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Transport and transcriptional regulation of oil production in plants

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ABSTRACT

Triacylglycerol (TAG) serves as an energy reservoir and phospholipids as build blocks of biomembrane to support plant life. They also provide human with foods and nutrients. Multi-compartmentalized biosynthesis, trafficking or cross-membrane transport of lipid intermediates or precursors and their regulatory mechanisms are not fully understood. Recent progress has aided our understanding of how fatty acids (FAs) and phospholipids are transported between the chloroplast, the cytoplasm, and the endoplasmic reticulum (ER), and how the ins and outs of lipids take place in the peroxisome and other organelles for lipid metabolism and function. In addition, information regarding the transcriptional regulation network associated with FA and TAG biosynthesis has been further enriched. Recent breakthroughs made in lipid transport and transcriptional regulation has provided significant insights into our comprehensive understanding of plant lipid biology. This review attempts to highlight the recent progress made on lipid synthesis, transport, degradation, and their regulatory mechanisms. Metabolic engineering, based on these knowledge-powered technologies for production of edible oils or biofuels, is reviewed. The biotechnological application of metabolic enzymes, transcription factors and transporters, for oil production and composition improvement, are discussed in a broad context in order to provide a fresh scenario for researchers and to guide future research and applications.

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Introduction

Lipids exhibit similar non-polar chemical properties and are easily soluble in non-polar organic solvents such as chloroform and hexane. These can be classified into many different types according to key features of their structure. Common classifications include FAs, galactolipids, phospholipids, sphingolipids, mono-, di-, or triacylglycerol (MAG, DAG, TAG, respectively), terpenoids, and Vitamins A, D, and E. Each group plays an important role in diverse physiological processes; for example, phospholipids are the major structural molecules in bilayer biomembranes; galactolipids are the predominant components of photosynthetic membranes in the chloroplast; and TAGs represent the major storage lipids in plant seeds. The cuticular lipids, produced by epidermal cells, provide a barrier against water loss, pathogen attacks and various environmental stresses. They are regarded as significant resources and potential to be manipulated for biofuels and bioproducts. FAs also serve as precursors for hormone biosynthesis.[1] In higher plants, TAGs serve as energy stores that support the germination and growth of young seedlings. Vegetable oils are of significant nutritional value to the human population, providing calories and essential substances to fuel human body.[2] The importance of FAs and the major metabolic end products of TAGs and membrane glycerolipids in terms of life activity have led to increased demand for them in food, chemical, and medical industries. Though several reviews have discussed advances in the field previously,[3,4] significant progress has been made in our understanding of lipid biosynthesis, transport,[5,6] storage,[7] degradation,[8] and transcriptional regulation.[9] With the aim of updating new findings, particularly those in lipid transport and transcription regulation for metabolic engineering, this article reviews and discusses transport and transcriptional regulation mechanisms that may hold keys and have great potential to be used for improvement of plant oil production and composition.

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Supplemental data for this article can be accessed here.

Lipid biosynthesis

Lipid biosynthesis includes multi-step reaction pathways that are governed by genetic and biochemical mechanisms and highly regulated at the transcription level (Figure 1).

FA biosynthesis

FAs are precursors for all lipids, whether for an energy storage or membrane structure. In the chloroplast, after acetyl-CoA carboxylase (ACCase) generated malonyl-CoA, the FA synthase complex transfers malonyl moiety to acyl-carrier proteins (ACPs) for the generation of long-chain FAs, mainly C16:0, C18:0 and C18:1. 3-Ketoacyl-ACP synthase (KAS), such as spinach KASIII, initiates the FA synthesis pathway through condensation of malonyl-CoA with an acyl acceptor.[10] KASI and KASII condensing enzymes serve to elongate the FA chain from C4:0 to C18:0 in a substrate specific manner. In addition with defects in chloroplast division and embryo development, *atkas1* mutants also show a reduction in polar lipid accumulation.[11] Fatty acyl-ACP thioesterase (Fat) is a core enzyme in the FA biosynthesis process that determines FA chain length. Based on amino acid sequence and substrate specificities, *Fat* genes are classified into *FatA* and *FatB*.[12] In contrast to a reduction of C16:0 and C18:0 FAs, FatB from *Chimonanthus praecox* (*CpFatB*) and *Lindera communis* (*LcFatB*) increase accumulation of C14:0 and C16:1 FAs in transgenic lines of tobacco and *L. communis*, respectively.[13,14] Down-regulation of *GmFatB* through RNAi enabled soybeans to produce high levels of oleic acid (85%) and to reduce the palmitic acid content (<5%), which are regarded as desirable for agronomy and biofuel performance.[15]

Plants, in which plastid/prokaryotic- and eukaryotic/ER-derived pathways equally contribute to the production of chloroplast membrane lipids, such as mono- and digalactosyldiacylglycerol (MGDG and

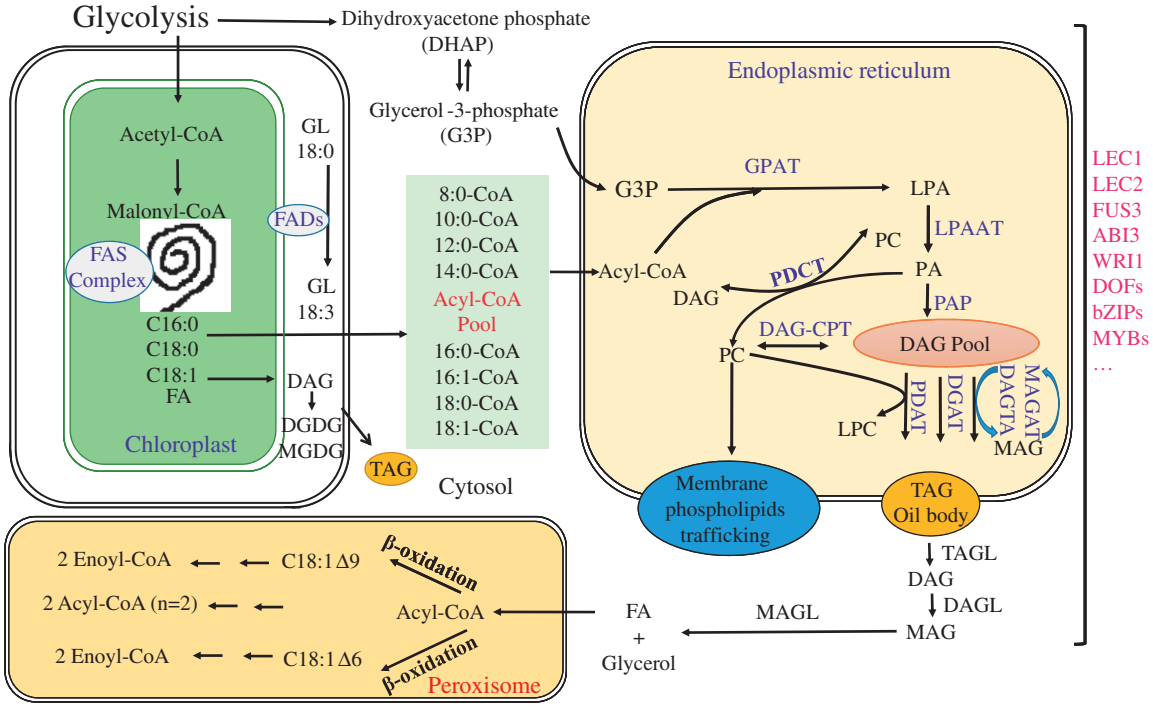

Figure 1. Multiple organelles involved in fatty acid and glycerolipid biosynthesis and catabolism. In plants, assimilated carbohydrates from photosynthesis are subject to glycolysis for either synthesis of proteins, storage starches or lipids. Lipid biosynthesis occurs in the chloroplast, also called prokaryotic pathway, including de novo biosynthesis of fatty acids (FA) from acetyl- and molonyl-CoAs through multiple cycles of elongation reactions catalyzed by a FAS complex, synthesis of PG and galactoglycerolipids (GLs), such as MGDG and DGDG for constructing photosynthetic membranes, as well as DAG and TAG under specific conditions. FAs synthesized in chloroplasts need to be exported to the ER for the biosynthesis of phospholipids and the major storage lipid. The lipid synthesis in the ER/eukaryotic pathway, starts from glycolytic product G3P and fatty acyl-CoAs from the cytosol by the action of GPAT to give LPA. LPA is then converted into PA, which is further catalyzed into DAG by PAP. PA and DAG are important precursors for synthesis of phospholipids such as PC and storage lipid TAG biosynthesis. DAG and PC are convertible by phosphatidylcholine transferase (PDCT) and DAG-choline transferase. DAG can be converted into TAG either by PDAT or DGAT and store in OBs surrounded by oleosin proteins. During germination, TAG is hydrolyzed sequentially by TAGL, DAGL and MAGL into FAs and glycerol. These FAs are imported by PXA1 for β-oxidation in peroxisome to release energy for various metabolic activities. All this process is tightly regulated by various transcription factors, such as LEC1, LEC2, ABI3, FUS3, and others during seed germination, embryogenesis and plant development.

DGDG, respectively) and phosphatidylglycerol (PG), are named as C16:3 plants. The plants where all glycerolipids, except PG, are synthesized by eukaryotic pathways are called C18:3 plants.[16,17] In both C16:3 and C18:3 plants after synthesis, FAs are esterified either in ER and plastid through a substrate and organelle specific FA desaturase (FAD) enzymes. With the exception of FAD2 and FAD3, which are localized in the ER, all plant FADs (FAD6, 7 and 8) are localized in the plastid. In the ER oleic to linoleic acid formation is catalyzed by FAD2, whereas in plastid, it is catalyzed by FAD6 (16). Linoleic to α-linolenic acid conversion in ER is mediated by FAD3 and in plastids either by FAD7 or FAD8 (17). Plant *FAD*, either alone or in combination with other plant *FAD* genes, can combat environmental conditions. At high temperatures, *fad3-fad7-fad8* triple mutant *Arabidopsis* leaves accumulated high concentrations of C16:0 and exhibited a decrease in C16:2 desaturated FAs, while no such change in the synthesis of C18:3 FAs was observed in wild types of the same leaves.[17]

Galactolipid biosynthesis in the chloroplast

Galactolipids are present in all organisms that perform oxygenic photosynthesis. Their biosynthesis requires the coordinated transport of lipids between different organelles and within the plastids themselves. In *Arabidopsis* chloroplasts, MGDG synthase 1 (MGD1), a peripheral protein of the chloroplast inner envelope membrane, catalyzes the formation of galactolipids and it is activated by trace amounts of phosphatidic acid (PA).[18] MGDG moves to the outer envelope of the chloroplast and is converted to DGDG by DGDG synthase (DGD1), which is embedded in the outer envelope membrane.[3] The *MGD2* and *MGD3* are up-regulated in parallel with *DGD2* under phosphate starved conditions.[19] As discussed in Section "Plastid-ER FA transport" of this review, some PAs from the ER are transported to the plastid for galactolipid production. The ER-plastid transporter *tgd1, 2, 3, 4* mutants shows an increased accumulation of trigalatosyldiacylglycerol (TGDG).[3,20] It has been proposed that DGDs control the overall production of glycolipids in chloroplast membranes because an exclusion of MGD1 is observed in membranes that are highly enriched in DGDs.[20]

Neutral and polar lipids biosynthesis in the endoplasmic reticulum

The canonical pathway of TAG, polar and neutral lipids biosynthesis, which is also known as the Kennedy pathway, is conserved in all organisms. Glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate are key intermediates of oil biosynthesis. G3P is acylated at sn-1 by glycerol-3-phosphate acyltransferase (GPAT) to generate lysophosphatidic acid (LPA), which is further acylated at the sn-2 position into PA by LPA acyltransferase (LPAAT).[21] PA is positioned in the center of lipid biosynthesis of TAG, with turnover of phospholipids, and lipid metabolism in the ER.[22] PAs are essential for synthesis of various membrane lipids, which are crucial to the life of the cell, PA transport to various locations and their regulation are essential for plant growth and oil production.[5,20] Isotope labeling is a more powerful tool to trace the transport processes and identify the intermediates directly linked to lipid production by serving as precursors for fatty acid biosynthesis and lipid assembly.[5] The enzymes involved in degradation of various phospholipids, such as phospholipase A, D, and C (PLA, PLD, and PLC) have been extensively studied in plants. For instance, research indicates that PLD-mediated phospholipid hydrolysis and PLD-generated PA plays a wide range of roles in lipid metabolism, such as TAG biosynthesis and acyl editing, plant response to abiotic and biotic stresses, and cellular dynamics such as vesicle trafficking and cytoskeleton dynamics.[23,24] The key enzymes for PA biosynthesis and degradation, including lysophosphatidylcholine (LPC) acyltransferase (LPCAT), LPAAT and phosphatidic acid phosphatase (PAP), essentially affect the biosynthesis of both TAG and phospholipids by modulating PA homeostasis.[22,25] PC (phosphatidylcholine) acts as an essential precursor for phospholipid remodeling and modifying the composition of TAGs through acting as preferred substrates for FADs, and acyl editing target through LPCAT and active conversion between PC and DAG.[5,25] Finally, acyl-CoA-dependent DAG esterization by diacylglycerol acyltransferase (DGAT) generates the major TAG.[22] DGAT1, 2 have a significant effect on TAG accumulation, oil production, and protein content in seeds.[26] RNA silencing of DGAT1 in tobacco decreases seed oil content and increases carbohydrate and protein.[27]

Acyl-CoA-independent TAG synthesis catalyzed by phospholipid: diacylglycerol acyltransferase (PDAT) or diacylglycerol transacylase (DAGTA) has been demonstrated. It occurs via transfer of an acyl chain between two molecules of DAG by DAGTA or DAG and PC by PDAT to form TAG and LPC.[28] A recent study revealed the dual roles of PDAT1 in enhancing FA synthesis and diverting FA from membrane lipids to TAG accumulation. Enhanced TAG accumulation, reduced chloroplast division and thylakoid biogenesis has been observed in *PDAT1* overexpression line in *tgd1* background, indicating that PDAT1 increases TAG at the expense of thylakoid membrane lipids reduction.[28] PDAT-mediated membrane lipid turnover and TAG biosynthesis is essential for vigorous growth under certain conditions and

for membrane lipid degradation with concomitant production of TAG to survive plants in a stressful environment.[29]

TAG storage in oil body

TAG is stored in cytosolic oil bodies (OBs) surrounded by a phospholipid monolayer embedded with integral proteins, mainly oleosins, caleosins and steroleosins, which sequentially deposit on the OB membrane. In the oilseeds, the size, number, and stability of OBs present in cotelydens are closely related to OB proteins; in particular, the predominant OB protein oleosin, confers the final OB size and TAG mobilization and degradation in several plant seeds.[30] Studies revealed that oleosin degradation is a prerequisite for lipid hydrolysis. A proteasome inhibitor reduced oleosin degradation and TAG mobilization in *Arabidopsis* seedling, and thereby enhanced oil production.[7] TAGs in OBs can be not only formed in the ER in seeds, they could also be generated from the chloroplasts in leaves through galactolipid pathway.[3,4]

The transporters involved in lipid degradation and beta oxidation

FAs and their derivatives have multiple functions in cells. FAs released from TAG degradation through the action of TAG, DAG, and MAG lipase, as well as from other sources, are subject to oxidation (primarily β-oxidation) for generation of energies and basic substances for simple metabolism and FA turnover to maintain lipid homeostasis.[31] Transport of FAs into peroxisomes for β-oxidation is mediated by a D-type ATP binding cassette (ABC) transporters, such as yeast peroxisomal ABC-transporter1 (PXA1) and PXA2 and plant comatose (CTS).[31] However, the transport mechanism by which these peroxisomal ABC transporters transfer FAs from the cytosol into peroxisomes has been a matter of debate. Recent studies have shown that CTS physically interacts with the peroxisomal long-chain acyl-coasynthetases (LACS) 6 and -7 (32). ATP-stimulated Fat activity is essential for CTS-mediated Fat degradation since a point mutation causes a significant reduction in thioesterase activity, which is accompanied by defective FA degradation.[32] Thus, CTS takes up FA acyl-CoA on the cytosolic side of peroxisome as an initial substrate, which is hydrolyzed by its intrinsic thioesterase activity to generate FAs. These FAs are converted by CTS-interacting LACS6 and 7 in the side of peroxisome lumen into fatty acyl-CoAs, which are subject to further β-oxidation. The peroxisomal membrane localized LACSs may act as a FA transporter working independently or together with CTS.[8]

Furthermore, a regulatory mechanism of plant PXA1 by a hydrolase comparative gene identification-58 (*CGI-58*) has been identified.[33] Disruption of the cytosol/peroxisomal membrane-localized CGI-58 in either mammals or plants results in a significant increase in TAG production.[34] The CGI-58 is not required for the PXA1-dependent TAG degradation in germinated seeds and positively regulates PXA1 activity, lipid metabolism and signaling particularly in vegetative tissues.[34] The barley homolog of CTS, HvABCD1, partially complemented yeast *pxa1/pxa2* mutant for FA β-oxidation.[35]

Cross-membrane lipid transport and trafficking

Lipids usually need to be trafficked from their site of synthesis to the destination for functions, such as biomembranes, organelles, intracellular or extracellular spaces. There are several potential routes for lipid transport such as, membrane transporter-mediated transport across membrane, vesicle trafficking, flip-flop of phospholipids across bilayer membranes under the aid of membrane transporters. The transporters responsible for ins and outs of FAs, phospholipids, sphingolipids, DAG/TAG, cuticle/wax precursor movements and deposition on aerial plant surfaces are summarized in Supplemental data Table 1.

Flip–flop movements

It is believed that the biomembranes are asymmetrically composed of two types of glycerolipids across the bilayer; aminophospholipids are enriched on the cytoplasmic surface of the plasma membrane and sphingolipids are enriched on the outer surface.[36] Such asymmetric distribution of phospholipids and sphingolipids in the biomembranes is important for proper function. An increasing body of evidence showed that P-type ATPases and ABC transporters are involved in the inward movement (flip) and outward movement (flop) of glycerophospholipids, e.g. mammalian ABCA1 and 4.[37] The mammalian plasma membrane proteins translocate phosphatidylserine from outer plasma membrane leaflet which protect cells from macrophages.[36] A homolog of yeast and mammalian P4-ATPases in *Arabidopsis*, aminophospholipid ATPase10 (ALA10), is recently characterized as a plasma membrane protein. ALA10 is expressed in the plasma membrane of root and guard cells to aid specifically the uptake of signaling lipid LPC, which is involved in plant development.[38] The oxidatively modified PCs enhance phospholipid flip-flop in liposomes by changing membrane biophysical properties.[39]

Phospholipids synthesized in the ER are transported to destination membranes via extensive vesicle trafficking.

Plastid-ER FA transport

FAs synthesized in chloroplasts are transported into the cytosol to form cytosolic acyl-CoA pools, which are then transported into the ER for assimilation into membrane structure phospholipids and storage neutral lipids, DAG or TAGs. The mechanisms of FA transport from the plastids to cytosol, and then to the ER, have been studied. [40,41]

A FA export 1 (FAX1), located in the inner envelope of the chloroplast, plays an essential role in the transportation of FAs out of the chloroplast. The mutation of *FAX1* results in pleiotropic effects on developments and metabolism, including retarded growth, impaired male fertility, reduced TAG and FA-derived phospholipids but increased accumulation of plastidic PG production.[40] However, over-expression of *FAX1* results in increased cytosol/ER-derived PC and TAG accumulation, but decreased plastidic lipids like MGDG and DGDG. There exists a genetic redundancy in the FA export from the chloroplast to the cytosol, since *fax1* does not completely abolish all phospholipid and TAG biosynthesis. The FAX1 alone is thus not enough to explain the overall chloroplast-cytosol FA export and to improve lipid/biofuel production in plants; several FAX1 homologs have been identified.[40]

An ABC transporter-mediated FA import from the cytoplasm into the ER was revealed for phospholipid and TAG biosynthesis. AtABCA9, an *Arabidopsis* ER-localized A-type ABC transporter was shown to function in the process.[41] The *atabca9* null mutant reduced seed TAG content by 35% and the developing *atabca9* seeds incorporated 35% less ^{14}C-labelled-oleoyl-CoA into TAG compared with WT. Moreover, overexpression of AtABCA9 enhanced TAG deposition by up to 40%.[41] This indicates that AtABCA9 may transport FA substrates for TAG biosynthesis at the ER during the seed-filling stage and some other transporters may be responsible for FAs transport from the cytosol into the ER.

ER-plastid phospholipid and FA transport

Genetic evidence suggests that lipid trafficking takes place from the eukaryotic pathway in the ER to the prokaryotic pathway in the chloroplast. An *Arabidopsis* mutant deficient in chloroplast G3P acyltransferase contains similar classes of glycerolipids to the wild type due to increased ER-plastid lipid trafficking.[3] Conversely, under phosphate starvation, phospholipids are replaced by galactolipids in both thylakoid and extraplastidic membranes.[4,42] The mechanism underlying these lipid trafficking processes is not fully understood. [3,4,43] In *Arabidopsis* and *Atriplexlen tiformis*, low temperatures up-regulate the chloroplast pathway and channel glycerolipids from the ER to the chloroplast, whereas high temperatures increase the contribution of the eukaryotic pathway but suppress the prokaryotic pathway.[43] *Arabidopsis* ABC lipid transporters are believed to be involved in the ER-chloroplast phospholipid trafficking consisting of TGD1, 2, and 3, which are located in the inner membrane envelope (Figure 2). In *Arabidopsis tgd* point mutants are used to check whether TGD1, 2 and 3 functions in the same pathway. The FA analysis of each mutant exhibited a reduction in C18 and an increase in C16 FAs, which implies an impaired ER-plastid lipid trafficking.[3,4] TGD4 is predicted β-barrel protein resides on outer chloroplast membrane involved in lipid precursor trafficking from the ER-Chloroplast.[4,20] A decrease in PA binding of *tgd4* and extraplastidic increases in PA accumulation in *tgd1-tgd4* mutants, suggested that TGD complex might be involved in the transportation of PA or other phospholipids across the plastidic membranes.[20] A recent study further added TGD5 to the TGD1–4 transport complex to facilitate lipid transport from the ER to plastid.[6] Together, a rich repertoire of lipid exchange mechanisms involves the thylakoid membranes, the chloroplast inner and outer envelope membranes, and the ER might play an important role in many physiological functions.

Isotope labeling experiments showed that lipids in the plastid are not only derived PAs from the ER, but also FAs from the ER. Therefore, ER-plastid FA transport process also plays an important role in lipid biosynthesis. The FAs are converted into metabolically active coenzyme A thioesters by LACSs. In yeast and mammals, LACSs are also reported to be FA transporters due to their second function in FA transport.[22] Expression of *Arabidopsis* LACS1, 2, and 3 in yeast facilitated the uptake of long chain fatty acid analogs in both yeast and plant.[22,44] *Arabidopsis* LACS4 and LACS9 have overlapping functions in FA trafficking from the ER to the plastid,[45] and LACS9 was localized in plastid outer envelope membrane for transport of ER-derived FA into the chloroplast.[46] The *lacs4-lacs9* double mutants strongly reduce the ER-derived lipid precursors for glycolipid synthesis in the chloroplast and significantly increased linoleic acid content in leaf tissue.[45] FA profiling on *Arabidopsis* double *lasc1-lacs9* and triple *lacs1-lacs8-lacs9* mutants showed the same decrease in seed FA content, suggesting that LACS9 also has an overlapping function with LACS1, but not with LACS8 in FA transport.[47]

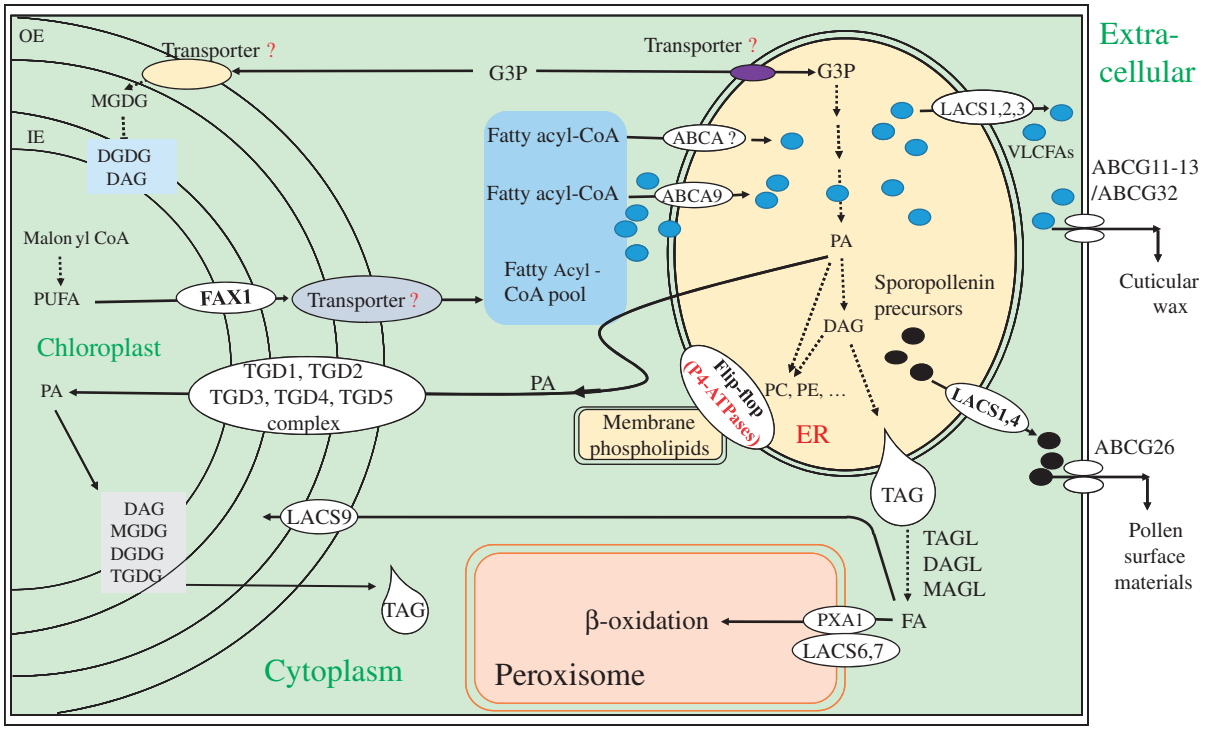

Figure 2. Lipid transport in plant cell. FAs synthesized in chloroplasts can be exported to the ER for the biosynthesis of phospholipids and the major storage lipid, triacylglycerol (TAG), and to provide lipid precursors for export to the extracellular cuticle. Fatty acid export1 (FAX1) localized in the inner envelope (IE) exports FAs out of chloroplast lumen. How FAs are transported across the outer envelope of the chloroplast is still unknown. Fatty acyl-CoA pools in the cytoplasm are further transported into ER by ABCA9 and other transporter(s) for glycerolipid biosynthesis. Meanwhile, the G3P generated from glycolysis in the cytosol is needed to be transported into the ER and chloroplast, but the mechanism is unknown. ER is the major organelle for biosynthesis of membrane lipids (phospholipids), surface lipids (wax, cutin) and storage lipids such as DAG and TAG. The presence of ER-derived chloroplast lipids requires the transport of ER lipid precursors to the chloroplast. TGDs transport complex and LACS9 present at chloroplast envelopes is involved in import of phospholipids and other glycolipid precursors into the chloroplast. The ER assembled precursors for cutin and wax biosynthesis are transported out of the ER by LACS1, LACS2 and LACS3 in *Arabidopsis*. At the plasma membrane ABCG11, ABCG12, ABCG13, ABCG32 export wax and cutin precursors out of epidermal cells for deposition on plant tissue surfaces. Sporopollenin precursors synthesized in the ER of tapetum cells are exported by LACS1 and LACS4, that are further transported by ABCG26 out of cells to gather on the pollen surfaces in developing microspores. Phospholipid turnover and transport are essential for membrane function and integrity. The mammalian flippase-like proteins, P4-ATPases ALA3, 6, and 10, are verified for the similar functions in plants. TAG degradation by various lipases to generate fatty acids, which are further subjected to β-oxidation are imported by PXA1 in the peroxisome. LACS 6 and 7 actively mediate transport of FAs into peroxisome for β-oxidation.

Transporters required for surface lipid deposition

LACSs export lipid precursors out of the ER

Aerial surfaces of a plant body are covered with and protected by a hydrophobic barrier that is known as the cuticle. The cuticle is mainly composed of polyesters cutin and suberin covered by cuticular waxes. Cuticular waxes are composed of a wide diversity of very long-chain FA (VLCFAs) with chains of C8–C24 and hydroxyl, epoxyl and carboxyl groups at various positions. Some of these derivatives such as alcohols, aldehydes, ketones, alkanes and wax esters are of great industrial interest for a range of products including resins, coatings, nylons, plastics, and biofuel.[48] The cuticle plays an important role in plant non stomata water loss and pathogen defense. A molecular genetics approach has led to breakthroughs in understanding of the assembly, transportation and accumulation of cuticular wax on non-woody aerial surfaces. Cuticle precursors have been found to be localized in the ER of epidermal cells. Several *Arabidopsis* LACSs have been reported functioning as transporters of these lipid polymer precursors out of the ER. *Arabidopsis* LACS1, 2 and 3 are localized to the ER and transport VLCFAs out of the ER for cutin synthesis.[44] LACS4 that is also localized to the ER, with LACS1, is required to transport sporopollenin precursors from the ER to the cytosol for pollen coat formation.[49] The *lacs1-lacs4* deficiency results in compromised pollen formation and male sterility. Thus LACS1 and 4 facilitate the transport of sporopollenin precursors from the ER for pollen coat formation. The cytosolic acyl-CoA binding proteins ACBP4, 5

and 6 can bind and shuffle long chain CoAs out of plasma membrane for pollen coat formation. Mutant *acbp4/5/6* pollen grains exhibit defects in oil bodies and the outer pollen cell wall.[50] Hence, LACS and ACBPs work in a combination to export the lipid precursors for cell wall deposition.

ABCGs export lipid precursors through the plasma membrane

In addition, transporters and enzymes (Supplemental data Table 1) must also be involved in further export of these VLCFAs or sporopollenin precursors to the plasma membrane, and finally to the outer cell wall. Two members of the ABCG's family of half transporters, ABCG11 and ABCG12, have been identified as being involved in the transportation of lipids from the epidermis to the aerial tissues of a plant.[51] An *Arabidopsis abcg11* mutant showed reduced wax and cutin level, while *abcg12* mutants exhibited a reduction in wax content only. Dwarfism, post-genital organ fusion, and sterility were also observed in *atabcg11* mutants in addition to defects in cuticle precursor transport. This study suggests that AtABCG11 may play a more extensive role than AtABCG12 (51). Researchers introduced AtABCG13 as a potential candidate for petal epidermis prototyping and flower cuticle secretion. They observed strong flower phenotype and inter-organ fusion in *atabcg13* mutants.[52] A full ABCG transporter (ABCG32), is also involved in cuticle layer formation. The *atabcg32* mutants' cuticle layer was more diffused, lost more water and was hypersensitive to herbicides.[53] Its homologs in rice (OsABCG31) and barley (HvABCG31) are required for low transpiration.[54] ACBP1 (acyl-CoA binding protein 1) functions in wax and cutin formation, by acting as intracellular carrier of acyl-CoA esters. ACBP1 possibly transports very long chain acyl-CoA from the ER to the plasma membrane since it has a transmembrane domain that attaches the ER and plasma membrane. [55] At the plasma membrane, ABCG11, ABCG12, ABCG13, and ABCG32 export wax and cutin precursors out of epidermal cells for deposition on plant tissue surfaces. Recently, three half size ABCG transporters ABCG2, ABCG6 and ABCG20 are reported in *Arabidopsis* to be involved in suberin layer formation in root endodermis and seed coat tissues. The triple mutant *abcg2/6/20* roots and seed coats have distorted suberin structures and high permeability.[56]

ABCGs export sporopollenin precursors for pollen wall formation

Sporopollenin provides strength and shields the exine of spores and pollen grains from terrestrial stresses. Exine precursors are synthesized in ER lumen in the epidermal cells and move to the tapetum cytosol through LACS1 and 4 (49) or transported through ER-Golgi transport network, together with other flavonoids.[42,57] AtABCG26 on the plasma membrane is required for export of sporopollenin from tapetum to the developing microspore. Similar to *atabcg11* and *atabcg12* mutants, lipid accumulation was observed in *atabcg26* mutants along with mature anther failing to release pollen.[58] Further, *abcg1/abcg16* double mutants are defective in the formation of inner exine and carbohydrate intine layers.[56] AtABCG26 monocot ortholog OsABCG15 exhibited male sterility in rice.[59] Dysfunction in AtABCG9 and AtABCG31 alters glycoside levels and the morphology of pollen coats. Double mutants are sensitive to dry air due to defects in pollen wall formation.[60] Therefore, sporopollenin precursors synthesized in the ER of tapetum cells are exported by LACS1 and LACS4, that are further transported by ABCG26, 1, 6 and 20 (56) out of cells with the help of ACBP4–6 (50).

Role of transcriptional regulation in lipid accumulation

TAG is the foremost energy reserve of oilseed crops that are primarily cultivated with seeds. Leafy cotyledon1 (*LEC1*), *LEC2*, abscisic acid insensitive 3 (*ABI3*), and fusca3 (*FUS3*) are considered at the core of seed development regulatory network (Figure 3). ABI3, FUS3 and LEC2 TFs control the seed oil or FA content of vegetative tissues by directly or indirectly regulating FA biosynthesis or TAG accumulation.[61,62] Recently, characterization of basic leucine zippers (bZIPs), DNA binding with one finger (Dof) and MYB TFs further revealed their functions in lipid accumulation.[63,64] All these genes are expressed primarily or specifically during seed development and have a significant impact on seed oil production (Supplemental data Table 2).

Leafy cotyledon 1 (LEC1)

LEC1 encodes a HAP3 subunit of the CCAAT binding TF. *Arabidopsis LEC1* and *LEC1-LIKE* (*L1L*) are expressed primarily during seed development and plays critical roles in embryogenesis. AtLEC1 globally regulate expression of genes that are involved in key reactions of glycolysis and condensation, chain elongation, and desaturation of glycerolipid biosynthesis.[65] The mutation of the genes encoding LEC1 TFs from different plants can have negative effects on seed maturation, including causing a drop in the level of seed oil. On the contrary, increasing the levels of their mRNA can lead to an increase in seed oil accumulation. For example,

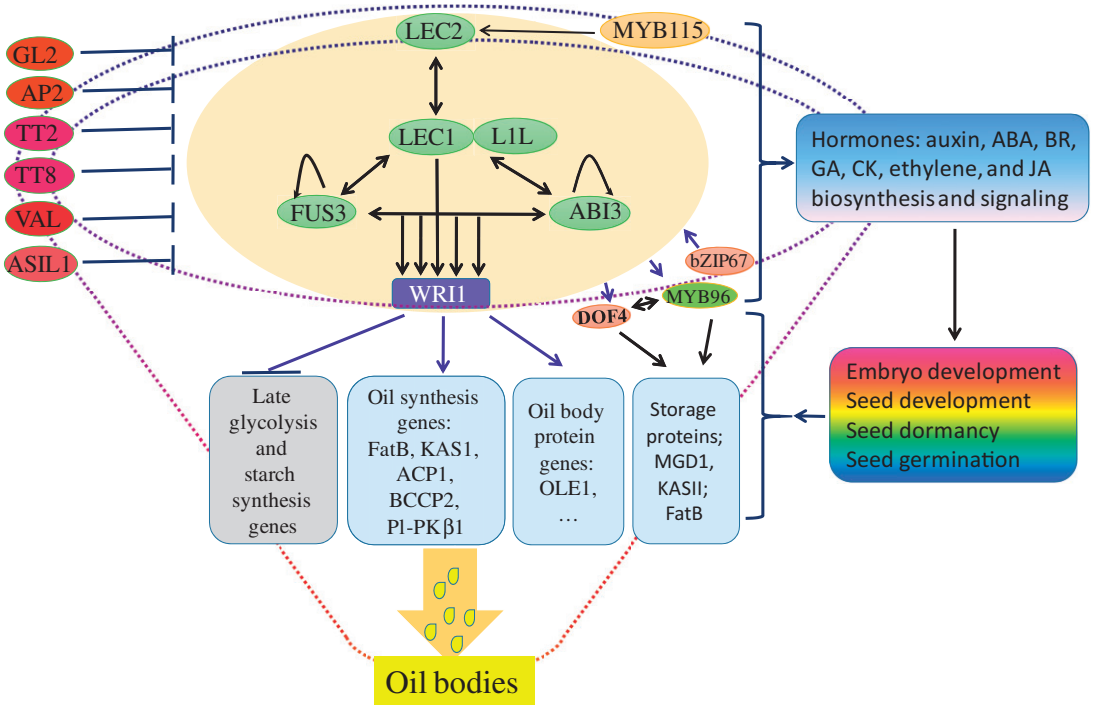

Figure 3. Schematic for transcriptional regulation of oil production in plant seeds. The B3 DNA binding domain transcription factors, such as ABI3, FUS3, and LEC2 (AFL-clade), and two HAP3 family transcription factors LEC1 and LEC1-LIKE (L1L) are major regulatory elements of regulation network for controlling plant embryo and seed development, seed dormancy and germination. These TFs modify biosynthesis and signaling of various hormones, such as auxin, ABA, GA, Cytokinin, JA, and ethylene at different stages of seed development. They thereby also regulate one of the integral parts of seed metabolic pathways, such as oil biosynthesis. LEC2, LEC1, L1L, ABI3, and FUS3 mutually regulate each other's expression to form a complex regulatory network. Overexpression of LEC2 up-regulates LEC1 and FUS3. ABI3 and FUS3 in turn are regulated by mutual positive interactions and also self-regulated. LEC1 can activate ABI3, FUS3, LEC2 and L1L; which all regulate auxin, ABA, GA, CK, JA, and ethylene biosynthesis and signaling during seed development, maturation, dormancy and germination. Wrinkled 1 (WRI1) is a another core downstream component more specifically regulating structural genes involved in early FA biosynthesis and meanwhile repressing genes involved in late glycolysis and storage starch synthesis. WRI1 is up-regulated by LEC1, LEC2, ABI3 and FUS3. Other downstream transcription factors, such as Myb96, Dof4, bZIP67 transcription factors, either feed back the core regulator complex, e.g. MYB115 up-regulates LEC2, or directly target on structural genes involved in oil biosynthesis in oilseed plants. TT2, TT8, GL2, VAL B3 domain factor, ASIL1, and APETALA2 (AP2) act as negative regulators of oil biosynthesis likely affecting the core regulatory complex, such as LEC genes and WRI1, or through other mixed regulatory mechanisms. Target genes of these transcription factors in oil biosynthesis include FatB, PKb1 (pyruvate kinase), BCCP2, ACP1, OLE1, KASI, and KASII.

seed-specific over-expression of maize *LEC1 (ZmLEC1)* resulted in an average 48% increase in seed oil but reduced germination and vegetative growth in the field.[66] Conversely, modest (2–3 fold) seed-specific increases in mRNA levels of *BnLEC1* or its close relative, *BnL1L*, increased seed oil content by up to 20% in field trials without incurring negative pleiotropic effects on agronomic traits such as yield and other nutritional values.[67] ABI3, FUS3 and WRINKLED 1 (WRI1) regulate *LEC1* during FA biosynthesis.[65] These transcriptional regulators work together to alter sucrose metabolism, reduce glycolytic activity and increase carbon flux toward FA synthesis and ultimately TAG production.

Leafy cotyledon 2 (LEC2)

LEC2, a B3 DNA binding domain TF, regulates downstream *TF* genes controlling the cotyledon developmental network and the structural genes involved in the biosynthesis of storage proteins and TAGs in seeds. LEC2 thus has been manipulated in plants for improving oil production.[68–70] When expressed in *Arabidopsis* seedlings, AtLEC2 positively regulates seed protein genes and oil bodies in the vegetative organs that normally activate during the maturation phase before the increases of LEC1 and FUS3 transcripts; hence it serves as a master regulator of the developmental network.[61] LEC2 regulates the seed filling and chemical composition. Loss-of-function mutant *atlec2* seeds contain a 30% reduction in oil and 15% less protein, while maintaining higher levels of sucrose and starch than the wild variation of the plant.[69] Besides regulating metabolic and cellular processes during embryo maturation, AtLEC2 also provokes somatic embryo formation by inducing auxin, GA, and other

hormone biosynthesis and signaling.[61] Since the constitutive expression of LEC2 usually causes somatic embryogenesis and defects in seedling growth, a senescence-inducible LEC2 was shown to increase TAG production by three-folds in transgenic leaves, at the cost of reduced biosynthesis of plastid-synthesized lipids, including MGDG, DGDG, and PG.[70] LEC2 inducible expression changes FA composition of vegetative tissues by up-regulating *LEC1, ABI3, FUS3, LEC2,* and *WRI1* that may further induce accumulation of *FA elongase 1 (FAE1)* and *oleosin* transcripts.[71] An ethylene response factor 022 (ERF022) interacts with LEC2 and regulates somatic embryogenesis. ERF022 negatively regulates ethylene synthesis and positively regulates somatic embryogenesis in *Arabidopsis*.[72]

Abscisic acid insensitive (ABI3) and FUSCA3 (FUS3)

Downstream of LEC2, the B3-domain TFs ABI3 and FUS3 promote seed maturation and dormancy by regulating ABA/GA signaling pathways, which also elicits several physiological processes such as stomatal movement, seed desiccation, drought/cold tolerance, and seedling growth inhibition.[73,74] ABI3 and viviparous 1(VP1) were initially considered to be restricted to seeds; 98 ABI3-targeted genes are identified in ABI3 regulon for seed oil storage and desiccation.[74] A Cedar ABI3 -interacting protein 2 (CnAIP2) promotes seed development, vegetative growth and reproductive initiation.[75] FUS3, a member of the *LEC2* group of genes, acts primarily as a transcriptional activator of many TFs associated with embryogenesis. FUS3 up-regulates *ABI3-LIKE1/VP1* repressors and *miRNA* encoding genes that repress indirect FUS3 targets.[76]

FUS3-overexpressing seeds are hypersensitive to high temperatures and do not germinate due to the increased ABA synthesis in transgenic seeds.[62] FUS3 interacts physically with AKIN10, an ortholog of *Arabidopsis* Sucrose Non-Fermenting-1-Related Kinase1 (SnRK1) and regulates phase transition and lateral organ growth.[77] AKIN10 and FUS3 functionally overlap in ABA signaling, and the seeds over-expressing both *TFs* exhibited hypersensitivity towards ABA and delayed germination.[77] Auto-regulation and cross- regulation relationships have been demonstrated among *ABI3* and *LEC* genes, also within the downstream pathways that they control (Figure 3).

Monitoring VLCFAs production in *Arabidopsis* reveals a reduction in the activity of FAE1 and KAS in *fus3* and *abi3* mutant seeds. A greater reduction of seed storage proteins than lipids was observed in *abi3*; however, a contrary effect was observed in *fus3* (9). A Mendel's mapped locus stay green1 (SGR1), responsible for cotyledon color, is regulated by ABI3.[78] The role of ABI3 in degreening mature seeds helps to solve the problem in plant oil qualities. In the light of these observations, ABI3 and FUS3 are key regulators in phase transition and seed development, maturation and dormancy, and thereby regulate oil biosynthesis.

Wrinkled 1

WRI1 is the member of the APETALA2 family of TFs, and an important regulator of oil production in mature *Arabidopsis* seeds.[79] *WRI1* is a target of LEC1 in oil biosynthesis pathway and *ZmWRI1* and *ZmLEC1* overexpression increases similar amounts of seed oil in maize.[66] WRI1 controls seed oil production by directly regulating metabolic genes involved in glycolytic pathway, amino acids, and FA biosynthesis.[66] In maize and *Arabidopsis,* WRI1 regulates the genes involved in FA biosynthesis, such as ACCase subunits, *ACP1*, and *KAS1*, through direct binding to AW box in their promoter regions.[66,79,80] *Arabidopsis PII* plays a role in the fine tuning of fatty acid biosynthesis and is directly controlled by WRI1. An increase in FA production was accompanied by a maximal content of PII *Arabidopsis*. Moreover, a minor modification in FA composition was also observed in *pII* mutant.[81] Over-expression of *AtWRI1* or its orthologs in different plants increase seed oil level in transgenic seeds.[66,79] Additionally, WRI3 and WRI4, which are closely related to WRI1 and belong to the AP2 protein family, have been identified in *Arabidopsis*.[82] The expression pattern of each of these three WRIs is markedly different; only WRI1 activates FA biosynthesis for TAG production in seeds, whereas WRI3 and WRI4 responded for providing acyl chains for cutin biosynthesis in floral tissues.[82]

Dofs increase the total FA content in transgenic plants

Dof is a plant-specific TF that is not found in other eukaryotes. Dofs control the synthesis of seed storage proteins and FAs, light regulation, plant defense, seed germination, auxin and gibberellins.[63] Overexpression of soybean *GmDof4* and *GmDof11* increased total FA content and seed yield. Both Dof TFs activated *ACCase* and *LACS* genes by binding with *cis*-element in their promoter regions and repress the cruciferrin-1 storage protein gene.[83] GmDof4 significantly up-regulated ACCase activity and increased lipid content by up to 52% in transgenic microalgae *C. ellipsoidea*.[64] Synthetic soybean Dof-like TFs elevated the expression of genes involved in FA, phospholipid and glycolipid biosynthesis, such as *enoyl-ACP-reductase*, *KASII*,

sulfolipid synthase, and *MGD1* in transgenic *C. reinhardtii*.[84]

Role of basic leucine zipper (bZIPs) in lipid accumulation

Basic leucine zipper (bZIP) proteins bind to ACGT *cis*-element and are classified on the basis of their binding affinities. bZIP TFs regulate genes involved in plant-environment interaction and stress responses. AtbZIP53 in the presence of C type *Arabidopsis* bZIPs interacts with ABI3.[85] Soybean GmbZIP123 participates in lipid accumulation in seeds by regulating the sucrose transporter genes *SUC1*, *5* and divert the sucrose from vegetative tissues to seed. In addition to regulating sucrose transporters, GmbZIP123 binds to the promoter regions of three cell wall invertases.[86] The regulation of sucrose synthase (SUS2) by bZIP proteins was previously reported in *Arabidopsis*. LEC1/L1L forms a protein complex with bZIP67 and regulates the expression of *SUS2* and *cruciferrin*.[87] Therefore, these bZIPs are crucial regulators of seed protein and carbohydrate metabolism, and lipid production.

MYB TFs regulate biosynthesis of VLCFAs

MYB TFs play important roles in multiple aspects of plant growth and development, and in their ability to respond to biotic and abiotic stresses.[88,89] *Arabidopsis MYB30* knockout and overexpression lines had altered levels of VLCFAs and accumulation of epidermal wax. MYB30 suppresses *FatB* and blocks VLCFAs biosynthesis.[89] The drought stress activated *Arabidopsis* AtMYB96, which in turn activated genes involved in VLCFA biosynthesis through direct binding to these genes' promoters.[90] The soybean GmMYB73 has been acknowledged to participate in regulating oil production through down-regulating *GLABRA2 (GL2)*, which is a negative regulator of oil biosynthesis.[88] GL2 directly regulates *mucilage modified 4* (*MUM4*) for seed mucilage production. The elevated seed oil content in the *gl2* mutant was related with loss of *MUM4* function, probably due to sucrose flux into oil biosynthesis in the absence of mucilage formation.[91] Overexpression of *GmMYB73* has been observed to elevate the total lipid contents in the leaves and seeds of transgenic Lotus and in transgenic hairy roots of soybean plants.[89]

Negative regulators of oil biosynthesis

TFs positively regulate seed development programs for oil biosynthesis. On the other hand, some repressor regulators can suppress oil production and related-gene expression during vegetative growth. PKL, a member of chromodomain/helicase family proteins, physically interacts with trimethylation of Lys 27 of histone H3 (H3K27me3)-enriched loci in the promoters of *ABI3*, *FUS3*, or *LEC2* to suppress their expression during germination. Roots of *pkl* mutants showed enhanced transcripts of embryogenic genes and TAG production. Mutations in components of polycomb repressive complex 2 that generates H3K27me3 result in up-regulated these master regulator genes and increased oil in seedlings.[92]

Another B3 domain TF, known as VAL, suppresses seed maturation and growth. Double mutants of high-level expression of sucrose inducible gene2 (HSI2)/VAL1 and HSI2-Like/VAL2 produce seedling expressing embryo specific genes, AFL clade genes, and accumulate high seed storage compounds.[93] Researchers recently found that a MYB and basic helix-loop-helix TFs, Transparent Testa 2 and 8 (TT2 and 8), respectively, inhibits FA biosynthesis in seed embryo by interacting with and inhibiting the FUS3 and LEC1, thereby repressing *KASII* and *BIOTIN CARBOXYL CARRIER PROTEIN2* (BCCP2); a subunit of ACCase.[94] *Arabidopsis* six-b-interacting protein 1-like 1 (ASIL1) is a regulator of seed filling. In developing *Arabidopsis* siliques, mutation of *ASIL1* led to earlier expression of *AFL-clade*, as well as genes for seed storage reserves.[95] *Arabidopsis* AP2 negatively controls seed mass. The *ap2* mutants produce larger seed with increased seed oil than wild type.[96]

Biotechnology tools for metabolic engineering of oil yield and composition

The need for improved oil production and optimized oil compositions of both edible oils for foods and health improvement and for biofuels or industries have driven a revolution in oilseed breeding. Both metabolic genes and regulatory factors have been used for improve oil composition and yields. Based on our understanding of lipid biosynthesis, increasing FA and TAG biosynthesis, repression of storage carbohydrate biosynthesis, TAG degradation and FA β-oxidation at certain stages provides comprehensive strategies for enhancing oil production.[97] Overexpression of *WRI1*, *DGAT1*, *oleosin 1* (*OLE1*) and suppression of *PXA1* and *ADP-glucose pyrophosphorylase* enhanced TAG production in transgenic sugarcane leaves and stems by 95 and 43 fold, respectively, compared to wild type.[98] Disruption of PDAT1 and SDP1 TAG lipase also increases TAG accumulation in *Arabidopsis*.[99]

Although α-linolenic acid (ALA; C18:3 cisΔ9, 12, 15) is desirable for human health, its chemical instability is one major reason for creation of side products trans-

fats during food processing. The high-oleic acid oil from major oilseed crops has been achieved by modifying *FAD2* and *FAD3* to solve the trans-fats issue.[100] The high-oleic soybean varieties come to the market,[15,101] and *WRI1*-overexpressing plants produce more vegetable oils and biofuels.[83] Efforts have also been made to produce omega-3 Long chain-PUFAs, such as eicosapentaenoic acid (EPA) and related omega-3 LC-PUFA eicosatetraenoic acid (ETA), in transgenic *Camelina* plants.[102] LuPDATs are able to preferentially catalyze the synthesis of TAG containing ALA acyl moieties. They may be used to genetically modify oilseed crops to enhance the production of ALA and other PUFAs for health-promoting foods and industrial applications.[103] Enhanced production of industrially important omega-7, epoxy or hydroxyl FAs have been achieved in several plants by redirecting the metabolic flux.[22] A further increase in omega-7 MUFAs (60–65%) in *Camelina* seeds was achieved by suppressing *KASII* and *FatB*.[104]

Microalgae oil is emerging as a potential source of feedstock for renewable biofuels. The compromised hydrolysis of triacylglycerols 7 is a repressor of cellular quiescence that is unable to degrade TAGs following nitrogen resupply and provides a target for the engineering of high-biomass/high-TAG microalgae.[105] Genomic and transcriptomic analysis of oleaginous microalgae *Fistulifera solaris* revealed that some unique patterns are responsible for high-rate lipid production in oleaginous photosynthetic organisms.[106] This novel gene expression pattern can lead to simultaneous oil accumulation phenotypes in algae for biofuel production. The substrate specificities and essential roles of lipid transporters in metabolic flux toward oil production may grant their efficient utilization in a precise metabolic engineering of certain lipid product for specific purposes. In summary, the lipid transport processes and the underlying mechanisms are just emerging, and our understanding on transcriptional factors is also not complete. Therefore, a more advanced understanding certainly drives the successful utilization of these factors in improvement of plant oil production.

Conclusions and perspectives

Metabolic engineering has emerged as a powerful tool for manipulating pathways in order to improve the existing products or creating novelties in a desirable way. One of the major challenges in lipid biology and metabolic engineering is revealing the elements that constitute or control a metabolic pathway such as structural enzymes, transporters, and TFs. Although they are quite complex and daunting in nature, there are potentials that they can be overcome by employing the fundamental principles and advanced approaches in lipid biology. This review examined the progress that has been made in the field of lipid biosynthesis, transport, and degradation. A particular focus was placed on subcellular transportation and transcriptional regulation (Figure 3). TFs provide a promising solution for altering complex traits such as oil yield and composition, yet undesirable traits are often shown up meanwhile.[66] Current understanding of subcellular lipid transport only represents the beginning, and the regulatory mechanisms involved in lipid biosynthesis and degradation remain full of mysteries. Given the indispensable roles of these known transporters and transcriptional regulators, as well as the intriguing connections between these integrated processes, utilization of these characterized transporters and TFs to improve plant oil production may hold great potentials. Even though the success of metabolic engineering of oilseed or biofuel plants with known TFs for desirable oil yields and composition is very limited, the obstacles may be removed by more profound understanding of transport and transcriptional regulatory mechanisms. For example, how to use these TFs to improve oil production without disturbed growth and yield? How to use transporters to improve oil composition? How are these lipid transporters regulated? Are these transporters also regulated by TFs? What additional transporters are involved in FA transport across chloroplast envelopes, ER membrane, or membranes of other organelles? How "flip-flop" mechanism is applied to phospholipid and TAG transport in plant cell? How do TFs and transporters integrate the biosynthesis and degradation of different types of lipids into the storage of starches and proteins in different oilseeds? How to utilize genome editing technology CRISPR/cas9 to manipulate these negative regulators to alter oil composition or oil production? Answering these questions may lead to a fundamental understanding of plant lipid biosynthesis, transport, catabolism, and transcriptional regulation, which will help scientists to use these principles to precisely manipulate traits while avoiding undesirable pleiotropic effects. The breakthroughs in these areas may open new gateways for exploring the great potentials of lipids as energy substitutes and more healthy foods, with more sophisticated technologies on lipidomics, lipid biology, and metabolic engineering.

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