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# Biochemical pathways in seed oil synthesis

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Oil produced in plant seeds is utilized as a major source of calories for human nutrition, as feedstocks for non-food uses such as soaps and polymers, and can serve as a high-energy biofuel. The biochemical pathways leading to oil (triacylglycerol) synthesis in seeds involve multiple subcellular organelles, requiring extensive lipid trafficking. Phosphatidylcholine plays a central role in these pathways as a substrate for acyl modifications and likely as a carrier for the trafficking of acyl groups between organelles and membrane subdomains. Although much has been clarified regarding the enzymes and pathways responsible for acyl-group flux, there are still major gaps in our understanding. These include the identity of several key enzymes, how flux between alternative pathways is controlled and the specialized cell biology leading to biogenesis of oil bodies that store up to 80% of carbon in seeds.

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## Introduction

Oils are the most energy-dense plant reserves, supplying humans with much of the calories and essential fatty acids required in our diet. Because they are composed of long chain hydrocarbons, plant oils can also replace petroleum in many applications, including as feedstocks for the chemical industry and as biofuels. The majority of the plant oils we consume are accumulated in seeds. World production from oilseed crops was approximately 100 billion kg of oil in 2011 [1] with a value near US\$120 billion [2]. Vegetable oil consumption is expected to double by 2040 [3]. The important uses, high value and growing demand are a major reason why oil biosynthesis in seeds has been extensively

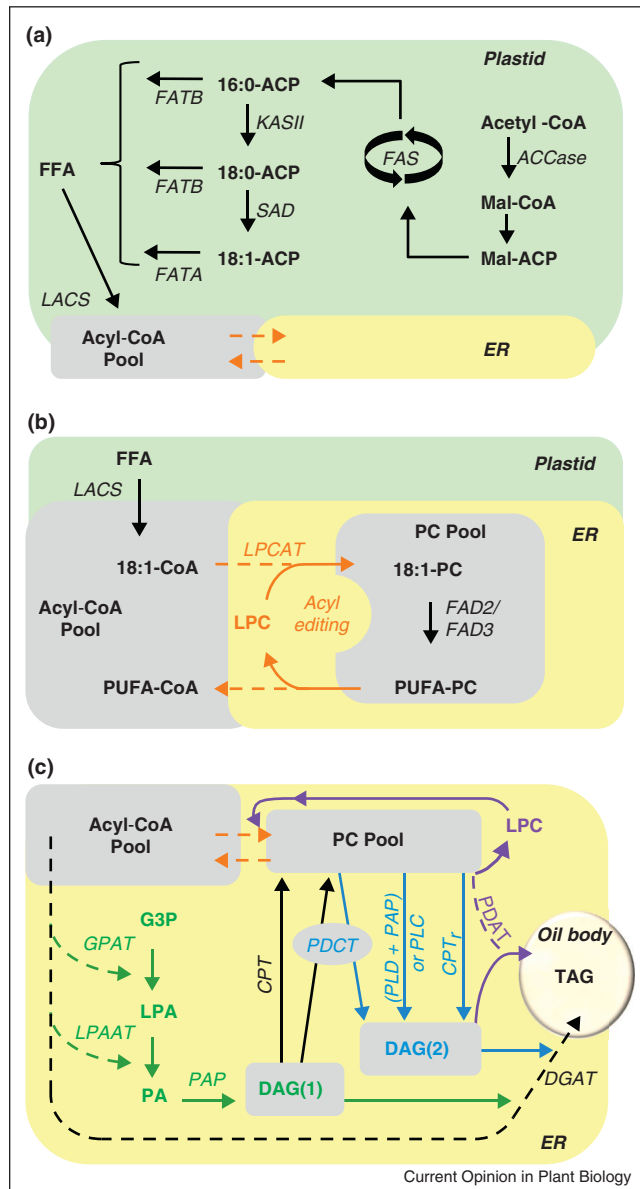
studied. A recent review [4<sup>••</sup>] and website ([aralip.plantbiology.msu.edu](http://aralip.plantbiology.msu.edu)) provide details on the very large number of genes involved in Arabidopsis oil synthesis and lipid metabolism.

## Seed oil biosynthesis synthesis begins in the plastid

Fatty acid (FA) synthesis is localized to plastids (Figure 1a), whereas assembly of the TAG molecule occurs outside the plastid and may be associated with both the endoplasmic reticulum (ER) and the oil body (Figure 1b,c) [5–7]. In most seeds, carbon is delivered to FA synthesis via glycolysis with hexose and/or triose as the predominant carbohydrate entering the plastid. However, green seeds can also use light to supply NADPH and ATP, which allows a ‘bypass’ of glycolysis via ribulose-1,5-bisphosphate carboxylase activity and pentose phosphate enzymes. This alternative pathway is more carbon efficient, resulting in 20% more acetyl-CoA available for oil synthesis, and also does not require reductant supply from the oxidative pentose phosphate pathway [8<sup>•</sup>]. The plastid FA synthesis pathway determines the chain length (up to 18 carbons) and the level of saturated FAs in seed oils. The first committed enzyme in the pathway is acetyl-CoA carboxylase (ACCase). As in yeast, animals and bacteria, plant ACCase is highly regulated and is a key control point over the flux of carbon into FAs [9]. In addition to control by phosphorylation, redox status and PII interactions [10<sup>•</sup>], feedback on ACCase by 18:1-ACP has recently been described [11<sup>••</sup>]. Assembly of FAs occurs on acyl carrier protein (ACP) via a cycle of 4 reactions that elongate the acyl chain by 2 carbons each cycle. After 7 cycles, the saturated 16 carbon acyl-ACP can either be hydrolyzed by the FATB acyl-ACP thioesterase or further elongated by KASII to 18:0-ACP, which is then desaturated to 18:1-ACP and hydrolyzed by the FATA thioesterase [4<sup>••</sup>]. The resulting 16:0 and 18:1 free acids are the main products of plastid FA synthesis, and their relative proportions are determined by the activities of FATA, FATB, 18:0-ACP desaturase (SAD) and KASII (Figure 1a). Transgenics and mutants have demonstrated that seed FA chain length and saturation can be altered by manipulation of any of these four enzymes [12]. For example, a dramatic demonstration of the control of chain length is the production of 60% lauric acid (12:0) in transgenic *B. napus* that expresses a FATB with specificity for this FA [13].

The transcription factor WRI1 [14] controls the expression of at least 15 enzymes including pyruvate dehydrogenase, ACCase and members of the FA synthesis and glycolytic pathways [15<sup>••</sup>]. Thus, WRI1 expression is

Figure 1



Overview of major reactions involved in fatty acid and triacylglycerol synthesis. **(a)** Plastid fatty acid synthesis; **(b)** Acyl editing and **(c)** TAG synthesis. Acyl transfer reactions are dashed lines. Green lines are de novo TAG synthesis, blue lines are PC-derived DAG synthesis, orange lines are acyl editing, and purple represents phospholipid:diacylglycerol acyltransferase (PDAT). DAG(1) is de novo synthesized DAG and DAG(2) is PC-derived DAG. *Abbreviations:* substrates are in bold: **ACP**, acyl carrier protein; **DAG**, diacylglycerol; **FFA**, free fatty acid; **G3P**, glycerol-3-phosphate; **LPA**, lyso-phosphatidic acid; **LPC**, lyso-phosphatidylcholine; **Mal**, malonate; **PA**, phosphatidic acid; **PC**, phosphatidylcholine; **PUFA**, polyunsaturated fatty acids; **TAG**, triacylglycerol. Enzymatic reactions are in italics: *ACCase*, acetyl-CoA carboxylase; *CPT*, CDP-choline:DAG cholinephosphotransferase; *DGAT*, acyl-CoA:DAG acyltransferase; *FAD*, fatty acid desaturase; *FAS*, fatty acid synthase; *FATA*, acyl-ACP thioesterase A; *FATB*, acyl-ACP thioesterase B; *GPAT*, acyl-CoA:G3P acyltransferase; *KASII*, ketoacyl-ACP synthase II; *LACS*, long chain acyl-CoA synthetase; *LPAAT*, acyl-CoA:LPA acyltransferase; *LPCAT*, acyl-CoA:LPC acyltransferase; *PAP*, PA phosphatase; *PDCT*, PC:DAG cholinephosphotransferase; *PLC*,

pivotal in directing the carbon flux that enters the seed toward the synthesis of FAs. Oil content is reduced to the extent of 80% in *wri1* mutants, and overexpression of *WRI1* increases maize embryo oil content by over 30% and oil yield per hectare by over 20% [16<sup>\*</sup>]. So far there is no evidence that *WRI1* controls expression of any of the acyltransferases or other 'downstream' enzymes involved in TAG assembly [81]. An intriguing observation is that the temporal patterns of gene expression for the enzymes of FA synthesis and for TAG assembly are very different during seed development. There is a general lack of understanding of the regulation of gene expression and enzyme activity for these later steps in oil biosynthesis [17<sup>\*</sup>,18<sup>\*</sup>].

### How do acyl chains move from the plastid to the ER?

After FA synthesis, the free FA products of the FATA/B thioesterases are exported from the plastid. Although there is evidence for a channeled pathway through the plastid envelope [19] a major unknown is whether there are specific transporters for this process. After export, it is presumed that long-chain acyl-CoA synthetase (*LACS*) on the outer plastid envelope forms the acyl-CoA that is the substrate for glycerolipid assembly (Figure 1a,b). However, it is puzzling that mutants in *LACS9*, the major plastid *LACS*, have no seed oil phenotype [20] suggesting that other members of the *LACS* family may be able to compensate [21]. Without more evidence, it cannot be ruled out that another type of enzyme, or reaction may activate FAs after their export from plastids. Regardless, it is likely that esterification of newly synthesized FAs to PC via the acyl-editing cycle can occur at the plastid envelope via acyl-CoA:lysophosphatidylcholine acyltransferase (*LPCAT*) [22] (Figure 1b). In this scenario we speculate that PC may act as a carrier of FAs from plastids to the ER, perhaps via direct connections between these organelles [23]. Inter-membrane transport of lipids can occur much more rapidly than diffusion through the cytosol via soluble carriers, and thus may provide an efficient plastid-ER acyl transfer mechanism. Recently, an ABC transporter has been proposed to be involved in the delivery of FA to the ER for oil synthesis [24]. However, both microarray and RNAseq data indicate that this transporter's expression in seeds is much lower than other lipid-related ABC transporters, and the radiolabeling evidence presented is uncertain because incubations were performed in the dark and without a supply of carbon. Thus, how this transporter modifies seed oil biosynthesis remains to be determined.

### Simple and complex pathways of TAG assembly

The de novo assembly of TAG from glycerol-3-phosphate and acyl-CoAs (also known as the Kennedy pathway)

phospholipase C; *PLD*, phospholipase D; *SAD*, Stearoyl-ACP desaturase.

involves only four enzymatic steps: first, two acylations of G3P by *sn*-1 glycerol-3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAAT), followed by phosphatidic acid phosphatase (PAP), and a third acylation by diacylglycerol acyltransferase (DGAT) (Figure 1c, green arrows). The pathway was first characterized in animals over 50 years ago [25,26] and soon after in plants [27]. Since that time biochemical, molecular genetic and metabolic flux analyses of plants have defined an additional more complex pathway in which the membrane lipid phosphatidylcholine (PC) is a central intermediate in the flux of FAs (Figure 1b) or diacylglycerol (Figure 1c), or both substrates into TAG [28]. The FA esterified to the *sn*-2 position of PC is the major site of ER localized FA modification (e.g. desaturation, hydroxylation, etc. [29,30]). Therefore, acyl fluxes into and out of PC are crucial for the production of TAG containing high levels of PC-modified FAs, such as the polyunsaturated FAs (PUFA) that are essential for human health [31]. The pathway of TAG synthesis appears to differ between plant species ranging from just a simple Kennedy pathway to a pathway where >90% of the FAs within the seed fluxes through PC before incorporation into TAG [28].

### Genes involved in *de novo* triacylglycerol assembly (Kennedy pathway)

Surprisingly, the *sn*-1 GPAT that initiates TAG assembly is not yet certain, but it may be the ER localized ‘GPAT9’ in Arabidopsis [32]. *GPAT9* is a homolog of mammalian and yeast GPATs involved in TAG production [33,34]. Arabidopsis also expresses an eight-member GPAT family (*GPAT1-8*) that is only found in land plants; however at least five of these encode *sn*-2 acyltransferases that are involved in synthesis of extracellular lipids (e.g. cutin, suberin) and almost certainly do not participate in membrane lipid or TAG synthesis [35]. Genes that encode LPAAT, the second step in *de novo* TAG assembly have been identified from several plants [36,37]. However, the isozyme(s) of PAP responsible for the majority of *de novo* synthesized diacylglycerol (DAG) (Figure 1c, DAG(1)) synthesis have yet to be identified. Arabidopsis contains at least 11 genes annotated as potential PAPs (aralip.plantbiology.msu.edu). A double knockout of two genes (*PAH1* and *PAH2*) only resulted in a 15% reduction in seed FA levels [38] indicating other PAP isozymes must also be contributing to TAG synthesis. For the last step of the Kennedy pathway, plants possess multiple unrelated DGAT enzymes, which appear to have differential involvement during TAG accumulation in oil producing tissues of different plants [7]. DGAT1 and DGAT2 are related to DGATs involved in TAG synthesis in animals and fungi [7,39,40]. Genetic manipulation of DGAT1 has demonstrated it to be the major enzyme catalyzing TAG production in Arabidopsis [41–43]. The function of DGAT2 in Arabidopsis remains unclear [43] but in some plants such as *Ricinus communis*

and *Vernicia fordii*, DGAT2 is more highly expressed than DGAT1 during seed maturation and DGAT2 appears to be the enzyme responsible for most TAG synthesis [44,45]. A distinct DGAT enzyme from *Euonymus alatus* is related to wax synthases, and utilizes acetyl-CoA rather than long chain acyl-CoAs to produce uniquely structured TAGs with an acetyl-group at the *sn*-3 hydroxyl of TAG [46]. Other enzymes with DGAT activity include a soluble DGAT3 first identified in developing peanut cotyledons [47] and that may have a role in TAG recycling within germinating Arabidopsis seedlings [48]. Two other Arabidopsis enzymes with DGAT activity have recently been identified but also do not appear to be involved in seed oil synthesis [49,50].

### The central role of phosphatidylcholine in TAG synthesis

Three mechanisms allow the flux of FA through PC for eventual TAG synthesis. First, ‘Acyl editing’ (Figure 1b, orange arrows) [51–53]. Second, direct transfer of a FA from PC to DAG producing TAG by the phospholipid:diacylglycerol acyltransferase (PDAT; Figure 1c, purple arrows) [54]. Third, utilization of a PC-derived DAG as the substrate for TAG synthesis (Figure 1c, DAG(2), blue arrows) [28].

### Acyl editing mechanisms

Acyl editing is a PC-deacylation and lysophosphatidylcholine (LPC)-reacylation cycle which exchanges the FA on PC with the acyl-CoA pool without net PC synthesis or degradation (Figure 1b, orange arrows). The acyl editing cycle allows: first, nascent FA from the plastid to be incorporated into PC while second, FAs that have been desaturated or otherwise modified on PC are released into the acyl-CoA pool where they can participate in the Kennedy pathway or other reactions. *In vitro* and *in vivo* metabolic analyses of acyl fluxes in/out of PC in multiple plant tissues [28,53] suggest that acyl editing may involve both the forward and reverse actions of LPCAT [55]. This mechanism is supported *in planta* by the inability of the Arabidopsis *lpcat1 lpcat2* double mutant to incorporate newly synthesized FA into PC through acyl editing in seeds [56\*\*]. These results indicate that LPCATs are involved in at least the LPC-reacylation half of the acyl editing cycle. Other broad specificity lysophospholipid acyltransferases, such as lyso-phosphatidylethanolamine acyltransferases [57], cannot compensate for the loss of LPCAT acyl editing in Arabidopsis seeds [56\*\*]. The molecular identity of the enzyme(s) responsible for the PC-deacylation half of the acyl editing cycle is a key unknown, and may involve the reverse activity of LPCAT [55,56\*\*] and/or a phospholipase A [58].

A second recent analysis of the *lpcat1 lpcat2* double mutant indicated increased total PC synthesis and degradation without a change in steady-state PC levels but with an increase in steady state levels of LPC [59]. The acyl

editing cycle does not require net PC synthesis, thus the increase in PC synthesis-degradation in the *lpcat1 lpcat2* double mutant was unexpected. However, the accumulation of LPC due to the lack of LPCAT activity (for acyl editing (Figure 1b) or PDAT activity (Figure 1c)) may induce LPC turnover due to its inherent detergent properties on membranes, and thus require synthesis of new PC in a futile cycle in these mutant plants.

#### The involvement of PDAT in TAG synthesis

The relative flux of acyl chains onto the *sn-3* position of TAG through either the DGAT or PDAT activities is unclear in most oilseed plants. The *dgat1* mutant of Arabidopsis only reduces oil content by 20–30% [41,42] and the *pdat1* mutant has no impact on TAG accumulation [60]. These results can be explained by overlapping functions of DGAT and PDAT that allow PDAT to partially compensate for a DGAT knockout, and DGAT to fully compensate for a lack of PDAT. A double knockout is pollen lethal and seed-specific RNAi of PDAT in the *dgat1* mutant reduced oil 80% [43]. Within the Arabidopsis *dgat1* mutant background PDAT expression is upregulated [61], perhaps to achieve ~80% of wild-type TAG levels [41–43]. It is possible that in some oilseeds PDAT has a primary role in maintaining membrane homeostasis, as in yeast [62], whereas in other species PDAT can play a more major role in TAG production [82]. PDATs from Arabidopsis and other plants have been shown to have high activity with PC containing unusual FA [63–65], suggesting that PDAT may be involved in removal of damaged or unusual FA from the membrane and sequestering them in TAG.

#### PC-derived DAG production

In addition to the above mechanisms, acyl chains on PC can also be incorporated into the *sn-1* and *sn-2* positions of TAG by remaining esterified to the glycerol backbone of PC until DAG is derived from PC for TAG synthesis. Three alternative enzymatic routes can generate DAG from PC (Figure 1c, blue arrows). First, phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) which exchanges phosphocholine between PC and DAG [66]; second, the reverse reaction of CDP-choline:diacylglycerol cholinephosphotransferase (CPT) [67,68]; or third, a lipase-based mechanism utilizing phospholipase C, or phospholipase D plus PAP.

#### Acyl fluxes through PC may or may not determine TAG FA composition

Different plants likely utilize different combinations of the above pathways of acyl flux through PC to accumulate PC-modified FA in TAG. For instance, characterization of *pdct1* [66] and the *pdct1 lpcat1 lpcat2* triple mutant indicates that LPCAT-based acyl editing and DAG produced by PDCT together are responsible for at least 2/3 of the flux of PUFA from PC to TAG in wild-type Arabidopsis seeds [56\*\*]. A residual PC-derived DAG

flux utilizing phospholipase D, may be involved in the remaining flux of PUFA from PC to substrates for TAG synthesis in Arabidopsis [56\*\*] and possibly higher levels in other plants such as that in soybean [69\*]. However, not all FA's that flux through PC are further modified. High flux of acyl chains into PC via acyl editing is likely to occur in oilseeds that modify very little FA in TAG (e.g. coriander [70]; and Thunbergia seeds [71]). Analysis of acyl fluxes in developing soybean embryos estimated that ~90% of all FA in TAG fluxed through PC either by acyl editing or by the predominant use of PC-derived DAG for TAG synthesis (regardless of whether the FA was further desaturated or not) [51]. Similar conclusions have been made in Arabidopsis which also contains high rates of PC acyl editing [22,56\*\*] and predominantly utilizes PC-derived DAG during TAG synthesis [72]. High rates of acyl editing without accompanying FA modification may imply the importance of acyl editing as a mechanism for the export of FA from the plastid [22] and PC-mediated 'FA trafficking' to specific sites of the ER for TAG biosynthesis, or for additional cellular processes such as maintenance of membrane homeostasis [52].

#### Multiple substrate pools and channeling

*In vivo* metabolic flux analysis of soybeans strongly suggests that de novo DAG (Figure 1c, DAG(1)) and PC-derived DAG (Figure 1c, DAG(2)) are distinct pools that do not intermix [51]. Additionally, many plants can produce unusual modified FA on PC, but these FAs do not accumulate in membrane lipids yet can accumulate to over 90% of the FA in TAG [73]. Together, these results suggest that plants may generate specific lipid substrate pools that can be channeled into TAG and kept separate from membrane lipid synthesis. How such channeling is achieved is unknown but may involve enzymes specific for unusual FAs [63,65,74–76] as well as separation of lipid biosynthetic activities to different subdomains of the ER membrane [44,77]. If there is spatial separation of Kennedy pathway de novo DAG synthesis from PC-derived DAG synthesis in different microdomains of the ER, then PC may serve as a 'DAG trafficking' molecule through the ER membrane.

#### What determines seed oil content?

Increasing oil production in seeds has been a target of plant breeders and metabolic engineers for decades. Even a few % increase in oil yield per ha in a crop such as soybean can add more than US\$1 billion to the crop's annual world value. How can this be achieved? In general, flux into metabolic pathways can be controlled either by increasing the supply of upstream substrates (source control) or by increasing 'demand' or 'sink' strength in the last steps of a pathway. Increased seed oil accumulation has been engineered at both stages. An increase in maize embryo oil by overexpressing the WRI1 transcription factor [16\*] and increased soybean and *B. napus* oil by expression of DGAT [78,79], represent successes at

source and sink levels, respectively. Although there are dozens of other reports of gene manipulations that increase seed oil [80], so far there are only a few examples where such increases have been demonstrated in multiple field trials. Further research is required to determine whether PC acyl flux reactions can also be utilized to modify oil content.

## Questions and unknowns

There is no shortage of questions that must be answered to better understand oil synthesis in plant seeds. These include identifying which genes are involved in: first, FA export from plastids; second, the GPAT and the PA to DAG reactions of the Kennedy pathway; third, PC deacylation during acyl editing. In addition, there is a fundamental lack of clarity about the cell biology of TAG assembly. Unknowns include: first, is PC a universal FA/DAG carrier between organelles? Second, to what extent are substrate channeling and distinct subcellular pools of DAG, PC, acyl-CoA (and other intermediates) involved in TAG biosynthesis? Third, do membrane and TAG synthesis require different locations of their biosynthetic enzymes and are oilbody:ER connections involved? Fourth, how are unusual FA excluded from membranes and channeled to TAG? Fifth, to what extent is PC involved in acyl fluxes of saturated or other FA that are not modified in the ER? Finally, how are levels of transcripts, proteins and activities of DGAT and other downstream enzymes of TAG assembly controlled?

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