Lipid Droplets in plants: more than a simple fat storage

Damien Le Moigne, Nolwenn Guéguen and Juliette Salvaing*

Laboratoire de Physiologie Cellulaire et Végétale, CEA, INRAE, CNRS, IRIG, CEA Grenoble, Université Grenoble Alpes, Grenoble, France

*juliette.salvaing@cea.fr

Abstract

Lipid droplets (LDs) are found in all lineages of living organisms, whether prokaryotes or eukaryotes. Their structure is rather simple with a core of hydrophobic molecules, mostly Triacylglycerols and Sterol Esters, surrounded by a monolayer of polar lipids with associated proteins. They are constitutively present in storage organs, such as seeds in plants, or adipose tissue in mammals, while they can form in response to various abiotic or biotic stresses in other plant organs and in microalgae. Their biogenesis has been mostly studied in yeast and animals, yet remains poorly understood in plants. In particular, the central role of the photosynthetic plastid in lipid metabolism raises questions about its role in LD biogenesis. Over the years, many functions of LDs have emerged in addition to the obvious role in carbon and energy storage. While some functions appear broadly distributed among organisms and organs, other appear specific to certain tissues and/or lineages. The present review aims at highlighting specific aspects of LD biogenesis and functions in all photosynthetic organisms, from the historic model plant *Arabidopsis thaliana* to emerging secondary endosymbiont models such as the diatom *Phaeodactylum tricornutum*.

Key words: Lipid Droplets, biotic and abiotic stress, energy storage, lipid remodeling, plants, algae, secondary endosymbiosis

Running title : Lipid Droplet roles in plants

1 Introduction

Lipid Droplets are intracellular lipid inclusions that can be found in very different organisms, and are present in almost all clades composing the tree of life, from Archaea and Bacteria to eukaryotic cells (Lundquist et al., 2020; Lupette & Maréchal, 2020; Murphy, 2012). These structures have been observed and described for the first time in the late 19th century (Altmann, 1894) and were originally called microsomes or liposomes, but they also have been referred to as lipid bodies, lipid droplets, adiposomes, granules, oleosomes or oil bodies (Murphy, 2012), depending on the research field, or on the considered organisms or tissues. We will adopt the increasingly used term Lipid Droplets (LDs) in the present review. LDs can be categorized in three different classes depending on their subcellular location (Lundquist et al., 2020). « Cytoplasmic LDs » are present in eukaryote's cytoplasms, « Prokaryotic LDs » in prokaryote's cytoplasms and Plastid LDs, also called plastoglobules, in the plastids of photosynthetic eukaryotes. We will restrict ourselves to the study of Cytoplasmic LDs in eukaryotic photosynthetic organisms, while plastoglobules are discussed in Chapter III.

Photosynthetic organisms are characterized by the presence of a plastid, which can derive from a primary symbiotic event (Archaeplastida that regroup land plants, green, blue and red algae) or from a secondary endosymbiosis event (Guéguen et al., 2021; Petroutsos et al., 2014) **(Figure 1)**. In pluricellular organisms, LDs can be found in various tissues. This great variety is reflected in the composition, biogenesis and main roles of LDs. Many recent reviews (Chapman et al., 2019; Guéguen et al., 2021; Ischebeck et al., 2020; Leyland, et al., 2020a) have explored the composition and biogenesis pathways of LDs in plants and/or microalgae, of which we give a rapid overview before discussing more extensively the functions of LDs in these organisms.

2 Lipid Droplets composition and biogenesis

LDs contain a hydrophobic core composed of neutral lipids such as Triacylglycerols (TAGs) and Steryl Esters (SEs) and surrounded by a glycerolipid monolayer surface with associated or embedded

proteins, which most often play structural or metabolic roles but can have other functions as we will discuss later (Huang, 2018) (**Figure 2**).

2.1 Lipid Droplet biogenesis

LD biogenesis is dependent on and starts with the core lipids synthesis. TAG synthesis takes place between the two leaflets of the ER membrane (Walther et al., 2017). They can be synthetized *de novo* by the Kennedy pathway, which is conserved across eukaryotes (Chapman & Ohlrogge, 2012). Briefly, fatty acids (FA) synthetized in the plastid are transported to the ER, where they are esterified with glycerol-3-phospate. Glycerol-3-phosphate acyl transferases (GPATs) catalyse the first esterification on position sn-1 to form lysophosphatidic acid (LPA), then lysophosphatidic acid acyl transferases (LPATs) esterify a second fatty acid on position sn-2 to generate phosphatidic acid (PA). PA's polar head can be further modified to form other phospholipids such as phosphatidylglycerol (PG) or removed by phosphatidic acid phosphatases (PAPs) to generate diacylglycerol (DAG), the latter serving as a platform for most glycerolipids synthesis. DAG can also be generated by recycling of other glycerolipids through different pathways that are presented on **Figure 3**. The last reaction to form TAG is then catalysed by Diacylglycerol Acyl Transferases (DGATs), which esterify a third fatty acid on the sn-3 position of DAG. These three successive acyl esterifications are using acyl-CoAs as donor substrates. Alternatively, TAG can be formed directly by transferring a fatty acid from the sn-2 position of a donor glycerolipid (generally Phosphatidylcholine (PC)) to the sn-3 position of DAG, a reaction catalyzed by Phospholipid:Diacylglycerol Acyltransferases (PDATs) **(Figure 3)**. Other core lipids include Sterol Esters (SEs) or wax esters (Baud, 2018; Chapman & Ohlrogge, 2012; Ferrer et al., 2017) and are also synthesised in the ER membrane of plant cells (Ischebeck et al., 2020).

When TAG and/or SE reach a certain concentration, they condense into lenses at specific nucleation sites (Choudhary et al., 2015; Thiam & Forêt, 2016). LD nucleation is favoured at points showing higher membrane curvature, especially in ER tubules, and can be modulated by several proteins, in particular Seipin (Santinho et al., 2020) that can trap TAG and DAG, according to *in silico* molecular

simulations (Prasanna et al., 2021; Zoni et al., 2021). In yeast, Seipin can associate with PEX30, a protein involved in peroxisome biogenesis, thus defining common nucleation sites for LD and peroxisome (Joshi et al., 2018; Wang et al., 2018). In mammals, Multiple C2 domain containing transmembrane protein 2 (MCTP2) plays a role similar to that of PEX30 (Joshi et al., 2018, 2021). In addition, Seipin can be recruited by LDAF1/Promethin, thus forming a complex that promotes LD formation; LDAF1/Promethin later dissociates from Seipin and covers the surface of LDs (Castro et al., 2019; Chung et al., 2019). Seipin function in LD biogenesis is conserved in plants (Cai et al., 2015) and algae (Lu 2017), although differences are observed. The three Seipin homologs identified in *Arabidopsis thaliana* have overlapping but not redundant functions (Cai et al., 2015; Taurino et al., 2018). Seipin 2 and 3, but not Seipin 1, can associate with the vesicle-associated membrane protein (VAMP)-associated protein VAP27, an interaction crucial for LD formation (Greer et al., 2020). LDAF1/Promethin homologs, as well have those of FIT2, another important protein in yeast and animals LD biogenesis (Choudhary et al., 2015), have not been identified thus far in plants. However, some specific plant proteins are involved in LD biogenesis such as Lipid Droplet Associated Proteins (LDAPs) (Gidda et al., 2016) and the associated LDAP-interacting protein (LDIP) (Pyc et al., 2017), and a recent study has shown that LDIP interacts with Seipin proteins and that this interaction has functional similarities with the Seipin/LDAF1 interaction (Pyc et al., 2021).

The monolayer surrounding LDs likely reflects the membrane composition of the compartment where LD biogenesis occurs. In plants, as well as in non-photosynthetic organisms, it mostly contains phospholipids (Tzen *et al.*, 1993; Tauchi-Sato *et al.*, 2002). However, other classes of lipids have been identified in microalgae. Upon nitrogen starvation, Sulfoquinovosyldiacylglycerol (SQDG) is found in the LDs of the green alga *Chlamydomonas rheinardtii* (Goold et al., 2016; C. H. Tsai et al., 2015) and the diatom *Phaeodactylum tricornutum* (Lupette et al., 2019), along with a betaine lipid, respectively 1,2- diacylglyceryl-3-O-4'-(N,N,N-trimethyl)-homoserine (DGTS) and 1,2- diacylglyceryl-3-O-2'-(hydroxymethyl)-(N,N,N-trimethyl)-β-alanine (DGTA). Mono- and digalactosylglycerol (MGDG and DGDG) are also found in the LD monolayer of *Chlamydomonas,* especially when algae are submitted

to high light stress (Goold et al., 2016). The simultaneous presence of ER lipids (betaine and phospholipids) and plastid lipids (SQDG, MGDG and DGDG) underlines a possible cooperation between the ER and plastid for LD biogenesis in microalgae. Electronic microscopy observations (Flori et al., 2016; Goodson et al., 2011; Jaussaud et al., 2020; Lupette et al., 2019) comfort this hypothesis, showing tight associations between the plastid and LDs. Interestingly, the role of the chloroplast in LD biogenesis could be conserved in the green tissues of higher plants. Indeed, older studies have shown an association of DGAT with chloroplast envelopes in spinach and *Arabisopsis* leaves (Kaup et al., 2002; Martin & Wilson, 1984), while contacts between LDs and chloroplasts were observed by electron microscopy in aging leaves (Brocard et al., 2017).

Altogether, the specificities of the proteins involved in LD biogenesis, as well as the increasingly obvious involvement of plastids, underline the interest in further exploring this process in plants, as well as in microalgae where the diversity of the plastid origins and architectures (cf. **Figure 1**) raises even more questions.

2.2 Lipid Droplet associated proteins

Proteins are an essential component of LDs and can be associated with LDs in different ways. Class I proteins are transferred from the RE to the LD and contain a hydrophobic domain forming a hairpin that anchors the protein in the LD (class I). Class II proteins are transferred to the LD surface from the cytosol. Some contain an amphipathic helix that associates with the LD surface while others interact more loosely with LDs through association either with other LD proteins or following post-translational modifications that add a lipid anchor, such as farsenylation (Bersuker & Olzmann, 2017; Dhiman et al., 2020; Olzmann & Carvalho, 2019) (**Figure 2A**). The most abundant proteins at the surface of LDs are often structural proteins, although they can have other functions as will be exposed later in the review. Land plants have four major families of LD proteins, with different tissue specificities and different associated roles (Huang, 2018) (**Figure 2C and D**). Oleosins (Huang, 1992) are the major LD proteins in plant seeds and pollen, but are absent from leaves LDs, which are

instead covered with LDAPs (Gidda et al., 2016; Horn et al., 2013). Oleosins are involved in the regulation of LD size, but also in TAG biosynthesis (Parthibane et al., 2012). The other two families, Caleosins (Næsted et al., 2000), and Steroleosins (Lin et al., 2002), are found in all plant tissues. Oleosins, caleosins and LDAPs homologs could be identified in green algae (Huang, 2018), but the most abundant LD protein in *Chlamydomonas reinhardtii* is the Major Lipid Droplet Protein (Moellering & Benning, 2010; Nguyen et al., 2011). Finally, the major LD proteins in secondary endosymbionts show little conservation: in the diatom *Phaeodactylum tricornutum,* the most abundant LD protein is Stramenopile Lipid Droplet Protein (StLDP) (Yoneda et al., 2016), while it is the Lipid Droplet Surface Protein in other stramenopiles belonging to the *Nannochloropsis* and *Microchloropsis* genera (Vieler et al., 2012). However, another abundant LD protein in *Phaeodactylum* called PtLDP1 (Lipid Droplet Protein 1) (Wang et al., 2017) is highly similar to the DOAP1 protein (diatom oleosome-associated protein 1) from another diatom, *Fistulifera sp.* (Guéguen et al., 2021; Nojima et al., 2013) (**Figure 2B**).

Thus, major LD proteins show specificities depending on the considered organism or tissue. Yet, it only gives a glimpse into the proteomic variety found in LDs, which in turns reflects the many roles of this fascinating compartment. Indeed, while energy storage has been the first identified role of LDs, many more have since emerged.

3 Functions for lipid droplets in normal conditions and abiotic stress

3.1 Lipid droplets as a source of carbon

In plant seeds, TAGs sored in LDs constitute a reservoir of energy and carbon skeletons to sustain germination and post-germination growth, when the plantlet cannot depend on photosynthesis (Graham, 2008; Huang, 1992). While energy storage can rely on other molecules, in particular proteins and starch, TAGs are widely used and can be stored in the embryo itself or in the endosperm (Miray et al., 2021). However, we will not distinguish here between the different tissues that compose the seed. LD mobilization can occur in two ways: lipophagy, a specialized form of

autophagy, and lipolysis. A recent study has shown that lipophagy plays a role in LD breakdown during seed germination and post-germination growth in *Ricinus communis* (Han et al., 2020). Yet, whether this is specific to certain species or a more general pathway remains to be determined. The most important LD degradation pathway in seeds is lipolysis (Graham, 2008). It starts by the degradation of Oleosins covering seed LDs by the 26S proteasome (Deruyffelaere et al., 2015, 2018; Kretzschmar et al., 2018). Then, the TAG lipases SUGAR-DEPENDENT1 (SDP1) and SUGAR-DEPENDENT1-LIKE (SDP1L) (Eastmond, 2006; Kelly et al., 2011) catalyze the first step of TAG hydrolysis that ultimately leads to the release of glycerol and free fatty acids. The control of seed LD size, which is dependent on Oleosins (Shimada et al., 2008; Siloto et al., 2006), is crucial to ensure lipolysis efficiency (Shimada et al., 2008). Indeed, smaller LDs have a higher surface/volume ratio that facilitates their degradation. Fatty acids can then be converted to acetyl-CoA by β-oxidation (Goepfert & Poirier, 2007) in specialized peroxisomes called glyoxysomes (Beevers, 1979), and used for gluconeogenesis (Goepfert & Poirier, 2007; Hayashi & Nishimura, 2003; Miray et al., 2021; Quettier et al., 2008). In most albuminous seeds, the TAGs stored in the endosperm are thus converted to sucrose before being transported to the seedling itself (Miray et al., 2021). Alternatively, fatty acids can be reused to build new membrane lipids.

Similarly to what occurs in seeds, the formation of LDs is essential for pollen development as disruption of TAG synthesis leads to pollen abortion (Zhang et al., 2010). Studies on olive pollen have shown that LD mobilization provides energy for pollen germination (Zienkiewicz et al., 2013). Moreover, impairment of fatty acid transport to peroxisome (Footitt et al., 2007), disruption of the TAG lipase OBL1 (Oil body lipase 1) (Muller & Ischebeck, 2018) as well as enlargement of LDs caused by disruption of the three SEIPINs (Taurino et al., 2018), reduce male fertility. However, the amount of TAGs in LDs is likely insufficient to store enough energy and lipid precursors to sustain the pollen tube growth. Moreover, there is a continuous synthesis of TAGs in this organ, raising the hypothesis that LDs essential role lies elsewhere (Ischebeck, 2016; Ischebeck et al., 2020).

In microalgae, LDs accumulate mainly in response to abiotic stress, in particular deprivation of nutrients, which induces growth arrest. While the photosynthetic apparatus and chloroplast lipids eventually get partially degraded, photosynthesis remains active (Abida et al., 2015; Simionato et al., 2013). In this situation, LDs allow the storage of the excess carbon produced, and provide energy and carbon to quickly restart when the conditions get better. In agreement with this, nitrogen resupply following nitrogen starvation leads to a very rapid degradation of TAGs, as well as other storage components such as starch (Mulders et al., 2015; Tsai et al., 2018).

3.2 Lipid droplets, lipid synthesis and lipid remodeling

The function of LDs in lipid membrane remodeling and homeostasis has been well described in yeast (Graef, 2018; Henne et al., 2020). Indeed, in *Saccharomyces cerevisiae*, the KO of the four genes involved in TAG (the *DGAT* ortholog *Dga1p* and the *PDAT* ortholog *Lro1*), and SE (the acyl-CoA:cholesterol O-acyltransferase orthologs *Are1p* and *Are2p*) syntheses severely impairs the yeast ability to respond to stress. Exposure of these mutants to unsaturated free fatty acids (FFA), which are normally taken in and incorporated into TAGs, leads to aberrant membrane formation and ultimately to cell death, suggesting that LD storage of TAGs and SEs acts as a buffer to protect the cell against toxic FFA (Petschnigg et al., 2009). Moreover, growth of these mutants at high temperature in the absence of Inositol leads to severe growth defects and the authors observed a decreased synthesis of membrane phospholipids even in normal growing conditions, suggesting a coordination of membrane lipids and storage lipid syntheses to maintain lipid homeostasis (Gaspar et al., 2011). In agreement with this idea, a more recent study has shown that LD formation and subsequent degradation by microautophagy are crucial for adaptation of yeast cells to lipid imbalance (Vevea et al., 2015). The quadruple TAG/SE synthesis mutants also show macroautophagy defects. This may be linked to general ER homeostasis defects or failure to supply the FA necessary for autophagophore formation (Henne et al., 2020; Velázquez et al., 2016), as it is the case for prospore membrane building during sporulation (Hsu et al., 2017). Finally, a recent study has shown an important function

for *Lro1,* the yeast homolog of *PDAT*, in the homeostasis of the nuclear envelop and localization of the nucleolus during the cell cycle or in response to stress (Barbosa et al., 2019).

3.2.1 Lipid Droplets, lipid synthesis and remodeling in microalgae

LD proteomes from green microalgae such as *Chlamydomonas reinhardtii* (Goold et al., 2016; Moellering & Benning, 2010; Nguyen et al., 2011; Tsai et al., 2015), as well as from secondary endosymbionts such as *Phaeodactylum tricornutum* (Leyland et al., 2020b; Lupette et al., 2019) have shown that their LDs contain high amounts of proteins involved in lipid synthesis. Most can be directly related to TAG biosynthesis (such as enzymes involved in FA synthesis, GPATs, LPATs and DGATs, see Figure 3), while others are involved in different lipid synthesis pathways. Interestingly, the DGTS synthase BTA1 has been identified in the LD proteomes of the green algae *Dunaliella bardawil* (Davidi et al., 2015), *Chromochloris zofingiensis* (X. Wang et al., 2019) and *Chlamydomonas reinhardtii*, in which it is one of the most abundant proteins (Goold et al., 2016; Nguyen et al., 2011; C. H. Tsai et al., 2015). It has been proposed that LD-resident BTA1 synthetizes the DGTS that surrounds *Chlamydomonas* LDs (Goold et al., 2016; Nguyen et al., 2011). However, while DGTA is found in the monolayer surrounding *Phaeodactylum* LDs (Lupette et al., 2019) the putative DGTA synthase has not been identified in the corresponding LD proteomes (Leyland et al., 2020b; Lupette et al., 2019). This could be explained by differences in experimentation time-courses, as LD proteomes from green algae were determined after 24-48h of nitrogen starvation while those of *Phaeodactylum* were established after 7 days. However, the different growth rates of these different algae make the comparison difficult. Alternatively, it could reflect metabolic differences between the considered organisms; DGTA is only a minor lipid in *Phaeodactylum*, while DGTS is major in *Chlamydomonas* where it replaces Phosphatidylcholine (PC).

Several proteins involved in sterol biosynthesis have also been identified in many LD proteomes. This is the case after nitrogen starvation in the previously cited green algae (Davidi et al., 2015; Goold et al., 2016; Nguyen et al., 2011; C. H. Tsai et al., 2015; X. Wang et al., 2019) and in the diatom

Phaeodactylum tricornutum (Leyland et al., 2020b), as well as in *Chlamydomonas* after exposure to high light. One of these proteins, the Sterol methyl-transferase 1, has also been identified in the proteome of *Nicotiana benthamiana* pollen tube LDs, along with cycloartenol synthase, another enzyme of sterol metabolism (Kretzschmar et al., 2018). Interestingly, a function of LDs in sterol metabolism has been shown in yeast (Sorger et al., 2004), but whether a similar function exists in algae or plants remains unknown.

Altogether, the presence of proteins involved in different lipid synthesis pathways in several independent LD proteomes raises questions as to potential roles of LDs in coordinating lipid production pathways in the considered organisms/organs.

In addition to proteins involved in lipid synthesis, proteins involved in membrane remodeling and lipid trafficking have also been found in the previously cited proteomes as well as in another diatom, *Fistulifera solaris* (Nonoyama et al., 2019), along with cytoskeleton and intracellular vesicle trafficking proteins. Several studies have pointed out the function of LDs in lipid remodeling. In the absence of stress, TAG synthesis in *Chlamydomonas reinhardtii* is mainly dependent on PDAT. This enzyme (CrPDAT) can not only use phospholipids but also the plastid lipids MGDG, DGDG and SQDG as fatty acid donors (cf. **Figure 2**). Moreover, reduction of CrPDAT levels results in a slightly slower growth, suggesting that lipid remodeling by this pathway is important for the cell fitness (Yoon et al., 2012). While this study shows that CrPDAT contribution is less important in stress conditions, degradation of glycerolipids is still important to feed TAGs under such situations. The disruption of the lipase PLASTID GALACTOGLYCEROLIPID DEGRADATION1 (PGD1), which hydrolyses MGDG, reduced TAG accumulation under nitrogen starvation (Li et al., 2012a), while other classes of glycerolipids are converted to TAGs under heat stress (Légeret et al., 2016). This recycling of glycerolipids to TAGs is likely conserved as high levels of very long-chain unsaturated fatty acids are found in TAGs in Stramenopiles (reviewed in (Guéguen et al., 2021)) as well as in the Haptophyte *Tisochrysis lutea* (B. Huang et al., 2019) following nitrogen or phosphate deprivation. Finally, study of the green microalga *Parachlorella kessleri* under salt stress has shown that LDs accumulate at the cell periphery and likely

contribute to remodeling the FA composition of the plasmid membrane lipids to modify its permeability (You et al., 2019).

3.2.2 Lipid Droplets and lipid remodeling in the pollen tube

In higher plants, lipid trafficking appears as a major function during pollen formation and later during pollen tube growth. Sporopollenin, a very resistant polymer that covers pollen, is synthetized and deposited from a specific tissue localized in the anther and called the tapetum. Tapetum cells form specific LDs associated with ER membranes, named tapetosomes, as well as specialized plastids filled with plastoglobuli, called elaioplasts. Upon programmed cell death, tapetal cells release the lipids contained in those structures that contribute to the formation of the pollen coat (reviewed by (Ischebeck, 2016)). Pollen germination and pollen tube growth require LD mobility. Indeed, in olive pollen after germination LD are no more found in the grain, but many are present in the pollen tube (K. Zienkiewicz et al., 2010). This localization change of LDs is hindered by the enlarged size of LDs in triple Seipin mutants of *Arabidopsis,* and leads to male fertility defects (Taurino et al., 2018). The continuous TAG synthesis during pollen tube growth is at least partly fed by the recycling of phospholipids. Indeed, mutants deficient for the non-specific phospholipases C2 and C6 (Bose et al., 2021) as well as for DGAT1 and PDAT1 (Bose et al., 2021; Zhang et al., 2010) (cf. **Figure 2**) show reduced TAG synthesis. In his 2016 review, Ischebeck proposed several interesting hypotheses as to the function of LDs in pollen tube, including the involvement of LDs in lipid transport, regulation of lipid synthesis, acyl-editing or adaptation to environmental changes occurring during the pollen tube growth (Ischebeck, 2016). These functions have been known in other organisms for some time, in particular mammals (Murphy, 2012), and may not be restricted to the pollen tube.

3.2.3 Lipid Droplets and lipid remodeling in leaves

In higher plants, TAGs represent a very low percentage of glycerolipids in healthy leaves and very few LDs are observed. However, disruption of TAG degradation leads to ectopic accumulation of LDs in the leaves (James et al., 2010), suggesting that TAG are constantly synthetized and degraded in this

tissue. Later studies have shown that LD abundance varies with the diurnal cycle, and that LDs are the most abundant at the end of the night phase (Gidda et al., 2016). As FA synthesis is promoted by photosynthesis, it seems unlikely that these TAG are synthetized *de novo* and more likely that they derive from recycling of membrane lipids. Mechanisms explaining TAG function in lipid homeostasis and remodeling in higher plants have been proposed in a recent review (Yu et al., 2021). Indeed, the *Arabidopsis* genome encodes three different DGATs and two PDATs with different substrate specificities in terms of acyl chain length and insaturation levels. Therefore, expression levels of the various DGATs may affect the acyl-CoA pool available for other lipid productions, while expression levels of the PDATs may promote recycling of certain PC species, thus modifying membrane compositions. As TAG storage organelles, LDs could just serve as a transitory sink or help to preserve certain fatty acids that are costly to produce or that can be used in case of rapidly changing conditions. Interestingly, a recent paper has enlightened the important role of autophagy for TAG formation and membrane lipid turnover in mature and senescent leaves, highlighting a possible role of LDs for the degradation of damaged organelles and recycling of their lipid contents (Fan et al., 2019).

Indeed, LD formation in leaves is mainly triggered by senescence and abiotic stresses. During senescence, the degradation of membrane lipids and subsequent formation of TAGs may allow the redistribution of FA to other tissues. Consistently, the proteome of LDs in senescent leaves contains several cytoskeleton proteins as well as proteins involved in lipid but also protein transports (Brocard et al., 2017). In addition to the formation of LDs, abiotic stresses trigger changes in the fatty acid composition of glycerolipids, in particular of the plastid lipids MGDG and DGDG, and of PC. These changes vary depending on the type of stress, as does TAG composition (reviewed in (Higashi & Saito, 2019)). Heat, drought and salt stress can induce a very rapid TAG accumulation, in contrast to cold, high light or osmotic stress that require a longer exposure (Mueller et al., 2015). The response to heat stress has been extensively studied and shows that TAG generation comes from the recycling of other lipids rather than from *de novo* synthesis (Higashi & Saito, 2019). Indeed, transcriptomic data

indicate a decrease of fatty acid and chloroplast glycerolipid syntheses (Higashi et al., 2015), while the generated TAGs contain high levels of unsaturated fatty acids, in particular C18:3 and C16:3 (Higashi et al., 2015; Mueller et al., 2015). Analysis of fatty acid desaturation mutants shows that these unsaturated fatty acids are likely recycled from MGDG (Mueller et al., 2017), after the initial removal of C18:3 from MGDG by the Heat-Inducible Lipase 1 (HIL1) (Higashi et al., 2018). The analysis of several TAG synthesis mutants has shown that PDAT1, but not DGAT1, is the major actor in TAG biosynthesis upon heat stress (Mueller 2017). Finally, the thermosensitivity observed in *pdat1* and *hil1* mutants enlightens the importance of MGDG remodeling and of TAG synthesis in this process (Higashi et al., 2018; Mueller et al., 2017). MGDG remodeling also appears essential in tolerance to freezing, albeit for different reasons. Indeed, Moellering and collaborators have identified Sensitive-to-Freezing2 (SFR2) as a galactolipid:galactolipid galactosyltransferase that can transfer galactose from MGDG to another galactolipid, thus depleting the MGDG pool and forming oligogalactolipids and DAGs, the latter being converted into TAGs (cf. **Figure 2**). The authors hypothesized that this reduction of MGDG and consequent increase in DGDG and other oligogalactolipids serve to stabilize membranes following freezing-induced desiccation (Moellering et al., 2010). Consistently, similar changes in galactolipids and TAGs were observed upon drought (Gasulla et al., 2013; Tarazona et al., 2015) and cold (Degenkolbe et al., 2012; Tarazona et al., 2015) stresses, and SFR2 was also associated with resistance to salt and drought stresses in tomato (Wang et al., 2016). More recent studies have shown that in contrast to heat stress, DGAT1 is the essential player in TAG formation upon freezing and is important for freezing tolerance (Arisz et al., 2018; Tan et al., 2018).

3.3 Lipid droplets and cell protection

In addition to their role in membrane lipid remodeling and transitory storage of fatty acids associated with stress conditions, leaf LDs appear essential to cell protection. Indeed, free fatty acids, as well as glycerolipids that cannot assemble into membranes such as PA or DAG, can be toxic for the cell by

disorganizing the complex intracellular membrane network (Li et al., 2020; Petschnigg et al., 2009). Storage into TAG serves as a transient but essential sink before degradation (Fan et al., 2014, 2017).

In addition to TAG and sterol esters, LDs can store other hydrophobic compounds such as carotenoids. In the marine green algae *Dunaliella bardawil* and *salina*, carotenoids are stored in plastoglobuli, while in freshwater green algae such as *Haematococcus pluvialis,* carotenoids are found in cytosolic LDs. In both cases, carotenoid production is induced by reactive oxygen species (ROS) that are generated following stress such as high light or nutrient deprivation, and they can serve both to protect the photosynthetic system against photodamage created by excessive irradiation and to quench the effect of ROS (review (Pick et al., 2019)). Carotenoids have also been found in *Phaeodactylum* LDs but whether they are stored in cytosolic LDs or plastoglobuli is not clear (Lupette et al., 2019).

Finally, several proteins of the ER associated protein degradation (ERAD) pathway, which serve to remove misfolded proteins and target them for degradation by the 26S-proteasome, have been identified in LD proteomes of the diatom *Phaeodactylum tricornutum* after 7 days of nitrogen deprivation (Leyland et al., 2020b; Lupette et al., 2019) as well as in *Arabidopsis* seeds (Deruyffelaere et al., 2018; Kretzschmar et al., 2018), senescent leaves (Fernandez-Santos et al., 2020) and *Nicotiana* pollen (Kretzschmar et al., 2018). Their function in *Arabidopsis* seeds seems to be mainly the degradation of Oleosins and Caleosins preceding the remobilization of oil reserves upon germination (Deruyffelaere et al., 2015, 2018). However, many additional proteins associated with the regulation of protein folding and ubiquitination have been identified in LDs from pollen tubes, leaves and *Phaeodactylum*, suggesting a broader function of lipid droplets in protein processing and catabolism.

3.4 Lipid droplets, a reserve to restart

In addition to proteins associated with lipid metabolism or LD structure that are expected to be present in LDs, much more unexpected proteins are found. In many cases, they are assumed to be

the result of contaminations, and, as such, are little discussed in the literature. Yet, there are lines of evidence suggesting that they should be considered with more attention.

Many of the microalgae LD proteomes performed under nutrient starvation conditions include proteins linked to DNA organization (histones), and regulation of protein translation (ribosomal proteins, translation elongation factors) (Davidi et al., 2015; Goold et al., 2016; Leyland et al., 2020b; Lupette et al., 2019; Moellering & Benning, 2010; Nguyen et al., 2011; Tsai et al., 2015; You et al., 2019). Interestingly, these are generally not found in higher plant seed proteomes.

Upon nutrient starvation, LDs can include all histones or a subset of histones, including core histones (H2A, H2B, H3 and H4), histone variants, as well as the linker histone H1. While histones are abundant proteins, they are mostly contained in the nucleus and are highly hydrophilic proteins, suggesting that their association with LDs in stressed microalgae is unlikely to result from contamination. The association of histones to LDs has been described in the model fly *Drosophila melanogaster*, where LDs serve as histone storage (Cermelli et al., 2006) and regulate histone supply during the early phases of embryonic development (Li et al., 2012b). As nutrient starvation severely impairs microalgae growth, it could be speculated that LDs, similarly to what occurs in *Drosophila*, could serve as a histone deposit during stress, when cell growth is severely impaired, to favor quick restart once the conditions become more favorable. The storage of ribosomal proteins and translation elongation factors could serve the same purpose. Ribosomal proteins are among the most abundant proteins in the cell and their presence in LD proteomes has long been considered as contaminations. Yet, similarly to histones, they are not found in plant seeds proteomes. Moreover, proteins associated with translation processes are the most strongly down-regulated upon nitrogen and phosphorus starvation (Hulatt et al., 2020), and storage in LDs could serve to preserve a minimal pool from degradation. In support of this possible role for LDs, neither histones nor translation-associated proteins are found in the LD proteome of *Chlamydomonas* grown under high light, a condition that triggers LD accumulation but not cell growth arrest (Goold et al., 2016).

Finally, some RNA-binding proteins have been identified in several microalgae proteomes (Lupette et al., 2019; Moellering & Benning, 2010; Nguyen et al., 2011; X. Wang et al., 2019), raising the possibility that LDs could serve to preserve a RNA pool.

3.5 Lipid droplets and brassinosteroid signaling

Together with Oleosins and Caleosins, Steroleosins are one of the three families of proteins associated with LDs in plants. They may be associated with the synthesis of brassinosteroids, which are plant hormones involved in different developmental processes as well as resistance to biotic and abiotic stresses (Divi & Krishna, 2009; Singh & Savaldi-Goldstein, 2015). The first two steroleosins have been identified in *Sesamum indicum* (Chen et al., 1998), and the first identified A*rabidopsis* homolog possesses 11$\beta-$ and 17β-hydroxysteroid dehydrogenase activities and was thus renamed HYDROXYSTEROID DEHYDROGENASE 1 (AtHSD1) (d'Andréa et al., 2007). Initial studies have shown that overexpression of AtHSD1 leads to reduced seed dormancy (Baud et al., 2009; Li et al., 2007) as well as to increased growth and resistance to salt stress (F. Li et al., 2007). How the association of AtHSD1 to LDs modulates its function and contributes to brassinosteroid signaling remain open questions.

4 LD and biotic stress

Interactions between LDs and pathogens have been mostly studied in mammalian cells and have highlighted two antagonist roles for LDs, which can either be used by the pathogen or participate in cell and organism defense.

4.1 Hijacking of the host metabolism

The current state of the art suggests that mammalian LDs can participate in the infection process, providing invaders with substrates for survival and/or growth (Bosch et al., 2020). This phenomenon is described as the "hijacking" of the host metabolism. Bacteria such as *Chlamydia trachomatis* (Kumar et al., 2006), eukaryotic parasites such as *Toxoplasma gondii* and *Neospora caninum* (Hu et

al., 2017), or even viruses such as Hepatitis C Virus (Herker et al., 2010; Miyanari et al., 2007) and Sars-Cov2 (Dias et al., 2020), can induce the accumulation of LDs in the host and use it as a lipid source. During viral infection, this hijacking can go even further. For example, the Hepatitis C Virus (HCV) targets the liver, where LDs are consumed for the production and assembly of new infectious virus particles. In a model proposed by Miyanari *et al,* the core HCV protein binds to the lipid monolayer surrounding the LD, brings ER membranes close to the LD and recruits virus proteins as well as replication complexes to this microenvironment in order to produce new viral particles (Miyanari et al., 2007).

Interestingly, this hijacking of the host metabolism by pathogens also occurs in viral infection of eukaryotic microalgae. Due to high ecological relevance, several studies have taken interest in the infection of Haptophytes, and in particular of the coccolithophore *Emiliana Huxleyi*, by large dsDNA viruses. An initial study showed that infection of *Emiliana* by the lytic virus EhV201, belonging to the Phycodnaviridae, leads to a complete rewiring of the host lipidome (Rosenwasser et al., 2014). An increase in *de novo* fatty acid synthesis proved crucial for viral production. Indeed, lipid biosynthesis reoriented to favor the production of specific glycosphingolipids at the expense of host sphingolipids. These virus-derived glycosphingolipids (vGSL) are central structural components of the virus membrane and can trigger programmed cell death of infected cell (Vardi et al., 2009). Finally, sterols appeared depleted in the host, feeding the new virions (Rosenwasser et al., 2014). Malitsky *et al* (2016) completed this study and showed that Ehv201 can induce specific TAG accumulation and LD formation by upregulating genes involved in *de novo* TAG synthesis (LPAT, PAP, DGAT2) and downregulating genes involved in TAG recycling from phospholipids (such as PDAT). Virus-induced TAG, characterized by a high enrichment in unsaturated and monounsaturated fatty acids, are later found in high amounts in the virions. All those results led the authors to speculate that virus-induced LDs play a crucial role in virus assembly (Malitsky et al., 2016) **(Figure 4, left panel)** as has been described for HCV (Miyanari et al., 2007). Other virus families may share this mechanism. Indeed, a recent study showed that the giant haptophyte-infecting virus, *Prymnesium kappa* virus RF01 (PkV

RF01) belonging to the Mimiviridae family, encodes for many proteins involved in lipid catabolism, among which a TAG lipase and other key enzymes of the β-oxidation pathway. This suggests that this virus is also capable to break down host LDs for its propagation (Blanc-Mathieu et al., 2021).

4.2 Lipid Droplets as defense organelles

While this LD hijacking by pathogens may paint LDs as weak points in the cell, their active role in innate defense have been equally reviewed (Pereira-Dutra et al., 2019). Indeed, in mammals, proteins with anti-pathogen properties such as viperin (Hinson & Cresswell, 2009) and interferon-γ (IFN-γ) inducible guanosine triphosphatase (GTPase) (IGTP (Bougnères et al., 2009; Haldar et al., 2013)) are localized in the LDs of infected cells, and similar roles of LDs have been shown in higher plants.

Several studies regarding LDs in *Arabidopsis thaliana* leaves highlight their function in defense against various pathogens (Brocard et al., 2017; Fernandez-Santos et al., 2020; Shimada et al., 2014; Shimada et al., 2018). Indeed, proteomics have shown that several enzymes involved in production of phytoalexins, compounds that participate in plant defense, are located in LDs. The most prominent example is that of Caleosins (CLO), which are structural components of plant LDs and exhibit a peroxygenase activity. CLO3, which is also known as Responsive to Dehydratation20 (RD20) can be induced by abiotic stresses, notably drought (Aubert et al., 2010) or salt, but also in response to the pathogenic fungus *Colletotrichum higginsianum* (Shimada et al., 2014). Upon infection, the authors have shown that CLO3 colocalizes with α-Dioxygenase 1 (αDOX1) on LDs of perilesional cells of the infected leaf, where they coordinately use α-linolenic acid (C18:3, ω-3) to produce the antifungal oxylipin 2-hydroxy-octadecatrienoic acid (2-HOT). A similar effect is triggered by senescence and the authors propose that phytoalexin-producing LDs that develop in dying cells of perilesional tissues or senescent leaves could serve to form a shield in order to protect the young healthy parts of the plant from infection (Shimada et al., 2014) **(Figure 4, right panel)**. A recent proteomics analysis comparing LDs from leaves infected by the bacterium *Pseudomonas syringae pv.*

Tomato (Pst) with senescent leaves has shown that they both contain enzymes involved in the biosynthesis of the antimicrobial compound camalexin, as well as GPAT4 and GPAT8, which are implicated in cutin biosynthesis (Fernandez-Santos et al., 2020). The overlap between LD proteomes in these two conditions confirms that they likely share common functions in cell defense against pathogens. Finally, the ectopic expression of stilbene synthases from *Vitis pseudoreticulata* (VpSTS29) in *Arabidopsis* shows that these enzymes, which play a critical role in powdery mildew resistance, can relocate from the cytoplasm to cytosolic LDs upon dark-induced senescence (Ma et al., 2018). Intriguingly, stilbene synthase-containing LDs can be found both in the cytoplasm and in the vacuole, where stilbenes seem to be stored, thus implying a trafficking role for LDs, an hypothesis supported by the association of many cytoskeleton proteins with LDs (Brocard et al., 2017; Fernandez-Santos et al., 2020). Finally, AtCPK1, which is a Ca2+ dependent protein kinase involved in the response to fungal pathogens through the salicylic acid-mediated defense pathway, shows a dual localization to peroxisomes and LDs (Coca & Segundo, 2010). However, the function of this localization remains unknown.

Altogether, these results suggest that LDs of higher plants can be seen as production platforms for anti-pathogenic compounds involved in various plant defense mechanisms. However, we could not find in the literature any evidence that LDs play such a role in microalgae. The only potential link identified thus far lies in the antibacterial function of Eicosapentaenoic acid (EPA), a very long chain polyunsaturated fatty acid (VLC-PUFA) that is very abundant in many microalgae (Desbois et al., 2009). However, VLC-PUFA are generally not very abundant in TAGs, and potential anti-pathogenic activities of LDs remain to be investigated in these organisms. This difference of functions, if it exists, may be linked with the multicellular nature of higher plants where an infected part of the plant can signal the danger to the rest of the organism and trigger defenses. It would be therefore very interesting to investigate whether similar signals could exist at the population scale in microalgae. Conversely, the hijacking of LDs has not yet been evidenced in plants even though it exists in

multicellular organisms such as mammals. The function of LDs in defense against pathogens in plants and algae thus retains many mysteries to unravel.

5 Conclusion

In the present review, we have explored the many roles of LDs, far from the simple fat stock that they initially represented **(Figure 5)**. It is not clear whether the same LDs carry all functions or if a cell can contain different types of LDs. Several lines of evidence suggest the latter. Analysis of LDs subpopulation in *Phaeodactylum* during nitrogen stress has shown that there are three waves of LD formation and that they exhibit different behaviours (Jaussaud et al., 2020). The coexistence of several LDs population with distinct function has also been suggested from the analysis of *Arabidopsis* aging leaves (Brocard et al., 2017). Finally, a refined analysis of LD at different stages of seed formation, germination and seedling development has shown many changes in their proteome, which may reflect production of different subsets of LDs (Kretzschmar et al., 2020). Our understanding of LD biogenesis as well as of their functions comes mostly from the study of non photosynthetic organisms, animals and yeast. Yet, while there are many common points, evidences show that this knowledge cannot be simply extrapolated to photosynthetic organisms. The primary characteristic of plants and algae is the presence of a photosynthetic plastid that is central in the overall metabolism and in particular in the metabolism of lipids. Moreover, the evolutionary origin of this plastid can considerably diverge when we start looking at secondary endosymbionts, and its role in LD biogenesis requires further investigations. The study of LD roles enlightens the specificities of the responses to abiotic stresses in one given organism, as well as the differences that can exist between a single cell organism or a pluricellular one, between a primary or secondary endosymbiont, or even between freshwater algae or marine ones **(Figure 5)**. Oleaginous microalgae are of great biotechnological interest, for the production of biofuels or high value-added molecules such as ω3-fatty acids or pigments, like EPA and astaxanthin, respectively. This has considerably increased the interest for these organisms. Unfortunately, many studies solely aim to increase the productivity of compounds of interest, without looking further into the biological or ecological relevance of the

results. Therefore, knowledge on many species remains sparse and few studies compare the effects of different stresses. Yet, increasing our knowledge of LD functions and of their potential adaptive roles would be of great interest in the context of global change we are facing. The combination of genomics, proteomics, lipidomics and microscopy tools that are increasingly powerful provide exciting opportunities for future research.

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Figures and Figure Legends

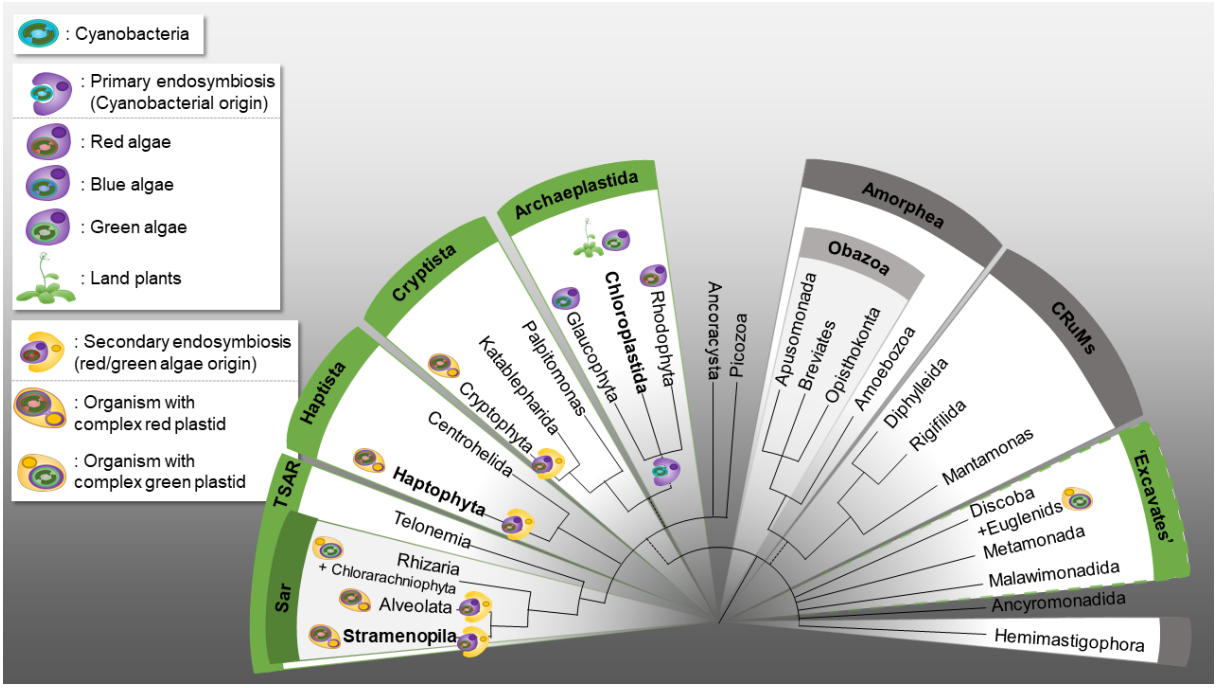


Figure 1: Photosynthetic organisms in the tree of Life

Different endosymbiosis events leading to the formation of photosynthetic organisms are represented on the eukaryotic tree of life (adapted from Burki *et al.,* 2020). Archaeplasida result from a single endosymbiotic event, engulfement of a cyanobacteria by a heterotrophic eukaryote. They are composed of blue algae (Glaucophyta), red algae (Rhodophyta) and Chloroplastida. The latter contains both land plants and green algae, such as *Chlamydomonas rheinardtii, Dunaliella sp.* and *Chromochloris zofigiensis*. Several independent secondary endosymbiosis events involving different heterotrophic eukaryotes and either green or red algae have later taken place and given rise to a great variety of photosynthetic organisms, commonly known as microalgae. Studies cited in the review concern mostly Stramenopila including *Nannochloropsis oceanica* and the diatoms *Fistulifera solaris* and *Phaeodactylum tricornutum*, but also Haptophyta such as the coccolithophore *Emiliana huxleyi*.

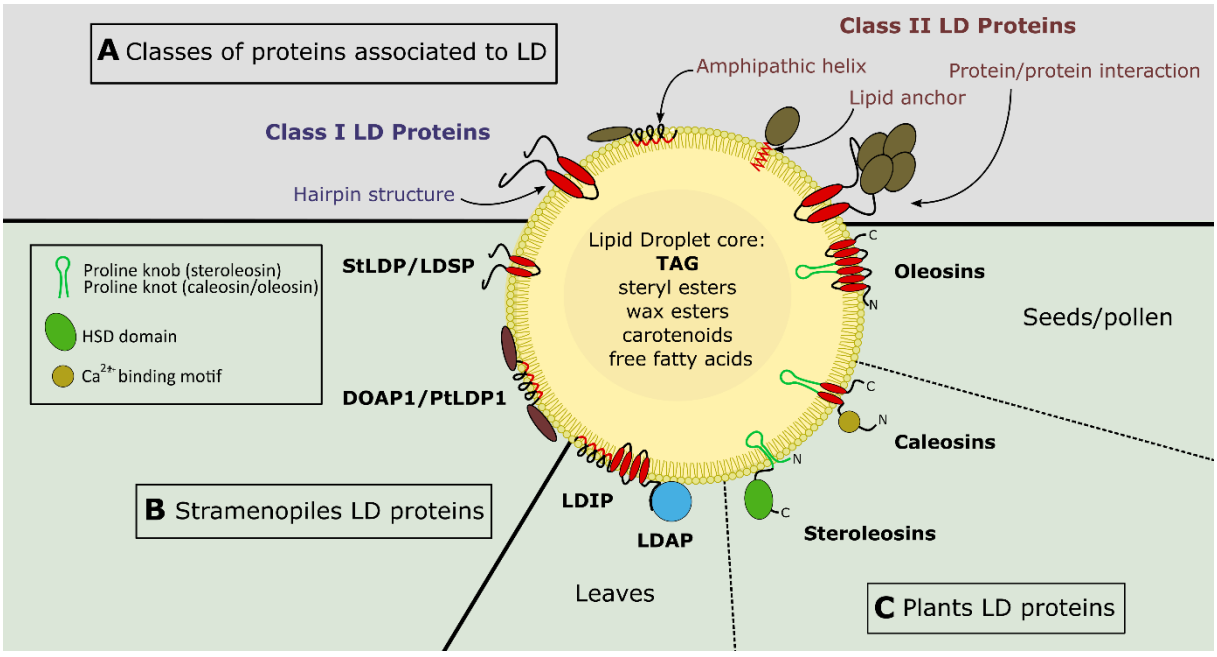


Figure 2: Structure and content of LDs

The LD core is mainly composed of TAGs with very diverse fatty acid compositions, and steryl esters. Other hydrophobic compounds can be found such as wax esters in certain land plants, and carotenoids and free fatty acids in some algae. Different classes of proteins can be found at the surface (A) and LD major proteins differ between organisms/tissues (B and C).

A: The different classes of proteins associated to the LD. Class I LD proteins are initially inserted via an hydrophobic hairpin structure in the ER membrane and are later transferred to the LD. Class II proteins are present in the cytosol and can become associated with the LDs. This association can be mediated by an amphipathic helix, or indirect, through protein-protein interaction or addition of a lipid anchor by post-translational modifications (adapted from (Dhiman et al., 2020)). Hydrophobic regions that mediate interaction with the droplet are indicated in red.

B: Major LD proteins in Stramenopiles. Predictions of major lipid droplet proteins in stramenopiles suggest that StLDP (*Phaeodactylum tricornutum*) and LDSP (*Nannochloropsis s.l.*) are class I proteins, with two hydrophobic α-helixes forming a hairpin. On the other hand, DOAP1 (*Fistulifera solaris*) and PtLDP1 (*Phaeodactylum tricornutum*) are class II proteins; they do not contain a clear hydrophobic region but the central region is predicted to form an amphipathic β-barrel (adapted from (Guéguen et al., 2021)).

C: Major LD proteins in plants. Oleosins are the major and the most abundant proteins of seeds and pollen LD, while Caleosins and Steroleosins are less abundant but found in all plant tissues. Oleosins and Caleosins are class I proteins, strongly anchored in the LD through several hydrophobic β-sheets that form hairpin structures. They also contain a central "Proline knot" that penetrates into the LD core and is essential for targeting the Oleosins to the LD. Steroleosins are anchored to the LD by a "Proline knob" that mainly associates with the LD surrounding monolayer (adapted from (Shao et al., 2019)). Plant leaves LDs are mainly covered by LDAPs and LDIP. LDIP is a class I protein with four transmembrane domains, while LDAPs are class II proteins that associate to the LD surface through their interactions with LDIP (adapted from (Chapman et al., 2019)).

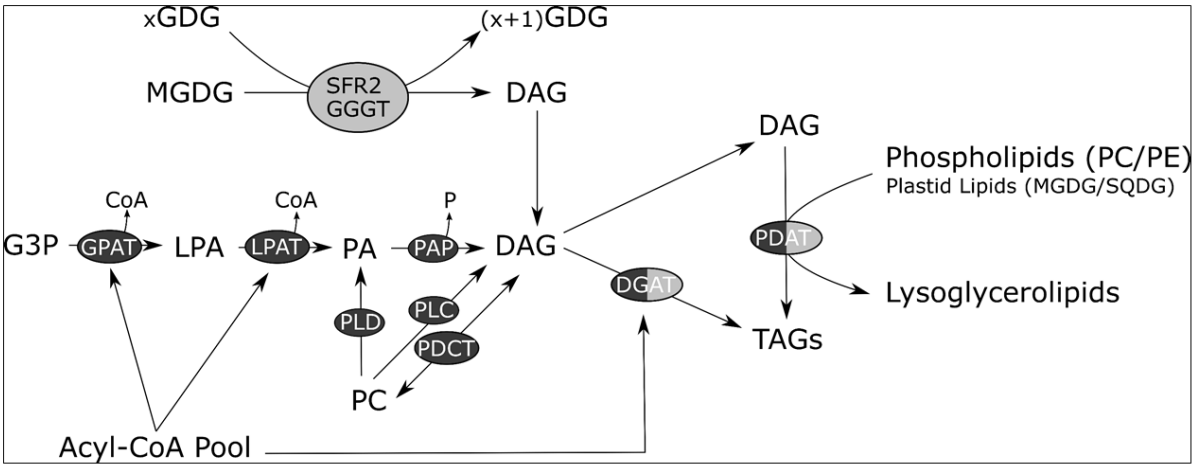


Figure 3: **TAG biosynthesis in photosynthetic organisms.**

Triacylglycerols (TAGs) can be generated either *de novo* or through the recycling of other glycerolipids. *De novo* synthesis starts with Glycerol-3-phosphate (G3P) and leads to diacylglycerol (DAG) formation after esterification of two fatty acids, respectively by a Glycerol-3-phosphate acyl-transferase (GPAT) and a lysophosphatidic acid acyl-transferase (LPAAT), and then dephosphorylation by a phospatidic acid phosphatase (PAP). DAG can also be generated by the recycling of phospholipids, either directly through the action of phospholipase C (PLC), or indirectly through phospholipase D (PLD) which generates PA. Diacylglycerol cholinephosphotransferase (PDCT) transfers the polar head from PC to DAG, thus leading to an exchange of the fatty acids contained in both molecules. Another recycling route involves the transfer of the galactose polar head of monogalactosyl-diacylglycerol (MGDG) to another galactolipid (xGDG) by a Galactolipid Galactolipid Galactosyltransferase, thus generating DAG as well as oligogalactolipids ((x+1)GDG)). The last step of TAG synthesis can involve a Diacylglycerol acyl-transferase (DGAT) or a phospholipid:diacylglycerol acyl-transferase (PDAT) that directly transfers a fatty acid from a glycerolipid to DAG thus generating lyso-glycerolipids and TAG. While most PDATs use phospholipids and, in particular, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the one from *Chlamydomonas*

reinhardtii (CrPDAT) can also use plastid lipids such as MGDG and sulvoquinovosyldiacylglycerol (SQDG) (Yoon *et al.,* 2012). Extra-plastidial enzymes are represented by black ellipses, and plastidial enzymes by light grey ellipses. DGATs and PDATs can have both localization depending on the considered isoform, organism or physiological state. Figure based on Ischebeck *et al.* (2020) and Higashi and Saito (2019).

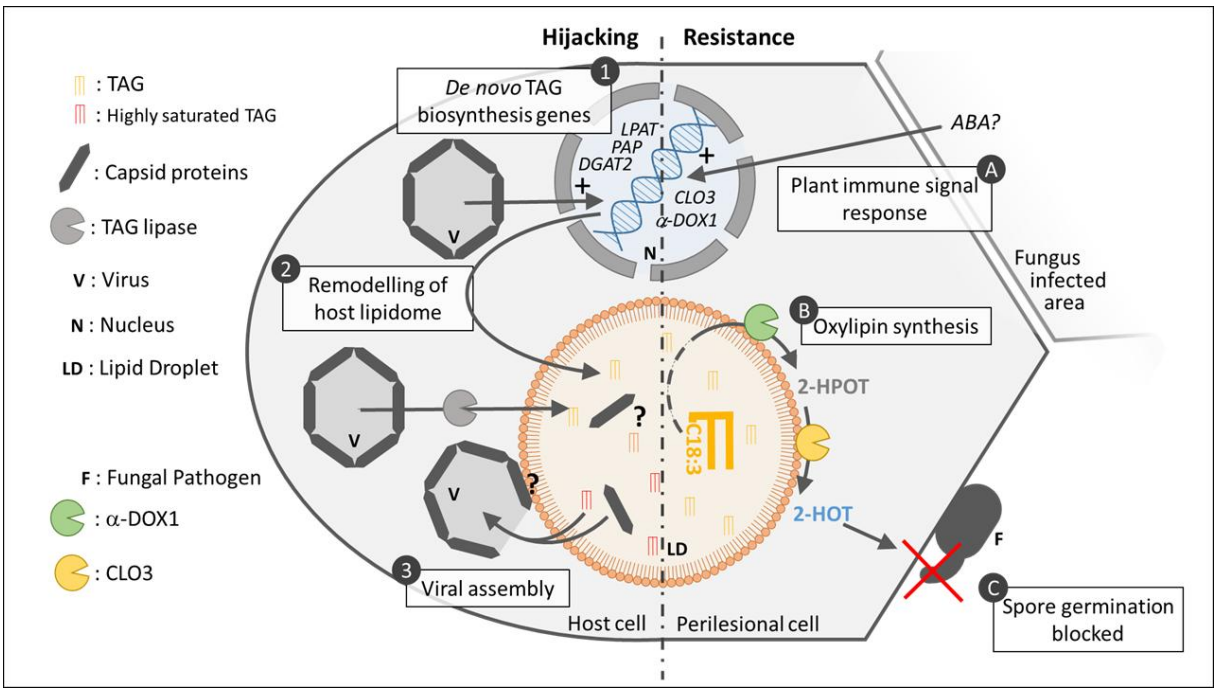
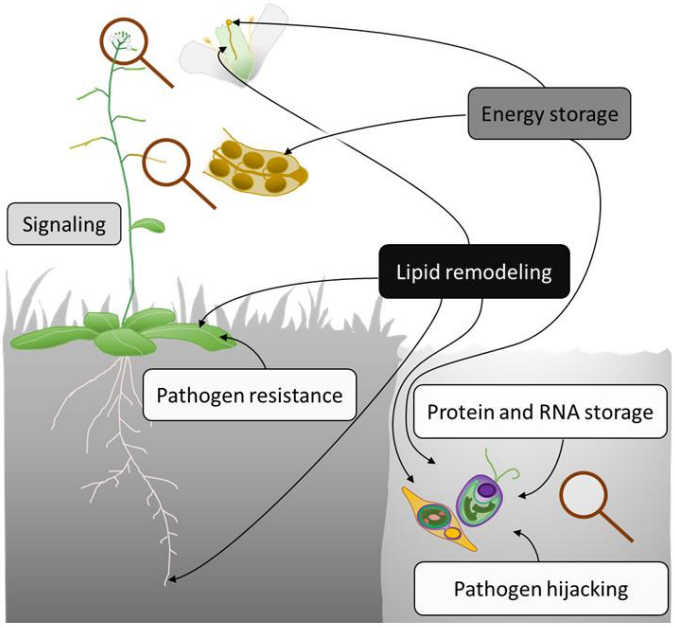


Figure 4: Functions of LDs in pathogen response: 2 examples.

Left panel: Hijacking of the host LD for the benefit of the pathogen (model based on Malitsky 2016 and inspired by Miyanari 2007): infection of the coccolithophore *Emiliana huxleyi* by the lytic *Emiliana huxleyi* virus (EhV201). At 24 hours post infection, the expression of genes involved in *de novo* TAG biosynthesis such as *LPAT*, *PAP* and *DGAT2* is upregulated **(1)**. This results in the remodelling of the host lipidome and the formation of lipid droplets **(2**), which could be associated with the storage of viral capsid proteins. The TAGs become progressively more saturated. In the later stage of the infection, high expression of virus-encoded lipases may lead to the disruption of the LD, and the formation of a new viral particle containing a high amount of highly saturated TAGs (**3)**.

Right panel: LD as a production platform for phytoalexins (based on Shimada *et al.,* 2014). Response of *Arabidopsis thaliana* leaves to fungi infection. Upon infection by fungi such as *Colletotrichum higginsianum*, the expression of Caleosin3 (CLO3) and α-Dioxygenase 1 (αDOX1) is induced in perilesional cells, possibly through the action of abscissic acid (ABA), an essential hormone involved in the plant immune response (**A**). α-DOX1 and CLO3 locate to the leaf LD and cooperatively produce the oxylipin 2-hydroxyoctadecatrienoic acid (2-HOT) from α-linolenic acid (C18:3, ω-3) (**B**). 2-HOT blocks the fungi (F) spore germination and its propagation to healthy tissues (**C**). A similar defense



system is induced by senescence.

Figure 5: The many functions of LDs

LDs have been studied in different organs from land plants, mainly seeds, pollen, leaves and to a lesser extent roots. They were also studied in different microalgae resulting from primary or secondary endosymbiosis, living in freshwater or marine environments. Functions that are common to most organs and organisms are indicated on a dark background. By contrast, functions that are only found in a group of organisms (plants or microalgae) or in a specific tissue are indicated on a light background.