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REVIEW

Engineering industrial fatty acids in oilseeds

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Abstract More than 300 types of modified fatty acids (mFA) are produced in triacylglycerols (TAG) by various plant species, with many of these unusual structures rendering unique physical and chemical properties that are desirable for a variety of bio-based industrial uses. Attempts to produce these mFA in crop species have thus far failed to reach the desired levels of production and highlighted the need to better understand how fatty acids are synthesized and accumulated in seed oils. In this review we discuss how some of the progress made in recent years, such as the improved TAG synthesis model to include acyl editing and new enzymes such as PDCT, may be utilized to achieve the goal of effectively modifying plant oils for industrial uses. Co-expressing several key enzymes may circumvent the bottlenecks for the accumulation of mFA in TAG through efficient removal of mFA from phosphatidylcholine. Other approaches include the prevention of feedback inhibition of fatty acid synthesis and improving primary enzyme activity in host transgenic plants. In addition, genomic approaches are providing unprecedented power to discover more factors that may facilitate engineering mFA in oilseeds. Based on the results of the last 20 years, creating a high mFA accumulating plant will not be done by simply inserting one or two genes; it is necessary to stack genes encoding enzymes with favorable kinetic activity or specificity along with additional complementary transgenes in optimized plant backgrounds to produce industrial fatty acids at desirable levels. Finally, we discuss the potential of *Camelina* as an industrial oilseed platform.

Keywords *Camelina*, metabolic engineering, modified fatty acids, oilseeds, triacylglycerol biosynthesis

Introduction

As the world's petroleum reserves dwindle and the human population grows, it is imperative to search for renewable resources to meet an ever increasing demand of energy and chemical raw materials. Plant oils, one of the most energy-rich and abundant forms of reduced carbon available from nature, have long been used by mankind not only as an essential nutrient but also in many other uses such as illumination and skin care. Modern transportation utilized vegetable oils as fuels in the form of straight vegetable oil (SVO) or processed biodiesel and jet fuels (Huber et al., 2006; Moser, 2010; Steen et al., 2010). Most plant oils are in the form of triacylglycerols (TAG) containing three fatty acids, which are immensely diversified in structures. While the majority of fatty acids in most vegetable oils are 18 carbons with up to three double bonds, there are more than 300 additional modified fatty acids (mFA) found in different plant TAG (Jaworski and Cahoon, 2003; Singh et al., 2005). Many of the unusual structures, such as hydroxy, epoxy, or conjugated groups, render unique physical and chemical properties (Gunstone, 1998; Voelker and Kinney, 2001). This opens up opportunities for the use of vegetable oils in a variety of bio-based industrial formulations, including lubricants and drying oils (Gunstone, 1998; Durrett et al., 2008; Napier and Graham, 2010; Lu et al., 2011), that are otherwise made from fossil petroleum.

Above all, the fatty acid composition in triacylglycerols determines the quality and thus the uses of plant oils. While seed oils to be used for human consumption should contain as little saturated fatty acids as possible and a significant proportion of polyunsaturated fatty acids (PUFAs) (Riediger et al., 2009), industrial oils require low polyunsaturated fatty acids for desirable oxidative stability or high homogeneity of certain fatty acids (Dyer and Mullen, 2008; Dyer et al., 2008; Pinzi et al., 2009). Many plants store high levels of industrial mFA in seeds, however most of these plants are not

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domesticated for agriculture. Molecular biologists have made many attempts over the past 20 years to introduce the capacity for producing specific mFA into crop species (Drexler et al., 2003; Jaworski and Cahoon, 2003; Lu et al., 2006; Napier and Graham, 2010). The results of these experiments have typically been disappointing, producing in most cases, plant lines with very low yields of the desired fatty acids (Broun and Somerville, 1997; Lee et al., 1998; Cahoon et al., 1999; Suh et al., 2002). These results underlie the need to understand the fundamental aspects of how plant fatty acids are synthesized and accumulated in seed oils. Here, we review some important progress made in recent years and discuss how these new discoveries may help achieve the goal of modifying plant oils for industrial uses in a predictable manner.

An evolving model of triacylglycerol biosynthesis in plants

The major pathways and enzymes involved in TAG synthesis have been unveiled in the past decades by biochemical and genetic means, especially by using mutants of *Arabidopsis thaliana*, the model oilseed plant. In plants, fatty acid synthesis occurs exclusively in plastids and produces mostly oleic acid (18:1, carbon number : double bonds) and a small amount of palmitic acid (16:0) and stearic acid (18:0) that are esterified to acyl carrier protein (ACP) (Ohlrogge and Browse, 1995). These fatty acids are incorporated into glycerolipids in two subcellular compartments: (1) acyl groups from acyl-ACP are directly used within the plastid by the "prokaryotic" pathway; and (2) fatty acids are removed from acyl-ACP to be exported into the cytosol, and converted into acyl-Coenzyme A (acyl-CoA) to be used in the "eukaryotic" pathway in the endoplasmic reticulum (ER) (Browse and Somerville, 1991; Ohlrogge and Browse, 1995). The proportion of newly synthesized fatty acids that goes into these two pathways varies widely among different plant species and even in different tissues of the same plant. In oilseeds, these acyl groups are used almost entirely ($>95\%$) by the eukaryotic pathway (Roughan and Slack, 1982; Browse and Somerville, 1991).

In seeds of *Arabidopsis* and some oilseed crops such as *Brassica napus* and *Camelina sativa*, fatty acids exported into the eukaryotic pathway may be modified in two major ways: carbon chain elongation and desaturation (Fig. 1; for simplicity, only 18:1 is shown). In the cytosol, 18:1-CoA may be elongated into 20:1- to 22:1-CoA esters by a fatty acid elongase FAE1 (Kunst et al., 1992). These acyl-CoAs, along with other acyl groups derived from *de novo* fatty acid synthesis or from other sources, will be available for incorporation into glycerolipids in the ER. The dominant flux of nascent fatty acids in oilseeds is to enter the membrane lipid phosphatidylcholine (PC) (Roughan and Slack, 1982), where they can be modified by the ER localized fatty acid desaturases (FAD, Fig. 1) including the oleate desaturase-FAD2 and the linoleate desaturase FAD3 (Browse et al., 1993; Sperling et al., 1993; Okuley et al., 1994) to produce the polyunsaturated fatty acids (PUFAs) linoleic acid (18:2) and α-linolenic acid (18:3). In plant seeds that accumulate many other modified fatty acids (mFA) with unusual chemical structures, the mFA are also synthesized on PC (Bafor et al., 1991; Jaworski and Cahoon, 2003).Therefore, understanding the mechanisms of fatty acids entering PC and, after they are modified, their subsequent channeling into TAG is critical for successful engineering of the mFA.

As shown in Fig. 1, it is traditionally thought that fatty acids are mainly incorporated into PC through the *de novo* pathway: Glycerol-3-phosphate (G3P) is sequentially acylated at the *sn*-1 and *sn*-2 positions using acyl-CoA exported from plastids to produce phosphatidic acid (PA). Removing the phosphate group at the *sn*-3 position of PA by PA phosphatases (PAP) produces diacylglycerol (DAG). Finally PC is formed from DAG by a CDP-choline: diacylglycerolcholinephosphotransferase (CPT) (Slack et al., 1983; Goode and Dewey, 1999). The TAG synthesis pathway shares most of the enzymes in the PC synthesis, and its committed step is the acylation of DAG by the *sn*-3 specific acyl-CoA: diacylglycerol acyltransferases (DGAT) (Hobbs et al., 1999; Routaboul et al., 1999; Zou et al., 1999). The sequential acylations of the *sn*-glycerol-3-phosphate is usually referred to as the Kennedy pathway (Kennedy, 1961). It was later discovered that an acyl-CoA independent phospholipid:diacylglycerol acyltransferase (PDAT) also directly transfers the *sn*-2 acyl group from PC into DAG to form TAG (Dahlqvist et al., 2000). DAG may also be converted into TAG by an elusive DAG:DAG transacylase (Stobart et al., 1997). Recent results demonstrated that DGAT and PDAT are responsible for the majority of TAG synthesized in seeds (Zhang et al., 2009).

However, this model was questioned by metabolic labeling experiments (Williams et al., 2000; Bates et al., 2007; Bates et al., 2009), which demonstrated that it should include acyl editing, also termed "remodeling" or "retailoring," a process that exchanges acyl groups between polar lipids but does not result in the net synthesis of these lipids. The work of Bates and coworkers (Bates et al., 2007; Bates et al., 2009) indicates that the majority of newly synthesized saturated (16:0) and monounsaturated (18:1) fatty acids enter PC by exchanging with polyunsaturated fatty acids (18:2, 18:3) on PC rather than proceeding through the Kennedy pathway. The PUFAs may be released from PC by either the reverse reaction of a lysophosphatidylcholine acyltransferase ($LPCAT^r$) or by the phospholipase A (PLA). The resulting lysophosphatidylcholine (LPC) can be reacylated by the forward reaction of LPCAT, and this reaction is speculated to be the major pathway for newly synthesized fatty acids entering PC. However, the genes encoding these enzymes have not been identified. The PUFAs released from PC in the forms of acyl-CoA (*via* LPCAT) or free fatty acids (*via* PLA), which are activated by the acyl-CoA synthases (LACS) (Shockey et al.,

2002), enter the acyl-CoA pool to be used for phospholipid or TAG synthesis. The acyl editing process thus enriches the PUFA in the acyl-CoA pool, which contribute to high levels of such fatty acids in PC and TAG.

Another breakthrough in understanding the TAG biosynthesis pathway was made by the discovery of a new enzyme, phosphatidylcholine diacylglycerol cholinephosphotransferase (PDCT), encoded by the *ROD1* locus in *Arabidopsis* (Lu et al., 2009). PDCT catalyzes a previously unknown reaction—the interconversion between DAG and PC by phosphocholine headgroup exchange. This mechanism generates a flux of fatty acids through PC, the site of desaturation and other modifying reactions, and results in the enrichment of PUFA or other mFAin DAG, and subsequently in TAG.

Expression of co-evolved enzymes to increase modified fatty acid content

The above model of TAG synthesis has been derived largely from *Arabidopsis*; however, it is envisioned that oilseeds which accumulate unusual mFAs in their TAGs also share most of the pathways illustrated in Fig. 1. Genes encoding many enzymes that synthesize mFA have been cloned, which also use PC as the substrate (Bafor et al., 1991; Cahoon et al., 1999; Thomæus et al., 2001; Bao et al., 2002; Cahoon et al., 2002; Jaworski and Cahoon, 2003). However, expressing the single catalytic enzymes required for mFA biosynthesis is insufficient to create transgenic plants producing large amounts of these fatty acids in seed oils (Jaworski and Cahoon, 2003; Lu et al., 2006). The occurrence of very high mFA content in native seeds is likely the result of collective effects of many genes that have co-evolved to facilitate the processing of mFA and their intermediate metabolites (Lu et al., 2006).

To validate this hypothesis, researchers from John Browse's laboratory initiated a high-throughput approach screening a full-length cDNA library from castor (*Ricinus communis* L.) by shotgun transformation into an engineered *Arabidopsis* line producing hydroxy fatty acids (Lu et al., 2006; Lu et al., 2007); and in parallel, isolated and tested by co-expression of several castor orthologous genes of the acyltransferases (Fig. 1). The hydroxylated ricinoleic acid (12-hydroxyoctadec-*cis*-9-enoic acid; 18:1-OH) accounts for 90% of the total fatty acids in castor oil. The biosynthesis of ricinoleic acid is catalyzed by the oleate Δ12-hydroxylase (FAH12) using PC as the substrate (van de Loo et al., 1995). Previous heterologous expression of FAH12 in *Arabidopsis* produced only up to 17% hydroxy fatty acids (HFA) in seed oils (Broun and Somerville, 1997; Lu et al., 2006). The efforts in the Browse group identified several genes from castor that boost HFA accumulation in transgenic *Arabidopsis* including an oleosin (Lu et al., 2006) and the DGAT2 and PDAT1A enzymes (Burgal et al., 2008; van Erp et al., 2011).

The acyltransferases DGAT and PDAT catalyze the final step of TAG assembly by the acylation of the *sn*-3 position on DAG (Fig. 1). The two enzymes have overlapping functions for embryo development and TAG biosynthesis in developing seeds and pollen (Zhang et al., 2009), and are considered the two major enzymes for oil accumulation in seeds. In the absence of a functioning DGAT in the *dgat1* mutant, it has been shown that PDAT1 is responsible for the remaining 65%–70% of TAG synthesized in *Arabidopsis* seeds (Xu et al., 2012). By co-expressing a castor diacylglycerol acyltransferase, RcDGAT2, along with FAH12 in *Arabidopsis*, the Browse group showed a significant increase in the accumulation of HFA from 17% up to 28% (Burgal et al., 2008). Similar effects were also shown for a PDAT that increased HFA levels in transgenic *Arabidopsis* (van Erp et al., 2011). The cDNA encoding the castor PDAT1A was transformed into the CL37 *Arabidopsis* line (also expressing FAH12) both alone and in addition to RcDGAT2. Expression of the castor PDAT1A raised HFA levels from 17% to 27%, similar to the increase observed with the co-expression of FAH12 with RcDGAT2 (Burgal et al., 2008). Expression of RcDGAT2 in the CL37 PDAT1A background increased HFA levels from 25.4%±0.3% to 26.7%±0.2%, a small but statistically significant amount, which translates to an increase of 19.6% in the mass of HFA per seed (van Erp et al., 2011). In contrast, overexpression of the *Arabidopsis* orthologs of DGAT2 or PDAT1A failed to produce such beneficial effects in both experiments (Burgal et al., 2008; van Erp et al., 2011). These are consistent with results that both RcDGAT2 and RcPDAT1A show a preference for ricinoleic acid-containing substrates, thus facilitating incorporation of high amounts of this fatty acid into TAG in castor (Dahlqvist et al., 2000; Burgal et al., 2008; van Erp et al., 2011). These results fuel the hypothesis that the limited mFA accumulation in seed oils may be due to inefficient utilization of these mFA substrates by the transgenic plant. Enzymes that have co-evolved to utilize mFA substrates for incorporation into TAG in plants such as castor may need to be co-expressed to accumulate these mFAs in the selected crop plant at levels that would be acceptable for industrial uses.

The theory of co-evolved enzymes has gained further support from species that accumulate other mFAs. Seeds of the tung tree (*Vernicia fordii*) produce large quantities of TAGs containing 80% eleostearic acid, a conjugated fatty acid. The tung DGAT1 is expressed at similar levels in various organs, whereas DGAT2 is strongly induced in developing seeds at the onset of oil biosynthesis. Expression in yeast showed the tung DGAT2 possessing an enhanced propensity for the synthesis of trieleostearin, the main component of tung oil (Shockey et al., 2006). It has also been shown that DGATs from *Vernonia galamensis* and *Stokesia laevis* have strong substrate preferences for vernolic acid, an 18-carbon Δ12-epoxy fatty acid (Yu et al., 2006). Expression of the *Stokesia* epoxygenase alone only resulted in 3%–7% epoxide levels. Co-expressing DGAT1 or DGAT2 from *V. galamensis* led to increased accumulation of up to 27.8% vernolic acid (Li et al., 2010). Researchers found that

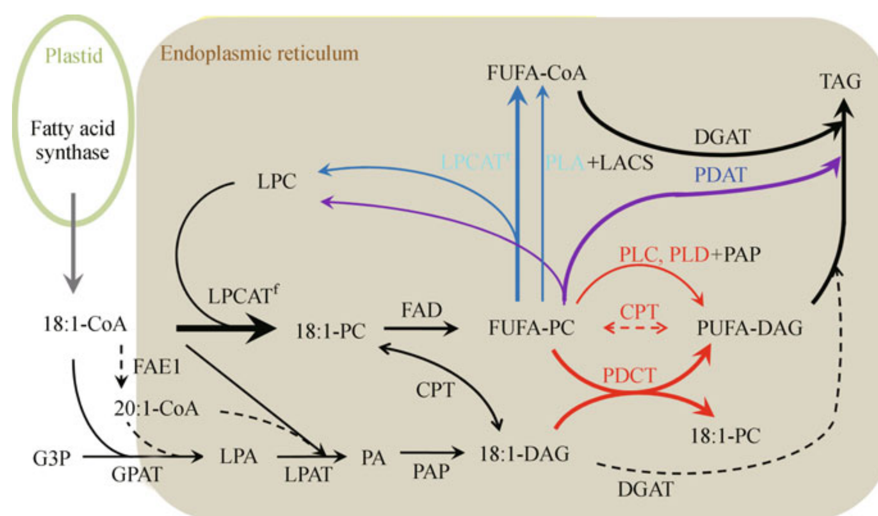

Figure 1 Metabolic network showing some of the pathway possibilities for the assembly of polyunsaturated fatty acids (PUFA) into TAG in *Arabidopsis*. Many other modified fatty acids (mFA) use similar mechanisms. Colored arrows denote different pathways for PUFA or other mFAs exiting PC; while heavy-weight arrows indicate major pathways, and dashed arrows indicate alternative or parallel routes for metabolites. Please note that the two 18 : 1-PCs are actually one pool, just arranged as two due to the spacing required to make different pathway reactions more easily distinguished. DAG: diacylglycerol; G3P: glycerol-3-phosphate; LPA: lysophosphatidic acid; LPC: lysophosphatidylcholine; PA: phosphatidic acid; PC: phosphatidylcholine; PUFA: polyunsaturated fatty acid; TAG: triacylglycerol; CPT CDP-choline: DAG cholinephosphotranferase; LPCAT acyl-CoA: LPC acyltransferase; DGAT acyl-CoA: DAG acyltransferase; PAP: phosphatidic acid phosphatase; FAD: oleate and linoleate desaturases; PDAT PC: DAG acyltransferase; FAE1: fatty acid elongase 1; PDCT PC: DAG cholinephosphotransferase; GPAT acyl-CoA: G3P acyltransferase; PLA: Phospholipase A; LACS: long-chain acyl-CoA synthetase; PLC: Phospholipase C; LPAT acyl-CoA: LPA acyltransferase; PLD: phospholipase D.

more vernolic acid accumulated in TAG with lower levels of vernolic acid left in PC which was similar to the ratio observed in native *Stokesia* and *Vernonia* seeds. Therefore DGATs from high epoxy fatty acid accumulators may selectively incorporate epoxy fatty acids into TAGs. These experiments indicate that high-level production of mFA in transgenic seeds requires co-expressing or stacking genes from the original accumulator species.

Overcoming bottlenecks – efficient removal of mFA from PC

The latest model of TAG synthesis highlights the central role of PC as the source of greatly diversified fatty acids in membrane and storage lipids. The metabolic labeling work (Bates et al., 2007; Bates et al., 2009) supports this model by finding that there are two functionally distinct pools of DAG in soybean. One pool of *de novo* DAG is utilized primarily for PC synthesis, while the other PC-derived DAG pool is used for TAG synthesis. It was revealed that 95% of TAG in soybean was synthesized using the PC-derived DAG pool (Bates et al., 2009). Similarly it has been shown that TAG synthesis in *Arabidopsis* also primarily utilizes PC-derived DAG (Bates and Browse, 2011). Therefore, the flux of fatty acids through PC and subsequent removal of mFAs from PC has been identified as a major bottleneck for mFA accumulation in TAG (Cahoon et al., 2006; van Erp et al., 2011; Bates and Browse, 2011). In transgenic soybean and *Arabidopsis* expressing a fatty acid conjugase, transgenic lines accumulated no more than 20% of the total FA as conjugated FAs in TAG, while displaying nearly 25% in PC compared to the $< 1\%$ typically seen in PC of native accumulating plants (Cahoon et al., 2006). Similar results were also seen in plants transformed with castor FAH12; the amount of HFA that accumulated in PC of the transgenics was more than twice that in castor (van Erp et al., 2011).

The efficient removal of mFA from PC to allow for their incorporation into TAG will be important in the creation of a high mFA accumulating transgenic plant. Enzymes specific for the removal of mFA from PC to break the bottleneck would be of the most use. Expression of a PDAT from castor, RcPDAT1A, was shown to increase HFA levels from 17 to 27% in the *Arabidopsis* CL37 line (van Erp et al., 2011). This increase in HFA content was most likely due to the direct transfer activity of RcPDAT1A succeeding in removal of HFA from PC, as the percent of HFA left in PC of the CL37 PDAT1A transgenics was lower and more comparable to that seen in castor (van Erp et al., 2011). The newly discovered enzyme, phosphatidylcholine diacylglycerol cholinephosphotransferase (PDCT), offers another possible solution for the removal of mFAs from PC so they can be incorporated into TAG. PDCT functions in the conversion between PC and DAG, enriching the DAG pool with mFAs prior to their incorporation into TAG. Through studies of the *Arabidopsis* *rod1* mutant that is deficient in the PDCT enzyme, it was

shown that at least 40% of the PUFAs present in TAG are derived from the PDCT-mediated DAG-PC conversions (Lu et al., 2009), so it stands to reason that PDCT could also be an important enzyme in mFA removal from PC. *Arabidopsis rod1* mutants expressing FAH12 only accumulated half the HFA that their wild type counterparts did. When a castor PDCT was co-expressed with FAH12, the RcPDCT enzyme was able to almost double HFA levels (Hu et al., 2012).

The results of PDAT and PDCT demonstrate that efficient metabolism of mFA through PC is an effective strategy to increase mFA accumulation in TAG. Additional enzymes in such roles may include those involved in acyl editing for mFA removal, and phospholipases C and D for PC-DAG conversion (Fig. 1). Given the importance of acyl-editing as discussed above, it should be interesting to test its roles in mFA metabolism. Genes encoding acyl editing enzymes have not been identified in oilseeds; however, they may include at least two candidates of LPCATs in *Arabidopsis* and *Brassica napus* (Ståhl et al., 2008; Stålberg et al., 2009; Zheng et al., 2012). It is interesting to note that the gene encoding LPCAT2 was upregulated by 50%–75% as measured by microarray and quantitative RT-PCR in the *dgat1* mutant, which contains a lesion at the *DGAT1* locus resulting in an oil content decrease by 30%–35% compared to wild type levels (Katavic et al., 1995; Xu et al., 2012). Co-incident knockout lines of *dgat1*/*lpcat2* displayed severe effects including deficient TAG accumulation to levels slightly lower than that of *dgat1*, as well as delayed plant development and seed set. Because PDAT1 was also upregulated by 65% in the *dgat1* mutant, researchers suggested that the LPCAT2 functioned to assist PDAT1 in biosynthesis of TAG by supplying PC as a substrate for the transfer of sn-2 acyl chains to the sn-3 position of the increased DAG pool (Xu et al., 2012). The increased levels of PUFAs and reduced very long chain fatty acids (VLCFA) present at the sn-3 position of TAG are evidence supporting the donation of acyl groups from the sn-2 position of PC to the sn-3 position of DAG for TAG synthesis. While the orthologous LPCATs from other native species remain to be isolated and tested, LPCAT2 shows promise as an enzyme to help increase the ratio of mFA in TAG either through cooperation with a PDAT1, or through the reverse reaction to release mFA from PC before being incorporated into TAG.

Feedback inhibition preventing accumulation of mFA products

The transgenic lines expressing mFA synthesizing genes fail to accumulate desired levels of products, and also often display decreased oil phenotypes (Dauk et al., 2007; van Erp et al., 2011). Comprehensive investigations of the FAH12 line (Bates and Browse, 2011) suggest that expression of FAH12 results in accumulation of disruptive intermediates and substantial reductions in both HFA and total oil content of seeds. The FAH12-expressing *Arabidopsis* line CL37 exhibits roughly 30% less oil than its parent *fae1* lines (van Erp et al., 2011). Similar detrimental effects were also observed in soybean expressing epoxy mFA transgenes (Li et al., 2010; Li et al., 2012).This reduced oil accumulation may have been the result of inefficient incorporation or utilization of mFA-containing lipids, which are subsequently degraded since fatty acids cannot accumulate in plant cells. The investigation of FAH12-expressing *Arabidopsis* lines indicated the fatty acid β-oxidation cycle is present even with low FAH12 gene expression levels (Moire et al., 2004). Similar results were also reported in *Brassica napus* expressing a lauroyl-acyl carrier protein thioesterase (Eccleston et al., 1996; Eccleston and Ohlrogge, 1998). To prevent a futile cycle of mFA synthesis and breakdown, enzymes that are selective for incorporation of mFA-containing DAG and acyl substrates into seed oil will need to be inserted. Utilization of native mFA accumulator DGAT2 and/or PDAT1A enzymes has been shown to improve the passage of intermediates into TAG and substantially (75%–80%) relieves the decline in mFA and oil content (van Erp et al., 2011; Li et al., 2012).The newly discovered enzyme PDCT has also been shown to partially restore low oil phenotype (Hu et al., 2012). Avoiding turnover of mFA lipids will hopefully aid in increasing the flux of mFAs into TAG.

Although breakdown of fatty acids by β-oxidation may contribute to the low oil content of mFA-transgenic seeds, recent results indicate that a reduction in fatty acid synthesis (FAS) rates is the main consequence of the bottleneck in TAG synthesis. This feedback inhibition of FAS may have a major role in limiting the productivity of plants engineered to produce mFA. FAS is believed to be controlled by activity of plastid ACCase which is a complex of four subunits in dicots and non-graminaceous monocots. The feedback involves inhibition of this complex by acyl-ACP (the end products of fatty acid synthesis) (Shintani and Ohlrogge, 1995; Andre et al., 2012). Overexpression of an *Arabidopsis* cytosolic ACCase, which is not subject to inhibition by acyl-ACP, in the plastid of *Brassica napus* resulted in a substantial increase in ACCase activity (Roesler et al., 1997). In native oilseeds, TAG synthesis is highly efficient so that feedback inhibition is possibly almost negligible. Therefore oil accumulation in the ACCase overexpressing lines increased by no more than 5% (Roesler et al., 1997). By contrast, the fatty acid synthesis rate is $>$ 30% reduced in FAH12 plants (Bates and Browse, 2011). If this is due to feedback downregulation of the plastid ACCase, expression of cytosolic ACCase in the plastid will restore fatty acid synthesis and may increase mFA accumulation.

Improving primary enzyme activities to maximize mFA production

One potential factor resulting in lower mFA accumulation in

the transgenic plants could be low activity of the enzymes derived from the original accumulator plant. While the enzyme activity may be sufficient when expressed in the native plant background, it might not be strong enough to compete with enzymes in the new host plant. An inability to compete for substrate to work upon or a slow turnover rate could lead to a low amount of desired products. In such cases, attempts at improving the enzyme activity through metabolic modification and mutations might prove effective to optimize mFA production.

Studies focused on improving enzyme activity have been previously performed with some success (Whittle and Shanklin, 2001; Broadwater et al., 2002; Nguyen et al., 2010). Combinatorial saturation mutagenesis of residues affecting chain length specificity was used to engineer a desaturase enzyme with 82 fold increase in specificity factor for the 16-carbon substrate while still retaining a K_m similar to that of the wild type enzyme with 18 carbon substrate (Whittle and Shanklin, 2001). This experiment was an important step in showing that it is possible to alter the substrate specificity of an enzyme without sacrificing favorable kinetic parameters. Simultaneous saturation mutagenesis at locations that were key to enzyme properties allowed researchers to identify mutations that altered the binding pocket to improve binding of the shorter 16:0 chain substrate. Site directed mutagenesis has also been used to make variant fatty acid desaturase (FAD2) enzymes, revealing certain residues that were important for specifying desaturation or hydroxylation function (Broadwater et al., 2002). Individual importance of these mutated residues could be confirmed by substitution to study the effects on enzyme function separately. Mutagenesis of FAD2 is especially interesting due to the fact that most of the enzymes that modify fatty acids on PC are diverged variants of the FAD2 desaturase.

The most successful improvement of enzyme function was done by the Shanklin group (Nguyen et al., 2010) in their attempts to synthesize ω-7 fatty acids. The *in vitro* activities of the required Δ9–16:0-ACP desaturase from native accumulators such as milkweed and *Doxantha unguiscati* were lower than desired, and resulted in low levels of ω-7 FA in *Arabidopsis* seeds of around 10%. To attempt to overcome this issue of low *in vitro* activity the researchers inserted an enzyme variant that arose from enzyme evolution experiments designed to enhance the 16:0-desaturase activity of castor Δ9–18:0-desaturase. The enzyme variant displayed a much higher specificity factor (K_{cat}/K_m) of 91.0 compared to Doxantha's 0.72, and effectively raised the total ω-7 FA from 10% to 14%. Additional metabolic modifications were made by expressing the new enzyme in a different background, *fab1*, containing higher 16:0 substrate levels which increased the substrate available for the enzyme and increased accumulation of ω-7 FA up to 39%. Expression of the variant enzyme in the *fab1fae1* double mutant background further decreased competition for the substrate, increased the level of 16:0 available for desaturation, and yielded ω-7 FA product levels up to 50%. Addition of an extraplastidial version of the desaturase was able to raise product levels to 71% in the highest lines. This 71% accumulation is nearly equivalent to the native accumulator plant, *Doxantha unguiscati* which has 72% ω-7 FA (Nguyen et al., 2010).

To perform effective metabolic engineering and create high mFA accumulating plants both the enzymes used and the plant background must be optimized. As the above researchers demonstrated, expression of an enzyme with favorable kinetic activity or specificity, additional complementary transgenes, increased substrate levels, and decreased enzyme competition are all important traits in optimizing a plant to produce mFAs at desirable levels. These qualities must be addressed for each type of mFA and plant host due to the inherent differences in their metabolic pathways and traits. Knowledge of specific metabolic pathways in both the native accumulator and new crop host plant will aid in their modification. Potential labeling experiments and additional studies using gene knockouts will help elucidate the flow of mFA through these pathways. Identification of enzyme variants and gene stacking will be vital engineering tools in the optimization of mFA accumulation following this method of metabolic modification wherein both the enzymes and plant background are tailored toward production of a modified fatty acid product.

Genomics approaches to uncovering additional factors in mFA metabolism

Greater knowledge has been obtained in recent years on enzyme functions and regulations that are involved in the accumulation of mFA in TAG. However transgenic experiments have shown that a complete understanding of the synthesis, modification and packaging of these mFA in seeds is required to allow for production of these useful fatty acids at commercially viable levels in agricultural crops. Genomics and bioinformatics tools have played important roles in the advancement of plant lipid research (Wallis and Browse, 2010). The most recent examples include the discovery of the PDCT enzyme encoded by the *Arabidopsis ROD1* gene (Lu et al., 2009) and a distinct DGAT with sn-3 acetyltransferase activity that synthesizes the unusual 3-acetyl-1,2-diacyl-sn-glycerols (acTAGs) in seeds of *Euonymus alatus* (Durrett et al., 2010). The advanced genomics tools including the next-generation sequencing capabilities and new databases will undoubtedly offer unprecedented opportunities to unravel the molecular and biochemical mechanisms of mFA accumulation, and provide new genes to engineer these mFAs in seed oils of transgenic crops.

Genomics studies in natural mFA-producing species will likely provide valuable insights on why these fatty acids are accumulated at high levels in seeds. Although genome sequences of such species are now only available in castor (Chan et al., 2010), global analyses of transcriptomes by ultra-deep sequencing are providing increasingly powerful

approaches to uncover biochemical functions and regulatory networks in TAG biosynthesis. By the 454 analysis of seed transriptomes of the bitter melon (*Momordica charantia*) that accumulates conjugated fatty acids, several candidate genes were identified for eleostearic acid metabolism such as DGAT1 and 2, and a PDAT1-related enzymes. Transcripts were also identified for a novel *FAD2* variant gene encoding a functional Δ12 oleic acid desaturase with potential implications for eleostearic acid biosynthesis (Yang et al., 2010). Another comprehensive RNA-Seq transcriptomic analysis was performed in castor using mRNA isolated from developing seeds and non-oleaginous tissues in order to identify differences in lipid-metabolic pathways and enzyme isoforms which could be important in the biosynthesis of TAG enriched in ricinoleic acid (Brown et al., 2012). This study identified several candidates, including the castor PDCT discussed above (Hu et al., 2012), that might improve the level of ricinoleic acid in transgenic plants.

As we discussed above, oilseeds that accumulate unusual mFAs share most of the major pathways illustrated in Fig. 1. It is therefore envisioned that transcriptional and post-translational regulations may also result in improved mFA metabolism. In this regard, a recent study of comparative transcriptomes between mFA- and non-mFA-accumulators yielded some valuable insights (Troncoso-Ponce et al., 2011). A large number of expressed sequence tag (EST) data sets was obtained from four stages of developing seeds of *Ricinus communis, Brassica napus, Euonymus alatus* and *Tropaeolum majus*, which differ in their storage tissue for oil and in the structure and content of their TAGs. This analysis revealed that while genes encoding core enzymes for fatty acid synthesis in plastids maintained a conserved stoichiometry and a strong correlation in temporal profiles throughout seed development, enzymes of TAG biosynthesis on ER displayed dissimilar temporal patterns indicative of different regulation. The EST levels for several genes potentially involved in mFA accumulation were also distinct. For example, much higher ESTs were found for orthologs of LPCAT, PLC, and PDAT-like/PDAT2 in castor than *B. napus*, suggesting that these are possible candidates associated with high accumulation of ricinoleate in TAG and its exclusion from membrane lipids in castor. The expression of *FAH* in castor is several fold higher than *FAD2* expression in other oilseeds, suggesting high *FAH* expression may be required to achieve the very high (>90%) ricinoleic acid content of castor oil (Troncoso-Ponce et al., 2011). Future studies of these candidates in transgenic experiments, and upregulation of FAH (e.g., promoter analysis and testing) may reveal their roles in hydroxy fatty acid accumulation.

Production of mFA in an industrial oilseed crop–*Camelina*

It is clear that the next generation of successful engineering of industrial fatty acids in oilseeds will require the incorporation of genetic resources from other native accumulator species into a host plant that is agronomically favorable. Choosing a broadly adaptable, non-food crop is essential to meet future demands of industrial oils. A successful industrial oilseed plant will most likely need to be grown across the region on many acres to meet demands for supply. More importantly, a good transformation method will need to be available in order to utilize the genetic resources for creation of these designer oils. Eliminating the food vs. fuel debate by choosing a non-food crop would allow more seed to be processed for oil and prevent the undesirable contamination of food lines with transgenic ones. To this end we propose *Camelina sativa* as an industrial oilseed platform for the engineering of plant seed oil for biofuels and biomaterials.

Camelina has many favorable agronomic attributes that make it desirable as an industrial platform for engineering mFA oil production (Zubr, 1997; Gehringer et al., 2006). *Camelina* contains 30%–40% oil in its seeds, which is comparable with widely grown oilseed canola (*Brassica napus*) and is much higher than the roughly 20% seen in soybean. *Camelina* is more cold and drought tolerant than some oilseeds, and is grown up into regions of Canada as well as across the US in states such as Montana (Pilgeram et al., 2007). *Camelina* has been proposed as a rotational crop to plant in place of a fallow year due to its low water and fertilizer requirements, short growing season (85–100 days), and few known specific diseases or pests (Moser, 2010). The potential of growing on ground that is less favorable for other oilseeds such as soybean, and incorporation of *Camelina* as a rotational crop would limit interference of food crop production. Despite the long history of cultivation, *Camelina* remains an under-developed crop. It is anticipated that rapid improvement can be made by traditional breeding and modern technologies. The short life cycle and ability to self-pollinate allow for multiple generations of plants to be grown per year which is advantageous both for development of breeding lines and for farmers with long or short growing seasons. The creation of adapted local varieties specific to the area's growing environment would also likely improve crop yields.

In addition to the advantageous agronomic features, its ease of transformation puts *Camelina* above other oilseed candidates for a biotechnology platform (Collins-Silva et al., 2011). Similar to *Arabidopsis*, *Camelina* can be transformed with a simple floral dip procedure under vacuum pressure which utilizes *Agrobacterium tumefaciens* for gene insertion (Lu and Kang, 2008), effectively eliminating cumbersome *in vitro* regeneration steps where risks such as deleterious somaclonal variation can occur (Visarada et al., 2009). The floral dip procedure allows many transgenics to be produced in a short period of time (six weeks) compared to many months in some species to regenerate plants via tissue culture. Markers such as DsRed allow for easy, quick, visual selection of transformed seeds (Stuitje et al., 2003; Lu and Kang,

2008). Although the fatty acid profile of *Camelina* oil does not meet any specific uses currently, it would be able to be modified through use of mutant and transgenic lines (Lu, 2008; Kang et al., 2011). *Camelina*'s close relation to *Arabidopsis* (Beilstein et al., 2006) also enables researchers to utilize *Arabidopsis* gene sequences to search for homologous genes, thus circumventing some of the problems with *Camelina* not yet being fully sequenced.

As we move into the next generation of engineering plant seed oils for biofuels and biomaterials, a good plant to focus our efforts on will be a necessity. In light of the reasons discussed above, the benefits of adopting *Camelina* as an industrial oilseed platform are numerous. Although each oilseed has its benefits, the ease of transformation of *Camelina*, its status as a non-food crop, room for improvement, and adaptability help place it above the others.

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