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REVIEW OPEN ACCESS

Lipid metabolism and accumulation in oilseed crops☆

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Abstract – Triacylglycerols (TAGs) serve as the most important storage form of energy and carbon in eukaryotic cells and thus are one of the fundamental macronutrients for animal and human diet. They are also used as a major feedstock for diverse industrial and energetic sectors due to their high energy density. Oilseed crops represent the most valuable source of TAGs and major world sources of edible oils. Originally, oilseeds of various species were used as a model to decipher plant lipid synthesis pathways. Given the continuous progress in research on plant lipid metabolism, here we provide an overview and update on the current state of knowledge related mainly to storage lipids in oilseeds. Moreover, we present the latest evidences on the molecular networks governing metabolism not only of TAGs but also of other seed lipids, like wax esters, sterols and sphingolipids. Finally, this review also provides a framework for understanding the complex lipid web existing in oilseeds.

Keywords: fatty acids / lipid droplets / lipid synthesis / oilseeds / triacylglycerols

Résumé – Métabolisme et accumulation de lipides dans les cultures oléagineuses. Les triglycérides (TG) représentent la forme de stockage d'énergie et de carbone la plus importante dans les cellules eucaryotes et donc l'un des macronutriments fondamentaux pour l'alimentation animale et humaine. Ils sont également utilisés comme matière première majeure dans divers secteurs industriels et énergétiques en raison de leur haute densité énergétique. Les oléagineux représentent la source la plus précieuse de TG et les principales sources mondiales d'huiles alimentaires. À l'origine, les oléagineux de diverses espèces ont été utilisés comme modèles pour déchiffrer les voies de synthèse des lipides végétaux. Étant donné les progrès continus de la recherche sur le métabolisme des lipides végétaux, nous fournissons ici une vue d'ensemble et une mise à jour de l'état actuel des connaissances liées principalement aux lipides de stockage dans les graines oléagineuses. Nous présentons aussi les dernières découvertes sur les réseaux moléculaires régissant le métabolisme non seulement des TAG mais aussi d'autres lipides des graines, comme les cires, les stérols et les sphingolipides. Finalement cette revue fournit également un cadre pour la compréhension du réseau lipidique complexe existant dans les graines oléagineuses.

Mots clés : acides gras / gouttelettes lipidiques / synthèse des lipides / oléagineux / triglycérides

1 Introduction

Oilseeds are a major global commodity as they contribute significantly to human and livestock nutrition and serve as a major feedstock for diverse branches of chemical industry as well as an important source of renewable energy in the form of biodiesel (Durrett *et al.*, 2008; Dyer *et al.*, 2008). World production of oilseeds has increased dramatically in the last decade and in 2021 it is estimated to reach nearly 600 million metric tons (https://usda.library.cornell.edu). Soybeans (*Glycine max*) are currently the leading type of oilseeds in the world, followed by rapeseed (*Brassica napus*), sunflower (*Helianthus annuus*) and peanuts (*Arachis hypogaea*), although palm (*Elaeis* spp.) oil is the world's leading vegetable oil.

☆ Contribution to the Topical Issue "Green and white biotechnologies in the fields of lipids and oil- and proteincrops / Biotechnologies vertes et blanches dans les domaines des lipides et oléoprotéagineux".

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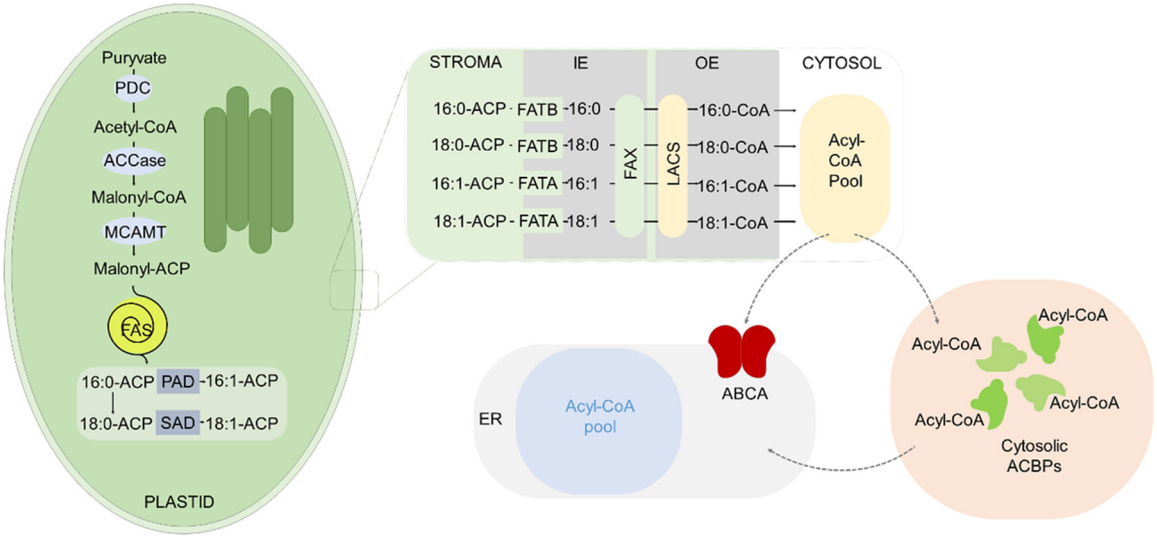

Fig. 1. General overview of fatty acid biosynthesis and eukaryotic pathway of CoAs origin in oilseed cells. Fatty acid synthesis takes place in plastids where malonyl-ACP is used as a substrate. FAs are synthesized by FAS complex in a series of condensation and elongation reactions which lead to formation of FA conjugates with ACP proteins. Their channeling to cytosol includes hydrolysis by thioesterases (FAT) to free fatty acids at the inner plastid envelope membrane (IE), followed by their transfer through the membrane mediated by FAX proteins. Once they reach the outer plastid envelope membrane (OE) they undergo vectorial acylation catalyzed by LACS enzymes. The resulting acyl-CoAs are transferred to the cytosol. The cytosolic pool of acyl-CoAs directly interacts with ACBP proteins, which serve as major transporters of acyl-CoAs from the cytosol to the ER. In the ER, acyl-CoAs are used as acyl donors for TAGs synthesis pathways. Abbreviations: ABCA: ATP-binding cassette (ABC) transporter subfamily A; ACBPs: acyl-CoA binding proteins; ACCase: acetyl-CoA carboxylase; ACP: acyl carrier protein; CoA: coenzyme A; FAs: fatty acids; FAS: fatty acid synthase complex; FATA/B: fatty acyl-ACP thioesterase; ER: endopsamic reticulum; FAX: fatty acid export protein; IE: inner plastid envelope; LACS: long chain acyl-CoA synthases; MCAMT: malonyl-CoA: acyl carrier protein S-malonyltransferase; OE: outer plastid envelope; PAD: palmitoyl-ACP desaturase; PDC: pyruvate dehydrogenase complex; SAD: stearoyl-ACP desaturase.

The most valuable components of oilseeds are triacylglycerols (TAGs), which are synthesized during seed development and reach the maximum content at seed maturity (Lung and Weselake, 2006). The process of seed formation in oil storing crops, like in all Angiosperms, starts from a successful pollination and fertilization. When a compatible pollen grain lands on the receptive stigma, it germinates into a pollen tube, which usually delivers two sperm cells into the female gametophyte – the embryo sac. During so called double fertilization, one of the sperm cells fuses with the egg cell to form the zygote, whereas the other sperm cell undergoes a fusion with the diploid polar nuclei and gives origin to a triploid endosperm. The zygote, after multiple mitotic divisions, transforms into the embryo, whereas endosperm serves as nutritive tissue for the developing embryo. In turn, the most external layers of the ovule, termed integuments, give rise to the seed coat (Bleckmann *et al.*, 2014; Dresselhaus *et al.*, 2016). The process of seed development is accompanied by a massive accumulation of reserve components, which will serve as energy and carbon sources during seed germination and post-germinative growth of the seedling. The major storage reserves are seed storage proteins (SSPs), carbohydrates (starch), and/or storage lipids (Baud *et al.*, 2008). The latter ones are the major storage reserves in oil crops and are represented mostly by TAGs (Voelker and Kinney, 2001). As this review focuses on lipid metabolism and accumulation in oilseeds, in further sections we characterize the key mechanisms related to these compounds during seed development. This includes both storage and non-storage lipids in diverse species, with a special emphasis on *Arabidopsis thaliana* being not only a leading model organism in plant biology and genetics but also a reference model for oil crops.

2 Everything starts in the plastid

In seeds and other plant tissues, *de novo* fatty acid (FA) biosynthesis takes place in plastids (Fig. 1). This biochemical pathway provides acyl chains necessary for the formation of diverse acyl lipids involved in the membrane synthesis or carbon and energy storage. *De novo* FA synthesis is catalyzed by the type II FA synthase and involves several enzymes and enzymatic complexes including the pyruvate dehydrogenase complex (PDC), acetyl-Co-A carboxylase (ACCase) or fatty acid synthase complex (FAS) (Troncoso-Ponce *et al.*, 2016b). The first committed step of FA synthesis involves formation of malonyl-CoA from acetyl-CoA and bicarbonate by ACCase. Assembly of FAs requires production of malonyl-ACP by transferring malonyl group from CoA to acyl carrier protein (ACP) catalyzed by a malonyl-CoA: acyl carrier protein S-malonyltransferase (MCAMT). The malonyl-ACP enters into a series of condensation reactions with acyl-CoA under control of FAS enzymes, which leads to production of saturated FA 16:0-ACP. Finally, 16:0-ACP is desaturated to 16:1-ACP or elongated to 18:0-ACP, which is then desaturated to 18:1-ACP (Ohlrogge and Browse, 1995; He *et al.*, 2020). The ratio between newly synthesized saturated and monounsaturated

FAs is regulated by the activity of stromal soluble acyl-acyl carrier protein desaturases (AADs). The *A. thaliana* genome contains seven genes encoding AADs including FATTY ACID BIOSYNTHESIS2 (FAB2)/SUPPRESSOR OF SALICYLIC ACID INSENSITIVE2 (SSI2) and ACYL-ACYL CARRIER PROTEIN DESATURASES1-6 (AADs) (Kachroo *et al.*, 2007). Depending on their regio- and substrate specificity, AADs belong to Δ9 stearoyl-ACP desaturases (SAD) responsible for desaturation 18:0-ACP to 18:1-ACP or Δ9 palmitoyl-ACP desaturases (PADs) involved in the production of 16:1-ACP from 16:0-ACP. Interestingly, analysis of single Arabidopsis*aad* mutants showed only slight changes in the FA composition, suggesting that AADs/SADs work redundantly in the production of 18:1-ACP. Indeed, recent studies demonstrated that four SAD desaturases – FAB2, AAD1, AAD5 and ADD6 play an important role in oleic acid biosynthesis in developing Arabidopsis seed (Kazaz *et al.*, 2020).

3 Fatty acid transporters – Transmembrane regulators of lipid homeostasis

The majority (62%) of *de novo* synthesized FAs are used for lipid synthesis in the ER *via* the eukaryotic pathway, whereas only 38% of FAs are incorporated into lipids in the prokaryotic pathway in the plastid (Li *et al.*, 2016). Before the first step of lipid synthesis in the eukaryotic pathway, free FAs need to be first exported from the plastid to the ER (Fig. 1). Therefore, acyl-ACPs are first hydrolyzed by two different classes of acyl-ACP thioesterases (FAT) at the inner plastid envelope membrane (IE) to free FAs (FFAs). In general, type A of FAT (FATA) prefers 18:1-ACP as a substrate and type B of FAT (FATB) shows preferences towards 16:0-ACP and 18:0-ACP (Salas and Ohlrogge, 2002; Bonaventure *et al.*, 2003). In oilseeds, free 18:1 (oleic acid) is the major FA exported along with small amounts of 16:0 (palmitic acid) and 18:0 (stearic acid). 18:1 and its derivatives are significantly accumulated during Arabidopsis seed development and their production is important for proper embryo development and seed maturation. Loss of the function of FATB proteins is associated with reduction of saturated FAs in oilseeds of Arabidopsis (Bonaventure *et al.*, 2003) or Camelina (Ozseyhan *et al.*, 2018).

Recently, it was well demonstrated that FATTY ACID EXPORT 1 (FAX1) protein anchored in the IE of the plastid plays an important role as a FFAs transporter in Arabidopsis leaves and flowers (Li *et al.*, 2015, 2016). Furthermore, detailed analysis of *fax1* mutant and *FAX1* overexpressing lines (*FAX1*ox) showed a significant decrease of TAG content in *fax1* leaves and flowers but not in seeds and increase of TAGs in both tissues in *FAX1*ox lines (Li *et al.*, 2015). These findings support the role of FAX1 in plastid FFAs transport for TAGs biosynthesis in Arabidopsis vegetative tissues. In Arabidopsis, the FAX transporters family includes seven members but only two of them, FAX2 and FAX4, are highly expressed during early stage of seed development. Moreover, recently (Li *et al.*, 2020) it was reported that *fax2 fax4* seeds contain 30% less TAGs compared with wild type seeds. Thus, the loss of FAX2 and FAX4 function affects FFAs transport, resulting in a drop of TAGs biosynthesis during seed development. On the other hand, small differences in the relative content (mol%) of TAG molecular species between these mutants and wild type suggest that other transporters may be also involved in TAGs synthesis in seeds (Li *et al.*, 2020).

In the next step, FFAs are shuttled across the plastid outer envelope (OE) most likely *via* vectorial acylation by long-chain acyl-CoA synthase (LACS), which catalyzes formation of acyl-CoA from FFAs (Fig. 1). In Arabidopsis, nine LACS proteins participate in lipid metabolism. However, overlapping function in the FAs transport from the plastid was reported only for three of them – LACS9, LACS1 and LACS4. Double mutants of *lacs1 lacs9* and *lacs4 lacs9* showed an 11% and 27% FA decrease of seed TAGs, respectively (Zhao *et al.*, 2010; Jessen *et al.*, 2015). Homologs of LACS genes were also identified in the *B. napus* genome. *BnLACS2* gene is highly expressed in developing seeds, regardless of whether expression was analyzed in high or low oil-content seeds (Ding *et al.*, 2020). Overexpression of *BnLACS2* in rapeseed was correlated with 6–8% increase in oil content and significant enhancement in the abundance of 18:2, 20:0, 20:1 and 22:0. On the other hand, in *BnLACS*-RNAi transgenic plants, a decrease of 3 to 6% in oil content was observed compared to wild type plants. Thus, *Bn*LACS2 may be involved in the lipid metabolism during *B. napus* seed development and can be used as a potential target for genetic manipulation, which leads to increase of rapeseed oil content. Two homologs of Arabidopsis *LACS9* and *LACS8* genes – *HaLACS1* and *HaLACS2*, respectively, were isolated from developing sunflower endosperm. Both genes exhibited high expression levels during sunflower seeds development (Aznar-Moreno *et al.*, 2014). Based on their subcellular localization and substrate activity, it has been proposed that *Ha*LACS1 might be involved in the activation of *de novo* synthesized FFAs in the plastid, whereas *Ha*LACS2 could be involved in the acyl turnover of ER glycerolipids (Aznar-Moreno *et al.*, 2014). FFAs activated to acyl-CoAs by the action of LACS are shuffled to the ER by the members of acyl-CoA binding proteins (ACBPs) (Fig. 1) (for review, see Raboanatahiry *et al.*, 2018). Plant ACBPs are divided into four separate classes according to their size and domains: Class I small ACBPs, Class II ACBPs containing ankyrin repeats, Class III large ACBPs and Class IV ACBPs. In Arabidopsis, *At*ACBP6 belongs to class I, *At*ACBP1 and *At*ACBP2 to class II, *At*ACBP3 to class III and *At*ACBP4 and *At*ACBP5 to class IV (Lung and Chye, 2016; Lai and Chye, 2021). Cytosolic ACBPs: *At*ACBP6, *At*ACBP4 and *At*ACBP5 have overlapping but distinct functions in Arabidopsis seed acyl-lipid homeostasis (Hsiao *et al.*, 2014). All of them are expressed during seed development, however, their binding affinity for acyl-CoA esters is different. Recombinant r*At*ACBP6 displayed strong affinity with long-chain acyl-CoA (16- to 18-CoA), whereas the affinity of r*At*ACBP4 and r*At*ACBP5 was much weaker for these acyl-CoAs (Hsiao *et al.*, 2014). Moreover, *acbp6* mutant was reported to accumulate 18:1-CoA in the embryos and both 18:1-CoA and 18:2-CoA in the seedlings. Thus, these ACBPs seem to play the most important role in acyl-CoA transport during seed and seedling development among the other cytosolic *At*ACBPs (Hsiao *et al.*, 2014). The ER-localized ACBPs: *At*ACBP1 and *At*ACBP2 are most probably involved in the transport of membrane-associated acyl pool from the ER to the plasma membrane (Raboanatahiry *et al.*, 2018). Homologs of all Arabidopsis ACBPs genes were

identified in *B. napus* genome (Raboanatahiry *et al.*, 2015a, b). Class I *Bn*ACBP is a homolog of *At*ABCP6 and is highly expressed in developing embryos and cotyledons of seedlings (Hills *et al.*, 1994; Engeseth *et al.*, 1996). It was also demonstrated that recombinant r*Bn*ACBP possesses the ability to bind 16:0-CoA and 18:1-CoA and to enhance the activity of the Kennedy pathway enzymes such as glycerol-3-phosphate acyltransferase (GPAT) (Brown *et al.*, 1998). So far, only few more homologs of *At*ABCP were identified and fully characterized in other oilseed crops. Strong expression in developing sunflower seeds was reported for genes encoding *Ha*ACBP6, a homolog of *At*ABCP6 and *Bn*ACBP, (Aznar-Moreno *et al.*, 2016) and *Ha*ACBP1 belonging to Class II ACBPs (Aznar-Moreno *et al.*, 2020). r*Ha*ACBP6 protein has been shown to have a higher affinity towards 16:0-CoA, 18:0-CoA and 18:1-CoA rather than 18:2-CoA. Ability of r*Ha*ACBP6 to bind different species of PC (dipalmitoyl-PC, dioleoyl-PC and dilinoleoyl-PC) suggests that this protein can be associated with TAGs biosynthesis (Aznar-Moreno *et al.*, 2016). In the following studies, r*Ha*ACBP1 displayed a high affinity to 16:0-CoA and 18:0-CoA and lower affinity to 18:1-CoA and 18:2-CoA. Similarly to r*Ha*ACBP6, r*Ha*ACBP1 has ability to bind to dioleoyl-PC and dilinoleoyl-PC (Aznar-Moreno *et al.*, 2020). The high affinity level of both proteins towards the products of *de novo* FAs synthesis clearly indicates their essential role in the transport and trafficking of acyl-CoAs in sunflower developing seeds. Based on *in silico* analysis of ACBPs, 11 members of soybean *Gm*ACBPs were identified and classified into four classes (Azlan *et al.*, 2021). Two genes encoding *GmACBP1* and *GmACBP2* proteins from Class I of ACBPs showed elevated expression during soybean seed development. However, more detailed analyses are needed in order to characterize the function of all members of soybean ACBPs in lipid trafficking in seeds.

In addition to ACBPs proteins, Arabidopsis *At*ABCA9 that belongs to ATP-binding cassette (ABC) transporter subfamily A, was characterized as a FA/acyl-CoA transporter which mediates transport of FAs into the ER (Kim *et al.*, 2013). *At*ABCA9 is localized in the ER and is highly expressed during seed development. Seeds of *abca9* mutants were characterized by reduced size, abnormal morphology and lower levels of TAGs, whereas the overexpression of *AtABCA9* increased seed TAG content when compared to wild type plants (Kim *et al.*, 2013). Moreover, feeding experiments showed significantly lower incorporation of FAs into TAGs in *abca9* seeds than in the wild type. Thus, it was concluded that *At*ABCA9 functions as a transporter of acyl-CoAs into the ER, where they can be used as substrates for TAGs synthesis. Interestingly, loss of function or overexpression of different transporters very often leads to the changes in the seed oil content. Therefore, use of lipid transporters could also be one of the strategies to increase oil content in seeds. Recently, it was demonstrated that overexpression of Arabidopsis *FAX1* and *ABCA9* affects oil production in Camelina seeds (Cai *et al.*, 2021). Moreover, simultaneous overexpression of both genes had a better effect on enhancing oil production in seeds than overexpression of *AtFAX1* and *AtABCA9* individually (Cai *et al.*, 2021).

4 TAG synthesis is not a simple linear pathway

After being transported from the plastid to the ER, the acyl-CoAs are utilized for TAGs or membrane lipid synthesis. TAGs, major storage lipids in oilseeds, can be synthesized *via* different pathways. The most common pathway to assembly FAs into TAGs is the acyl-CoA-dependent Kennedy pathway (Fig. 2). In this pathway, acyl-CoAs are incorporated into the sn-1 and sn-2 positions of glycerol-3-phosphate (G3P) by G3P acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAAT), respectively, to form phosphatidic acid (PA). Next, a phosphate group is removed from PA by phosphatidic acid phosphatase (PAP) and the resulting diacylglycerol (DAG) is acylated on the sn-3 position by acyl-CoA: diacylglycerol acyltransferase (DGAT) (Chapman and Ohlrogge, 2012). This reaction completes the *de novo* synthesis of TAGs and indicates DGATs as the key enzymes for oil accumulation in oilseeds. Plant DGATs identified so far belong to three distinct families. DGAT1 and DGAT2, which are integral ER proteins and play non-redundant roles in TAGs biosynthesis, and DGAT3 being a cytosolic enzyme (Turchetto-Zolet *et al.*, 2016). DGAT1 is a key enzyme involved in TAGs formation in developing seeds. Disruption of Arabidopsis DGAT1 resulted in decrease in seed TAG content and changes in their profile, characterized by the higher level of 18:3 when compared to wild type plants (Katavic *et al.*, 1995; Routaboul *et al.*, 1999; Zou *et al.*, 1999). In turn, its overexpression resulted in increased seed oil accumulation (Jako *et al.*, 2001). In contrast, *dgat2* mutation had no impact on seed TAGs deposition (Zhang *et al.*, 2009). However, later studies on plants accumulating unusual FAs showed that DGAT2s are responsible for incorporation of unusual FAs into TAGs (Li *et al.*, 2010), such as ricinoleic acid in *Ricinus communis* (Burgal *et al.*, 2008) or eleosteric acid in *Vernicia fordii* (Shockey *et al.*, 2006). Studies on *B. napus* revealed that DGAT2s isoforms have different substrate specificities and display highly variable activity towards 18:3 and from very little to high activity towards 22:1 (Demski *et al.*, 2019). Interestingly, *Camelina sativa* DGAT1 and DGAT2 have complementary substrate specificities. *Cs*DGAT1 possesses high specificity towards acyl donors with saturated and monounsaturated FAs, whereas *Cs*DGAT2 prefers acyl acceptors containing only polyunsaturated FAs (Lager *et al.*, 2020). The third family of DGAT enzymes, DGAT3 consists of soluble cytosolic enzymes identified for the first time in peanut (Saha *et al.*, 2006). In Arabidopsis, DGAT3 is involved in recycling of 18:2 and 18:3 FAs into TAGs when storage lipids are not catabolized (Hernández *et al.*, 2012). Recently, it was shown that Arabidopsis DGAT3 is a metalloprotein with the ability to synthetise TAGs *in vitro* (Aymé *et al.*, 2018). Among three DGAT3 genes identified in *C. sativa*, *CsDGAT3-3* was highly expressed in developing seeds. Moreover, overexpression of *CsDGAT3-3* leads to accumulation of TAGs in yeast and *Nicotiana benthamiana* leaves. *Cs*DGAT3-3 showed preference towards unsaturated FAs, particularly eicosenoic acid (20:1n-9) (Gao *et al.*, 2021). Since the terminal step of the Kennedy pathway is one of the rate-limiting steps of TAGs synthesis, DGATs are one of the main targets of genetic

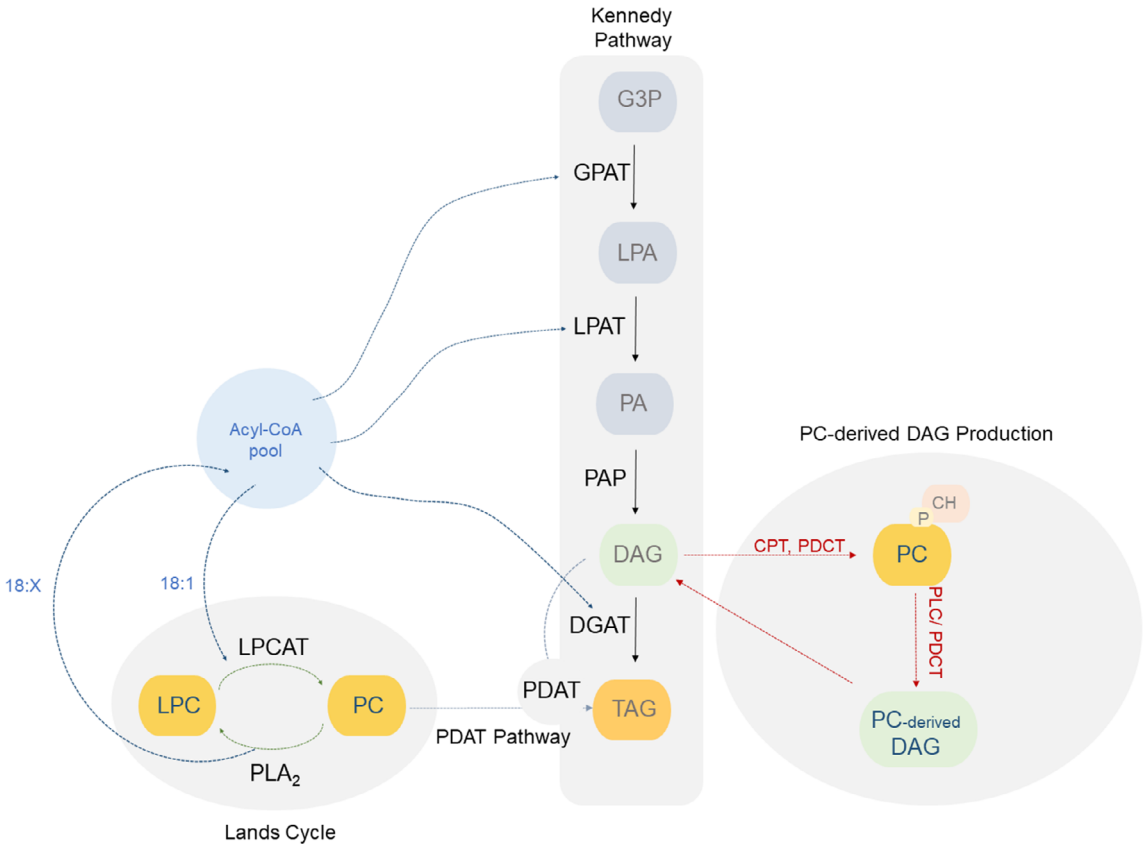

Fig. 2. Schematic presentation of ER localized TAG formation pathways in oilseeds. Acyl-CoAs from the plastid are exported to the cytosol and used as substrates in glycerol transesterification reactions of the Kennedy pathway. The final product of these reactions, TAGs formed by esterification of DAG by DGAT enzyme. DAGs can also be transformed to TAGs by the action of PDAT enzyme, which uses PC as the acyl donor (PDAT pathway). The cycles of deacylation/reacylation of PC/LPC catalyzed by phospholipases (PLA2) and Acyl-CoA: Lysophosphatidylcholine Acyltransferase (LPCAT), respectively, deliver acyl-CoAs. These reactions are known as the Lands cycle and produce new or modified acyl-CoAs. PC can also serve as a donor of DAG moiety used for synthesis of TAGs (PC-derived DAG production). Detailed description of the presented pathway can be found in the text. Abbreviations: CoA: coenzyme A; CPT: CDP-choline: diacylglycerol phosphotransferase; DAG: diacylglycerol; DGAT: acyl-CoA: diacylglycerol acyltransferase; G3P: glycerol 3 phosphate; GPAT: acyl-CoA: glycerol-3-phosphate acyltransferase; LPA: lysophosphatidic acid; LPAT: acyl-CoA: lysophosphatidic acid acyltransferase; LPCAT: acyl-CoA: lysophosphatidylcholine acyltransferase; PA: phosphatidic acid; PAP: phosphatidic acid phosphatase; PC: phosphatidylcholine; PDCT: phosphatidylcholine diacylglycerol cholinephosphotransferase; PLC: phospholipase C; PLA2: phospholipase A2; TAG: triacylglycerol.

manipulations towards increasing oil production in plants (reviewed in Xu *et al.*, 2018). Overexpression of DGAT enzymes led to enhanced oil deposition in rapeseed (Weselake *et al.*, 2008; Taylor *et al.*, 2009), *Jatropha curcas* (Zhang *et al.*, 2021), soybean (Lardizabal *et al.*, 2008; Roesler *et al.*, 2016), cottonseed (Wu *et al.*, 2021) and maize (Oakes *et al.*, 2011).

TAGs synthesis is not a simple linear pathway as TAGs composition depends on the flux of acyl groups in and out of phosphatidylcholine (PC) prior FAs incorporation to the TAGs. Most *de novo* 18:1-CoAs are first incorporated into membrane PC and then desaturated by the action of fatty acid desaturases. FAD2 (ω-6 fatty acid desaturase) catalyzes the conversion of 18:1-CoA into 18:2-PC, which is then desaturated to 18:3-PC by FAD3 (ω-3 fatty acid desaturase) (Lou *et al.*, 2014). By the action of FAD2 and FAD3 most of oil crop seeds contain high concentrations of polyunsaturated FAs (PUFAs), such as 18:2 and 18:3 (Dar *et al.*, 2017; Du *et al.*, 2018). However, the high concentration of PUFAs reduces the oxidative stability of oil and lowers its quality. Thus, the recent genetic engineering strategies focus on generation of transgenic seeds containing reduced amount of PUFAs and higher content of monounsaturated fatty acids (MUFAs), such as oleic acid. RNAi and CRISPR-Cas9 technologies were employed to create transgenic plants with mutated FAD2 and FAD3. CRISPR-Cas9 editing of the soybean *GmFAD2-1A*, *GmFAD2-1B* and *GmFAD2-2* led to relevant increase in 18:1 and decrease in 18:2 content (Al Amin *et al.*, 2019; Do *et al.*, 2019). Similarly, the seeds of Camelina triple *Cs*FAD2 knockout mutants generated by CRISPR-Cas9 system showed reduced content of total PUFAs and significant increase in MUFA levels reaching 80% of dry weight. Unfortunately, the transgenic lines showed inhibited growth and produced less seeds compared to wild type plants (Lee *et al.*, 2021). However, Camelina plants expressing only one copy of

CsFAD2 gene showed normal growth rates and increased levels of MUFA reaching 60% of dry weight (Lee *et al.*, 2021). Acyl-CoAs can be also elongated by fatty acyl elongase complex, which leads to production of very long-chain FAs (VLCFAs). FAE1 gene encodes 3-ketoacyl-CoA synthase (KCS), which catalyzes the first rate-limiting step of acyl-CoA elongation and is commonly used to modify acyl-CoA pool towards higher levels of VLCFAs (Mietkiewska *et al.*, 2007; Ma *et al.*, 2021).

Acyl-editing mechanisms, including the land cycle or reversible LPCAT (acyl-CoA: lysophosphatidylcholine acyltransferase) activity, are responsible for exchange of FAs between PC and acyl-CoA pool (Fig. 2). In the Land cycle, FAs are released from PC by the action of phospholipase A2 (PLA2), which leads to production of lysophosphatidylcholine (LPC) and FFAs. Before entering the acyl-CoA pool, FFAs are activated to acyl-CoAs by LACS (Fig. 1). Meantime, reacyclation of LPC and incorporation of 18:1 acyl-CoAs are catalyzed by the LPCAT (see review Bates, 2016). On the other hand, the reverse activity of LPCAT leads to a direct transfer of FAs from PC into acyl-CoA pool (Bates, 2016). A third pathway of TAGs synthesis implies the acyl flux through PC *via* acyl-CoA-independent mechanism governed by a phospholipid: diacylglycerol acyltransferase (PDAT). This enzyme catalyses an acyl transfer from sn-2 position of PC to DAG leading to formation of TAGs (Dahlqvist *et al.*, 2000). PDAT contribution to TAGs biosynthesis in seeds is still not well understood. In the PDAT knockout lines of Arabidopsis no changes in seed TAG content were observed (Mhaske *et al.*, 2005), which was attributed to the ability of DGAT1 to fully compensate for the loss of PDAT activity (Zhang *et al.*, 2009). Since a double *dgat1 pdat* mutant produced sterile pollen, downregulation of *PDAT* in *dgat1* background was achieved using RNAi silencing and resulted in reduction of seed oil content up to 80% and impaired embryo development (Zhang *et al.*, 2009). The ratio between PDAT and DGAT activities differs among oilseed plants. For example, it was significantly higher in membrane fractions obtained from safflower developing seeds compared to sunflower. However, the specificities of PDATs from both species were similar as in both cases the substrate preference for 18:2-PC over 18:1-PC as an acyl donor was observed (Banas *et al.*, 2013). Interestingly, overexpression of *CsPDAT* significantly increased 18:2 on the expense of 18:3 and 20:1 in transgenic *C. sativa* seeds (Marmon *et al.*, 2017). In addition, recent studies on *Cs*PDAT showed that its activity depends on available DAG species. In *in vitro* analysis of *Cs*PDAT specificity performed with di-18:2-DAG, 18:3-PC was rather preferred than 18:2-PC, whereas with di-18:3-DAG the opposite pattern was observed (Lager *et al.*, 2020). It has also been shown that PDATs play an important role in the incorporation of unusual FAs into TAGs. PDATs from castor bean or *Crepis palaestina* showed a preference for phospholipids containing hydroxylated and epoxidated FAs, typical for these plant species (Dahlqvist *et al.*, 2000). Co-expression of the gene encoding seed-specific castor bean fatty acid hydroxylase (*RcFAH12*) together with *RcPDAT* enhanced accumulation of hydroxy FAs up to 27% in Arabidopsis seeds (Kim *et al.*, 2011; van Erp *et al.*, 2011). Finally, production of TAGs implicates the flux of DAG moieties between DAGs synthesized by the Kennedy pathway and PC. *De novo* synthesized DAGs are incorporated into PC by the action of CDP-choline: diacylglycerol cholinephosphotransferase (CPT) and PC-derived DAGs are generated by the cleavage of PC by phospholipase C (PLC) (Bates, 2016). The phosphatidylcholine: 1,2-sn-diacylglycerol choline phosphotransferase (PDCT) is another enzyme involved in the transfer of the phosphocholine headgroup between DAG and PC (Lu *et al.*, 2009). Recently, the interactions between *At*DGAT1 and *At*LPCAT2, *At*PDCT and *At*PDAT1 were confirmed in yeast two-hybrid system (Regmi *et al.*, 2020). The interaction between these enzymes supports the hypothesis about the crosstalk between different metabolic networks leading finally to TAGs synthesis. This finding proves as well that *At*DGAT1 uses both PC-derived acyl-CoA and DAG as substrates (Regmi *et al.*, 2020).

5 Seed lipid droplets – The final destination for TAGs

In seeds, the TAGs synthesized by the above mentioned enzymes are packed into specialized organelles called lipid droplets (LDs), which consist of a neutral lipid core surrounded by a phospholipid monolayer harbouring specific structural proteins, such as oleosins, caleosins and steroleosins (recently reviewed in Huang, 2018; Shao *et al.*, 2019; Zienkiewicz and Zienkiewicz, 2020). Oleosins are small proteins (15–30 kDa) that contain cytosolic-facing N- and C-termini and a large hydrophobic domain essential for their targeting to LDs. Oleosins play an important role in the regulation of LDs size and stability. In the *A. thaliana* genome, 16 oleosin-encoding genes have been identified and five of them are specifically expressed in seeds (Kim *et al.*, 2002). *At*OLE1 accounts for approximately 65% of the LD-associated proteins in seeds, while *At*OLE2 and *At*OLE4 together represent around 30% of the LDs proteins. *At*OLE3 and *At*OLE5 are less abundant in the seed LD fraction (Siloto *et al.*, 2006; Miquel *et al.*, 2014; Huang, 2018). Caleosins comprise an N-terminal hydrophilic domain with an EF-hand calcium binding motif, a central hydrophobic region containing a proline knot that anchors the protein to the LDs membrane and a C-terminal hydrophilic region with several phosphorylation sites (Hanano *et al.*, 2006; Purkrtova *et al.*, 2007). Among eight Arabidopsis caleosin-encoding genes, *AtCLO1* and *AtCLO2* are expressed abundantly in developing seeds (Naested *et al.*, 2000; Poxleitner *et al.*, 2006). Steroleosins are hydroxysteroid dehydrogenases (HSD) involved in metabolism of brassinosteroids. A sterol-binding dehydrogenase/reductase domain is localized on the C-terminus of the protein, whereas N-terminal hydrophobic region with a proline knob motif anchors the protein to the LDs membrane (Lin and Tzen, 2004; d'Andréa *et al.*, 2007). Other LD-associated proteins, such as oil body associated protein 1 (OBAP1), LDAP-interacting protein (LDIP) or different low-abundance LD proteins were also identified in oilseeds (López-Ribera *et al.*, 2014; Pyc *et al.*, 2017; Kretzschmar *et al.*, 2020).

The mechanism of LD biogenesis in plants (reviewed in Chapman and Ohlrogge, 2012; Ischebeck *et al.*, 2020) is still not well characterised. LD formation starts on the ER membrane. Storage lipids are synthesised by lipid biosynthetic enzymes associated with the ER and accumulate between the two leaflets of the ER bilayer to form lens-like structures.

Table 1. Approximate fatty acid composition of TAGs from selected oilseeds. The values are presented as % of total fatty acids (TFAs) and the given ranges represent the data from diverse literature sources (soybean: Kanai *et al.*, 2019; Vogel *et al.*, 2019; rapeseed: Baud and Lepiniec, 2010; Lu *et al.*, 2020; Arabidopsis: Li *et al.*, 2006; Kelly, 2018; Camelina: Ciurescu *et al.*, 2016; Ozseyhan *et al.*, 2018).

Fatty acid (% TFA)	Soybean	Rapeseed	Arabidopsis	Camelina
16:0	10–13	3–4	7–8.5	6–7
18:0	3–5	1–2	3–3.5	1.5–4
18:1	18–24	16–24	15–18.5	11–16
18:2	53–55	13–16	27–29	18–19
18:3	8–13	7–9	17–19	33–37
20:0	–	1–2	1.7–2	0–3.5
20:1	–	9–15	17–20	12–13
20:2	–	–	–	1.4–1.7
20:4	–	–	–	0–1
22:1	–	40–45	1.5–2	2.5–5

Oleosins are co-translationally inserted into the ER and move to nascent LDs. Mature LDs detach from the ER or are formed by the fusion of nascent small LDs (Chapman and Ohlrogge, 2012; Huang, 2018). To date, several proteins orchestrating LDs formation have been identified. Among them, SEIPINs play a crucial role in controlling the number and size of LDs. In the *A. thaliana* genome, there are three SEIPIN homologs (Cai *et al.*, 2015). SEIPIN2 and SEIPIN3-deficient mutants produced enlarged LDs (Taurino *et al.*, 2018), whereas silencing of SEIPIN1 led to reduced seed size and lower oil accumulation (Cai *et al.*, 2015). In plants overexpressing *SEIPIN1*, an increased number of LDs and higher neutral lipid content compared to the wild type was observed (Cai *et al.*, 2015). SEIPINs are localized at ER-LD junctions and cooperate with other proteins from LD biogenesis machinery (Greer *et al.*, 2020). A series of recent studies identified SEIPIN-interaction partners, which included the vesicle-associated membrane protein (VAMP)-associated protein (VAP) family member *At*VAP27-1 (Greer *et al.*, 2020), lipid droplet-associated protein (LDAP) and LDAP-Interacting Protein (LDIP) (Pyc *et al.*, 2021). Based on these findings, Pyc *et al.* (2021) proposed a new and more detailed model of LD biogenesis.

Overexpression of different genes encoding LD-associated proteins can lead to enhanced LDs assembly. In rice, the expression of two soybean oleosin genes under an embryo-specific promoter resulted in smaller and more numerous LDs compared to the wild type and increased the seed lipid content up to 46% (Liu *et al.*, 2013). It was also shown that mouse fat-storage-inducing transmembrane protein (FIT2), which is involved in LD biogenesis, can promote LDs accumulation in Arabidopsis seeds (Cai *et al.*, 2017). The possibility of enhancing LDs assembly *via* overexpression of genes encoding plant LD-associated proteins was investigated in yeasts (Froissard *et al.*, 2009; Jacquier *et al.*, 2013) and microalgae (Zulu *et al.*, 2017). Yeasts were engineered for the accumulation of increased neutral lipid levels by promoting LDs formation by introducing genes encoding LDs proteins, such as *At*OLE1 (Jacquier *et al.*, 2013) or *At*CLO1 (Froissard *et al.*, 2009).

6 Endosperm or embryo – This is a question

Depending on species, LDs accumulate in different parts of the seeds. Some of the oilseed crops, like castor bean store TAGs mainly in endosperm, whereas in rapeseed, Arabidopsis, soybean or sunflower most of these lipids are present in embryo (Baud and Lepiniec, 2010). The seed oil content varies between diverse species of oil crops and ranges from about 20% for soybeans (Clemente and Cahoon, 2009) to over 40% for sunflowers or canola (rapeseed) (Matthaus *et al.*, 2016; Premnath *et al.*, 2016). Importantly, the oilseed crops vary not only in the seed oil content but also in its composition (Tab. 1). The most common FAs found in seed TAGs include palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and α-linolenic acid (18:3) (Voelker and Kinney, 2001). However, many other FAs have been described in TAGs accumulated in the seeds of diverse Angiosperms. These include longer FAs composed of 20 to 24 carbon atoms identified in Brassicaceae family, like gondoic acid (20:1) or erucic acid (22:1) as well as modified common FAs, like hydroxylated ricinoleic acid (18:1-OH) found in castor bean (Baud and Lepiniec, 2010). Interestingly, the studies of Woodfield *et al.* (2017) in *B. napus* seeds by using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) revealed highly diverse distribution patterns of TAGs molecular species between different tissues of the seed. For example, the authors showed that palmitate-containing PC and TAGs are particularly abundant in the embryonic axis. In contrast, seed coat/aleurone layer is mostly enriched in lipid species containing 18:2 and 18:3 (Woodfield *et al.*, 2017). Such heterogeneous spatial distribution patterns of lipids likely indicate differences in lipid metabolic pathways between diverse tissues of the seed.

7 Wax esters – Unique seed storage lipids

Wax esters (WEs) are unusual type of storage lipids in plants. They are accumulated in seeds of *Simmondsia chinensis* (jojoba), a perennial desert shrub native to North America.

Jojoba oil accounts for approximately 60% of the seed weight and contains WEs composed of very long-chain (C20, C22, and C24) monounsaturated FAs and alcohols (Miwa, 1971; Ohlrogge *et al.*, 1978). WEs are synthesised in developing embryos by a three-step pathway, which involves the activity of fatty acid elongase (FAE), fatty acyl reductase (FAR) and wax synthase (WS). FAE is a complex of four membrane-associated enzymes with activities similar to FAS, which elongate 18:1-CoA by two carbons producing 20:1-CoA, 22:1-CoA and 24:1-CoA (Lardizabal *et al.*, 2000). Formed long-chain acyl-CoAs are further reduced by FAR to corresponding alcohols (Metz *et al.*, 2000). The final step of WE synthesis is catalyzed by WS, fatty acyl-CoA: fatty alcohol acyltransferase, which esterifies long-chain fatty acyl-CoAs to long-chain fatty alcohols (Lardizabal *et al.*, 2000).

WEs are stored in so called wax bodies with a structure resembling plant LDs (Rost and Paterson, 1978). Wax bodies are formed at about 55–60 days after pollination and WE content increases during the entire maturation stage (Benzioni *et al.*, 2007). Analysis of MS/MS data for polypeptides isolated from the wax body fraction resulted in matches with oleosins from castor bean and grape (Rajangam *et al.*, 2013). In the recently published jojoba genome, the sequences of six oleosins, one caleosin and two lipid droplet-associated protein (LDAPs) have been identified. Based on the abundance of LDAP1 in wax bodies localized in cotyledons, it was suggested that this protein may be important for the proper packaging of WEs (Sturtevant *et al.*, 2020). Jojoba WEs have a variety of industrial applications. However, due to the expensive and limited production of jojoba oil, they are mainly used as a component of pharmaceuticals and cosmetics. Metabolic engineering approaches enabled the transfer of the jojoba WEs biosynthetic pathway to bacteria (Kalscheuer *et al.*, 2006), yeast (Wenning *et al.*, 2017, 2019) and plants (Iven *et al.*, 2016; Li *et al.*, 2019; Yu *et al.*, 2018). Jojoba-like WEs were produced in TAG-accumulating non-food Brassicaceae species, such as Arabidopsis, *C. sativa*, *Brassica carinata*, *Crambe abyssinica* (Iven *et al.*, 2016; Zhu *et al.*, 2016) and *Lepidium campestre* (Ivarson *et al.*, 2017), reaching up to 100 mg of WE per 1 g of seeds. Such engineered oilseed crops are promising platforms for future renewable and sustainable WEs production for commercial purposes.

8 Transcriptional factors as puppet masters

Accumulating evidence suggests that a transcriptional cascade of diverse transcription factors (TFs) controls seed oil deposition (Baud and Lepiniec, 2010). The master regulator of FAs biosynthesis is WRINKLED1 (WRI1), a member of the APETALA2 (AP2) family of transcription factors (Kong and Ma, 2018). The Arabidopsis *wri1* mutant produced wrinkled seeds with reduced oil content by up to 80% when compared to wild type plants (Focks and Benning, 1998; Cernac and Benning, 2004). In turn, overexpression of *AtWRI1* or other *WRI1* orthologs elevated oil content in transgenic seeds (Cernac and Benning, 2004; Liu *et al.*, 2010; An and Suh, 2015; Vogel *et al.*, 2019). *At*WRI1 regulates the transcription of multiple genes directly involved in glycolytic and FAs biosynthesis pathways such as ACP3, KASI or FAD2. The activation of *AtWRI1* depends on the action of LEAFY COTYLEDON1 (LEC1), LEAFY COTYLEDON2 (LEC2) and FUSCA3 (FUS3) TFs located upstream of *AtWRI1* in the regulatory network (Baud *et al.*, 2007; Mu *et al.*, 2008). These TFs together with ABSCISIC ACID INSENSITIVE 3 (ABI3) are known as a LAFL proteins (LEC2, ABI3, FUS3, LEC1) (Boulard *et al.*, 2017; Tian *et al.*, 2020). LAFL TFs play an important role in different aspects of seed development, including oil accumulation. In addition to positive regulators of FAs synthesis in seeds, few negative regulators of oil synthesis were identified as well. MYB89 TF was reported as an important repressor of *AtWRI1* (Li *et al.*, 2017). In turn, TRANSPARENT TESTA8 (TT8) as a repressor of Arabidopsis *LEC1*, *LEC2* and *FUS3* expression (Chen *et al.*, 2014) and TRANSPARENT TESTA GLABRA1 (TTG1) negatively regulates the transcription of *AtFUS3* (Chen *et al.*, 2015). In the maturating endosperm of Arabidopsis, the transcription of two PADs desaturases *AAD2* and *AAD3* is regulated by two TFs: MYB115 and MYB118 (Troncoso-Ponce *et al.*, 2016a). While LEC2 activates the transcription of both TFs, MYB18 negatively regulates the transcription of *LEC2*. Both MYBs work simultaneously in regulation of *AAD2* and *AAD3* transcription (Troncoso-Ponce *et al.*, 2016a). In addition to transcriptional regulation of the genes involved in FAs synthesis, Lee *et al.* (2018) reported that R2R3-type MYB96 TF activates expression of *AtPDAT1* and *AtDGAT1*. Overexpression of *MYB96* resulted in higher content of accumulated TAGs in seeds, while in *myb96*-deficient mutant the deposition of TAGs was lower compared to the wild type (Lee *et al.*, 2018).

9 Best supporting characters – Sterols and sphingolipids

In general, sterols are isoprenoid-derived lipids playing essential role in membrane fluidity and permeability and serve as precursors for brassnosteroids synthesis (Valitova *et al.*, 2016; Bajguz *et al.*, 2020). Sterols, together with sphingolipids, form membrane microdomains called lipid rafts, which are involved in diverse cellular processes (Xu *et al.*, 2001). In plants, cycloartenol serves as a common substrate for sterols synthesis including cholesterol, sitosterol, stigmasterol and campesterol. Interestingly, during Arabidopsis seed development, expression of the genes encoding enzymes responsible for sterol biosynthesis is positively correlated with the expression of the genes associated with FAs and TAGs biosynthesis (Yu *et al.*, 2021). Moreover, accumulation of significant levels of total sterols is observed in oilseeds (Harker *et al.*, 2003). On the other hand, storage form of sterols–sterol esters are together with TAGs accumulated in seed LDs. Recently, Yu *et al.* (2021) revealed that mutations in sterol synthesis pathway in *A. thaliana* resulted not only in reduced seed TAG content and seed yield, but also in the presence of enlarged, but less numerous LDs. Moreover, the impairment of sterol synthesis was also accompanied by the reduced levels of seed oleosin. Based on their results, the authors suggested that sterols might be involved in regulating essential physico-chemical properties of the ER membrane required for a proper LDs formation and/or controlling the recruitment of LD-associated proteins necessary for formation of LDs during seed development (Yu *et al.*, 2021).

Sphingolipids, together with sterols are structural and functional membrane lipids. In plants, sphingolipids are classified into four main classes: 1) long-chain base (LCBs), 2) ceramides (Cer), 3) glucosylceramides (GluCer) and 4) glycosyl inositolphosphoceramides (GIPCs) (Michaelson *et al.*, 2016). Detailed sphingolipid profiling was performed on Arabidopsis and Camelina seeds and oil (Tellier *et al.*, 2014). Interestingly, Cer and hCer (hydroxylated Cer) were accumulated in both, seed and oil, meanwhile GluCer and GIPCs were absent in the oil fraction. Moreover, Arabidopsis seeds are characterized by the accumulation of the specific GIPCs such as NH2-GIPCs and series B GIPCs. Regarding to Camelina seeds, less Cers and hCers were observed when compared to Arabidopsis seeds, while both Camelina and Arabidopsis seeds showed comparable levels of GluCer, GIPCs or NH2-GIPCs (Tellier *et al.*, 2014). Significant decrease in GlcCer and Cer content was observed during soybean seed development (Wang *et al.*, 2006). Recently, it was demonstrated that loss of serine palmitoyltransferase (SPT) regulation in the CRISPR/Cas9 knockout mutant of OROSOMUCOID-LIKE PROTEINS (ORMs) leads to production of nonviable and abnormal seeds (Gonzalez-Solis *et al.*, 2020). The analysis of lipid content demonstrated that TAG content was reduced in abnormal seeds up to 85–95%, when compared to wild type seeds. Moreover, the reduction in TAG content was accompanied by a high concentration of ceramides in the abnormal seeds (Gonzalez-Solis *et al.*, 2020). These results suggest that homeostasis in sphingolipid content is essential for proper seed development, however, the progress in understanding of this phenomena is still in its infancy. Overall, our knowledge on both sterol and sphingolipid involvement in oil accumulation in seeds is still scarce and more research is needed in order to elucidate the role of non-TAG-related compounds in oilseed lipid metabolism.

10 Concluding remarks

Although seed lipid metabolism is thus far the best characterized among all the plant tissues and organs, many questions remain to be elucidated. Definitively, sequencing of the oil crops genomes boosted the progress in our understanding of this phenomena and has set the stage for identifying and characterizing genes and their protein products involved in diverse aspects of lipid metabolism. The research of the last decade only delivered plenty of valuable data on transcriptional network governing TAGs synthesis in oilseeds, novel intracellular transporters of FAs or on the functional nature of lipid modifying enzymes. Moreover, cutting-edge lipidomic profiling of oilseeds and mapping of diverse lipids *in situ* by MALSI-MSI continuously deliver big data sets on the complexity of lipids in oilseeds. This knowledge is of essential meaning not only to fill the gaps in our understanding of the key lipid pathways existing in plant cells, but also for development of biotechnological and genetic engineering of oil crops in order to increase their energetic density. The latter aspect is currently extremely important because of negative climatic changes occurring currently on Earth, progressive reduction of arable land and constantly growing human population, which together could face us with a global food crisis in the nearest future. Thus, future work should focus mechanistic studies on oilseed lipid metabolism, combining diverse omics technologies, molecular biology and biotechnology. Such a combined approach will guarantee the substantial progress in deciphering yet unknown but crucial avenues that underlie oil synthesis in seeds.

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