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REVIEW

Exploiting lipid droplet metabolic pathway to foster lipid production: oleosin in focus

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Abstract

In the past decade, there has been an emerging gap between the demand and supply of vegetable oils globally for both edible and industrial use. Lipids are important biomolecules with enormous applications in the industrial sector and a major source of energy for animals and plants. Hence, to elevate the lipid content through metabolic engineering, new strategies have come up for triacylglycerol (TAG) accumulation and in raising the lipid or oil yield in crop plants. Increased levels of energy density can be achieved by single and multiple gene strategies that re-orient the carbon flux into TAG. Transcription factors and enzymes of the metabolic pathways have been targeted to foster lipid production. Oleosin, a structural protein of the lipid droplet plays a vital role in its stabilization and subsequently in its mobilization for seed germination and seedling growth. Maintenance of increased lipid content with optimal composition is a major target. Knowledge gained from genetic engineering strategies suggests that oleosin co-expression can result in a significant shift in carbon allocation to LDs. In this review, we present a detailed analysis of the recent advancements in metabolic engineering of plant lipids with emphasis on oleosin with its distinct patterns and functions in plants.

Keywords Oleosin · Lipid droplet · Triacylglycerol · Lipid degradation · Lipase · Metabolic engineering

Introduction

Plant seeds accumulate energy-intense biomolecules to support their growth and development during germination. Neutral lipids such as triacylglycerols (TAGs), being energy rich, anhydrous and composed of three fatty acyl groups esterified to the glycerol backbone, form an ideal reserve energy material for nearly all life forms from lower (yeast, actinomycetes, *Nocardia,* etc.) to complex eukaryotes including mammals (Murphy and Vance 1999). Due to their highly hydrophobic nature, these non-polar TAG molecules along with other sterol esters are densely packed at the core of independent storage organelles/bodies referred to as lipid droplets (LDs). In plants, LDs are enveloped with phospholipid monolayer where specific structural proteins such as oleosin, caleosin and steroleosin are embedded in them (Shao et al. 2019). It has been speculated that densely packed LDs help in providing protection against lipases possibly by resisting their action (D'Andrea 2016). Oleosin forms 80–90% of LD protein content with specific regulatory functions in seed development and germination (Tzen 2012). Attempts made at improving seed oil content involve overexpression of genes including those for oleosin. In the strategy named "push–pull–protect", wrinkled 1 (*WRI1*), diacylglycerol acyltransferase (*DGAT*) and oleosin genes were targeted to enhance the overall TAG content in plants (Vanhercke et al. 2019). This uses WRI1, a transcription factor that enhances transcription of lipid anabolism genes as the "push", DGAT an enzyme catalyzing the rate-limiting step of TAG synthesis as the "pull", and oleosin that protects TAGs by sequestering it into stable droplets as the "protect".

The oleosin gene was first identified and characterized in maize and it was reported that oleosin may serve as the recognition signal for the specific binding of lipase to LDs

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in the lipid degradation pathway (Vance and Huang 1987). Both oleosin and TAGs are simultaneously synthesized in the endoplasmic reticulum (ER) membrane (Thakur and Bhatla 2015). It has also been suggested that as soon as TAG accumulates, it forms a growing bud within the bilayer where co-translationally synthesized oleosin is inserted in specific segments of the ER bilayer membrane (Huang and Huang 2017). This newly formed oleosin extends to TAG-rich regions, thereby assisting in LD budding (Ojha et al. 2021; Huang 2018; Chapman et al. 2012). The electrostatic repulsion and steric hindrance conferred by oleosin on LDs prevent their aggregation. Widely observed in desiccation-tolerant seeds, the oleosin proteins are thus known to maintain LD size by preventing their coalescing under various conditions, especially during seed germination (Tzen. 2012; Huang 2018). For instance, RNA interference (RNAi) employed to disrupt *Arabidopsis* seed oleosin resulted in aberrant LD morphology and delayed germination (Siloto et al. 2006). Apart from this abundantly characterized role, the oleosin protein has been observed to show enzyme activity as a monoacylglycerol acyltransferase (MGAT) and a phospholipase (PLA_2) during seed germination (Parthibane et al. 2012). In peanut, oleosin 3 (OLE3) constitutes a part of the 14S multiprotein complex which is an active enzyme complex involved in the acylation of monoacylglycerols (MAG). Therefore, the heterologous expression of peanut OLE3 in yeast (*Saccharomyces cerevisiae*) led to an increase in diacylglycerols (DAGs) and a concomitant decrease in phospholipids (Tzen. 2012; Parthibane et al. 2012). Despite the extensive identification and characterization of oleosin in plants such as rapeseed (Chen et al. 2019), safflower (Mosupiemang et al. 2022), soybean (Xu et al. 2021), and sesame (Chen et al. 1997), their functions in lipid homeostasis are still under investigation. Furthermore, alongside crops already employed for biodiesel production, the identification and characterization of oleosin in crop plants such as cotton (Yuan et al. 2021), apricot (Hu et al. 2023), and duckweed (Liang et al. 2023) would introduce fresh opportunities within the biofuel sector. The lipids stored in LDs are mainly mobilized by lipases after germination to support the pre-photosynthetic growth of seedlings along with their organogenesis (Quettier and Eastmond 2009; Sinha et al. 2020; Bhunia et al. 2021a). Postgerminative mobilization of stored TAGs as LDs is preceded by the degradation of oleosins via post-translational modifications like ubiquitination and phosphorylation (Deruyffelaere et al. 2015). While ubiquitination of the oleosin protein induces proteasomal degradation, it greatly reduced oleosin breakdown and lipid hydrolysis when the proteasomal inhibitor MG132 was used to investigate the process (Deruyffelaere et al. 2015). Hence, it can be inferred that oleosin might play an important role in LD stability, besides conferring a certain degree of protection over the TAG at the core.

In this review, considering the functional and structural aspects of LD-associated proteins with a focus on oleosin, a detailed analysis of LD biosynthesis to degradation has been discussed. While consolidating recent results, we discuss that despite the complexity rendered by lipid and carbon metabolism, modulating the expression of lipid genes including oleosin can enhance the quality and quantity of storage lipids.

Biosynthesis of LDs: toward energy conservation

LDs are 0.5–2.0 µm-sized organelles with a TAG-rich core that is enveloped by a phospholipid monolayer, embedded with structural proteins (Shao et al. 2019; Huang 2018; Shimada et al. 2018). Plant seeds are the major lipid storage tissues where they constitute up to 70% of the dry seed weight (Kretzschmar et al. 2020), besides the abundance in pollen and fruits (Kretzschmar et al. 2018). At the seed post-germination stage, stored TAGs are mobilized and utilized to support plant growth and development (Huang 2018). The metabolic pathways for TAG synthesis and degradation are majorly conserved across organisms. In general, two major pathways are known to play an important role in TAG biosynthesis, viz., acyl CoA-dependent (known as the Kennedy pathway) and acyl-independent pathway (Caligari et al. 2011). Sucrose represents the major form of assimilated carbon which is transported to the oil-accumulating sink, i.e., seeds. Many mechanisms control sugar unloading and cleavage in plant seeds and release phosphosugar, which is metabolized through glycolysis or the oxidative pentose phosphate pathway. Both these pathways produce precursors for fatty acid (FA) biosynthesis, where two glycolytic intermediates serve as the precursor for TAG biosynthesis. Firstly, the glycerol backbone necessary for TAG biosynthesis is provided by the conversion of cytosolic dihydroxyacetone-phosphate (DHAP) to glyceraldehyde-3-phosphate (G3P) by G3P-dehydrogenases (G3PDH). A transgenic approach based on heterologous expression of yeast cytosolic *G3PDH* gene in oilseed rape resulted in a 3-4 fold increase in G3P which resulted in 40% increase in oil accumulation (Baud and Lepiniec 2010). Secondly, pyruvate dehydrogenase complex (PDC) catalyses the decarboxylation of pyruvate producing CO_2 and acetyl-CoA which is used in the de novo FA synthesis pathway (Baud and Lepiniec 2010). FA synthesis in chloroplasts have two fates, either to remain in the chloroplasts and form complex lipids, or to get transported to ER for TAG assembly (via any of the three pathways). FA synthase is a crucial enzyme complex involved in the synthesis of long-chain unsaturated FAs from acetyl-CoA and malonyl-CoA. The FAS monomer (270 kDa) has six catalytic activities: beta-ketoacyl synthase (KS), acetyl/malonyl

transacylase (AT/MT), beta-hydroxyacyl dehydratase (DH), enoyl reductase, beta-ketoacyl reductase (KR), acyl carrier protein (ACP), and thioesterase (TE). The acetyl-CoA carboxylase (ACC) catalyses the carboxylation of acetyl-CoA, resulting in malonyl-CoA production. Following that, malonyl-CoA is transported to the acyl carrier protein (ACP) (Baud and Lepiniec 2010). The fatty acyl-ACP esters are hydrolyzed by thioesterase and exported from the chloroplast to generate an acyl-CoA pool. These acyl-CoAs are used in the Kennedy pathway which is an acyl CoA-dependent pathway in the ER for G3P acylation.

Acyl CoA-dependent pathway

This pathway is the primary pathway for TAG biosynthesis in plants. The initial acylation is catalysed by glycerol-3-phosphate acyltransferase (GPAT), which results in lysophosphatidic acid (LPA). Lysophosphatidic acid acyltransferase (LPAAT) catalyses the second acylation to produce phosphatidic acid (PA). Phosphatidic acid phosphatase (PAP) then converts PA into 1,2-*sn*-DAG. Finally, DGAT or phospholipid:diacylglycerol acyltransferase (PDAT) catalyses the rate-limiting step by transferring the acyl moiety to the *sn-3* position of DAG, forming TAG (Baud and Lepiniec 2010; Bhunia et al. 2016).

Acyl-CoA-independent pathway

An alternative to the Kennedy pathway is the acyl-CoA-independent pathway where, instead of the incorporation of nascent FA in phosphatidylcholine (PC) via PA or DAG, it is incorporated directly via acyl editing. This can be done either by conversion of TAG back to DAG or by PDAT enzyme that utilizes different phospholipids as acyl donors and accepts acyl groups which ultimately results in contributing to TAG accumulation (Dahlqvist et al. 2000; Cagliari et al. 2011). Another acyl-CoA-independent pathway is MAG pathway which is mediated by MGAT (Yen et al. 2002). This pathway is more common in the intestine where MAG is acylated by MGAT to form DAG which is further acylated by DGAT to form TAG. Up to 75% of the TAGs in enterocytes are formed via this pathway (Pan and Hussain 2012). This is not a typical route for TAG biosynthesis in plants. However, there have been attempts to recruit the MAG pathway in plants by facilitating heterologous expression of mouse MGAT in *Nicotiana Benthamiana* which increased the TAG accumulation by 6-fold in seedlings (Petrie et al. 2012).

LD biogenesis

The complexities of LD biogenesis in plants are poorly understood. The organization of LDs is often controlled by various ER-resident proteins as they emerge from the ER. The neutral lipids are deposited between the ER leaflets by two routes, TAG and cholesterol ester biosynthesis pathway. Deposited neutral lipids undergo a process known as demixing, which causes them to condense into a lens-like shape when a specific concentration is reached (Olzmann and Carvalho 2019). Several LD biogenesis factors are recruited to this structure that facilitate the growth of nascent LDs. The lens expansion leads to LD budding from the ER surface. Earlier studies have suggested that the ER membrane phospholipid composition plays a key role in LD budding (Olzmann and Carvalho 2019). This emergence is affected by the surface tension possessed by the ER leaflets. The LD acquires a circular shape to minimize the contact of the TAG core with the aqueous cytoplasm.

The protein and phospholipid composition induces tension which is responsible for the direction of LD budding. Moreover, these phospholipids also mask the oil–water interface. The formation of LD-associated proteins (LDAPs) takes place simultaneously on the ER surface and are added to the phospholipid which induces LD to bud off from the surface (Chapman et al. 2012). Numerous approaches to identify new proteins involved in LD production are being explored that include identification of the protein machinery via genetic screens of LD mutants in yeast and homology-based searches in plants (Chapman et al. 2012).

The pillars of LD

Seed LDs are associated with three major proteins including oleosin, caleosin, and steroleosin (sterol dehydrogenases), each with distinct structures and functions (Huang 2018; Bersuker et al. 2018). Oleosin proteins are the most widely and abundantly expressed proteins having their N- and C-terminals embedded in the phospholipid bilayer of LD as alpha-helices. Oleosin-like proteins are ubiquitously expressed in lower plants and algae such as *Chlamydomonas* and *Volvox* (Huang 2018), thereby suggesting that oleosin in higher plants might have evolved from oleosin-like proteins (Fang et al. 2014).

Oleosin

Oleosin is a 15–26 kDa alkaline protein (Tzen 2012; Huang 2018). Apart from seeds, it is weakly expressed in tapetal cells, pollen grains, and oleaginous fruits (Chapman et al. 2012). N- and C-terminal amphipathic arms flank a hydrophobic core region known as the H-domain. The N- and C-terminal arms lie on the LD surface, whereas the central H-domain is embedded in the phospholipid monolayer, forming a hairpin structure (Huang 2018). The H-domain hairpin structure, which is almost 72 amino acid long,

consists of two arms of around 30 amino acid residues connected by a proline knot motif (PKM; PX5SPX3P) (Beaudoin and Napier 2002; Huang and Huang 2017; Board et al. 2022). With no cleavable signal sequence detected so far in oleosin, the multiple regions within its hydrophobic domain including PKM are considered indispensable for their localization to LDs (Tzen 2012; Beaudoin and Napier 2002; Huang and Huang 2017). Despite the abilities of protein folding software such as AlphaFold (Jumper et al. 2021) and RaptorX (Xu et al. 2021) to predict protein structures with considerable accuracy, the modelling of oleosin from various plants including *Arabidopsis*, almond, hemp, and sunflower indicates that the N- and C-terminals are disordered. The H-domain is observed to form an alpha-helix, yet the proline knot remains challenging to predict accurately. This indicates that oleosin is a protein whose structure necessitates experimental determination through methods like Fourier transform infrared spectroscopy and circular dichroism spectroscopy (Board et al. 2022). Besides oleosin that cover the entire surface of LD, a small number of other proteins are also associated with it. Caleosin and steroleosin proteins are also found on the surfaces of LDs (Huang 2018).

Caleosins

Caleosins are a family of proteins that are ubiquitous in land plants and also present in some fungi and green algae. Structurally, they are different from oleosin in having a characteristic EF-hand (helix loop helix) calcium-binding domain at the N-terminal region, a hydrophobic central domain and a C-terminal consisting of conserved phosphorylation sites. The hydrophobic region of caleosin is shorter than that of oleosin, but retains the proline-rich region forming PKM that inserts into the phospholipid monolayer. Two isoforms of caleosin are found in seeds—27 kDa ER and the 25 kDa LD types (Huang 2018; Chapman et al. 2012). The first caleosin was discovered in response to osmotic stress in rice seeds (Frandsen et al. 1996). Initially, caleosins were believed to have a primary function in seed, and subsequent studies have shown its expression in various tissues. In *Arabidopsis*, eight caleosin genes were identified which were then divided into two classes on the basis of their molecular weight. H-caleosin contained *AtCLO1*, *AtCLO2*, *AtCLO3* and *AtCLO8*, while L-caleosins contain *AtCLO4*, *AtCLO5*, *AtCLO6* and *AtCLO7* (Shen et al. 2014). Out of these, *AtCLO1* showed high expression in root tips, developing embryos and mature seeds, while *–AtCLO2-5* have shown low expression in various tissues (Næsted et al. 2000; Shen et al. 2014). Caleosins have now been identified as a family of multifunctional proteins exhibiting their participation in various aspects of metabolism in plants ranging from oil accumulation to stress mitigation (Table 1). The varied functions of caleosins, along with their distribution across different organs and organelles in plants beyond seeds and pollen, have prompted researchers to propose novel classification systems incorporating bioinformatics and biochemical findings (Shen et al. 2014; Saadat 2023). Enhanced comprehension of caleosins holds promise not only for the development of improved oil-accumulating crop plants, but also for leveraging their biochemical roles in diverse physiological processes to address various environmental stresses.

Steroleosin

Steroleosin, also called sterol dehydrogenases, are frequently associated with LDs (Lin et al. 2002). Steroleosin is a member of the hydroxysteroid dehydrogenase (HSD) family found in a wide variety of organisms; it contains both

Table 1 Functions of caleosins in the plant system

Caleosin type	Functions	References
CLO1	Peroxygenase activity, affects the genes affecting oil accumulation and embryo development, LD degradation during germination, LD turnover	Næsted et al. (2000); Shen et al. (2014); Hanano et al. (2006); Miklaszewska et al. (2023); Liu et al. (2022); Hanano et al. (2023)
CLO2	Peroxygenase activity, affects the genes affecting oil accumulation and embryo development	Hanano et al. (2023); Liu et al. (2022)
CLO3	Interacts closely with dioxygenases (*α-DOX1*) which are triggered by pathogens or senescence, plays a role in the production of phytoalexin for plant defence mechanisms, *CLO3* negatively regulates lateral root growth in response to ABA	Shimada et al. (2014); Huang (2018); Hanano et al. (2023); Brunetti et al. (2023)
CLO4	Negative regulator of ABA responses in *Arabidopsis*, embryo development	Kim et al. (2011); Liu et al. (2022)
CLO5	Negative co-regulator of salt stress tolerance during germination	Jing et al. (2021)
CLO6	Embryo development	Liu et al. (2022)
CLO7	Interacts with G-protein signalling and affects seed germination and embryo viability	Brunetti (2022)

a sterol-binding site and an NADPH-binding site (Lin et al. 2002; Huang 2018). They possess a significantly shorter hydrophobic domain than oleosin (nearly 1/3rd in length) and a C-terminal HSD domain. While steroleosin and caleosin are expressed ubiquitously, their level is only near 2.0–4.0% of oleosin and < 5.0% of total LD proteins (Huang 2018). LDs possess isoforms of steroleosins with distinctive sterol-binding sites which are regulated with distinctive sterols that carry out functions that are primarily involved in degradation and formation of LDs (Pasaribu et al. 2016). Another study in sesame demonstrated the presence of another kind of steroleosin with sesamin binding site (Tera et al. 2019). This indicated its putative role in functions related to maturation and germination of seeds as sesamin in plants has been reported to possess functions related to the aforementioned processes. It has also reported that steroleosins might have key roles in brassinsteroid metabolism and plant signalling (Li et al. 2007; Aziz et al. 2020).

LD-associated proteins (LDAP)

To understand the diverse functions of LDs, other LDAP have also been identified in oilseed and non-seed tissues. During pollination, a vigorously energy-demanding and fiercely competitive race is observed between the pollen tubes towards the ovule. Given the vital significance of this process for survival, the swift elongation of pollen tubes to facilitate the delivery of sperm cells necessitates membrane lipid precursors. These precursors can be sourced from LDs, which serve as reservoirs of acyl chains. The mobilization of neutral lipids occurs through the activity of lipases and lipoxygenases present within LDs (Guzha et al. 2023; Hernández et al. 2020). Caleosins work closely with dioxygenase, an enzyme that is triggered by pathogens or senescence and plays a significant role in the production of signalling chemicals like phytoalexins for plant defence mechanisms (Huang 2018). The LDAP family, which is ubiquitously expressed in *Arabidopsis* and mostly abundant in siliques (LDAP1 and LDAP3) and seedlings (LDAP2), has shed light on the role of LDs in leaves and seeds (Shimada et al. 2018). For efficient compartmentalization and LD stability during post-germinative seedling growth, the LDAP1 is crucial (Gidda et al. 2016). Additionally, it was shown that *Arabidopsis* LDAP-interacting protein (LDIP) affects neutral lipid homeostasis and LD size in both leaves and seeds (Pyc et al. 2017). In a study, LDIP was shown to play an important role to enhance LD biogenesis in cooperation with ER-localized seipin protein and LDAP in *Arabidopsis* (Pyc et al. 2017) where seipin interacts with LDIP via a conserved hydrophobic helix and therefore functions with LDAP to establish a new model for LD synthesis in plants, especially in oilseeds.

Formation, classification and multifunctional features of oleosin in concordance with LD synthesis and degradation

The enzymes of TAG synthesis are localized in different regions of the ER membrane that are associated with oleosin synthesis (Beaudoin and Napier 2002; Jacquemyn et al. 2017). Oleosin mRNA is translated into a nascent polypeptide that is co-translationally inserted in the ER membrane via the signal recognition particle (SRP) pathway (Beaudoin and Napier 2002; Hsieh and Huang 2004). Yeast mutants lacking the SRP pathway showed incorrect targeting after heterologous expression of oleosin (Hsieh and Huang 2004). The nascent polypeptide of oleosin is translated from mRNA on the rough ER that stops growing once it binds to SRP. When the peptide inserts into the ER via the SRP receptor, the translation resumes. Various in vitro synthesis studies of oleosin have been conducted using microsomes, oil emulsion of TAG with phospholipids (retrieved by sonication) and SRPs from different sources in attempts to reveal the sequence features needed for its localization and targeting (Tzen 2012). Even though oleosin is directly targeted to the LDs after synthesis and LDs emerge from the ER via the budding process, there is no Golgi body-assisted modification such as glycosylation on oleosin as is observed with other familiar proteins (Wahlroos and Petri 2015). The disruption of ER and Golgi traffic by using brefeldin-A did not affect the oleosin protein activity. This confirms the Golgi-independent synthesis of oleosin as well as the fact that oleosin does not require glycosylation and other Golgi-mediated modifications (Wahlroos and Petri 2015).

The oleosin gene from *Zea mays* was expressed in *B. napus* to study oleosin targeting. *Agrobacterium*-mediated transformation was used to transfer a monocotyledonous oleosin gene under the promoter/terminator of a seed storage protein (napin) to dicotyledonous *B. napus* (Beaudoin and Napier 2002; Lee et al. 1991). Immunoblotting analysis of the transgenics indicated the presence of oleosin proteins in seed LDs, thereby suggesting that maize and *B. napus* have a similar oleosin targeting mechanism. This study showed accurate targeting of maize oleosin to the LDs which is temporal and tissue specific (Beaudoin and Napier 2002; Lee et al. 1991). Green fluorescent protein (GFP) fusions with oleosin variants having deletions and modifications in specific segments were used to conclude that the N- and C-terminal regions do not have any role in oleosin targeting and the central hydrophobic region is solely responsible for its localization (Beaudoin and Napier 2002; Huang and Huang 2017). Since there is no specific signal sequence in this domain, it has been concluded that multiple sequence regions confer this property

on this domain. While the initial residues of the hairpin (six in *P. patens*, four in *A. thaliana*) were found to be inevitably crucial for the oleosin targeting into ER and LDs (Beaudoin and Napier 2002; Huang and Huang 2017), the N- terminal allows the bulky hydrophobic domain to bind the microsomes or ER (Huang and Huang 2017).

The importance of proline and serine residues (PSPP) in proline knot motif was also confirmed by the substitution of these residues to PYPP (serine substituted with tyrosine) and LSLL (proline substituted with lysine) which ultimately led to failed oleosin targeting (Tzen 2012; Hsieh and Huang 2004; Huang and Huang 2017). The topology of oleosin is determined by its hydrophobic or hydrophilic interactions with the ER bilayer. The hydrophobic central hairpin stretch is buried within the hydrophobic acyl portion of the ER bilayer. This central hydrophobic region is unable to take its most stable configuration due to the lack of hydrophobic volume possessed by the phospholipid bilayer. On the contrary, the LD matrix is capable of providing suitable conditions for its stable conformation (Hsieh and Huang 2004). Thus, the oleosin central hydrophobic domain probably assumes a bent hairpin or extended conformation that is parallel to the ER bilayer. Once the TAG accumulation increases, the region of ER within the bilayer expands and this region being largely hydrophobic allows oleosin to fold into the proper hairpin structure. Fluidity of the membrane assists localization of oleosin to TAG-rich segments. Once stable, the LDs emerge by budding out of the ER (Hsieh and Huang 2004; Huang and Huang 2017).

Classification of oleosin and their distinct spatio-temporal expression patterns

Oleosin is classified into different lineages depending on their evolutionary development as well as their expression patterns. To date, six families of oleosin have been identified that include primitive (P), universal (U), seed low molecular weight (SL), seed high molecular weight (SH), tapetum (T), and mesocarp (M) oleosin. Most seeds generally express the SL and SH isoforms of oleosin (Huang 2018; Huang and Huang 2016). While the U lineage is ubiquitously distributed, the P, T and M lineages are specific to the green algae, *Brassicaceae* and *Lauraceae*, respectively. U oleosins diverge into SL oleosins which are found in all seed plants, while the SH oleosins are found in angiosperms (Huang 2018). Typically, two to four isoforms of oleosin from the SH and SL lineages are expressed concurrently in seeds and the expression level of SL and SH oleosin isoforms is generally equal, implying that they may form dimers/multimers (Huang 2018; Huang and Huang 2016). Among the oleosins, SH oleosin isoform is predominantly expressed before seed maturation, and as the maturation continues the SL oleosins dominate. Generally, the SH oleosins are expressed early in seed development and their level is reduced at maturation. The oleosins at germination belong to the SL lineage and it has been observed that LDs with SL-oleosin are more stable (Winichayakul et al. 2013). This suggests that the SL oleosins have a positive role in seed germination.

LDs can be found in all parts of a seed, including the embryo axis, cotyledon, endosperm, and aleurone layer (Huang 2018). TFs such as leafy cotyledon 2 (LEC2) and abscisic acid insensitive 3 (ABI3) have been shown to induce oleosin expression (Zafar et al. 2019). The expression of oleosin in rice embryo and aleurone is usually dependent on abscisic acid (ABA) (Ali et al 2021). ABA regulates the expression of the *DOG1-LIKE 4 (DOGL4)* gene and increases the expression of oleosin during the *Arabidopsis* seed maturation process. The transcripts of oleosin are upregulated in both the seed and endosperm of *Arabidopsis* due to the higher expression pattern of *ABI3* (Crowe et al 2000; Penfield et al 2006). Apart from the seeds that act as primary lipid storage organs in plants, the vegetative tissues, pollens and tapetum also have LDs, albeit serving different functions. While the LDs of vegetative tissues serve to sequester toxic free FA from the cytosol, there is no evidence of oleosin covering the surface of these LDs (Xu and Shanklin 2016). LDs of pollens and tapetum confer hydrophobicity on pollen grains, but unlike vegetative tissues, they have evolved distinct oleosin isoforms. Pollens express the universal isoform all along with the angiosperms. Apart from that, the tapetosomes of the *Brassicaceae* have the tapetum isoform which is known to be distinctly tolerant to dehydration. This isoform, also known as T-oleosin, is highly expressed at the maturation stages of tapetosomes. T-oleosins in *Brassica* are subjected to proteolytic cleavage that results in the formation of mature T-oleosins which is also known as pollenin that constitutes the major protein component of the pollen coat (Murphy 2001). Unlike *Brassica* T-oleosins which have been studied extensively, T-oleosins of *Arabidopsis* are yet to be explored. However, a report by Lemay et al. (2016) has indicated the role individual domains of T-oleosins in *Arabidopsis* tapetosome formation. Additionally, the mesocarp of some fruits like avocados (*Persea americana*), olives (*Olea europaea*), oil palm fruits (*Elaeis guineensis*), tung tree fruit (*Vernicia fordii*) and some sweet tropical fruits also accumulate LDs. However, these do not have oleosin except for the *Lauraceae* family such as the avocados, which are known to express the mesocarp lineage that differs from pollen, tapetum and seed oleosin (Huang 2018; Huang and Huang 2016).

In *Arabidopsis*, evolution and function of a tandem oleosin gene cluster have been studied. *Arabidopsis* has 17 oleosin genes, five of which are expressed in seeds, three in both seeds and pollens and nine in the tapetum of flower anthers (Huang and Huang 2016). Relative transcript levels of

oleosin in *Arabidopsis* were low during early silique development, but quickly increased as seed maturation began; mRNA levels thereafter gradually decreased throughout the maturation phase (Miquel et al. 2014). Different plants have different lifecycle patterns and, thus, the exact time of oleosin expression in days varies across the plant kingdom. As observed in sunflower seed development, oleosin showed high levels of expression from 20 to 30 days after anthesis (DAA), followed by desiccation and seed maturation (Thakur and Bhatla 2015). In *Paeonia ostii*, the oil content was observed to increase from 60 days after flowering (DAF) to 100 DAF and decrease slightly at 120 DAF. An overlapping expression pattern of *P. astii* was observed for oleosin where protein analysis showed an increase in OLE17.5 from 60 to 100 DAF and a slight decrease at 120 DAF. *Carthamus* seeds demonstrated that oleosin expression progressively increased after flowering till the seed maturation stage (Lu et al. 2018). Oleosin expression analysis in *Coffea Arabica* revealed that mature seeds had higher levels of oleosin expression. However, during germination, the accumulation of oleosin transcript decreased significantly, thereby suggesting degradation of the oleosin protein (Marin et al. 2020). A comprehensive genome-wide identification and differential expression analysis of the oleosin gene family within the Theaceae species delineated that clade SL and SH showed higher expression in comparison to U oleosins which was consistent with the patterns exhibited by *Arabidopsis*, *Zea mays*, soybean and *Brachypodium*. This study also showed that most tea species contained more oleosin members in each clade and within each clade, about ~ 1–2 oleosin genes were highly expressed than other oleosin gene and this observation was also consistent with previous studies which substantiates the evolutionary hypothesis that one to two oleosin genes in each lineage have undergone activation and achieved dominance during evolution (Zhang et al. 2023). The systematic investigation into the expression divergence of oleosin genes across diverse plant taxa promises to yield pivotal insights into the molecular underpinnings governing plant growth and developmental paradigms.

Multifunctional features of oleosin protein in concordance with LD synthesis

Seed-specific oleosin is involved in numerous regulatory functions and influences various aspects of storage lipids in seeds. As the lipids accumulate in the ER bilayer, oleosin begins to cover the entire surface of LDs and maintain their size and shape. This can be extrapolated from the fact that N- and C-terminals of oleosin proteins have negatively charged residues exposed to the cytosol that provide an overall negative charge to the LDs (Winichayakul et al. 2013). There are various physical manifestations of oleosin that impart charge on LDs and their insertion into the LD membrane. These include an overall reduction in LD size, regularity and uniformity in their shape, enhanced stability, maintenance of size rendering LDs stability to desiccation and cold temperature which otherwise would lead to coalescence of droplets (Chen et al. 2019; Lu et al. 2018).

In *Arabidopsis*, amongst the reported 17 oleosin genes, five are seed specific (Huang and Huang 2016) where *OLE1*, *OLE2* and *OLE4* are the major seed oleosins involved in lipid accumulation and rendering freezing tolerance, while *OLE3* is involved in LD degradation and *OLE5* is the minor seed protein controlling LD dynamics (Shao et al. 2019). In earlier studies, RNAi-mediated suppression of two major rice oleosin isoforms, 16 kDa and 18 kDa, led to an aberrant morphology of LDs with large, irregular clusters of lipids (Wu et al 2010). Similar results were obtained in RNAi-directed knockdown of *Arabidopsis* oleosin that led to larger or abrupt LDs in seeds (Chapman et al. 2012; Siloto et al. 2006). In *Arabidopsis*, single mutations in oleosin (*ole1, ole2* and *ole3*) displayed larger average LD size and delayed germination (Shimada et al. 2018). A similar result was also obtained in double mutants of oleosin including *ole1/ole2, ole1/ole3* and *ole2/ole3*. While normal seed cells have LDs surrounding the central nuclei and protein storage vesicles (PSVs), their enlargement and irregular structure lead to the disruption of orderly arrangement of PSVs and nuclei that are pushed to the periphery in seeds. Such disordered arrangement is responsible for the germination defects in seeds (Shimada et al. 2008).

During seedling development following seed germination, the TAG reserve is mobilized and hydrolyzed. While the highly abundant OLE1 isoform of *Arabidopsis* degrades post-germinatively, other forms including OLE2, OLE4 and OLE5 degrade at early stages. In *Arabidopsis*, oil body mobilization during post-germinative seedling growth is involved in ubiquitin-mediated proteasomal degradation of oleosins (Deruyffelaere et al. 2015). Degradation of oleosins and digestion of phospholipid memberane are the two key processes involved in lipid mobilization. Oleosins are not partially proteolysed, but instead are extracted from the LD surface leading to an increase in the surface/interfacial tension which allows the docking of new proteins such as lipases needed for lipid degradation (Deruyffelaere et al 2015; Thiam et al. 2013).

Apart from seed germination and development, many other demonstrated roles of oleosin have been summarized in Table 2. Oleosin being structural proteins also possess bifunctional enzyme activity. When peanut *OLE3* gene was overexpressed in yeast, it resulted in an increased accumulation of DAGs and TAGs, and decreased phospholipids, thereby indicating that oleosin had MGAT and a phospholipase A_2 (PLA2) activity as well (Parthibane et al. 2012). However, except for peanut oleosin, this functionality has

Table 2 Role of oleosin protein in plants

	Functions	Experimental analysis	Result	References
1	Bifunctional enzyme	Overexpression of *OLE3* in yeast	Increased TAGs and DAGs due to the monoacylglycerol acetyl transferase activity of OLE3 and decreased phospholipids due to its phospholipase activity	Parthibane et al. (2012)
2	Cold Tolerance	Double mutants (*ole1 ole2*, *ole1 ole3* and *ole2 ole3*) were generated Freezing treatment followed by imbibition was given	Oleosins increased the viability of over-wintering oilseeds by preventing abnormal fusion of oil bodies	Shimada et al. (2008)
3	Oleosome stability	Colloidal stability and the interfacial activity of native and enzymatically digested soybean oleosomes was performed	After digestion, the ability of oleosins to stabilize oil–water or air–water interfaces was lost	Maurer et al. (2013)
4	Oleosome size	RNAi-mediated suppression of oleo1 in *Arabidopsis* Double mutants (*ole1 ole2*, *ole1 ole3* and *ole2 ole3*) were generated electron microscopy was done	Aberrant sized oleosomes Inverse relationship between oil body sizes and total oleosin levels	Shimada et al. (2008); Siloto et al. (2006)

not been confirmed in any other oleosin to date. The enzymatic activity of oleosin has been shown to be modulated by its phosphorylation state where the phosphate group enhances PLA2 activity and reduces the MGAT activity. In soybean, high off-flavours have been associated with their oleosin content where it exhibits PLA2 activity that contributes to the off-flavours. Certain biological elicitors like jasmonic acid and chitosan have been identified as potential activators of the PLA2 activity, since they affect the phosphorylation state of oleosin through various kinases (Kumari et al 2016).

In concordance with the above functions, another novel role associated with oleosin is its importance in rendering cold tolerance to the seeds. In the absence of oleosin, the LDs fuse and form expanded oil structure which is detrimental for the oil seeds. These expanded oil structures push the nucleus to the periphery and pose a hindrance towards its nuclear activities (Shimada et al. 2008).

Role of oleosin in LD degradation

The period of accelerated growth and development is supported by the degradation of storage TAG reserve. At the time of LD degradation, lipases and proteases degrade the monolayer and oleosin undergoes proteolysis after some modifications. A core retromer guides sugar-dependent 1 (SDP1) lipase from peroxisomes to the LDs that helps in lipid mobilization (Thazar et al. 2015). However, the lipase–LD interaction is yet to be explored in detail.

In *Arabidopsis,* all the five seed-specific oleosins undergo proteolysis sequentially prior to lipid degradation (Deruyffelaere et al. 2015). Oleosin proteins are target of different post-translational modifications like ubiquitination and phosphorylation which are involved in switching the LD catabolic process. The topology of ubiquitinated oleosin is very distinct and follows three different ubiquitinations during the onset of LD mobilization (Deruyffelaere et al. 2015). During germination, *Arabidopsis* OLE1 was the only oleosin that degraded at the same rate as storage lipids (66%), unlike others that degraded at a faster rate (90%). Therefore, it can be inferred that oleosin degradation began before the mobilization of LD, while OLE1 was the last oleosin to degrade concurrently with lipid hydrolysis. Moreover, immunodetection of oleosin showed significant rise in the molecular mass of OLE1–OLE5 between 32 and 50 h of germination, clearly indicating that oleosins are modified post-germinatively (Deruyffelaere et al. 2015). The ubiquitination profile of *Arabidopsis* was examined to look for distinct types of ubiquitination modification of oleosin during seed germination and growth. Three different and predominantly exclusive motifs, monoubiquitin, K48-linked diubiquitin (K48Ub2) and K63-linked diubiquitin, were used

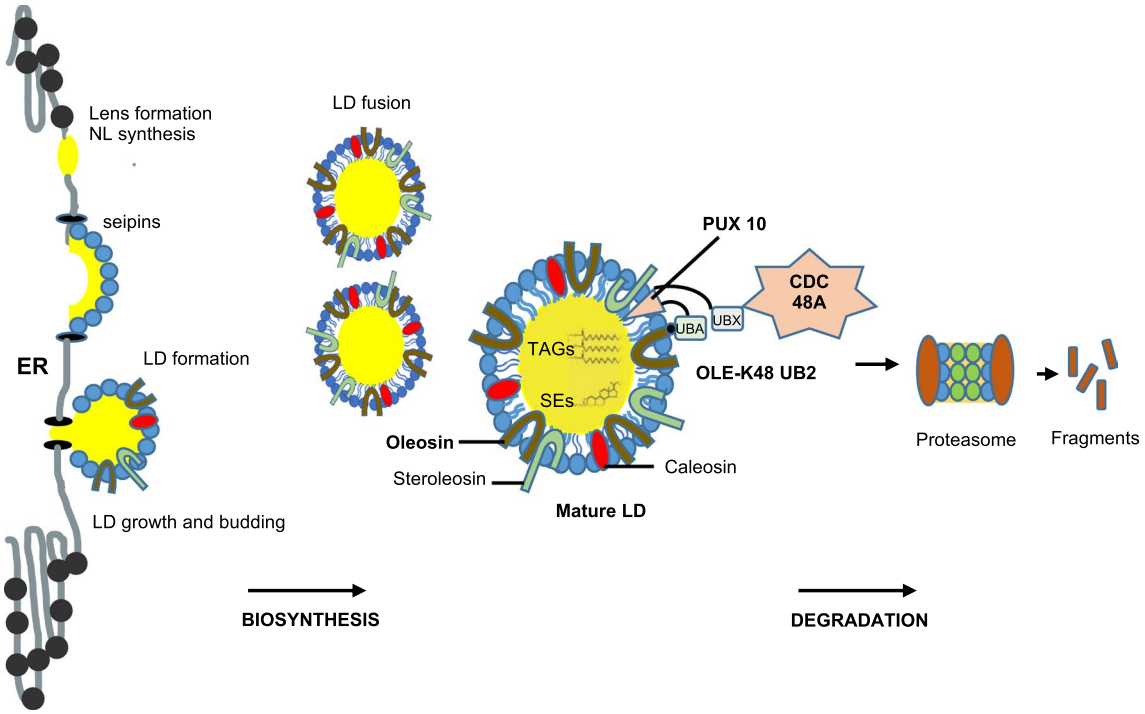

Fig. 1 LD biogenesis begins with the synthesis of TAGs like neutral lipids in the ER, which accumulate for the formation of lens-like structure within the leaflets of the ER membrane. Proteins including seipins help facilitate the budding of LD into the cytosol, surrounded by the phospholipid monolayer. Oleosin, caleosin and steroleosin embed into the outer layer of LD during its formation and promote ER growth and help in additional functions. Shown here is the proposed model for PUX 10-mediated breakdown of LD. CDC 48A, a key component of ubiquitin-mediated pathway, links PUX10 embedded in the membrane, which helps in the degradation of ubiquitinated oleosin. These LD-associated proteins are further degraded in the proteasomal pathway

to modify *Arabidopsis* OLE1 and OLE2 proteins (Deruyffelaere et al. 2015). The K48Ub2-linked ubiquitination on oleosin aids an eventual degradation after exposure to the 26S proteasome complex (Fig. 1) (Deruyffelaere et al. 2015; Kretzschmar et al. 2018). The ubiquitin regulatory X protein (UBX), known as UBX domain containing protein-10 (PUX-10), mediates extraction of modified oleosin from the LD monolayer along with CDC48A (a cell division cycle 48 homolog A) for guiding to the cytosolic proteasome (Deruyffelaere et al. 2018). In contrast to K48-linked ubiquitination, K63- linked ubiquitination aids in vacuolar degradation of ubiquitinated protein. The involvement of proteasome degradation system and vacoule in the catabolic process was confirmed by using a proteasome inhibitor MG132; an inhibitor of vacuole acidification, concanamycin A (ConA); and an inhibitor of cysteine proteases, E64d. While these did not affect seed germination, MG132 decreased oleosin degradation for 40 h of seed germination and E64d reduced lipid hydrolysis to some extent. Although no spherical LDs were observed in MG132-treated seedlings, cytosolic accumulation of oleosin–K48Ub2 aggregates was suspected due to their hydrophobicity (Deruyffelaere et al. 2015). This led to the hypothesis that oleosins must be extracted from the LD for the proteasome degradation system to act on them, and that in the presence of MG132, oleosins are extracted but not degraded. However, a recent study employed forward genetics to suggest the probable role of MIEL1 (MYB30-interacting E3 ligase 1) in oleosin degradation. LDs are closely associated with peroxisomes. MIEL encodes for a ubiquitin-protein ligase and was not previously implicated in LD or peroxisome dynamics. They observed a decrease in oleosin ubiquitination and stablized oleosins with decrease in TAG degradation in *miel1* mutants. On the other hand, when overexpressed, MIEL increased oleosin degradation, subsequently leading to increase in LD size as well. This along with other data including co-localization of MIEL1 and peroxisomes suggests that MIEL1 ubiquitinates seed oleosins in the vicinity of peroxisome for proteosomal degradation during germination (Traver and Bartel 2023).

Another work by Tailor and Bhatla (2023) is the first ever report to hint towards the role of oleosin degradation in regulating the association between polyamine metabolism and LD mobilization in plants under stress and normal conditions. Based on their data, they designed a model proposing that PA deficit causes enhanced oleosin degradation and faster LD mobilization to facilitate rapid TAG degradation.

In mammalian LDs, perilipin protein is a major LD-associated protein that acts as a docking site for the

hormone-sensitive lipase (HSL) as shown by the physical interaction between these proteins (Sztalryd and Brasaemle 2017). Similarly, mammalian protein comparative gene identification 58 (CGI-58) that has an α/β hydrolase fold, activator of lipases and is an inactive protein interacts with the perilipins on the LD surface. Once mobilization signals are received, the CGI-58 protein is replaced by active lipases. In *Arabidopsis*, disruption of the CGI-58 gene resulted in 10-fold higher accumulation of LDs in mature leaves than in wild-type plants (James et al. 2010). CGI-58 activates *Arabidopsis* lipases by direct protein–protein interaction (Quettier and Eastmond 2009). Similarly, different regulators are responsible for different interactions in LD biosynthesis, dynamics and turnover in different systems. In algae, LDs are synthesized in highly stressful conditions and they are known to get sequestered into vacuole via microautophagy. In plants, a retromer subunit, e.g. vacuolar protein sorting 29 (VPS29), is known to mediate the movement of SDP1 from peroxisome to LD for degradation. In yeasts, the ESCRT system plays a significant role in the regulation of LD turnover (Huang et al. 2019). Therefore, LDs are important organelles for development and long-term stored energy reserves conserved across species. Table 3 highlights the proteins that interact with LDs and are involved in several physiological functions that range from lipid synthesis, stress response to maintenance, modulations and lysis of LDs during different developmental stages in plants.

Exploiting potential genetic targets for augmenting TAG accumulation

The oil content was increased using genetic approaches involving overexpression/downregulation of one or multiple genes. The enzyme of TAG synthesis was best targeted to improve TAG yield is DGAT which catalyses the final commitment step of TAG synthesis by adding a third fatty acyl group to the DAG (Chawla et al. 2020; Bhunia et al. 2021b). Numerous reports have concluded that there exists a positive correlation between seed oil content and oleosin expression. Overexpression of soybean oleosin led to an overall 10.6% increase in the seed oil content (Zhang et al. 2019). In a genome-wide association study, soybean *OLE1* was found to lie in a quantitative trait locus (QTL) region, GqOil20, and this single nucleotide polymorphism was consistently observed in oil variants positively affecting TAG metabolism (Zhang et al. 2019). The stable expression of *Brassica* oleosin genes resulted in a variation in the FA profile with an overall increase in the oil content of seeds and a concomitant increase in the seed weight. Two of the *Arabidopsis* oleosin genes (*OLE1* and *OLE2*) lie in one of the QTLs responsible for seed oil content (Gu et al. 2017 Elevating the expression levels of rapeseed oleosin genes in Arabidopsis resulted in a minor enhancement in seed oil content. However, the study also noted alterations in the FA compositions, particularly a rise in linoleic acid content to approximately 13%, alongside

Table 3 LD-associated proteins and their proposed functions in plants

Protein	Function	References
Oleosin, steroleosin, caleosin	Structural proteins	Huang et al. (2019); Walther and Farese. (2012)
Diacylglycerol acyltransferase (DGAT)	TAG synthesis	Walther and Farese (2012)
Phosphoserine aminotransferase (PSAT)	Sterol ester synthesis	Walther and Farese. (2012)
Phospholipid:diacylglycerol acyltransferase (PDAT)	TAG synthesis, stress response	Li et al. (2010); Yuan et al. (2017)
Vesicle-associated membrane protein (VAMP)-associated proteins (VAPs)	Lipid transfer, interacts with seipin for LD formation	Esnay et al. (2020); Greer et al. (2020)
Sugar-dependent phospholipase (SDP1)	Lipid degradation	Zienkiewicz and Zienkiewicz (2020)
Vacuolar protein sorting 29 (Vps29)	Regulates lipid storage and degradation	Durand et al. (2019)
UBX domain containing (PUX10) and cell division cycle 48 (CDC48A)	Ubiquitination of oleosins	Deruyffelaere et al. (2018); Huang et al. (2019)
Autophagy-related genes (ATG)	Lipophagy	Tran et al. (2019); Zienkiewicz and Zienkiewicz (2020); Zhao et al. (2020)
Lipid-droplet associated protein (LDAP)	Maintains and regulates LDs, responds to stress and regulates post-germinative growth	Gidda et al. (2016)
LD-associated protein [LDAP]-interacting protein (LDIP) and seipin	Modulates LD size and abundance and maintains neutral lipid homeostasis in leaves and seeds	Pyc et al. (2017); Pyc et al. (2012)
Seed LD protein (SLDP) and LD–plasma membrane adaptor (LIPA)	Form a LD–plasma membrane tethering complex that anchors LDs to the plasma membrane during post-germinative seedling growth	Krawczyk et al. (2022)

an increase in seed weight within the overexpressed lines (Chen et al. 2019). In another study, the overexpression of *Carthamus* oleosin in *Arabidopsis* enhanced the oil yield as well as average seed weight. The diameter of individual LDs was found to be less than the wild-type seeds, but these were uniformly distributed throughout the seed (Lu et al. 2018). Recent investigations of *Carthamus* oleosins have verified an inverse relationship between the size of LD and oil content. Furthermore, they proposed a positive correlation between oil content and the presence of oleosin genes. These findings imply that the expression of oleosin genes could serve as a crucial parameter for characterizing oilseed crops based on their oil content (Mosupiemang et al. 2022).

Studies in yeast have suggested that using microorganisms, apart from plant organs as factories for lipid production can serve as an alternative method for several applications (Chawla et al. 2020). *Yarrowia lipolytica,* an oleaginous yeast, utilizes a broad range of substrates and is easy to cultivate. Extensive research on lipid metabolism and the role of oleosin in its biogenesis have enabled the use of different metabolic engineering studies and heterologous expression of various enzymes to enhance the lipid titre and productivity up to several folds (Park and Nicaud 2020). In yeast, phosphatidic acid phosphorylase 1 (PAH1) enzyme dephosphorylates PA to DAG during TAG biosynthesis (Shao et al. 2019). Ectopic overexpression of *Arabidopsis OLE1* in yeast *pah1* mutant enhances the TAG and steryl ester accumulation and LD formation (Shao et al. 2019). In addition to oleaginous yeast, microalgae like marine diatom *Phaeodactylum tricornutum* serve as an excellent host for enhancing oil quantity using heterologous expression of yeast *DGAT* and *Arabidopsis OLE3*. This was accompanied with a 3.6-fold increase in TAG content (Zulu et al. 2017). This illustrates that microbial systems can be used as promising cell factories for numerous valuable products including biofuels, besides the use of oleosin as an efficient tool for increasing oil quantity and production of biologically active compounds in seeds (Parmenter et al. 1995). Additionally, in a multigene strategy named push–pull–protect, the lipid pathway is metabolically engineered by targeting multiple genes in parallel. This involves pushing the pathway towards lipid fate by WRI1, pulling the carbon and fixing into TAG by DGAT and finally protecting the lipids by oleosin (Zafar et al. 2019; Vanhercke et al. 2014). The push–pull–protect strategy employing WRI1, DGAT and oleosin has been carried out in vegetative tissues like leaves, stems and tubers. In a recent attempt in potato leaves, the TAG content was raised by more than 30-fold (Xu et al. 2020). In a very recent attempt, co-expression *Sesame indicum* oleosin L with *AtWRI* and *AtDGAT1* increased the leaf oil content up to 2.3-fold (Yee et al. 2021). A similar attempt in sorghum and tobacco led to a 100-fold and 76-fold increase, respectively, in the leaves of the transgenic plants (Vanhercke et al. 2019, 2014). Additionally, suppression of enzyme ADP-glucose phosphorylase (AGPase) in sugarcane along with overexpression of WRI1, DGAT and oleosin resulted in a 9.5-fold higher TAG content in leaves and stem of the transgenic plants than the wild-type plants (Zale et al. 2016). Overexpression of multiple genes has also been used for high TAG accumulation where the involvement of oleosin as one of the key targets shows a significant increase in oil yield (Zafar et al. 2019). Due to their rapid growth rate among higher plants, duckweeds are considered a promising candidate for biofuel feedstock production. Liang and colleagues (2022) revealed that employing a strategy involving the engineering of a sesame oleosin variant (*SiOLE(*)*), in conjunction with *Arabidopsis WRI1* and mouse DGAT following a push–pull–protect approach could significantly boost triacylglycerol (TAG) accumulation by a staggering 45-fold. Recent advancements have seen the development of metabolically engineered sugarcane varieties, dubbed 'oilcane,' characterized by their enhanced storage of energy-rich TAG within their vegetative tissues. Kanan et al. (2022) reported the first report of agronomic performance, stable co-expression of lipogenic factors and TAG accumulation in transgenic sugarcane under field conditions. This study demonstrated that the co-expression of *WRI1*, *DGAT1*, *OLE1* and RNAi-mediated knockdown of *PXA1* remained consistent over a two-year field trial, leading to TAG levels reaching 4.4% of leaf dry weight (DW). Such TAG levels represent a 70-fold increase over non-genetically modified counterparts and a more than twofold enhancement compared to earlier greenhouse-based assessments of the same genetic line. A strong positive correlation was identified between TAG accumulation and *WRI1* expression levels. Nonetheless, the constitutive expression of *WRI1* inversely impacted biomass production. Transgenic variants lacking *WRI1* expression achieved TAG concentrations up to 1.6% of leaf DW without compromising biomass yield in the initial growth cycle. These results underscore the potential of sugarcane as a viable source for the generation of vegetative lipids and offer insights for refining strategies to optimize lipid and biomass outputs in the future.

Table 4 summarizes the strategies that have been reported in the last two decades that used oleosin to increase the lipid content of plants.

Apart from the strategies which involve oleosin overexpression, modification of oleosin structure is also gaining attention. It has been demonstrated that LD integrity was enhanced in both plants and animals when seed LD proteins were incorporated with recombinant tandem head-to-tail repeats of oleosin protein (one, three or six oleosin sequence repeats), also known as polyoleosin. Such LDs also displayed freezing tolerance and delayed germination due to the

Table 4 Examples of metabolic engineering strategies involving oleosin expression to enhance the triacylglycerol (TAG) content in plants

Name	Expression	Change in lipid content	Plant	References
Prunus sibirica oleosin	Overexpression	~1.3 fold oil accumulation in seeds	*Arabidopsis thaliana*	Hu et al. (2023)
Cyperus esculentus oleosin	Overexpression	upto 2.33 fold increase in TAG content	*Nicotiana benthamiana*	Zou et al. (2023)
Sesame modified oleosin variants with mouse DGAT2	Co-expression	54% increase in leaves and 13% in seeds	*Arabidopsis thaliana*	Anaokar et al. (2024)
Sesame Cys-oleosin and *Arabidopsis* DGAT	Co-expression	2-5 fold increase in total fatty acid content	*Arabidopsis thaliana*	Winichayakul et al. (2013)
Sesame oleosin L with *Arabidopsis* WRI1 and DGAT1	Co-expression	2.3 fold increase in leaf oil content	*Nicotiana benthamiana*	Yee et al. (2021)
Sesame poly-oleosin	Overexpression	<2% increase in LD protein accumulation	*Arabidopsis thaliana*	Scott et al. (2010)
Sesame oleosin with *Arabidopsis* WRI1 and DGAT1	Co-expression	15% increase in leaf TAG content	*Nicotiana tabacum*	Vanhercke et al. (2014)
Sesame oleosin L with *Zea mays* WRI1 and *Umbelopsis ramanniana* DGAT2a	Co-expression	3–8.4% increase in leaf TAG content	*Sorghum bicolor*	Vanhercke et al. (2019)
Sesame oleosin variant with *Arabidopsis* WRI1 and mouse DGAT2	Co-expression	7–45 fold increase in TAG content	*Lemna japonica*	Liang et al. (2023)
Sesame oleosin 1 with *Arabidopsis* WRI1 and *Zea mays* DGAT	Co-expression	400 fold increase in leaf TAG content	*Saccharum officinarum*	Parajuli et al. (2020)
Arabidopsis oleosin with WRI1 and DGAT1	Co-expression	79% increase in total leaf oil content	*Zea mays*	Alameldin et al. (2017)
Sesame Cys–oleosin and *Arabidopsis* DGAT	Co-expression	6-7% increase in leaf lipid content	*Lolium perenne*	Beechey-Gradwell et al. (2019)
Arabidopsis oleosin 1 and DGAT1-2 with *Zea mays* WRI1	Co-expression	1.5- to 9.5-fold increase in TAG content	*Saccharum officinarum*	Zale et al. (2016)
Sugarcane AGPase and PXA1	Co-suppression			

inhibition of access to the lipases (Scott et al. 2010). Since polyoleosin was only found in seeds but not in the leaves, it is a major limiting factor for its functional validation in vegetative tissues (Winichayakul et al. 2013). Modification of the amphipathic arms of oleosin with strategically placed cysteine residues (Cys-oleosin) and coexpression with DGAT1 in *Arabidopsis* increased CO_2 assimilation per unit leaf area by 24%, leaf biomass by 50%, and FA content by up to five fold in vegetative tissues (Winichayakul et al. 2013). The SH-oleosins undergo ubiquitination during germination in sesame, while SL-oleosins remained unchanged and were essentially degraded by Cys proteases (Hsiao and Tzen 2011). Since these proteases are abundant at germination, the creation of a cysteine Cys bridge between the amphipathic arms has been thought to protect oleosin from degradation (Winichayakul et al. 2013; Hsiao and Tzen 2011). Since the wild-type oleosins rarely have cysteine residues in sequence, SDM was used to create recombinants with one to seven Cys residues in both N- and C-terminal helices. The variant with o3-3 Cys residues (the first and second numbers indicate the number of Cys residues in the N- and C-terminal amphipathic arms, respectively. "o" stands for oleosin) in both arms was shown to be most resistant to TAG degradation and stable during senescence (Winichayakul et al. 2013). In a recent study, this variant of (*Sesamum indicum*) oleosin, termed o3-3 Cys-OLE (SiCo), served as the basis for investigating whether alterations in presumed ubiquitin conjugation sites could facilitate the stabilization of oleosin proteins, thereby enhancing lipogenesis. An SiCo-variant, designated SiCOv1, was engineered through the substitution of all five lysine residues with arginines. Concurrently, another variant, SiCOv2, was created by excising six cysteine residues from the o3-3 Cys-OLE framework. Additionally, a composite variant, termed SiCOv3, was synthesized by amalgamating the genetic modifications present in SiCOv1 and SiCOv2. The experimental results indicated that the constitutive expression of SiCOv3, in synergy with mouse DGAT2, precipitated a significant enhancement in TAG accumulation, evidencing a 54% increase in foliar tissues and a 13% rise in seed tissues when compared with

control lines co-expressing SiCo and mouse DGAT2 (Anaokar et al. 2024). This investigation underscores the potential of targeted genetic modifications in oleosin variants for the strategic enhancement of lipid accumulation in plant systems. Another very interesting perspective was put forward recently where the surface of LDs were enveloped with Cys-oleosin capable of preventing the feedback inhibition of the pathways for carbon fixation, thereby obtaining a greater efficiency of fixation (Beechey-Gradwell et al. 2020). Since vegetative organs like leaves make up a large fraction of the crop biomass, modifications in the enzymes and proteins involved in lipid metabolism could help in raising oil yields per hectare. Perennial ryegrass (*Lolium perenne*) is a widely used forage crop in temperate regions due to its benefits in rendering tolerance to defoliation and balanced seasonal dry matter (Chapman et al. 2012). Therefore, the co-expression of Cys-oleosin/DGAT was able to benefit animals in a cost-effective manner. Perennial ryegrass transformed with DGAT and modified Cys-oleosin led to increased efficiency of foliar lipid concentration and plant biomass (Beechey-Gradwell et al. 2019, 2020). Recently, this high-metabolizable energy (HME) perennial ryegrass generated by Winichayakul and co-workers as mentioned above was further analysed for its feeding value and its suitability for adoption into the pastoral system. Incubation of HME ryegrass plant material in rumen fluids collected from four nonlactating Jersey × Holstein cows indicated a significant decrease in butyrate emission and a drop of 10–15% CH_4 proportion of overall gas production. Oleosin demonstrated strong potential for increasing forage feeding value and offering 10–15% drop in the methane proportion of overall gas production in dairy cattle (Winichayakul et al. 2020). Forage crops used for livestock grazing are often associated with microbial biohydrogenation of unsaturated FA due to fermentation in rumen along with enteric methane production (Ojha et al. 2021). The lipid degradation rate of endogenous lipases in the rumen bacteria and yeast has been shown to be reduced by oleosin, while lipid mobilization decreases drastically when oleosin gene was suppressed in *Arabidopsis* (Ojha et al. 2021; Siloto et al. 2006).

While many gene combinations have been investigated to increase seed and non-seed lipid titre, oleosin modification in co-expression remains prominant in studies that have helped in raising plant biomass and oil quantity predominantly. However, our recent study showed that *DUG1* (*DGAT upstream gene 1*), a gene upstream of *DGAT2*, which has higher expression in leaves, can enhance oil content in leaf tissues without involving oleosin. Genome editing was employed to fuse the *DGAT2* gene under the control of the *DUG1* gene promoter in the *Arabidopsis sdp1* mutant by deleting the 5′UTR region of *DUG1* to the 5′UTR region of *DGAT2* (Bhunia et al. 2022). Compared to *sdp1* mutant leaves, total lipid content (% cell dry weight) increased by twofold and TAG content by 30-fold (Bhunia et al. 2022). Hence, the application of oleosin to achieve an increase in storage oil production can also be explored.

Conclusion and future perspective

The need for the accumulation of TAG content has become an issue of real concern. In this review, different metabolic strategies have been discussed which can help foster lipid production. Oleosin has an integral role in the protection of LDs from degradation. Since targeting different genes in the TAG biogenesis and degradation pathway helps in increasing the TAG content, modulation of oleosin genes can have a significant impact in raising the oil quantity. Although green forages are an important source of nourishment for the livestock, their lesser energy content is one of the greatest drawbacks faced today. The additional release of methane emmission from cattles, roughly accounting for more than 30% of the total emission, makes them a primary contributor in ground-level ozone formation, which is a hazardous air pollutant and a greenhouse gas responsible for millions of premature deaths. It is noteworthy that cattle, buffaloes, sheep and goats in India produce an estimated 9.25–14.2 million tonnes (mt) of methane annually, out of a global total of 90 mt-plus from livestock. While methane production corresponds with livestock feed consumption, increasing the proportion of lipids in feed can reduce methane emissions by increasing caloric density and decreasing dry matter intake. So, to meet energy needs and reduce methane emissions, approach like generation of high-metabolizable energy (HME) perennial ryegrass could be advantageous. Using similar approaches and modifying Cys residues of oleosin in mesophyll tissues of leaves in fodder crops like sorghum would not only help in improving the oil accumulation in the feed, but also help in reducing the methane emission in cattles. Additionally, the higher TAG content is desirable for food and biodiesel applications as well. The increase in the current TAG level with the cumulative modifications of oleosin could be envisaged in both vegetative and non-vegetative parts of the plants. Besides, it will be worthwhile to determine if the "push–pull–protect" strategy can be superimposed in the whole plant, thereby using the whole biomass in lipid accumulation for industrial as well as livestock consumption. Apart from different biotechnological interventions highlighted above, a key concern is whether the TAG accumulation can be enhanced by diverting the carbon flow of different metabolic pathways towards lipid biogenesis. This can enable raising the TAG content several folds, thereby being beneficial for human needs and demand.

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Data availability The data used to support the findings of this study are included within the article.

Declarations

Conflict of interest All the authors declare that they have no financial or competing interests.

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