCR

Cells were ground to a fine powder in liquid nitrogen or broken by use of MagNa Lyser green beads (Roche) in Trizol reagent (Invitrogen) for RNA extraction. Crude RNA in Trizol reagent was purified with the use of an illustra RNAspin mini RNA isolate kit (GE Healthcare). For S. grevilleana, RNA was first extracted by the pine tree method (Chang et al., 1993) and then purified with an illustra RNAspin mini RNA isolate kit. RNA sequencing was performed with the use of Illumina Genome Analyzer Ix at Yurgene Bioscience. Briefly, mRNA was isolated from 10 µg of total RNA with the use of oligo(dt) beads and randomly fragmented by use of cations and heat. The cleaved mRNA fragments were converted to double-stranded complementary DNA (cDNA) with the use of random primers and ligated with Multiplex-Identifyer tags after adenylation. The tagged fragments were size selected on an agarose gel (for 200–400 bp), and the selected fragments were enriched by PCR amplification. Sequencing was carried out with the use of Illumina Genome Analyzer Ixks to yield more than 25 million 101-nucleotide fragments, which were subjected to paired-end reads.

For RT-PCR, a sample of 2.5 µg of RNA was converted to first-strand cDNA with the use of Thermoscript reverse transcriptase (Invitrogen). PCR was carried out with the use of 50 ng of the cDNA as template and DNA polymerase in a total volume of 15 µl. Gene-specific primer pairs were designed according to the sequences of specific genes (Supplemental Table S1). For detecting the oleosin1 RNA fragment of C. reinhardtii, we used nested PCR with cDNA (or genomic DNA for PCR) as template, primers Oleosin-1-F and Oleosin-1-R (Supplemental Table S1), and dimethyl sulfoxide (9%) was added to break open the DNA secondary structures. The PCR product of the first PCR was used as template for nested PCR with the primers Oleosin-1-F and Oleosin-nested-R (Supplemental Table S1). Amplified DNA fragments from RT-PCR and PCR were analyzed on 1% agarose gels, and the candidate fragments were subcloned into pGEM-T Easy (Promega) for DNA sequencing analysis.

Transient Expression in P. patens

A full-length fragment of DNA encoding C. reinhardtii oleolike and a full-length fragment of DNA encoding S. grevilleana oleosin3 were generated by use of PCR with gene-specific primer pairs containing the BamHI site (Supplemental Table S1). After BamHI digestion, the PCR fragment was cloned into the BamHI site of a GFP expression vector (Chiu et al., 1996). The DNA construct was transformed into P. patens as described (Huang et al., 2009).

Nucleotide and amino acid sequences of all the genes and proteins reported in this article are shown in Supplemental Figure S2 and have been deposited in NCBI.

Supplemental Data

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

Supplemental Figure S1. TEM images of C. reinhardtii and S. grevilleana.

Supplemental Figure S2. Nucleotide and amino acid sequences of oleolike and oleosins.

Supplemental Table S1. Primer sequences for RT-PCR, PCR, and construction of the DNA expression cassette.

Supplemental Table S2. Proteins in S. grevilleana LD fractions identified by MS/MS.

ACKNOWLEDGMENTS

We greatly appreciate assistance from Dr. Wann-Neng Jan for electron microscopy (Academia Sinica); Drs. Tsuan-Nan Wen and Shu-Yu Lin (Academia Sinica) for proteomics; Drs. Yubing Li (University of California, Riverside), Jiunn-Tzong Wu (Academia Sinica), Su-Chiung Fang (Academia Sinica), and Ruth Timme and Charles Delwiche (University of Maryland) for advice on algae biology and genomics; Drs. Wen-Dar Lin and Caroline Hsing (Academia Sinica) for advice on database studies; and Drs. Sean Coughlan and Anthony Kinney (DuPont) for antibodies against the castor bean calreticulin.

Received December 9, 2012; accepted February 4, 2013; published February 7, 2013.

LITERATURE CITED

Beller M, Thiel K, Thul PJ, Jäckle H (2010) Lipid droplets: a dynamic organelle moves into focus. FEBS Lett 584: 2176–2182

Bickel PE, Tansey JT, Welte MA (2009) PAT proteins, an ancient family of lipid droplet proteins that regulate cellular lipid stores. Biochem Biophys Acta 1791: 419–440

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911–917

Chang S, Puryear J, Cainey J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep 11: 113–116

Chen YR, Su YS, Tu SL (2012) Distinct phytochrome actions in nonvascular plants revealed by targeted inactivation of phyto bilin biosynthesis. Proc Natl Acad Sci USA 109: 8310–8315

Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. Curr Biol 6: 325–330

Coughlan SJ, Hastings C, Winfrey R Jr (1997) Cloning and characterization of the calreticulin gene from R. communis L. Plant Mol Biol 34: 897–911

Davidi I, Katz A, Pick U (2012) Characterization of major lipid droplet proteins from Dunaliella. Planta 236: 19–33

Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.
Frandsen GI, Mundy J, Tzen JT (2001) Oil bodies and their associated proteins: oleosin and oleosin. Physiol Plant 112: 301–307
Goodman JM (2008) The gregarious lipid droplets. J Biol Chem 280: 28005–28009
Goodson C, Roth R, Wang ZT, Goodenough U (2013) Structural correlates of cytoplasmic and plastocyanin: their sequence in the photosynthetic electron transport chain of Chlamydomonas reinhardtii and stimulation of lipid body production with acetate boost. Eur Biol Cell 16: 1592–1606
Gorman DS, Levine RP (1992) Oil bodies and oleosins in seeds. Annu Rev Plant Physiol Plant Mol Biol 17: 582–596
Huang CY, Chung CI, Lin YC, Hsing YIC, Huang AHC (1965) Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of Chlamydomonas reinhardtii. Proc Natl Acad Sci USA 54: 1665–1669
Greenspan P, Mayer EP, Fowler SD (1991) Oleosin is a bifunctional enzyme that has both monoacylglycerol lipase and acyltransferase activities. J Biol Chem 267: 596–603
Hsieh K, Huang AHC (2008) The gregarious lipid droplets. J Biol Chem 283: 97–106
Huang et al. (2012) Oleosin is bifunctional enzyme that has both monoacylglycerol lipase and acyltransferase activities. J Biol Chem 287: 1946–1954
Peled E, Leu S, Zarka A, Weiss M, Pick U, Khozin-Goldberg I, Boussiba S (2011) Isolation of a novel oil globule protein from the green alga Haematococcus pluvialis (Chlorophyceae). Lipids 46: 851–861
Robenen H, Hofnagel O, Buer S, Robenen MJ, Troyer D, Severs NJ (2006) Adipophilin-enriched domains in the ER membrane are sites of lipid droplet biogenesis. J Cell Sci 119: 4215–4224
Ueno T, Sasaki K (1978) Light dependency of the mating process in Closterium acerosum. Plant Cell Physiol 19: 245–252
Vieler A, Brubaker SB, Vick B, Benning C (2012) A lipid droplet protein of Nannochloropsis with functions partially analogous to plant oleosins. Plant Physiol 158: 1562–1569
Wang ZT, Ulrich N, Joo S, Waffenschmidt S, Goodenough U (2009) Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starless Chlamydomonas reinhardtii. Eur Biol Cell 18: 1856–1868
Wu SSH, Platt KA, Ratnayake C, Wang TW, Ting JTL, Huang AHC (1997) Isolation and characterization of neutral-lipid-containing organelles and globuli-filled plastids from Brassica napus tapetum. Proc Natl Acad Sci USA 94: 12711–12716

Lechtreck KF, Luro S, Awata J, Witman GB (2009) HA-tagging of putative flagellar proteins in Chlamydomonas reinhardtii identifies a novel protein of intraflagellar transport complex B. Cell Motil Cytoskeleton 66: 469–482
Lee WS, Tzen JTC, Kridl JC, Radke SE, Huang AHC (1991) Maize oleosin is correctly targeted to seed oil bodies in Brassica napus transformed with the maize oleosin gene. Proc Natl Acad Sci USA 88: 6181–6185
Liu Q, Sun Y, Su W, Yang J, Liu X, Wang Y, Wang F, Li H, Li X (2012) Species-specific size expansion and molecular evolution of the oleosins in angiosperms. Gene 509: 247–257
Moellerling ER, Benning C (2010) RNA interference silencing of a major lipid droplet protein affects lipid droplet size in Chlamydomonas reinhardtii. Eur Biol Cell 9: 97–106
Murphy DJ (2012) The dynamic roles of intracellular lipid droplets: from archaea to mammals. Protoplasma 249: 541–555
Parthibane V, Rajakumari S, Venkateshwar V, Iyappan R, Rajasekharan R (2012) Oleosin is bifunctional enzyme that has both monoacylglycerol acyltransferase and phospholipase activities. J Biol Chem 287: 1946–1954