Current Topics

Intracellular Lipid Droplet-Associated Proteins: Unique Members and Their Biological Functions

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Oil-Body-Membrane Proteins and Their Physiological Functions in Plants

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Oilseeds accumulate a large amount of storage lipids, which are used as sources of carbon and energy for seed germination and seedling growth. The storage lipids are accumulated in oil bodies during seed maturation. Oil bodies in seeds are surrounded with three oil-body-membrane protein families, oleosins, caleosins and steroleosins. These proteins are plant-specific and much abundant in seeds. Here we show a unique function of oleosins in preventing fusion of oil bodies and maintaining seed germination. Reverse genetic analysis using oleosin-deficient mutants shows the inverse proportion of oil-body sizes to total oleosin contents. The double mutant *ole1 ole2* with the lowest levels of oleosins has irregularly-enlarged oil bodies throughout the seed cells, and hardly germinates. Germination rates are positively associated with oleosin contents, suggesting that the defects of germination are related to the expansion of oil bodies due to oleosin deficiency. Interestingly, freezing treatment followed by imbibition at 4 °C inhibits seed germination of single mutants (*ole1* and *ole2*), which germinate normally without freezing treatment. The freezing treatment accelerates the fusion of oil bodies and generates eccentric nuclei in *ole1* seeds, which caused seed mortality. Taken together, our findings suggest that oleosins increase the viability of oilseeds by preventing abnormal fusion of oil bodies for overwintering. Knowledge of oleosin contributes a great deal to not only an insight into freezing tolerance of oilseeds, but also creating genetically modified plants for developing a bioenergy and biomass resource.

Key words *Arabidopsis thaliana*; oil body; oleosin; freezing; oilseed

1. INTRODUCTION

Recently, oilseeds crops have been getting a lot of attention as a bioenergy and biomass resource. Oilseeds accumulate a large amount of storage lipids, mainly triglycerides, which are used as sources of carbon and energy for seed germination and subsequent seedling growth. The lipids are stored in a specific organelle, oil body. The oil bodies, which accumulate storage lipids (triglycerides) inside, are usually 0.5 to 2.0 μm in diameter and are surrounded by a phospholipid monolayer overlaid with oil-body-membrane proteins (Fig. 1).[1—5]

A lot of researches have been done for a mechanism of oil-body formation and lipid accumulation. It is thought that the most major oil-body-membrane proteins, oleosins have an important role on the mechanism. Recent studies showed that oleosins had a molecular function as a size-regulator of oil bodies.[6—10]

To investigate a physiological function of oleosins, we generated an oleosin-deficient mutant series of *Arabidopsis thaliana*, one of the oilseed plants. Interestingly, we found that oleosin contents affected germination and freezing tolerance of seeds.[10] These results contribute a great deal to not only an insight into freezing tolerance of oilseeds, which is required for overwintering, but also creating genetically modified plants for developing a bioenergy and biomass resource.

2. OIL-BODY-MEMBRANE PROTEINS OF SEEDS

Confirming lipid-storage system is important for organisms.[11] In plant seeds, three oil-body-membrane-protein families (oleosins, caleosins and steroleosins) were identified

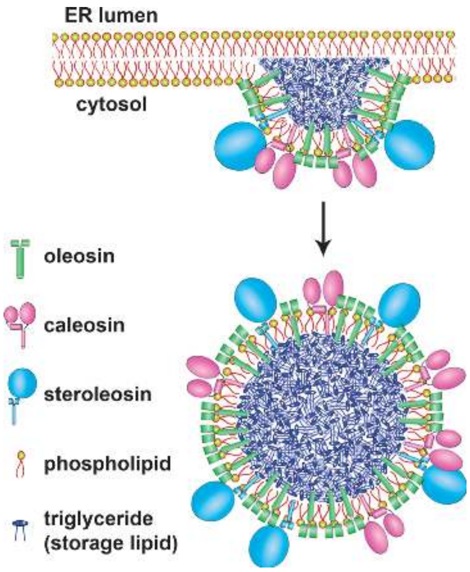

Fig. 1. A Schematic Model of Forming Oil Body during Seed Development and Mature Oil Body Surrounded by Oil-Body Proteins

Oil bodies accumulate triglycerides (storage lipids) inside and is surrounded by a phospholipid monolayer overlaid with oil-body-membrane proteins, oleosins, caleosins and steroleosins. Oil bodies are generated from endoplasmic reticulum (ER) in developing seeds of plants.

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according to proteomics analysis of isolated oil bodies from seeds (Fig. 1).[2,6,12,13] These proteins are plant-specific. The most abundant proteins in oil bodies of seeds are oleosins, which are structural proteins. All oleosins have three domains: a hydrophilic N-terminal domain, a hydrophobic central domain, and a hydrophilic C-terminal domain.[14] The central domain is highly conserved[15] and forms a hairpin-like structure[16] that is composed of two anti-parallel β-strands connected by a proline knot motif. This proline knot motif consists of the amino acid sequence, Pro-5 hydorophobic amino acids-Ser-Pro-3 hydrophobic amino acids-Pro. This structure is inserted into the triglyceride matrix of oil bodies, and enables oleosins to target to oil bodies through endoplasmic reticulum (ER) (Fig. 1).[17,18]

Oleosins are expressed in seeds and floral anther. Seed-type oleosins with molecular masses of 15 to 30 kDa regulate both oil-body sizes and seed germination (discuss below). Anther-type oleosins with molecular masses of 10 to 50 kDa have glycine-rich domains which do not exist in seed-type oleosins.[19] Anther-type oleosins function in stabilizing pollen-oil bodies,[20] and are important for the formation of pollen and pollen coat.[21]

Caleosins have molecular masses about 30 kDa. Caleosins are also composed of a hydrophilic N-terminal domain, a conserved hydrophobic central domain with a proline knot motif, and a hydrophilic C-terminal domain.[22] Unlike oleosin, caleosin has a calcium-binding site, EF-hand motif, in an N-terminal domain[22] and has an enzyme activity as peroxygenase.[23] Both EF-hand motif and calcium are necessary for peroxygenase activity of caleosins.[23] Some caleosin homologues might be involved in defense to environmental stresses, because they are induced in leaves and stems by drought and osmotic stresses.[24]

Steroleosins have molecular masses about 40 kDa and have a dehydrogenase/reductase.[3] At the N-terminus, steroleosin has a proline knob motif that consists of Pro-Pro/Phe/Ile/Leu -10 hydrophobic amino acids-Pro-Pro/Phe. Although steroleosins is supposed to be related to sterol-biosynthesis, their physiological functions are unknown.

3. MOLECULAR FUNCTION OF OLEOSINS

Oleosins have an important role on oil-body formation. Oil bodies surrounded by oleosins have negative charge on their surface (Fig. 1).[25] Because the negative charge causes the steric hindrance and electrical repulsion, oleosins stabilize oil bodies.[15]

Oleosin contents are correlated with the size of oil bodies in several oilseeds.[7,8,26] High-oil seeds of maize have a high ratio of oil to oleosin and produce large oil bodies, while low-oil seeds have a low ratio of oil to oleosin and produce small oil bodies.[8] An avocado mesocarp cell, which contains no oleosins, has only one very large oil body (20 μm in diameter).[27]

Reverse genetic analysis using *Arabidopsis thaliana* revealed that oleosins function as a size-regulator of oil bodies. *Arabidopsis* has 16 oleosins; five seed-type oleosins, eight anther-type oleosins and three seed-and-anther-type oleosins.[28] The most abundant oleosin in seeds is OLEOSIN1 (OLE1/OLEO1, AT4G25140), and OLEOSIN2 (OLE1/OLEO2, AT5G40420) in the second. T-DNA insertion mutants of *OLE1* and *OLE2* are isolated as oleosin deficient mutants *ole1* and *ole2*, respectively.[9,10] And a double mutant *ole1 ole2* is generated by crossing the single mutants.[10]

ole2 seeds have larger oil bodies than wild type seeds, and *ole1* seeds have larger oil bodies than *ole2* seeds (Figs. 2A—C).[9,10] Significant phenotypes are shown in the seeds of double mutant *ole1 ole2*, which has lower content of oleosins than *ole1* and *ole2* (Figs. 2B—D).[10] Oil bodies of *ole1 ole2* are much larger than wild type, *ole1* and *ole2*. This result is consistent with the fact that avocado mesocarp cell, which has no oleosins, forms one very large oil body.[10] These data clearly show the inverse proportion of oil body sizes to total oleosin contents. Lack of oleosins may promote fusion of oil bodies in the cells during seed maturation. In contrast, seeds of oleosin overexpression have smaller oil bodies than wild type.[29] These facts show that oleosin is a size regulator of oil bodies in seeds.

4. PHYSIOLOGICAL FUNCTION OF OLEOSINS

1) Oleosins Have an Important Role in Germination and Freezing Tolerance of Seeds The physiological meaning of oleosins in oilseeds has been clarified recently. Some experiment show that oleosin contents are related to seed germination.[9,10,30] Seed-germination analysis reveals that seeds of *ole1 ole2* are hardly germinated (only *ca.* 10%), al-

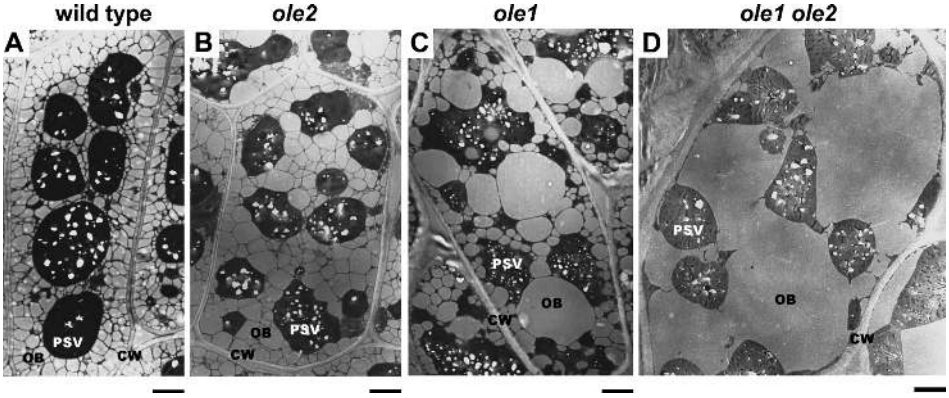

Fig. 2. Seeds of Oleosin Deficient Mutants Have Enlarged Oil Bodies

Electron micrographs of seeds of wild type (A), *ole2* single mutant (B), *ole1* single mutant (C) and *ole1 ole2* double mutants (D). The sizes of oil bodies are in reverse order of the oleosin contents in the mutant seeds. OB, oil body; PSV, protein storage vacuole; CW, cell wall. Bars=2 μm.

though both seeds of *ole1* and *ole2* are normally germinated same as wild type (Figs. 3A, C, E).[10] The defect of *ole1 ole2* germination is related to the extreme expansion of oil bodies. This result suggests that the double mutant *ole1 ole2* significantly exhibits the defect of seed germination, while single mutants and wild type do not (Fig. 4).

Interestingly, storing *ole1* seeds at −30 °C for 1 d (freezing treatment) reduced the germination rate (Fig. 3D).[10] The freezing treatment causes the delay, but not reduction, of germination to *ole2* seeds.[10] None of *ole1 ole2* seeds that were exposed to freezing are germinated (Fig. 3F).[10] In contrast, wild-type seeds that are exposed to freezing are germinated normally (Fig. 3B).[10] These data suggests that seeds with low levels of oleosins are sensitive to freezing stress, and that oleosins provide seeds with freezing tolerance (Fig. 4).

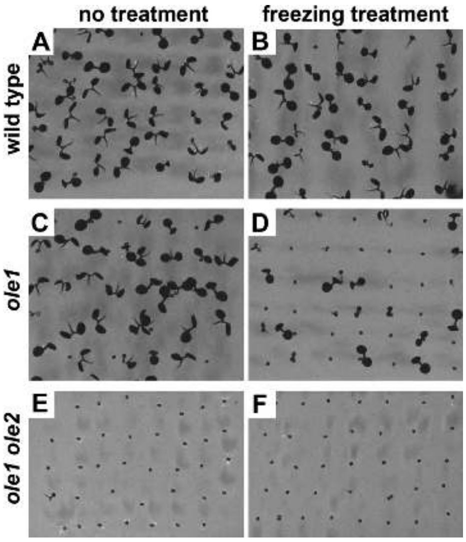

Fig. 3. Germination Rates Are Reduced in Seeds of Oleosin Deficient Mutants

(A, C, E) Seeds of wild type (A), *ole1* (C) and *ole1 ole2* (E) were imbibed at 4 °C for 3 d (stratification) before germination in the absence of sucrose. Photographs were taken at 5 d of germination. (B, D, F) Seeds of wild type (B), *ole1* (D) and *ole1 ole2* (F) were stored at −30 °C for 1 d (freezing treatment) and then were treated with stratification before germination in the absence of sucrose. Photographs were taken at 5 d of germination.

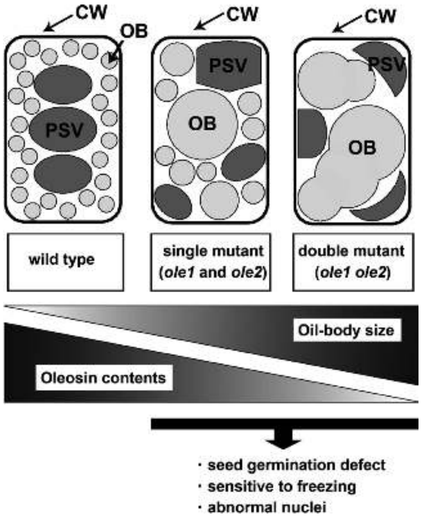

Fig. 4. Effects of Oleosin Contents on Oil-Body Formation and Seed Germination

Wild type, oleosin a single mutant (*ole1* or *ole2*) and a double mutant (*ole1 ole2*) are classified into three groups. Wild-type seeds with the high-level-oleosin content have small oil bodies, and germinate normally. Single mutant seeds with the middle-level-oleosin content have larger oil bodies than wild-type seeds, and are sensitive to freezing. Double mutant seeds with low-level-oleosin content have the largest oil bodies, and hardly germinate. Oleosin deficiency causes irregular formation of nuclei in seed cells. OB, oil body; PSV, protein storage vacuole; CW, cell wall.

The oil bodies of *ole1* seeds treated with freezing are enlarged during imbibition at 4 °C for 3 d (stratification).[10] Freezing treatment results from multiple stresses including cold-stress, mechanical stress, and severe dehydration stress.[31] These stresses damage membrane of plant cells.[32,33] Freezing stress may cause undetectable damage to oleosin-deficient membrane of oil bodies, which causes oil bodies to fuse additionally. The additionally enlarged oil bodies of *ole1* seeds are similar to oil bodies of *ole1 ole2* seeds. We suggest that the additionally fusion of oil bodies cause the germination defect of *ole1* seeds treated with freezing.

These insights of oleosins apply to not only oilseeds but also other plants including starchy seed plants. Rice, which is one of starchy seed plants, is abundant in storage lipids (*ca.* 34%) in the embryo and in the aleurone layer.[34] Rice embryo accumulates a lot of oil bodies and oleosins as do oilseeds.[34—36] It appears that oleosins of starchy seed plants also function as a size-regulator of oil bodies and is responsible for freezing tolerance.

2) Enlarged Oil Bodies Damage Nuclei of Seeds How do the enlarged oil bodies cause the germination defects? One of possibilities is that the enlarged oil bodies are difficult to be degraded and exchanged to energy source. *sdp1* mutant, which lacks a lipase for degradation of storage lipids, dies at the seedling stage in the absence sucrose, while the mutant can grow in the presence of sucrose.[37] Unexpectedly, sucrose supply cannot recover the defects of oleosin mutants.[10] It is suggested that the defects of oleosin mutants are independent of energy source.

Enlarged oil bodies have a harmful effect on subcellular organization of seeds, especially nuclei. Nuclei of wild type dry seeds are spherical and located in the center of the cells, and the shape and location remain stable either after stratification or after freezing followed by stratification.[10] In contrast, *ole1 ole2* seeds have irregularly shaped nuclei on the periphery of the seed cells.[10] Unexpectedly, nuclei of *ole1* dry seeds are spherical and located in the center of the cells same as wild type.[10] However, 4 out of 10 *ole1* seeds treated with freezing followed by stratification have irregularly shaped nuclei on the periphery of the cells, as have *ole1 ole2* seeds.[10] These irregularly shaped nuclei gradually disappear after temperature shift to 22 °C, which may show that seed cells are dead.[10] Our findings suggest that the irregularly shaped nuclei result in the defects of seed germinations and freezing tolerance of seeds (Fig. 4). Abnormal enlargement of oil bodies within cells might give some damage to the nuclei.

3) Prospect for Gain of Function of Oleosins Taken together, we suggest that overexpression of oleosins contribute a great deal to applied science of agriculture by up-regulating freezing tolerance of seeds. Some plants that live in temperate- and cool-temperate-zones survive the harsh winter. The seeds need to acquire resistant systems against freezing. Much effort has been done on cryopreservation, freezing-tolerance and desiccation-tolerance of oilseed crops.[38—40] Clarification of the mechanism underlying the freezing tolerance of oilseeds is important from agricultural and ecological points of view.

In vegetative tissue, there is evidence that cold acclimation is associated with changes in membrane composition, synthesis of proteins having cryoprotective properties, and increases in osmoprotectants, all of which are likely to contribute to an increase in freezing tolerance.[33,41,42] On the other hand, oleosins significantly contribute freezing tolerance of *Arabidopsis* seeds by preventing fusion of oil-body membranes.[10] The result suggests that oil bodies must be densely surrounded with oleosins because abnormal fusion of oil bodies is dangerous for oilseeds. Consequently, seeds of autumn-flowering oilseed plants might need large amounts of oleosins to overwinter. We suggest that oleosins increase the viability of overwintering-oilseeds by preventing abnormal fusion of oil bodies during imbibition in the spring time. Here, we propose oleosin as a key protein to conferring freezing tolerance to oilseeds. We suggest that overexpression of oleosins increases freezing tolerance of seeds, especially freezing-sensitive seeds.

An increase in production of seed-oil contents has been attracting much attention. In *Brassica napus*, seeds with high oil contents accumulate oleosins more highly than those with low oil contents.[30] In *Arabidopsis*, seeds of oleosin deficient mutants have lower oil contents than wild type.[9,10] These results suggest that overexpression of oleosins increases oil contents because oleosins stabilize oil bodies.

5. CONCLUSION

In plant seeds, oleosins keep oil-body size small by preventing fusion of oil bodies. The molecular function contributes healthy germination and freezing tolerance to seeds by maintaining nuclear structure (Fig. 4). Oleosins must be accumulated abundantly as increasing the viability of seeds. We expect that the studies of oleosin give a lot of approaches to creating useful crops.

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