Leaf oil bodies are subcellular factories producing antifungal oxylipins

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Abstract

Oil bodies act as lipid storage compartments in plant cells. In seeds they supply energy for germination and early seedling growth. Oil bodies are also present in the leaves of many vascular plants, but their function in leaves has been poorly understood. Recent studies with oil bodies from senescent *A. thaliana* leaves identified two enzymes, peroxygenase (CLO3) and α-dioxygenase (α-DOX), which together catalyze a coupling reaction to produce an antifungal compound (2-hydroxy-octadecanoic acid) from α-linolenic acid. Leaf oil bodies also have other enzymes including lipoxygenases, phospholipases, and triacylglycerol lipases. Hence, leaf oil bodies might function as intracellular factories to efficiently produce stable compounds via unstable intermediates by concentrating the enzymes and hydrophobic substrates.

Oil bodies

Lipids have an essential role for living organisms. In plants, lipids function as components of biomembranes (phospholipids and phytosterols), phytohormones (jasmonic acid), antifungal compounds (oxylipins), and storage lipids (triacylglycerols). Lipid metabolic pathways are localized in organelles such as endoplasmic reticulum (ER), plastids, peroxisomes, and mitochondria [1]. Some lipids are stored as lipid droplets in the cytosol or plastids. In this review, we define cytosolic lipid droplets as oil bodies, and plastidial lipid droplets as plastoglobules.

Oil bodies are surrounded by a phospholipid monolayer membrane with associated proteins, and contain neutral lipids inside [2]. Oil bodies are found in tissues such as seeds, tapetum in anthers, and leaves [3]. Seed oil-body membrane protein families include oleosins, caleosins, and steroleosins [4]. Oleosins are the major protein family in seed oil bodies, and function to inhibit oil-body fusion [5]. Oleosin action prevents formation of large oil bodies and preserves small oil body sizes. In *Arabidopsis thaliana* oleosin mutants, oil bodies are larger than those in wild type because the oil bodies easily fuse with each other [6,7]. The germination rate of oleosin mutant seeds is lower than that of wild-type seeds, and oleosin mutant seeds are susceptible to freezing [7]. These results suggest that oleosins have an important role in seed germination by preventing oil-body fusion. Some oleosin homologs are expressed in tapetum and are important for pollen formation [8-10].

Oleosin is abundant in seeds and tapetum, whereas oleosin levels are low in leaves [11], although leaf cells have oil bodies [12]. The functions of leaf oil bodies are unclear. However, recent research revealed the protein components of leaf oil bodies and their function. This review will explore leaf oil bodies.

Leaf oil-body-localized proteins

Leaf oil-body proteins have been identified as shown in Figure 1 and Table 1. Caleosins have the calcium-binding domain EF-hand and have a peroxygenase activity [13,14]. The *CALEOSIN3/RESPONSIVE TO DESSICATION20/PEROXYGENASE3* (*CLO3/RD20/PXG3*) gene in *A. thaliana* is expressed in response to abiotic stresses [15]. The CLO3-GFP fusion protein localizes on leaf oil-body membranes [13], indicating that CLO3 is a leaf oil-body protein. *CLO4* is expressed in leaves and the protein localizes on oil bodies [16], showing that CLO4 is a leaf oil-body protein. *A. thaliana* α-dioxygenase1 (α-DOX1) was identified as a

leaf oil-body protein by proteomics analysis of isolated leaf oil bodies [13]. Lipoxygenases [17], phospholipases [18,19], and triacylglycerol lipases [20] are oil-body proteins in seedlings. Lipoxygenases are involved in oil-body lipid metabolism. Phospholipases catalyze the conversion of oil-body membrane phospholipids to free fatty acids. Triacylglycerol lipases catalyze the conversion of oil-body storage lipids to free fatty acids. The *A. thaliana* triacylglycerol-lipase SUGAR-DEPENDENT1 (SDP1) localizes on oil bodies [21].

Caleosins

Caleosins are 20–30 kDa proteins containing a proline-knot motif, which is an oil-body binding domain, and an EF-hand motif, which is a calcium-binding domain [23]. The *A. thaliana* genome encodes at least eight caleosins. *CLO1* (At4g26740) and *CLO2* (At5g55240) are expressed in seed, whereas *CLO3* (At2g33380) and *CLO4* (At1g70670) are expressed in several tissues, including leaf [24]. According to the AtGenExpress database, *CLO5* (At1g23240) is expressed in flower, *CLO6* (At1g70680) is ubiquitously expressed in various organs, and *CLO7* (At1g23250) is expressed in flower and seed, although *CLO8* (At5g29560) has no expression data. CLO1, CLO2 and CLO3 have peroxygenase enzymatic activity [13,14]. *CLO3* is expressed in response to the pathogenic fungus *Colletotrichum higginsianum* [13], abscisic acid, drought, and NaCl [15]. A study of seed-type caleosins reported that caleosins have peroxygenase activity, which catalyzes the oxidation of an unsaturated fatty acid by a hydroperoxy fatty acid to form a hydroxyl fatty acid and epoxy fatty acids [14]. This result suggests that caleosins function as enzymes involved in oxylipin metabolism.

Both CLO3 and α-DOX1 are localized on leaf oil bodies during *C. higginsianum* infection [13]. CLO3 and α-DOX1 cooperatively function to synthesize 2-hydroxy-octadecatrienoic acid (2-HOT) from α-linolenic acid (Figure 2a) [13]. 2-HOT is an oxylipin with antifungal activity against *Colletotrichum*, and 2-HOT levels increase during senescence and *C. higginsianum* infection [13]. These results suggest that 2-HOT is an *A. thaliana* phytoalexin. Leaf oil bodies may function as a scaffold for the production of 2-HOT. In *Nicotiana attenuata*, 2-HOT is required for defense responses against larvae [25,26]. 2-HOT has an additional function in inhibiting cell death [27,28]. Caleosins are conserved in several plants including angiosperms (SALAD Database [29], http://salad.dna.affrc.go.jp/salad/), *Cycas revoluta* [30], *Physcomitrella patens* [31], and

Chlorella species [32]. These results suggest that land plants have an oil-body mediated defense mechanism that utilizes oxylipin production

Analysis of *CLO3* mutant and overexpression lines indicates that CLO3 produces 13-hydroxyoctadecatrienoic acid (13-HOT) and 15,16-epoxy-13-HOT (Figure 2b) [33]. The 13-HOT oxylipin has antimicrobial activity against oomycete (*Phytophthora parasitica*) and fungi (*Cladosporium herbarum*, *Botrytis cinerea*) [34]. It is thought that 13-HOT is produced by caleosins from 13-hydroperoxyoctadecatrienoic acid (13-HPOT), which is produced by 13-lipoxygenase (13-LOX) using α-linolenic acid (Figure 2b). *A. thaliana* has four 13-LOXs (LOX2, LOX3, LOX4 and LOX6) [35] that are expressed in rosette leaves (ATTED-II [22], http://atted.jp/). According to subcellular localization prediction by WoLF PSORT (http://www.genscript.com/psort/wolf_psort.html), LOX3 and LOX4 localize in cytosol. These cytosolic 13-LOXs might be associated with leaf oil bodies together with caleosins to produce the antimicrobial oxylipin 13-HOT.

α-Dioxygenase

α-Dioxygenase adds a hydroperoxy group to a branched chain of fatty acids and synthesizes 2-HPOT from α-linolenic acid (Figure 2a) [36]. 2-HPOT is unstable and easily degrades to the aldehyde [36]. Because both CLO3 and α-DOX1 are localized on leaf oil bodies, they efficiently synthesize 2-HOT utilizing the unstable intermediate 2-HPOT [13]. These results suggest that leaf oil bodies function as subcellular factories to efficiently produce the antifungal oxylipin 2-HOT.

A. thaliana has two α-dioxygenase homologs, α-DOX1 and α-DOX2 [37,38]. α-DOX1 is localized on leaf oil bodies, whereas α-DOX2 is localized on ER [13]. The 2-HOT content in senescent and infected leaves of *α-dox1* mutants is less than that of wild type, whereas the 2-HOT content of *α-dox2* mutants is similar to that of wild type [13]. These results suggest that only α-DOX1 is necessary for 2-HOT production *in vivo*. The fact that α-dioxygenase is localized on oil bodies may be important for 2-HOT production. Defective α-dioxygenase also reduces 2-HOT content in *N. attenuata* [25]. Silencing of α-dioxygenase in *N. attenuata* produces plants that are smaller than wild type [25], whereas no phenotype was observed for *α-dox1 α-dox2* double mutants of *A. thaliana* [39]. *A. thaliana* plants lacking *α-DOX1* enhances susceptibility to green peach aphid (*Myzus persicae*) [40]. These results suggest that α-dioxygenase and 2-HOT have functional diversity among different plant species. The

question regarding whether α-dioxygenases in other plants are localized on leaf oil bodies should be the subject of future work. Infection of the pathogenic fungus *Botrytis cinerea* and treatment with elicitors of the pathogenic bacterium *Pectobacterium carotovorum* induce *α-DOX* in *P. patens* [41], suggesting that *α-DOX*-dependent defense mechanisms are conserved in land plants.

Triacylglycerol lipase

Fatty acids are the substrates of oxylipin production and energy sources. In oil bodies, fatty acids are bound with glycerol to produce triacylglycerol. Triacylglycerol lipases catalyze triacylglycerol metabolism to fatty acids. The triacylglycerol-lipase SDP1 [21], which is localized on oil bodies, is expressed in *A. thaliana* rosette leaves (ATTED-II [22], http://atted.jp/). Triacylglycerol contents in *sdp1* leaves are slightly higher than those of the wild-type leaves, whereas triacylglycerol contents in *sdp1* roots are much higher than those of the wild-type roots [42]. SDP1 may function on leaf oil bodies.

Leaf oil bodies in senescent leaves

Senescent leaves contain more leaf oil bodies than young leaves (Figure 3) [43]. During senescence, *CLO3* and *α-DOX1* expression and the amount of 2-HOT increase [13]. These results suggest that senescence is a key factor in leaf oil-body function. Old membranes and organelles are digested in senescent leaves, and some products of digestion can be translocated to new tissues. Peroxisomes in senescent leaves become specialized for fatty acid beta-oxidation and the glyoxylate cycle [44-46]. It is believed that lipids from old membranes and organelles are sequestered into leaf oil bodies, and then are metabolized by peroxisomes to provide an energy source.

Leaf oil bodies have an important role in defense mechanisms through oxylipin production, suggesting that senescent leaves containing abundant leaf oil bodies function as a defensive shield to inhibit pathogen invasion into new or healthy tissues [43]. Leaf oil bodies actively function in oxylipin production and defense response as well as lipid storage (Figure 3). This mechanism may protect the whole plant from pathogens [43].

Sterol ester storage in leaf oil bodies

Leaf oil bodies may have a role in storage of sterol esters. Over-production of sterols in plant cells enhances the formation of oil bodies [47]. Application of squalene, which is a sterol precursor, to the leaf surface elevates the sterol ester content and the number of leaf oil bodies [48]. By contrast, the amount of free sterols after squalene application is similar to that before application [48]. Phospholipid:sterol acyltransferase (PSAT) catalyzes the transfer of phospholipid fatty acyl groups to sterols [48]. Treatment of the *A. thaliana psat1* mutant with squalene enhances leaf senescence but has no effect on the number of oil bodies [48]. These results suggest that excess sterols (which are toxic to plant cells) may be converted to sterol esters by PSAT and stored in leaf oil bodies (Figure 1). In the *A. thaliana psat1* mutant, irregular cell death occurs in response to infection from the oomycete *Phytophthora infestans* [49]. Sterol storage in leaf oil bodies may have an important function in defense responses.

Conclusion

Plants produce bioactive lipids (oxylipins), usually in chloroplasts, to defend against pathogens and herbivores. A recent study provided evidence of bioactive compound production on leaf oil bodies of plants [13]. Plants have leaf oil body-mediated defense. Leaf oil bodies have an important role in plant defense by producing antimicrobial oxylipins [13,33,34], one of which is the stable compound 2-HOT with antifungal activity against *Colletotrichum*, a fungus that causes significant damage to crops [43]. Senescent leaves contain a massive amount of leaf oil bodies. Leaf oil bodies produce 2-HOT in dying cells to accumulate high levels of the stable antifungal compound in dead cells, which could otherwise provide a source of fungal proliferation. Abscised leaves accumulating a large amount of antimicrobial compounds might prevent pathogenic fungi from replicating then spreading into healthy and young tissues. Considering the fact that leaf oil bodies and genes similar to *CLO3* and *α-DOX1* are widely found in various land plants, plants might have evolved the leaf oil body-mediated phytoalexin production for defense against fungal proliferation.

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- • • of outstanding interest

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Figure Legends

Figure 1. A schematic view of a leaf oil body and associated proteins. Enzyme families localized on leaf oil bodies and their substrates and products. PSAT, phospholipid:sterol acyltransferase; 2-HOT, 2-hydroxy-octadecatrienoic acid; 13-HOT, 13-hydroxy-octadecatrienoic acid.

Figure 2. Lipid metabolic pathways of leaf oil bodies. **(a)** Two leaf oil-body proteins, caleosin and α-dioxygenase, cooperatively produce 2-HOT from α-linolenic acid. 2-HPOT, 2-hydroperoxy-octadecatrienoic acid. Fatty acid epoxides are possible products. **(b)** A biosynthetic pathway to produce 13-HOT from α-linolenic acid.

Figure 3. Induction of leaf oil bodies during senescence. Leaf oil bodies are few in green leaves and then are induced in senescent leaves. Leaf oil bodies actively function in oxylipin biosynthesis and defense response, although seed oil bodies function in lipid storage.

Figure in original format

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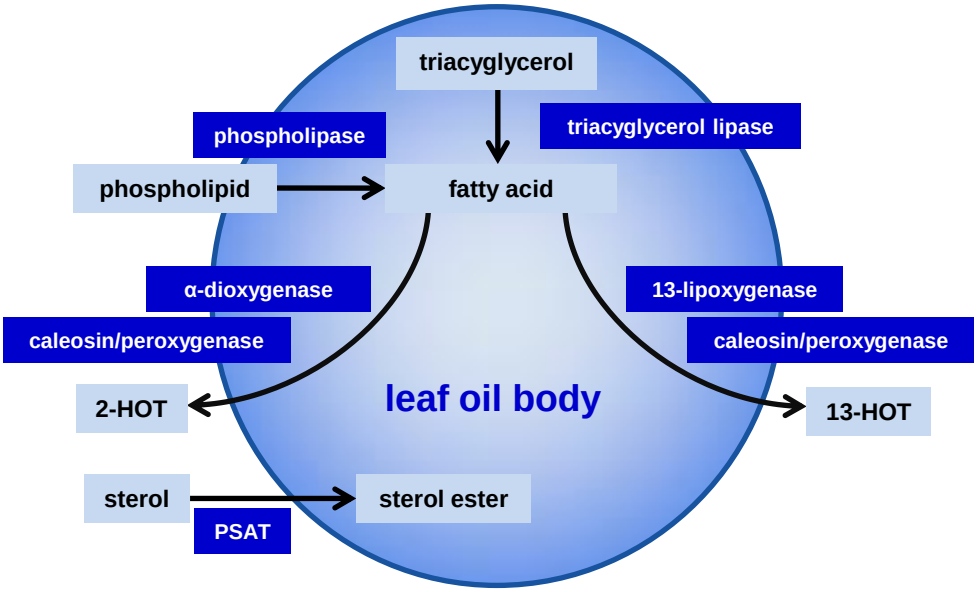

Figure 1

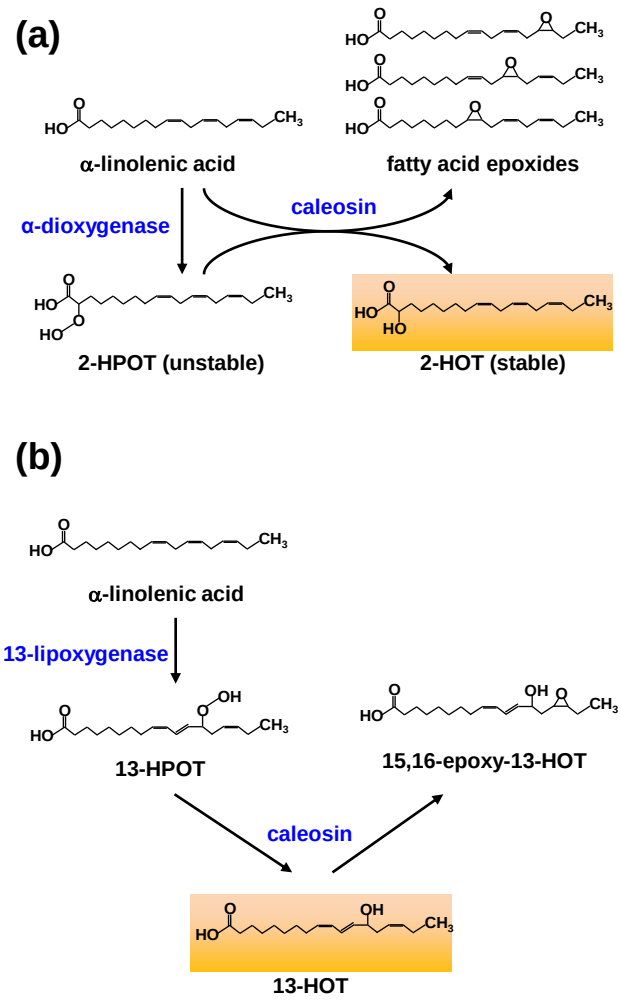
(a)
α-linolenic acid
fatty acid epoxides
α-dioxygenase
caleosin
2-HPOT (unstable)
2-HOT (stable)
(b)
α-linolenic acid
13-lipoxygenase
13-HPOT
15,16-epoxy-13-HOT
caleosin
13-HOT

Figure 2

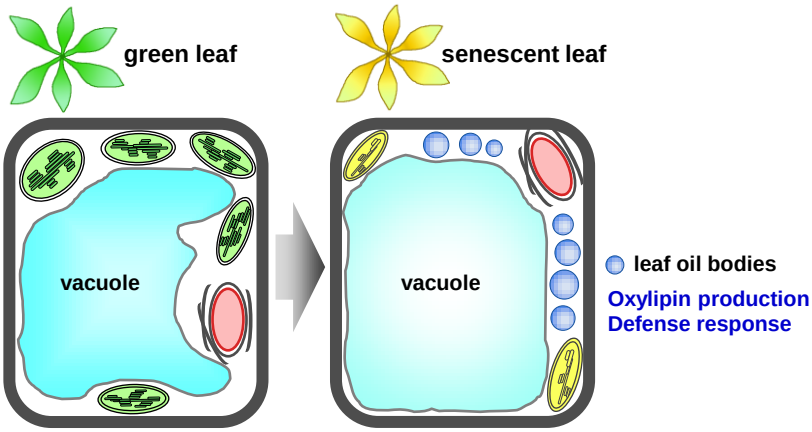
green leaf
senescent leaf
vacuole
vacuole
leaf oil bodies
Oxylipin production
Defense response

Figure 3

Table1 | Leaf oil-body proteins

Protein	Enzymatic activity	Species	Accession	Reference
CLO3/RD20/PXG3	peroxygenase	*Arabidopsis thaliana*	At2g33380	[13, 15, 24, 33]
CLO4	peroxygenase[2]	*Arabidopsis thaliana*	At1g70670	[16, 24]
CLO6	peroxygenase[2]	*Arabidopsis thaliana*	At1g70680	[24]
α-DOX1	α-dioxyganase	*Arabidopsis thaliana*	At3g01420	[13, 36, 37, 38]
SDP1	triacylglycerol-lipase	*Arabidopsis thaliana*	At5g04040	[21, 42]
patatin-like protein[1]	phospholipase A_2	*Cucumis sativus*	Y12793	[18, 19]

[1]induced in seedling, [2]possible roles