

Suppression of Soybean Oleosin Produces Micro-Oil Bodies that Aggregate into Oil Body/ER Complexes

Monica A. Schmidt^a and Eliot M. Herman^{b,1}

^a Donald Danforth Plant Science Center, 975 N Warson Rd, St Louis, MO 63132, USA

^b Plant Genetics Research Unit, USDA/ARS, Donald Danforth Plant Science Center, 975 N. Warson Rd, St Louis, MO 63132, USA

ABSTRACT Using RNAi, the seed oil body protein 24-kDa oleosin has been suppressed in transgenic soybeans. The endoplasmic reticulum (ER) forms micro-oil bodies about 50 nm in diameter that coalesce with adjacent oil bodies forming a hierarchy of oil body sizes. The oil bodies in the oleosin knockdown form large oil body–ER complexes with the interior dominated by micro-oil bodies and intermediate-sized oil bodies, while the peripheral areas of the complex are dominated by large oil bodies. The complex merges to form giant oil bodies with onset of seed dormancy that disrupts cell structure. The transcriptome of the oleosin knockdown shows few changes compared to wild-type. Proteomic analysis of the isolated oil bodies of the 24-kDa oleosin knockdown shows the absence of the 24-kDa oleosin and the presence of abundant caleosin and lipoxxygenase. The formation of the micro-oil bodies in the oleosin knockdown is interpreted to indicate a function of the oleosin as a surfactant.

INTRODUCTION

The triglyceride (TAG) oils accumulated in seeds are important agricultural commodities. Seed TAG is a high-energy carbon reserve that is utilized by germinating seeds as a source of energy and nutrition before the onset of autotrophic growth. Seed TAG is sequestered in sub-cellular organelles termed oil bodies (OBs) (Herman, 1994; Hsieh and Huang, 2003; Huang, 1992, 1996; Napier et al., 1996 for reviews) that are spherical organelles 0.2–2.0 μ m in diameter with a simple structure consisting of a TAG core encased in a half-unit membrane (Jacks et al., 1990; Yatsu and Jacks, 1972) consisting of phospholipids and a few different proteins. OBs are accumulated in maturing seeds and in high-oil seeds, such as soybean and peanut, and fill much of the cytoplasmic space by onset of dormancy. TAG is synthesized in the endoplasmic reticulum (ER) (Lacey et al., 1999, for example) and serves as a major storage substance. The accumulation of OB TAG is linked to carbon allocation by the accumulation of the other classes of storage substances, protein, and nonstructural carbohydrate. The relative ratio of the seed storage substance accumulation has a genetic basis (Chung et al., 2003) with some superimposed environmental and nutritional plasticity (Wilson, 2004 for review). In soybean it has long been known by breeders that there is about a 2:1 ratio of seed protein to TAG (Wilson, 2004 for review).

Structural evidence for an ER origin of oil bodies dates to the early ultra-structural observations of seeds (Frey-Wyssling

et al., 1963). Initial observations with conventional aldehyde/osmium fixation indicated that the forming OBs appeared to originate from the ER and that the OBs appeared to have a half-unit membrane (Yatsu and Jacks, 1972) originating from the outer half of the ER bilayer. Other observations indicated that OBs appear to originate from the distal tips of tubular extensions of the ER that is an ER subdomain (Herman, 1987; Herman et al., 1985). The isolation, characterization and cloning of the deduced cDNA and genomic sequences of the proteins associated with OBs have shown that these are members of a single family of proteins first termed oleosins by Huang (Herman, 1994; Hsieh and Huang, 2003; Huang, 1992, 1996; Moloney, 1999 for reviews). The sequence/structure of oleosins consists of three distinct domains: first, an amino-terminal domain, which may be either hydrophilic or hydrophobic; second, a central 70–77 amino acid hydrophobic domain (Abell et al., 2002; Alexander et al., 2002; Li et al., 2002) with a conserved proline knot in the center of the domain (Abell et al., 1997; Huang, 1992); third, a carboxyterminal

¹ To whom correspondence should be addressed. E-mail eheman@danforthcenter.org, fax 314/587-1392, tel. 314/587-1292.

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amphipathic domain of variable length. The signature characteristic of the oleosins is that they all possess the central hydrophobic domain. The central hydrophobic region constitutes the longest continuous region of hydrophobic amino acids known in any protein and is unique to these proteins (Huang, 1992, 1996). This hydrophobic domain has been modeled as an anti-parallel helical structure anchored in the TAG core with the proline knot reversing the direction of the polypeptide (Huang, 1992). The C-terminal amphipathic domain is largely conserved as an amphipathic alpha-helical structure but the sequence of this domain is highly variable.

Loer and Herman (1993) and Hills et al. (1993) tested whether oleosins are co-translationally inserted into ER-derived microsomes using soybean and *Brassica* cDNAs as template for translation. The results showed that oleosins are co-translationally inserted into the ER bilayer and partially protected from the action of exogenous proteases unless the membrane was disrupted by the action of detergents. Oleosins can only be inserted into the ER microsomes, consistent with the EM observations, and not into already formed OBs (Loer and Herman, 1993). Additional experiments have shown that there are specific sequence requirements for oleosin insertion into the ER membrane and that this insertion is dependent on the ER signal recognition protein (Thoyts et al., 1995; Beaudoin et al., 2000). The N-terminal domain and hydrophobic domain have been shown to have essential sequence and structural features leading to the oleosin's insertion into oil bodies in the ER (Beaudoin and Napier, 2002; van Rooijen and Moloney, 1995). Oleosin proteins from one species will correctly associate with forming OBs of another species; for example, the soybean oleosin gene transferred into *Brassica napus* resulted in soybean oleosin being correctly expressed in seed development and the soybean oleosin protein was inserted into the *Brassica* OBs as a mixed population of proteins with the intrinsic *Brassica* oleosin (Sarmiento et al., 1997).

Although OBs are found in other plant tissues such as fruit, only pollen and seeds produce oleosins where the OBs are subjected to developmentally regulated desiccation and hydration. There is experimental evidence to support a functional role of the oleosins to impede OB aggregation during seed desiccation maintaining them as distinct organelles and impact final size of the oil body. Several lines of evidence support this function, including variability of oleosins and oil body size in maize lines with different oil content (Ting et al., 1996), the effects of low temperature on oil bodies (Leprince et al., 1998), and characterization of isolated oil bodies (Tzen and Huang, 1998; Tzen et al., 1992). Rodrigo et al. (2006) showed that silencing *Arabidopsis* oleosins resulted in the formation of enlarged OBs compared to the wild-type. Taken together, these results support the proposal that oleosins have a function as a barrier to coalescence, especially during desiccation. Since oil bodies can be formed in fruit and other organs that do not undergo developmental cycles of desiccation and hydration, it is assumed that oleosins do not have a significant role in oil body ontogeny. However, only by analyzing oleosin null

seeds, whether resulting from genetic modification or mutation, can possible roles for oleosins be tested. In this paper, it is shown that the knockdown of one of the major oleosins of soybean seeds results in the formation of aberrant oil bodies. The interpretation of the resulting data indicates that oleosins function during oil body formation to lower the surface energy/tension of the newly formed triglyceride droplet, permitting it to enlarge to the final size of seed oil bodies.

RESULTS

Knockdown of 24-kDa Oleosin Produces a Phenotype of Aggregation of Oil Bodies and ER and Low Viability of the Seeds upon Onset of Desiccation

The synthesis of the major 24-kDa oleosin was suppressed using an RNAi construct driven by the oleosin isoform A promoter (Rowley and Herman, 1997; GenBank U09118) (Figure 1). The sequence used for the RNAi was obtained from the carboxy-terminal domain of 24-kDa oleosin isoform A and is highly conserved among the 24-kDa oleosin cDNAs but is also highly divergent in comparison to the oleosins of other plant species. By using the oleosin's own promoter, the construct was designed to suppress the 24-kDa oleosin in the same developmental pattern as oleosin synthesis. Transgenic soybeans were produced by biolistic transformation of somatic embryos (Parrott and Clemente, 2004 for review). The resulting transformation events were selected by hygromycin and somatic embryos regenerated and tested for oleosin suppression by immunoblot using a 24-kDa anti-oleosin antibody (Herman, 1987). Embryos from lines that apparently lack 24-kDa oleosin were germinated and T0 plants produced.

Five transgenic soybean lines of the 24-kDa oleosin knockdown were obtained and all plants grew normally and set seeds similar to controls. SDS-PAGE immunoblot assay of the seeds showed an essentially total knockdown of the 24-kDa oleosin with three of the lines shown in Figure 2. Mature seeds were chipped for additional SDS-PAGE immunoblots, 2D gels, light and electron microscopic observations. Light microscopic observations using Nile Red stain (Greenspan et al., 1985) to stain OBs of the oleosin knockdown and wild-type showed that the OBs of the 24-kDa oleosin knockdown

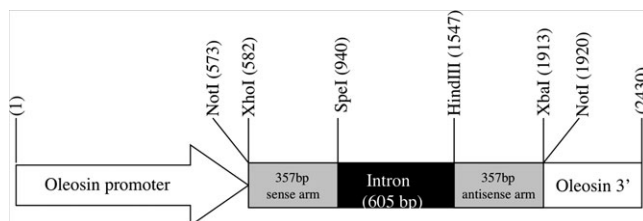


Figure 1. A Graphical Representation of the Construct Used for the Suppression of 24-kDa Oleosin in Soybean Seeds.

RNAi technology was used with a 357-bp cloned segment of the oleosin isoform A gene driven by the seed-specific oleosin isoform A promoter.

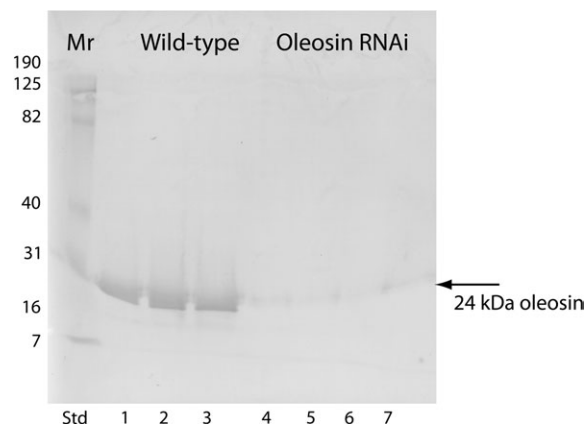


Figure 2. The Knockdown of 24-kDa Oleosin Screened from the Chips of Multiple Seeds by SDS-PAGE Immunoblot Assay.

Lanes 2, 3, and 4 show control lysates obtained from wild-type seeds and lanes 5, 6, and 7 show the suppressed accumulation of 24-kDa oleosin in the knockdown.

cotyledons were much larger in diameter—average 7 micrometer of μm in diameter, compared to average >1 micrometer of μm in wild-type (Figure 3)—results similar to that observed in an oleosin suppression in *Arabidopsis* (Rodrigo et al., 2006). In maturing and fully mature cotyledon storage parenchyma cells, the OBs are aggregated in discrete domains of the cell. The rehydrated seeds exhibited poor growth. The Nile Red stained cells and parallel EM observations indicated that the cotyledon cells contained large oil bodies in apparently disrupted cells. Higher-resolution assays using conventional TEM resulted in the observation of giant OBs with some smaller adjacent OBs, indicating that, in desiccated seeds, the OBs have merged. The ultra-structure of the cell surrounding the giant OBs lacks the compartments observed in conventional soybeans, particularly the protein storage vacuoles, PSVs, which are disrupted with its matrix contents present in the cytoplasm surrounding the OBs (Figure 4).

The 24-kDa oleosin knockdown seeds germinated, but the subsequent growth is greatly retarded compared to the control wild-type, with only a fraction of the slowly growing 24-kDa oleosin knockdown seedlings surviving to establish an autotrophic plant. Figure 5 shows examples of the seedling growth of the 24-kDa oleosin knockdown in comparison with the wild-type. After 7 d of growth, the wild-type seeds have an elaborated root system and have started to extend the hypocotyl and apical axis to form the primary leaves. In contrast, after 7 d of growth, the 24-kDa oleosin knockdown exhibited radial protrusion and only slight growth (Figure 5A). As the seedling growth of the 24-kDa oleosin knockdown proceeds, the embryo continues to slowly elongate and differentiate while the cotyledon appears to be static and unchanging. Figure 5B shows that the cotyledons of an emerging seedling of 24-kDa oleosin knockdown open as in the control wild-type; however, the cotyledons that retain the yellow color of the imbibed seed do not appear to have undergone any of the normal post-germination alteration. The cotyledons of the wild-

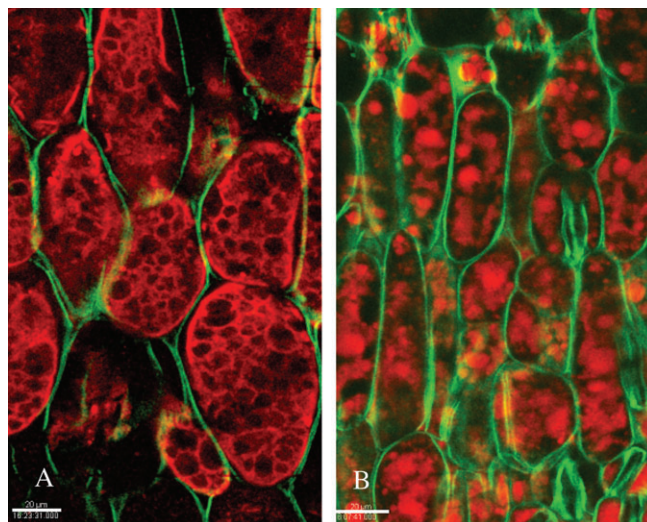


Figure 3. The Comparative Morphology of OBs Stained with Nile Red of Hydrated Cotyledon Chips of the Wild-Type (Panel A) and 24-kDa Oleosin Knockdown (Panel B).

The OBs stained in the 24-kDa oleosin knockdown are much larger than the OBs stained in the wild-type seeds. Bar = 20 micrometer of μm .

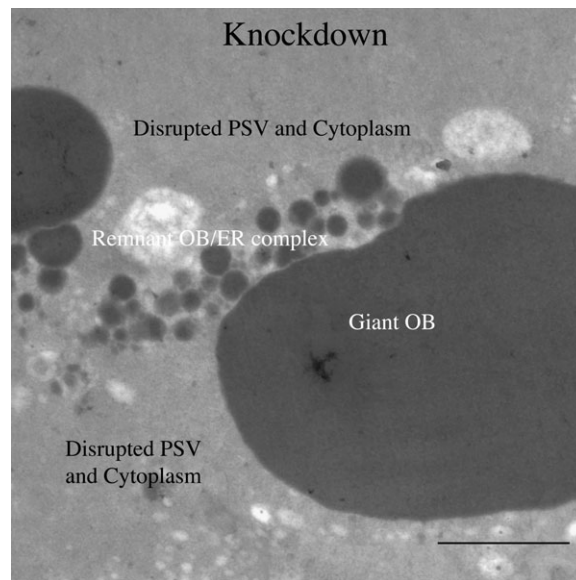


Figure 4. The Morphology of a Cell of a Hydrated 24-kDa Oleosin Knockdown Cell from Material Prepared for Conventional Transmission Electron Microscopy.

Giant osmophilic OBs dominate the cellular space, while the other cellular compartments appear disrupted. Bar = 1 micrometer of μm .

type seed re-green and remain attached to the seedling for more than 2 weeks (Figure 5C, arrows). In contrast, in a comparatively sized, although older because of slow growth, 24-kDa oleosin knockdown plant, the cotyledons do not re-green and abscise (Figure 5D), indicating that the 24-kDa oleosin knockdown cotyledons are not viable.

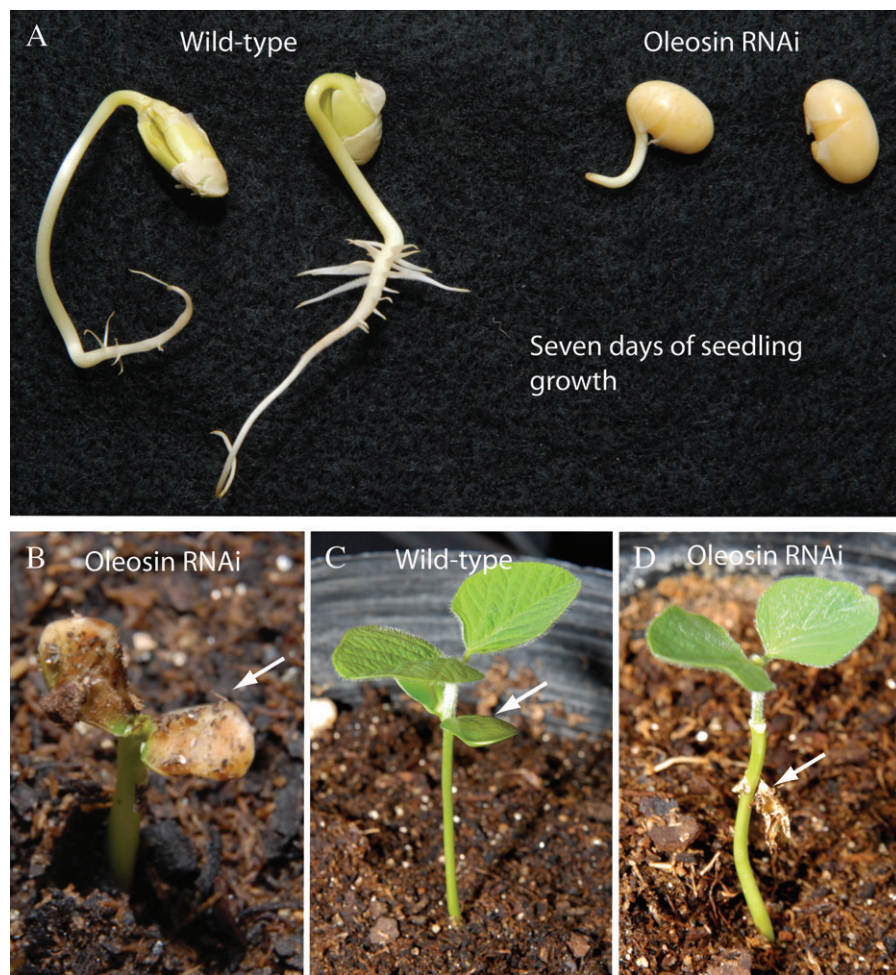


Figure 5. Photographs of Germinated Seeds of the Wild-Type and 24-kDa Oleosin Knockdown Illustrating the Slow Growth that Results from the Formation of Giant Oil Bodies in the Cotyledon Cells.

(A) Comparison of wild-type and 24-kDa oleosin knockdown seedlings after 7 d of growth. The wild-type have well elaborated root systems, establishing the new plant's growth; in comparison, the 24-kDa oleosin seeds grow very slowly, with a 7-day-old plant appearing similar to that of a 1–2-day-old wild-type plant.

(B–D) Examples of young plants. (B) shows a newly emerged 24-kDa oleosin plant with the two cotyledons appearing to be dead tissue. (C) shows a wild-type plant after the emergence of the primary leaves with the cotyledons remaining attached and re-greened. In comparison, (D) shows a plant of comparable size, although older, where the two dead cotyledons have dropped off the plant.

In order to test the oleosin knockdown cell's viability, hydrated cotyledon seed chips were stained with a fluorescent dye that assays for cellular viability. Figure 6A shows that hydrated wild-type cotyledon cells are fluorescent and hence viable, while parallel assays on hydrated chips of the oleosin knockdown (Figure 6B) indicate that few, if any, of the cotyledon cells retain viability upon hydration and germination. The low viability presents difficulty in propagating a subsequent generation from 24-kDa oleosin knockdown plants.

The 24-kDa Oleosin Knockdown Results in Formation of a Complex Structure of Interconnected ER and OBs

OBs possess a simple structure of a half-unit membrane of proteins and phospholipids encasing a hydrophobic core (Yatsu and Jacks, 1972). The aldehyde fixation chemistry will cross-react with free amino groups of proteins that are on the OB membrane and it is only in the second postfixation with OsO_4 that the core triglycerides are chemically fixed. This presents the potential that the unfixed components of OBs might be altered during fixation that could appear to be a specific oleosin knockdown phenotype. To address this potential problem, parallel samples were prepared using high-pressure

cryofixation (Craig and Staehelin, 1988; Kiss et al., 1996; Staehelin et al., 1990). This technique has the advantage of rapidly immobilizing all cellular components in amorphous-phase ice that is subsequently removed by freeze substitution using an acetone/osmium solution. This protocol is the best possible approach for preserving cellular structures and often results in observations of fine detail not well preserved by conventional chemical fixation protocols. The OBs in freeze-substituted samples appear empty due to the partition of the TAG into acetone prior to cross-linking by the OsO_4 and therefore the TAG is not preserved. The OsO_4 does fix and enhance the visualization of the OB membrane that is difficult to visualize in conventional aldehyde/ OsO_4 chemically fixed material (see Herman, 1987, for example). The OBs in the wild-type are discrete organelles that do not exhibit any interconnectivity as previously observed and shown in Figure 7A (see Herman, 1987, for soybean TEM and Herman et al., 1985, for freeze fracture of soybean seed cells). The 24-kDa oleosin knockdown OBs are aggregated into a large complex of OBs and ER, with the OBs varying widely in size (Figure 7B). The aggregation of the OBs depletes the adjacent cytoplasm of OBs. Within the OB complex of the 24-kDa oleosin knockdown,

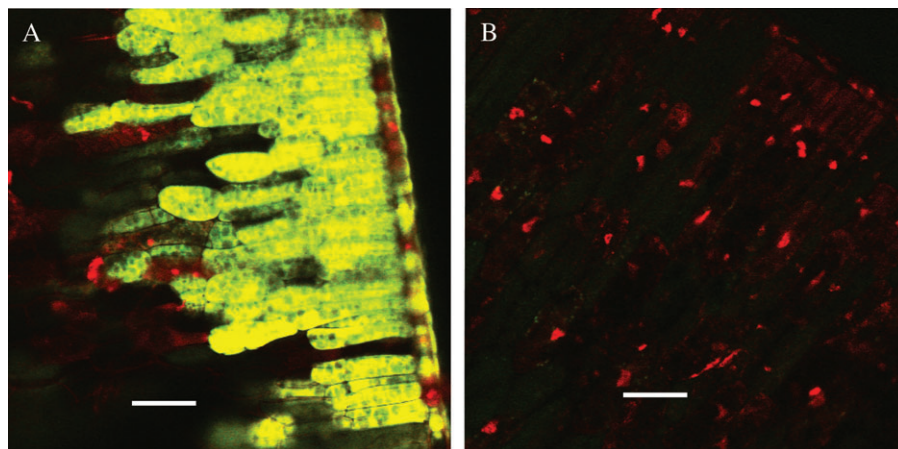


Figure 6. The Viability of the Oleosin Knockdown Cotyledon Cells Was Evaluated with a Vitality Stain of Hydrated Seed Chips in Comparison with the Control Wild-Type Samples.

(A) Wild-type cells take up the dye and are fluorescent, indicating that the cells are viable. (B) In contrast, the cells of the 24-kDa oleosin cotyledon are not fluorescent, indicating a complete absence of viable cells. Bar = 25 micrometer of μm .

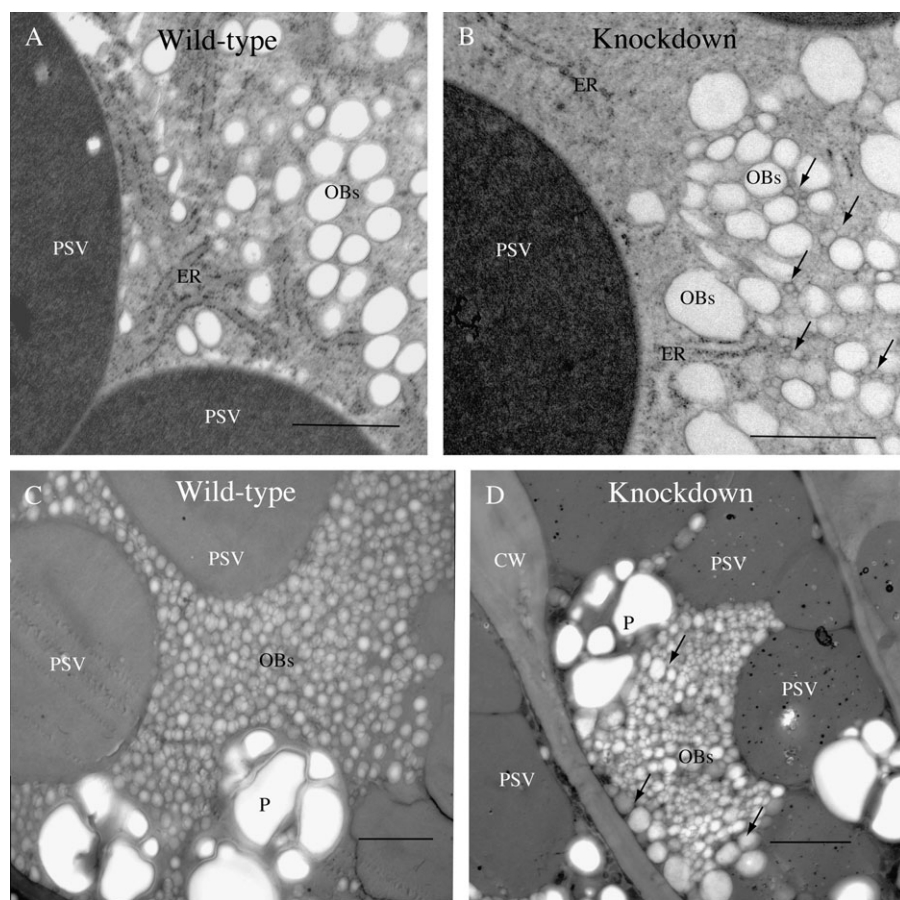


Figure 7. The Morphology of the OB/ER Complex that Results from 24-kDa Oleosin Knockdown of the Wild-Type and Knockdown of Cells from Mid-Maturation Cotyledon Tissue Prepared by Cryofixation.

(A) Uniform-sized OBs of the wild-type. (B) Diverse-sized OBs of the knockdown. Note the RNAi knockdown's OBs are distributed so that the larger OBs are on the periphery of the complex while the interior of the complex is dominated by ER and smaller OBs (B).

(C) Semi-thick sections visualized using energy-filtered electron microscopy illustrating the even size distribution of OBs in the wild-type.

(D) Semi-thick sections visualized using energy-filtered electron microscopy illustrating the 24-kDa oleosin knockdown's size distribution with the larger OBs on the periphery of the OB/ER complex (D). (A, B) Bar = 1 micrometer of μm , (C, D) Bar = 5 micrometer of μm .

there are numerous small OBs (Figure 7B) that are novel variants of OB morphology not observed in the wild-type (Figure 7A). The other cytoplasmic organelles, the protein storage vacuoles (PSV), Golgi, nucleus, plastids, and mitochondria all are unaffected in the 24-kDa oleosin knockdown, with the observed ultra-structure of the organelles appearing identical to the controls.

Semi-thick-section TEM observations provide a different perspective of sub-cellular structures and, by using an energy-filtering mode, the resolution and detail of thicker sections can be greatly enhanced (Lutz-Meindi and Aichinger, 2004). Semi-thick sections were cut from the same cryofixed material as used for the thin sections, stained with uranyl acetate, and examined with the TEM. The 350–450-nm thick

sections may completely encompass organelles such as OBs and ER so that the structural relationships between organelles can be examined. For organelle complexes such as the OB/ER complex produced as a 24-kDa oleosin knockdown phenotype, semi-thick-section TEM is ideal to visualize the structural relationships that can only be inferred with conventional thin-section TEM. Figure 7C and 7D, respectively, show portions of wild-type and 24-kDa oleosin knockdown storage parenchyma cells imaged from semi-thick sections. The wild-type OBs are relatively uniform in size and are observed as discrete organelles seen one on top of another because the section thickness is sufficient to completely encompass part or all of one or more OBs (Figure 7C). In contrast, the semi-thick section shows the diverse size range of the OBs contained within the OB/ER complex resulting from the 24-kDa oleosin knockdown. The semi-thick sections show that the largest OBs tend to be located on the periphery of the OB/ER complex (Figure 7D, arrows) while the interior of the complex is dominated by smaller OBs.

To examine the initial formation of the OB/ER complex, additional tissue samples of the 24-kDa oleosin knockdown cotyledons from the early stage of storage substance accumulation were processed by high-pressure cryofixation. The resulting material was examined with the TEM and observed to contain numerous examples of the initial stages of the OB/ER complex formation. Figure 8A shows an example of the early stage of the OB/ER complex where the structure of closely spaced and parallel sheets of ER encompassing a domain of oil bodies of varying sizes is observed in cross-section. Several OB-forming regions are present in the center of the ER/OB complex (Figure 8A, arrows) that has the morphology of an origin center of closed packed small OBs. The OBs in the 24-kDa oleosin knockdown are formed by the distal end of an ER segment, shown in Figure 8B (arrow) with a cluster of small OBs associated with ER segment end.

The Oleosin Knockdown Forms Novel 50-nm Micro-OBs that Fuse to Form a Hierarchy of OB Sizes Forming the OB/ER Complex

In order to better resolve the OB origin structures and its association with the OB/ER complex in the 24-kDa oleosin knockdown cotyledon cells, a series of high-magnification/high-resolution observations were conducted with ultra-thin-section and semi-thick-section high-pressure cryofixed material. Figure 9A, 9B and 9D show examples of the wild-type and the 24-kDa knockdown of semi-thick sections visualized for the high-resolution portion of the OB/ER complex. The OB/ER complex formed in the 24-kDa oleosin knockdown is an extended structure, 5 micrometer of μm or more across, containing OBs of diverse sizes from 50-nm micro-OBs to 0.5-micrometer of μm large OBs in various stages of aggregation and coalescence. The larger OBs tend to be located on the periphery of the OB/ER complex, while the interior ER is associated with the OBs of varying size, including the 50-nm micro-OBs. At high-resolution, image depth shows that the 50-nm micro-OBs are abundant in the interior of the OB/ER complex. In par-

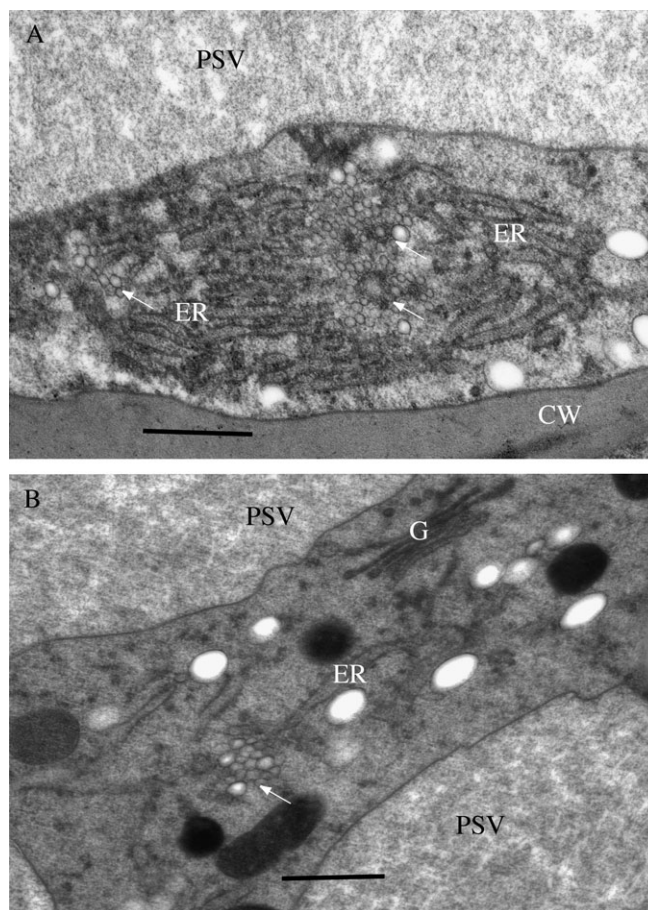


Figure 8. The Initial Stages of the Formation of the OB/ER Complex Early in the Process of Reserve Substance Accumulation.

(A) ER segments encompassing a cytoplasmic domain containing diverse-sized OBs with three centers of OB formation (arrows). The OB formation centers are characterized as a discrete domain with many micro-OBs.

(B) Formation of micro-OBs at the distal end of an ER segment (arrow) illustrating the ER origin of OBs in the oleosin knockdown. The protein storage vacuoles (PSV) at an early stage of seed maturation contain disperse protein deposits and are not yet subdivided. Bar = 1 micrometer of μm .

allel wild-type controls, the 50-nm micro-OBs were not observed (Figure 9D). The 50-nm oil droplets often are observed distributed as radial symmetric micro-OBs in the apparent process of coalescence with adjacent OBs to form larger organelles (Figure 9A and 9B, black arrows). Figure 9A shows multiple sites where the 50-nm micro-OBs are present embedded within the OB/ER complex, while Figure 9B shows high magnification of two clusters of 50-nm micro-OBs. The semi-thick section imaging of the 24-kDa oleosin knockdown OB/ER complex shows that there is a hierarchy of OB sizes, with the smaller OBs in various stages of coalescence to form larger OBs (Figure 9A). The OBs in the wild-type do not interact and coalesce (Figure 9D), remaining a fixed size. Ultra-thin sections of parallel samples and imaging the OB/ER complex resulted in observing multiple 50-nm oil droplets originating from the ER contained within

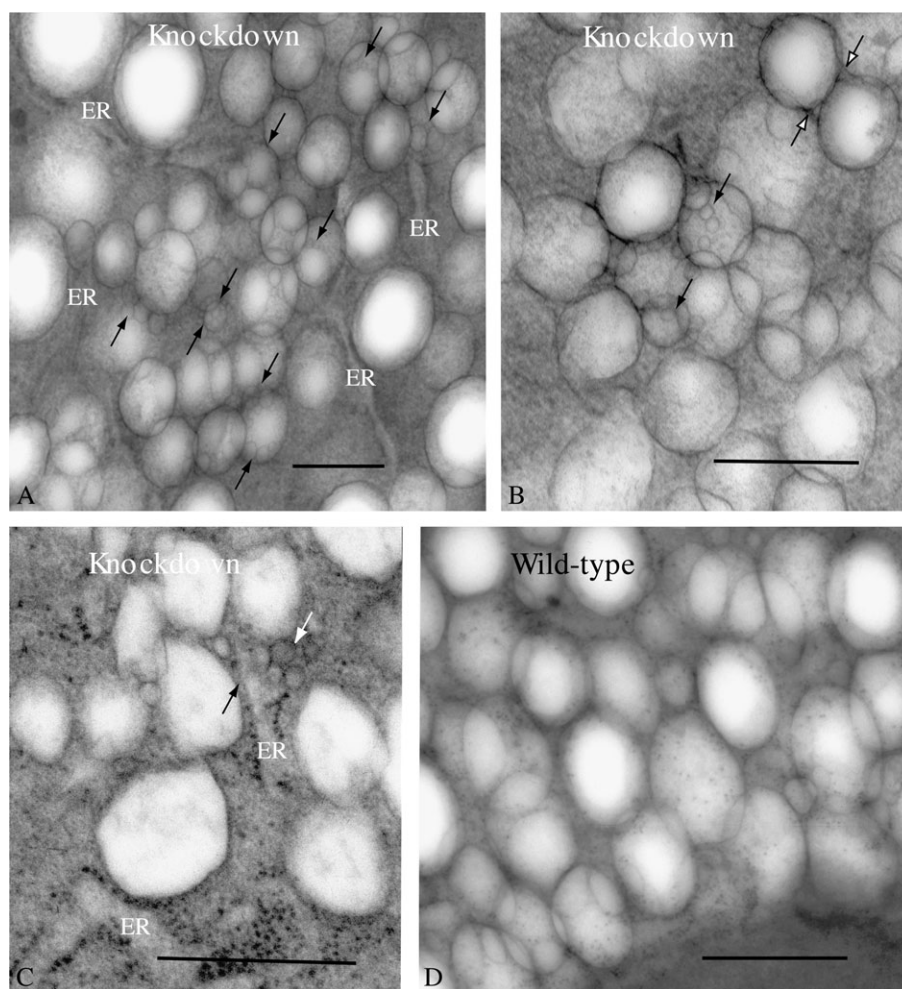


Figure 9. High-Magnification Images of the Interior Structure of the OB/ER Complex and Associated Micro-OBs Using Semi-Thick-Section and Ultrathin-Section Material.

(A, B) Clusters of micro-OBs distributed within the OB/ER complex (black arrows). At higher magnification, the micro-OBs are observed as a group of radially distributed structures with some of the micro-OBs in the process of merging with adjacent micro-OBs.

(B) The OB/OB fusions that enlarge the OBs forming the OB/ER complex is visualized in semi-thick sections (white arrows).

(C) Correlative ultra-thin-section observations of the distal end of an ER segment forming micro-OBs shows the presence of multiple micro-OBs simultaneously being formed (arrows).

(D) Semi-thick sections of the wild-type control show a relatively uniform size and distribution of OBs without either the micro-OBs or large OBs as observed in the 24-kDa oleosin knockdown. Bar = 0.5 micrometer of μm .

the OB/ER complex (Figure 9C), indicating that the formation of the 50-nm micro-oil bodies occurs at the same distal end of the ER domain where OBs are normally assembled in the wild-type (see Herman, 1987; Loer and Herman, 1993).

Proteome and Transcriptome Analysis of 24-kDa Oleosin Knockdown Shows Limited Alterations Compared to Controls

The protein polypeptide distribution in the oleosin knockdown lines was evaluated using two-dimensional gel electrophoresis by broad-range pH 3–10 IEF first-dimension separation and SDS-PAGE for the second dimension shown in Figure 10A. The 24-kDa oleosin knockdown's total protein distribution is dominated by the seed storage proteins and other protein stor-

age vacuole proteins that are similar to the wild-type (Figure 10B) and published soybean seed proteome maps (Herman et al., 2003; Hajdуч et al., 2005), indicating that the structural alterations of the oil bodies does not have a significant impact on the abundant proteins of the proteome.

In order to acquire more detailed data on possible collateral changes that result from the knockdown of the 24-kDa oleosin, the transcriptome of the knockdown was compared to the wild-type using the Affymetrix Genechip™ soybean DNA array platform. Mid-maturation green seeds of both the wild-type Jack and 24-kDa knockdown from plants grown in parallel were used as RNA source material. Both sample and biological replicate array experiments were conducted, with one experiment of both wild-type and the oleosin knockdown assayed in triplicate as

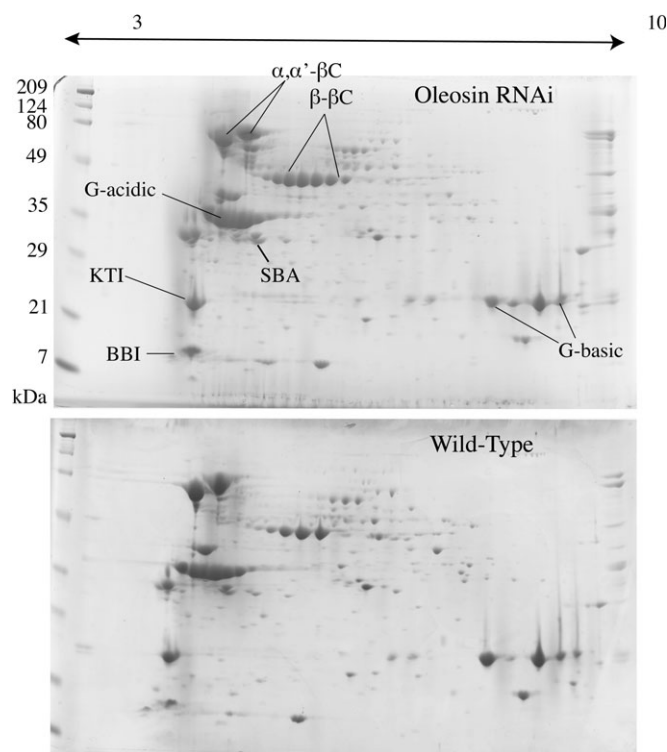


Figure 10. The Two-Dimensional Gel Electrophoresis Using Broad-Range pH 3–10 IEF First Dimension and SDS–PAGE Second Dimension of the Total Proteins of the 24-kDa Oleosin Knockdown and Wild-Type.

The knockdown of the 24-kDa oleosin shown in (A) has no apparent impact on the accumulation of storage and other major seed proteins compared to the wild-type shown in (B).

technical replicates and a second experiment with pairs of arrays assayed with probes from two biological (different individuals) replicates with an oleosin knockdown control. The raw array data are available with accession numbers GSE12300. The resulting arrays and both experiments exhibited consistency of data when evaluated using Stratagene ArrayAssist™ software.

Using cut-off $p = 0.05$ for significance, the arrays resulted in the identification of 29 up-regulated and 184 down-regulated transcripts at two-fold or greater; including 25 and 159, respectively, without identifying annotation and four and 27 that are identified. Table 1 shows a summary of the identification and averaged, corrected two-fold changes up and down of selected annotated array spots. The down-regulated transcripts include high-proline proteins, proteins related to iron metabolism and storage, a lipid transfer protein, components of electron transport systems, and lipoxygenase. The up-regulated transcripts include a calcium-binding oleosin-related protein termed caleosin (Chen et al., 1999; Frandsen et al., 1996, 2001; Naested et al., 2000). Notable in its absence is any apparent effect on the transcript abundance encoding the proteins involved in lipid/oil synthesis. Further, there is no apparent impact of the oleosin knockdown on genes encoding the synthesis of the other classes of reserve substances, the storage proteins and car-

bohydrates, either starch or oligosaccharides. The underlying dataset of the Affymetrix soybean array is derived from the public soybean EST project. As a consequence, many of the spots on the Affymetrix array are not annotated beyond the correlation to an expressed transcript with its database linkage to source organ and developmental stage.

Isolation of Oil Bodies from 24-kDa Oleosin Knockdown Soybeans and Mass Spectroscopy of the 1D SDS–PAGE Fractionated Proteins Show that there Is Abundant Caleosin in the Isolated Oil Bodies

To evaluate whether knockdown of the 24-kDa oleosin and formation of large OBs result in alteration of the OB-associated proteins, OBs were isolated and the identities of the OB-associated were determined. The isolated oil bodies from the 24-kDa knockdown when compared with the wild-type exhibited obvious differences in surface cohesive properties, with the wild-type tending to remain aggregated in large clumps as an oil pad on top of the post-centrifugation lysate while the OBs from the oleosin knockdown line were easily dispersed and exhibited little tendency to remain as clumps. The OB-associated proteins from the 24-kDa oleosin knockdown and the cv Jack control isolated by cold-acetone precipitation were fractionated by one-dimensional SDS–PAGE (Figure 11). The stained gel confirmed that both the monomer and dimer of the 24-kDa oleosin protein are absent in the 24-kDa oleosin knockdown OBs. The oleosin protein has long been observed to tend to fractionate as dimers on SDS–PAGE; whether this represents the in-vivo conformation or whether this is a SDS–PAGE artifact remains unknown. Individual protein bands were excised, subjected to tryptic digestion, and the fragments subjected to mass spectroscopy analysis and identification of fragment patterns using the soybean database. The summary results of the protein identification are shown in Table 2. Two proteins of interest were identified as enriched in the OBs of 24-kDa oleosin knockdown seeds: lipoxygenase and caleosin.

DISCUSSION

The knockdown of one of the two soybean seed oleosins results in an altered phenotype of aberrant formation of OBs. The knockdown results in producing novel 50-nm micro-OBs that progressively coalesce to form a hierarchy of larger OBs and an OB/ER complex. The 24-kDa oleosin knockdown also results in an altered protein content with accumulation of caleosin (Chen et al., 1999; Frandsen et al., 1996, 2001; Naested et al., 2000), a protein that has been previously associated with OB mobilization (Poxleitner et al., 2006). There is no significant change in the soybean seed proteome as a consequence of suppressing 24-kDa oleosin synthesis. Similarly, using stringent corrections and statistical analysis of mRNA populations assayed using array data shows that there are only limited changes in the transcriptome as a consequence of the feedback from the 24-kDa oleosin knockdown. It is curious that although there are identified transcripts that are differentially

Table 1. The Three-Fold or Greater Up- and Down-Regulated Transcripts of the Maturing 24-kDa Oleosin Cotyledon in Comparison with the Wild-Type.

Annotation	Fold change
Ca ²⁺ -binding EF hand protein (caleosin)	4.1
Cystathionine-gamma-synthase precursor (cys) mRNA	3.9
MRNA from stress-induced gene (H4)	3.5
Hydroxyproline-rich glycoprotein (sbHRGP2) mRNA, 3' end	3.2
28-kDa protein	−3.0
Glutamate dehydrogenase 1	−3.0
Lipoxygenase	−3.0
Ferritin light chain	−3.0
Cytochrome P450 71A10	−3.1
Peroxidase precursor (GMIPER1)	−3.2
Glucosyltransferase	−3.2
Hydroxyproline-rich glycoprotein (HRGP)	−3.6
Hydroxyproline-rich glycoprotein (HRGP)	−3.7
24-kDa oleosin isoform (partial) (clone P24/89)	−3.9
Pyruvate kinase	−3.9
Subtilisin-type protease precursor	−4.0
Cytochrome P450 mono-oxygenase CYP72A68 (CYP72A68)	−4.0
Ferritin light chain	−4.1
Hydroxyproline-rich glycoprotein (sbHRGP2) mRNA, 3' end	−4.2
24-kDa seed coat protein	−4.2
Glucosyltransferase	−4.3
SferH-2 mRNA for ferritin	−4.4
Ferritin light chain	−4.6
24-kDa oleosin isoform	−5.3
Lipoxygenase	−7.0
Peroxidase, pathogen-induced	−7.3
Soybean 24-kDa oleosin isoform	−7.5
Repetitive proline-rich protein	−8.0
Repetitive proline-rich protein	−9.9
Hydrophobic seed protein precurs	−18.7

Only four up-regulated and 27 down-regulated genes were identified from the DNA array assay.

expressed as a result of the 24-kDa knockdown, none of these transcripts is associated with the synthesis of any of the three classes of seed reserve substances. The identified transcripts that are regulated include diverse genes that would appear to be unrelated to the formation of oil bodies and its complex with the ER. However, with a majority of the up- and down-regulated transcripts not being annotated beyond being expressed genes, the interpretation that the altered transcripts are not related to OB ontogeny is still tentative. The structural changes that do occur as a consequence of the 24-kDa oleosin

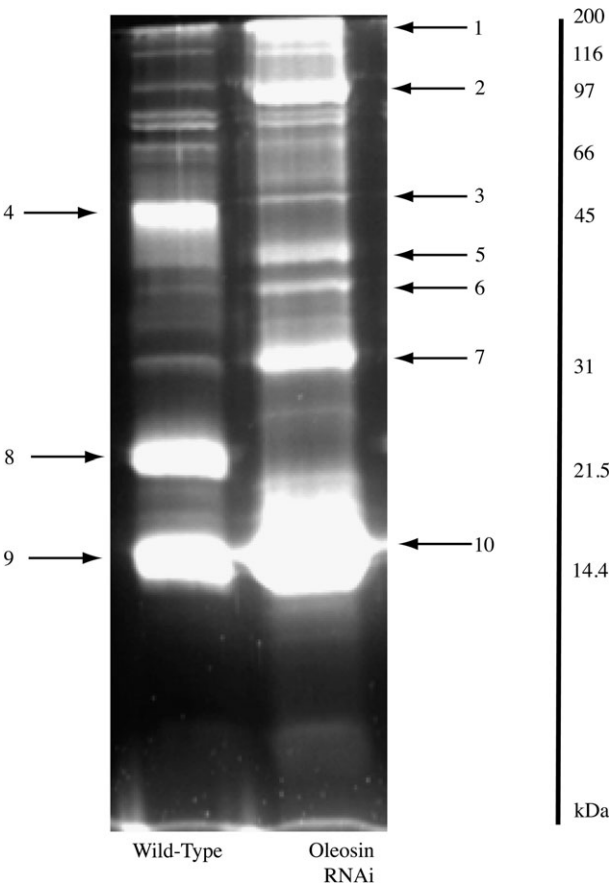


Figure 11. SDS-PAGE Fractionation of Proteins Associated with Isolated Oil Bodies of the 24-kDa Oleosin Knockdown and Wild-Type Control.

There is a nearly complete suppression of the 24-kDa oleosin in the knockdown with both the 24-kDa oleosin monomer and dimer absent as a consequence of the RNAi. The polypeptide distribution of the wild-type (Lane A) and 24-kDa oleosin knockdown (lane B) differ with other polypeptides present in the RNAi OBs not present in the wild-type. The arrows denote polypeptide bands excised and subjected to mass spectroscopy analysis, with the results shown in Table 2.

knockdown provides new information on the role of the oleosins in OB formation and the proposal of a new model for the processes leading to seed OB formation.

The Absence of 24-kDa Oleosin Results in the Formation of Novel 50-nm Micro-OBs

It is striking that the phenotype of the 24-kDa oleosin knockdown is the formation of micro-OBs. This indicates that the 24-kDa oleosin has a role in forming the correct size of the nascent OB during its formation. Electron microscopy of cryo-fixed material shows that micro-OBs and wild-type OBs both form at the same domain at the distal end of the ER domain. This is interpreted to indicate that the process of TAG formation by the OB-producing domain of the ER is not impaired and that the ER is not reorganized by the absence of 24-kDa oleosin

Table 2. The Identifications of the Proteins of Isolated Oil Bodies Obtained from Digestion and Mass Spectroscopy Assay of the Bands Identified by Arrows in Figure 11.

Band number	GenBank accession number	Protein identification	Mascot score	Number of peptides
1	Gi1794172	Lipoxygenase-2	243	10
2	Gi1794172	Lipoxygenase-3	596	21
3	Gi9967359	β -subunit conglycinin	358	11
4	Gi21465628 Gi476214 Gi266689	β -subunit conglycinin	202	16
		P24 oleosin isoform A	135	7
		P24 oleosin isoform B	109	6
5	Gi18637 Gi18635	Glycinin G2	195	6
		Glycinin G1	159	7
6	Gi18637 Gi18635	Glycinin G2	275	10
		Glycinin G1	196	6
7	Gi2270994	Calcium-binding EF (caleosin)	206	8
8	Gi 476214 Gi 266689	P24 oleosin isoform A	137	3
		P24 oleosin isoform B	136	3
9	Gi 21311554	15.8-kDa oleosin (cocoa)	120	3
10	Gi 21311554	15.8-kDa oleosin (cocoa)	120	3

The isolated 24-kDa oleosin oil bodies were determined to have two proteins (caleosin and lipoxygenase) that are not abundantly present in the isolated wild-type oil bodies.

synthesis. This indicates that the ER domain that produces OBs is formed in the absence of the 24-kDa oleosin and that the remaining components of the OB assembly continue to operate normally. The micro-OB formation shows that it is the 24-kDa oleosin that precludes this outcome of TAG synthesis from normally occurring. This then implies that it is the 24-kDa oleosin that is a, if not the, key protein involved in assembling the correctly sized OBs. If the oleosin/TAG OB structure is considered as a simple two-component system, then this can be used to develop a model consistent with the TEM observations of the micro-OB formation. Although there are a small quantity of phospholipids in the half-unit membrane, and perhaps some other minor proteins, the oleosins and TAG constitute most of the structure and therefore should specify the OB's morphology. Without the 24-kDa oleosins on the OB surface, small quantities of TAG synthesized by the ER self-assemble into 50-nm micro-OBs that are much smaller than any OBs normally produced by plant cells. The simultaneous assembly of multiple micro-OBs by distal tip ER produces the radial symmetrical micro-OBs observed enface.

By considering the micro-OBs in the 24-kDa oleosin knock-down seed cells as the smallest stable volume unit of TAG that can be self-assembled by hydrophobic interactions into a meta-stable droplet, a model can be developed for OB ontogeny. For a very small vesicle or droplet about 50 nm in diameter, the surface-to-volume ratio will be high, with a high angle of curvature. For a small vesicle, the surface pressure ($p = 4T/r$) will be high where T = surface tension and r = radius for a two liquid interface. This estimates high surface pressure for the micro-OBs that would drive them coalesce to form

stable structures that are the larger OBs with lower surface pressure. The TAG molecules in the micro-OBs, with their high hydrophobic-cohesion properties, coalesce with adjacent micro-OBs, producing an increasing hierarchy of OB size, observed as the 'popcorn' structure in cryofixed tissue.

The Formation of OB/ER Complexes during Seed Maturation

The increasing size with higher volume-to-surface of the oil droplets within the OB complexes lowers the free energy state, to reach a steady state in size when the system's free energy is sufficiently reduced to produce a stable population distribution of OBs. This is the OB complex at its lowest surface energy at the extent temperature and cytoplasmic solute conditions. As more micro-OBs are produced during the course of seed maturation, it is interpreted that this produces a constant flux of new higher-energy material that merges into the pre-existing population, enlarging the OB/ER complex. The OB/ER complex appears to have an underlying structure that corresponds to the dynamics of the OB formation processes. The micro-OBs and smaller OBs tend to be within the interior of the OB/ER complex, while the larger OBs tend to be located on the OB/ER complex periphery. This suggests that much of the OB formation occurs within the interior of the OB/ER complex, mediated by the ER and, as the complex enlarges during seed maturation, the older, larger OBs are located in the complex's periphery. As seed maturation proceeds, each storage parenchyma cell will form a quantity of OB/ER complexes, each independent from the other, that sequester all the diversely sized OBs into the complexes, with the adjacent cytoplasm depleted of OBs. In the fully mature seed, the 24-kDa oleosin knockdown phenotype is distinct from

the wild-type, with the several OB/ER complexes contrasting with the dispersed, relatively uniformly sized population of OBs filling the cytoplasm of the wild-type.

The formation of giant OBs in desiccating/dormant seeds is likely derived from each one of these complexes coalescing into a single giant OB. This too should be the consequence of the physical chemistry of the TAG core and the surrounding membrane of OB. As the seed desiccates, this will produce a lower water potential and higher solute concentration that should induce the surface energy of the OBs within the OB/ER complex to increase. As a consequence, the previously stable OB population becomes unstable and this results in further aggregation and coalescence, forming giant OBs with a lower surface energy. This result is consistent with the observations that in a knock-down as well as an insertion mutant of an *Arabidopsis* seed, oleosin results in the formation a population of enlarged oil bodies (Rodrigo et al., 2006). The formation of giant OBs appears to disrupt the cells, resulting in reduced viability for the 24-kDa oleosin knockdown seeds. Lower viability was also reported for the suppression of oleosin in *Arabidopsis* seeds (Rodrigo et al., 2006), although the degree of lethality appears to be higher in soybean, which may be due to the larger cell and oil body size.

Oleosins May Function as a Surfactant

The oleosin molecular structure with a hydrophobic face anchored in the TAG (Huang, 1992, 1996 for review) and an amphipathic domain has the overt characteristics of a macromolecular surfactant, or a surface-active agent. Although surfactants are usually small molecules such as detergents, if oleosins are modeled as possessing surfactant activity, this yields a model for the role of oleosins in OB formation. Surfactants function to lower surface tension that is also defined as lowering surface energy. The presence of a surfactant should extend the diameter of the micelle size of TAG as it is formed, resulting in a larger-diameter OB and precluding the formation of the micro-OBs observed in the 24-kDa oleosin knockdown. The oleosins have been modeled as forming a surface cage structure on the oil bodies (see Figure 1 in Huang, 1992). The interlocked oleosin cage has the overt characteristics of a surfactant to provide an encasing layer surrounding the oil body. In this model, the co-synthesis of oleosins and TAG results in the oleosin functioning as a surfactant and chaperone of the TAG. With each oleosin monomer interacting with each other oleosin monomer, this would tend to form OBs of relatively similar sizes. This models the important properties in the physiological adaptation of plants to varying environments and hence a selective role underlying the conservation of oleosin gene expression in seeds. The surfactant and chaperone activity of oleosin as a macromolecule should be relatively independent of ambient temperature over the physiological range compared to a low M_i detergent. The consequence of oleosins as a macromolecular surfactant is that, over the temperature range tolerated by the plant, the OBs formed would be a relatively constant size.

Without Oleosin, Giant OBs Are Formed during Dormancy that Impedes Post-Germination Growth

In the absence of oleosin, the cotyledon cells forming giant OBs appears to be the cause of cellular disruption and cell death. The giant OBs form during and as the result of dormancy and are observed with Nile Red stain. Germination studies have shown that the embryonic axis of the 24-kDa oleosin knockdown seeds appears to be viable but that the plants grow very slowly due to the lack of viability of the cotyledons. The overt appearance of the cotyledons of the 24-kDa oleosin knockdown post-germination seeds is that of a dead tissue. The 24-kDa oleosin knockdown cotyledons do not re-green like the wild-type. Viability stain confirms that the 24-kDa oleosin knockdown cotyledon cells are not viable post germination. Parallel EM observations show that the giant OBs are sequestered within cells whose sub-cellular structure is disrupted. Taken together, these results indicate that as the seed desiccates and enters dormancy, the decreasing water drives the 24-kDa oleosin minus OBs to further aggregation and coalescence, forming the giant OBs that appear to disrupt the cellular integrity of the cotyledon cells. This observation may have some implications on the preservation of long-term seed viability. If OBs can be induced to aggregate and coalesce in storage, especially under less than optimum storage conditions, this would presumably result in similar cellular disruption. As a possible mechanism of loss of seed viability, the potential of OB-mediated cellular disruption has not been investigated. One possible hint that such a mechanism may exist is the increase in oxygenated fatty acids on TAG that occurs in parallel to loss of viability. The oleosin/phospholipid membrane of the OB should function as a barrier to storage-induced changes in the TAG, so an interpretation is that if the TAG is so modified, then this barrier must be disrupted. Such a model is readily testable and the results may contribute to enhance seed storage viability.

Lipoxygenase and Caleosin Are Abundant Proteins of the OB/ER Complex

The 24-kDa oleosin knockdown seeds possess high levels of caleosin and lipoxygenase in the isolated OBs. Lipoxygenases include cytosolic, vacuolar forms and have been shown to associate with OBs using transgene expression using an epitope tag (Hause et al., 2000). The presence of lipoxygenase could indicate that the seed's response to producing the OB/ER complex is to initiate processes normally associated with post-germination TAG mobilization events.

Caleosin possesses a hydrophobic domain similar in structure to the hydrophobic domain of oleosins (Chen et al., 1999; Naested et al., 2000) that would presumably require that the protein be inserted into the TAG core of the OBs in the OB/ER complex. The association of caleosin in the OB/ER complex is particularly interesting because, like lipoxygenase (Hause et al., 2000; Liu et al., 2004), caleosin has been associated with OB/TAG mobilization during post-germination seedling

growth rather than during seed maturation (Poxleitner et al., 2006). That caleosin is associated with post-germination mobilization implies that it should be synthesized during early seedling growth and inserted into the OBs formed during seed maturation. Caleosin possesses a Ca^{+2} -binding domain that presumably is used to mediate some OB-related process by interacting with a Ca^{+2} -regulated protein during OB mobilization. Knockdown of caleosin has been shown to affect OB mobilization, indicating a specific role in TAG mobilization (Poxleitner et al., 2006). This suggests two alternate interpretations of accumulation of caleosin in the 24-kDa oleosin knockdown. Either there is an up-regulation of soybean OB mobilization mechanisms in response to the producing aberrant micro-OBs and the OB-ER complex, or, alternately, caleosin has a function in normal OB formation and the process of producing malformed OBs results in caleosin being synthesized and preserved in the OB/ER complex because of its aberrant structure. Differentiating between these two alternatives and defining the roles of caleosin and lipoxygenase will require further research, currently in progress.

METHODS

Construction of Oleosin RNAi Cassette

Total RNA from 150-mg soybean (*Glycine max* cv. Jack) cotyledons was extracted as described by Cashmore et al. (1978). Residual genomic DNA was degraded by DNase treatment at 37°C and then 2 mg RNA was used in a cDNA reaction using Superscript II (Invitrogen) and primers specific to the 24-kD oleosin open reading frame. Primer pairs 5'-Ole-XbaI/XhoI (5'-TTCTAGACTCGAGCGTGGCCGGGTTCTGAC-3') and 3'-Ole-HindIII/Spel (5'-TAAGCTTACTAGTCATGCGGTTGCGGTTGTT-3'), including restriction sites XbaI, XhoI (underlined and bold, respectively) on the 5' primer located at 928–946 bp, and HindIII and Spel (underlined and bold, respectively) on the 3' primer located at 126–285 bp were used to amplify a 357-bp region of the 24-kD oleosin gene (oleosin isoform A; Genbank accession U09118). RT-PCR reaction conditions were: 50 ng cDNA, 1X Vent DNA polymerase buffer, 2U VENT polymerase (New England Biolabs), 250 nM of each dNTP and 100 mM of each primer with PCR conditions of an initial denaturation (94°C, 2 min), and 45 amplification cycles (94°C, 35 s; 55°C, 40 s; 72°C, 80 s) followed by a final elongation step (72°C, 5 min) (PTC-225 MJ Research). The 357-bp product from the oleosin gene was gel excised (Qiagen) and cloned into TOPO™ vector (Invitrogen), this vector hereby referred to as pOLE. The cloned oleosin fragment was sequenced by using M13 universal primers, Big Dye ver. 3.1 (Applied Biosystems) with cycling parameters 94°C, 30 s; 50°C, 15 s; 60°C, 90 s, for 34 cycles. The resultant amplification products were cleaned by SigmaSpin™ post reaction clean-up columns (Sigma) dried in a speed vacuum and analyzed (Washington University, St Louis, MO). For the RNAi cassette, the intron from pKannibal (Wesley et al., 2001) was amplified using the primer pair: 5' primer (5'-GGTACCGCGGCCGCTCGAGTTACTAGTACCCCAATTGGTAA-

GGAAAT-3') containing NotI (italic), XhoI (bold), and Spel (underlined) and 3' primer; (5'-TTAGAGCTGCGGCCGATTAA-GCAGGACTCTAGAGGAAGCTTCC-3') containing NotI (italic), XbaI (bold) and HindIII (underlined). The amplification product of the intron was cloned into TOPO™ vector, as outlined above, to create pHan-intron plasmid. Two 357-bp fragments were excised from the pOLE vector with two separate restriction enzyme reactions: XhoI/Spe and XbaI/HindIII. The pOLE-XhoI/Spe fragment was first inserted into the new restriction sites at the 5' end of pKan-intron. This plasmid is named pHan-intron-OLE1. After the insertion of the first arm of the RNAi inverted repeat was confirmed through restriction digests, the second arm pOLE-XbaI/HindIII was inserted into pHan-intron-OLE1, thereby generating the cassette containing a 357-bp oleosin hairpin flanking the modified pKan-intron; this vector is hereby named phairpinOLE. This cassette was then digested with NotI to move the entire oleosin hairpin under the control of regulatory elements for the oleosin 24-kD isoform A promoter and terminator (Rowley and Herman, 1997). Correct orientation of the hairpin between the regulatory elements was confirmed by sequencing using an oleosin promoter primer (5'-TATGATGAAAAATACCACCAACACC-3') (Rowley and Herman, 1987, Genbank accession U09118) and the above sequencing parameters. The vector now containing the hairpin for oleosin, under the regulatory control of the oleosin promoter, also contains the hygromycin resistance gene (kindly provided by N. Murai, Louisiana State University) under the control of potato ubiquitin 3 promoter and terminator (Garbarino and Belknap, 1994); this vector is hereby referred to as pRNAiOLE (Figure 1).

Transgenic Soybean

Soybean (*Glycine max* (L.) Merrill cv. Jack) somatic embryos were initiated and transformed as in Trick et al. (1997) and regenerated into fertile plants as in Schmidt et al. (2004). Cotyledonary embryos were screened by immunoblot analysis for the suppression of oleosin 24-kDa and only transgenic lines with the desired phenotype were regenerated into plants.

Immunoblotting of Oleosin 24-kDa Suppressed Seeds

Seed protein extracts were analyzed by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) immunoblot. Total soluble protein was extracted from chips from soybean seeds by a Tris-EDTA, pH 7.4, 0.1% (w/v) SDS extraction buffer (Sambrook et al., 1999) and denatured at 95°C for 5 min. After a brief centrifugation to pellet cell debris, protein were dissolved in sample buffer (50 mM Tris HCl, pH 6.8, 2% (w/v) SDS, 0.7 M β -mercaptoethanol, 0.1% (w/v) bromophenol blue and 10% (v/v) glycerol) (Sambrook et al., 1999) and then denatured at 95°C for 5 min. Protein content was determined by Bradford (1976) assay. 10 mg protein was loaded onto 12% SDS-PAGE gels and electrophoretically separated. Gels were electroblotted to Immobilon-P transfer membranes (Millipore, Bedford, MA). Blots were blocked with a 3% milk solution in TBS for at least

1 h. Primary antibody reaction was performed using a polyclonal antibody specific to the oleosin 24-kD protein (Herman, 1987) in a 1:1000 ratio in TBS buffer for at least 1 h at room temperature. Blots were washed with three buffer washes, each 15 min in duration, prior to the incubation with the secondary antibody (anti-rabbit IgG (Fab specific) alkaline phosphatase conjugate (Sigma)) in a 1:10 000 ratio in TBS buffer for at least 1 h. The presence of the 24-kD oleosin was detected using a color substrate system (BCIP/NBT: final concentrations 0.02% (w/v) 5-bromo-4-chloro-3-indolyl phosphate and 0.03% (w/v) nitro blue tetrazolium in 70% (v/v) demethylformamide) (KPL). Non-transformed soybean seed was used as a positive control.

Two-Dimensional Protein Analysis

Total protein was isolated from mature seeds according to Joseph et al. (2005). The soluble protein extract (150 mg) from both a wild-type soybean seed and a seed determined by immunoblot analysis to be suppressed in the production of oleosin 24-kDa was loaded onto 11-cm immobilized pH gradient (IPG) gel strips (pH 3–10 non-linear) (BioRad, Hercules, CA) and allowed to hydrate overnight. Isoelectric focusing (IEF) was performed for a total of 40 kVh using Protean IEF Cell (BioRad). The IPG strips were equilibrated according to the manufacturer's procedures (BioRad). Second-dimension SDS-PAGE gels (8–16% linear gradient) were run on a Criterion Cell (BioRad) for 15 min at 60 V and then at 200 V for 1 h. Gels were stained in 0.1% (w/v) Coomassie Brilliant Blue R250 in 40% (v/v) methanol, 10% (v/v) acetic acid overnight and subsequently destained for approximately 3 h in 40% methanol, 10% acetic acid.

DNA Array Analysis

Total RNA was extracted from 150 mg cotyledons from both wild-type and 24-kDa oleosin knockdown cotyledons as previously described (Joseph et al., 2005). The total RNA samples were then passed through a spin column from RN-easy kit (Qiagen). Both quality and quantity of the RNA were determined by spectrophotometric readings and then hybridized to Soybean Arrays (Affymetrix) by Iowa State University Service Center. Two separate array experiments were performed; in the first experiment, three technical replicates of RNA from the 24-kDa oleosin knockdown were compared to three wild-type control replicates. In the second experiment, the RNA used in the array experiment was from the biological samples: a non-transformed control and two individual RNAi oleosin cotyledons, with each sample performed in duplicate. The array data were analyzed by ArrayAssist (Stratagene). The array data were deposited (accession numbers to be determined) in the public repository.

Isolation of OB-Associated Proteins and Identification of Proteins by Mass Spectroscopy

OBs were isolated from three mature seeds of wild-type and oleosin knockdown seeds (each seed used was chipped and the 24-kDa oleosin knockdown phenotype individually confirmed by SDS-PAGE immunoblot assay). OBs were isolated

from seeds hydrated for 12 h and wild-type control and 24-kDa oleosin knockdown seeds were ground with a mortar and pestle in 0.1 M Tris HCl, pH 8.6. The resulting lysate was passed through several layers of cheesecloth and subjected to high-speed centrifugation at 25 000 rpm using a Beckman Optima XL-100k ultracentrifuge and SW28 rotor. Centrifugal floatation forms an oil pad on the surface of the lysate that was removed, transferred to ultracentrifuge tubes, and re-suspended in the same buffer. The lysate was then centrifuged at 39 000 rpm for 90 min at 4°C in a SW41 rotor using a Beckman XL100K ultracentrifuge. The oil pad was removed and then washed, re-suspended, and centrifugation repeated. The washed oil pad was then extracted with cold (0°C) acetone to remove TAG and the resulting protein precipitate re-suspended in SDS-PAGE sample buffer. The OB proteins (2 mg) were fractionated by SDS-PAGE using 12% gel and stained with Sypro Ruby™ (Molecular Probes) according to the manufacturer's instructions and photographed under blue light.

Protein bands of interest were manually excised and in-gel digestion of the proteins with a 37°C 10-hr incubation in 50 mM NH_4HCO_3 containing 6 mg ml^{-1} modified trypsin (Promega). Peptides were subsequently extracted by 1% (v/v) formic acid/2% (v/v) acetonitrile and then with 60% acetonitrile. The peptides were dried and re-suspended in 1% (v/v) formic acid/2% (v/v) acetonitrile and then a ZipTip (Millipore) was performed according to the manufacturer's instructions. A QSTAR XL (Applied Biosystems) hybrid quadrupole TOF MS/MS system was used for peptide sequence data acquisition. The peptide electrospray tandem mass spectra were processed using Analyst QS software (ABI) and searched against the NCBI EST database using Mascot (ver. 1.9) with the following parameters: oxidation of methionine and carbamidomethylation of cysteine. Positive identifications were determined by considering the following parameters: (1) number of peptide sequences; (2) protein sequence coverage; (3) total Mascot score; (4) quality of fragmentation data whereby a positive is considered to have at least six consecutive amino acids in the full-length y-ion series.

Light Microscopy Analysis

Tissue from both non-transformed and oleosin knockdown 150-mg cotyledons were simultaneously tested by immunoblot analysis for the 24-kDa oleosin knockdown phenotype and fixed in 4% (v/v) formaldehyde in 0.05 M phosphate buffer, pH 7.4. Visualization of OBs was performed using Nile Red stain (Polysciences) with an excitation of 543 nm and a 565–615-nm filter, and, for contrasting perspectives, calcofluor (Polysciences), a cell wall stain, was used at excitation 710 nm and a 435–485-nm filter. Tissue from both wild-type control and 24-kDa oleosin knockdown seeds was tested for viability by using staining with fluorescence diacetate (Polysciences) with an excitation of 488 nm and a 500–530nm filter and propidium iodide (Sigma) with an excitation of 543 nm and a 565–645-nm filter simultaneously. Viable cells will appear green with the inclusion of fluorescence diacetate while non-viable cells will

be red due to propidium iodide inclusion. All fluorescent micrographs were obtained on a two-photo excitation using a Zeiss LSM 510 microscope.

Transmission Electron Microscopy (TEM)

Conventional TEM material was prepared by cutting cotyledon tissue into approximately 1-mm² cubes and fixing the tissue in 4% (v/v) formaldehyde, 2% (v/v) glutaraldehyde in 0.05 M phosphate buffer pH 7.4. The tissue was then post-fixed with 1% (v/v) OsO₄, dehydrated and embedded in Epon plastic (electron microscopy sciences). Parallel tissue samples were cryofixed with a Balzer's high-pressure device, freeze substituted with acetone/OsO₄ and embedded in Epon plastic. For thin-section TEM, ultra-thin sections were stained with both saturated uranyl acetate and lead citrate (33 mg ml⁻¹) prior to observation. For semi-thick TEM observations, 300–450-nm thick sections were cut from the same material with a diamond histology knife and stained with saturated uranyl acetate. All TEM was performed with a LEO 912AB microscope, with imagery captured using a 2 × 2-k CCD camera operated in montage mode. Visualization of the semi-thick sections was aided by operating the TEM in the energy-filtering mode that improves the resolution of the material.

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