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RESEARCH REVIEW

Toward sustainable production of value-added bioenergy and industrial oils in oilseed and biomass feedstocks

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Abstract

Plant fatty acids are used for food, feed, fuel, and industrial materials. Structurally and chemically diverse fatty acids, referred to as unusual or specialized fatty acids, are found in the seed oils of diverse plant species. Many unusual fatty acids have potential use as alternative and renewable sources of biofuels and bio-based industrial feedstocks due to their variant structures' physical or functional properties. Oils enriched in these fatty acids can increase the value of oilseed crops and provide co-products that can be readily extracted from lignocellulosic materials in biomass crops. Here, we describe recent progress in strategies for enhancement of oil production and quality in oilseed crops and the extension of this knowledge for metabolic engineering of biomass crops. Successful implementation of these strategies, in part, through the use of emerging synthetic biology tools, will provide added product value for the economic sustainability of biomass crops as bioenergy feedstocks.

KEYWORDS

hydroxy fatty acid, medium-chain fatty acid, metabolic engineering, sustainable oil production, unusual fatty acid

1 | INTRODUCTION

Vegetable oils extracted from plant seeds have been widely used for food, feed, and industrial materials. This collection of molecules is composed principally of triacylglycerols (TAGs) in seeds or mesocarps of most oil crops, including soybean and oil palm. In seeds, TAGs function as storage molecules for photosynthetically derived fatty acids linked to each of the three carbon atoms of a glycerol backbone. The stored carbon provides energy

and carbon skeletons to support seed germination and seedling establishment. Plant fatty acids are often classified into “common” and “unusual” fatty acids depending on their frequency of occurrence in the plant kingdom (Cahoon & Li-Beisson, 2020). Common fatty acids, including palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1 Δ^9), linoleic acid (18:2 $\Delta^{9,12}$), and α -linolenic acid (18:3 $\Delta^{9,12,15}$), are ubiquitous in plant tissues as membrane and storage lipids. In contrast, unusual fatty acids have limited occurrence in plants, often as major

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components of seed oils of selected species or families. Unusual fatty acids also have divergent chemical structures compared with common fatty acids. Structural variations in unusual fatty acids can include diversity in carbon chain lengths (shorter than 16 or longer than 18), unsaturation (different positions/orientation of double and triple bonds), and functional groups (e.g., hydroxyl, epoxy, and cyclopropane groups). While unusual fatty acids are typically found as major components of seed oils, they can also be found in other plant organs, tissues, and cells, including trichomes and roots (Busta et al., 2018; Ohlrogge et al., 2018; Segura Munoz et al., 2020). Because of the physical and functional properties conferred by their structures, many unusual fatty acids have high value for applications such as dietary nutraceuticals and feedstocks for biofuels, cosmetics, and industrial chemicals (lubricants, emulsifiers, and detergents; Dyer et al., 2008). As a result, intensive research has been conducted to identify sources of unusual fatty acids, elucidate their specialized biosynthetic and oil storage pathways for basic and applied knowledge of variant lipid metabolism.

Attempts to develop plants that naturally produce oils with high levels of unusual fatty acids as agronomic crop plants have had only limited success because these plants often have unfavorable agricultural traits that can include toxic by-products, seed dormancy, seed shatter, tropical adaptation, indeterminate flowering, and unsuitable morphologies (Jaworski & Cahoon, 2003). Plant biotechnological efforts have been directed at transferring these pathways to established oilseed crops to address these limitations. More recently, research has also been directed at transferring unusual fatty acid metabolism to biomass feedstocks, such as sorghum and energy cane, to create a high-value co-product that can be easily separated from lignocellulosic material (Reynolds et al., 2017; Vanhercke et al., 2019; Yurchenko et al., 2017). Here, we review recent studies of unusual fatty acid biosynthetic and metabolic enzymes and their use in metabolic engineering to enhance the value of oilseed crops. We also discuss the possible use of vegetative organs as platforms for the production of vegetable oils and specialty oils to promote the economic viability of biomass crops as renewable bioenergy feedstocks. While the focus of this review is on land plant-based production of oils enriched in unusual fatty acids, the research described can also be applied toward metabolic engineering of the biosynthesis of these oils in microbial and algal production platforms. The economic potential of these platforms may be comparable or greater than that of seed or plant vegetative tissue production of unusual fatty acid-enriched oils for high-value, low-volume markets.

2 | OVERVIEW OF THE UNUSUAL FATTY ACID AND VEGETABLE OIL PRODUCTION IN OILSEED AND BIOMASS CROPS

2.1 | Unusual fatty acid biosynthesis

The fatty acid biosynthetic and modification reactions that generate unusual fatty acids are distributed between plastids and the endoplasmic reticulum (ER; Figure 1). These reactions are catalyzed by functionally and structurally divergent forms of enzymes found widely in the plant kingdom. These variant enzymes can be classified largely into four categories: (1) FatB acyl-acyl carrier protein thioesterase variants that generate medium-chain fatty acid (MCFA; C8–C14) by premature termination of *de novo* fatty acid biosynthesis prior to the C16 and C18 stages of carbon chain elongation; (2) fatty acid desaturase variants that can introduce double bonds at atypical positions or with *trans* stereochemistry, catalyze alternative oxygenation outcomes (e.g., hydroxylation, epoxygenation), or introduce triple bonds; (3) β -ketoacyl-CoA synthase- or FAE1 variants that extend fatty acid carbon chains beyond C18 to lengths of up to C28; and (4) cytochrome P450s that catalyze oxygenation reactions that produce carbon chain modifications including hydroxyl and epoxy groups. While these are the primary enzyme types associated with unusual fatty acid biosynthesis, other enzymes catalyze the biosynthesis of selected unusual fatty acids, such as an S-adenosylmethionine methyltransferase variant that produces cyclopropane fatty acids (Bao et al., 2002). Collectively, these enzymes confer differences in carbon chain lengths and carbon chain modifications (e.g., hydroxylation; Figure 2). Unusual fatty acid biosynthesis may use fatty acyl-ACP substrates from *de novo* fatty acid biosynthesis, as is the case for FatB and $\Delta 9$ -stearoyl-ACP desaturase variants (Cahoon & Schmid, 2008). Other unusual fatty acid biosynthetic reactions use acyl-CoA or fatty acids linked to lipids, particularly phosphatidylcholine (PC), in the ER following fatty acid export from plastids. In addition, the biosynthesis of possibly hundreds of other unusual fatty acids remains to be elucidated, including those with keto groups and variations in unsaturation and hydroxylation (Badami & Patil, 1980; Ohlrogge et al., 2018). Identification of the genes for the biosynthetic and associated metabolic enzymes for these fatty acids offers additional opportunities to generate enhanced biofuel and other bioproduct functionalities.

2.2 | Triacylglycerol biosynthesis

Seeds that accumulate high levels of unusual fatty acids sequester these molecules following their biosynthesis

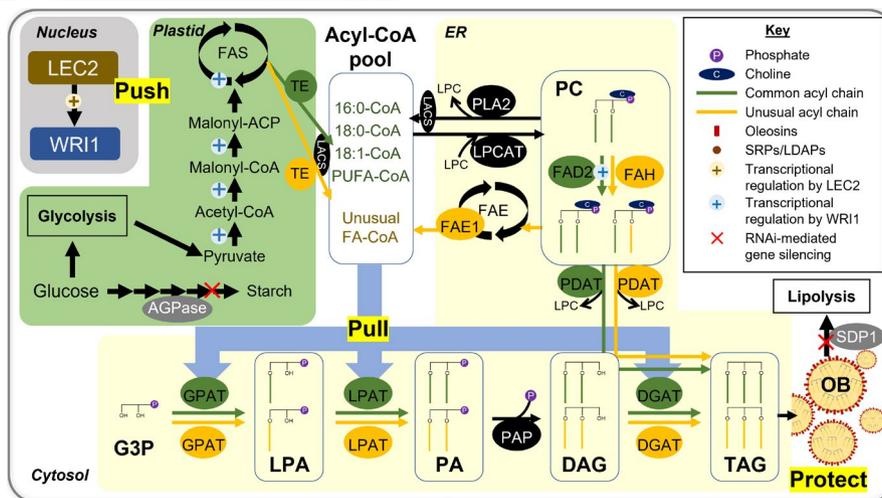


FIGURE 1 Overview of metabolic engineering strategies for the production of oils containing common or unusual fatty acids. Triacylglycerol (TAG) is synthesized by interconnected metabolism in two cellular compartments: plastid and endoplasmic reticulum (ER). TAG production and accumulation is mediated by sequential flux from fatty acid biosynthesis, TAG sequestration, and oil body formation and maintenance by “push–pull–protect” metabolic modules as noted in the figure. Orange and green lines/ovals represent metabolic flux and enzymes for unusual and common fatty acids, respectively. Transcriptional regulation by WRINKLED1 (WRI1) and LEAFY COTYLEDON2 (LEC2) is shown, as representative of the function of these and additional transcription factors (e.g., LEC1). ACP, acyl carrier protein; AGPase, ADP-glucose pyrophosphorylase; CoA, coenzyme A; DAG, diacylglycerol; DGAT, DAG acyltransferase; FAE, fatty acid elongase; FAD2, fatty acid desaturase2; FAH, fatty acid hydroxylase; FAS, fatty acid synthase; G3P, glycerol-3-phosphate; GPAT, G3P acyltransferase; LPA, lysophosphatidic acid; LPAT, LPA acyltransferase; LPC, lysoPC; LPCAT, LPC acyltransferase; OB, oil body; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PDAT, phospholipid:DAG acyltransferase; PLA2, phospholipase A2; SDP1, sugar-dependent lipase1; TE, thioesterase. Fatty acid nomenclature: $X:Y$, X = numbers of carbon atoms in fatty acid chain; Y = numbers of double bonds in fatty acid chain

in an inert storage form as TAGs (Cahoon & Li-Beisson, 2020). TAGs are comprised of three fatty acids linked to a glycerol backbone and are formed by numerous acyltransferases that incorporate fatty acids onto glycerol using primarily acyl-CoAs as substrates. In the case of seeds that accumulate very high levels of unusual fatty acids, specific acyltransferases have also evolved alternative substrate specificities to accommodate unusual fatty acid accumulation at all three stereospecific positions of glycerol. These include lysophosphatidic acid acyltransferases (LPATs) that catalyze the incorporation of fatty acids at the *sn*-2 position of glycerol. LPATs found in seeds of most plants have strict substrate specificity for $\Delta 9$ double bond-containing mono- or poly-unsaturated fatty acids (e.g., oleic, linoleic, and α -linolenic acids) and have little activity with a saturated fatty acid or hydroxylated fatty acid substrates (Cahoon & Schmid, 2008). In the case of seeds from species such as those from the *Cuphea* genus that produce oils with $\geq 90\%$ of saturated C8 and C10 fatty acids or castor bean that accumulates $>90\%$ of the $\Delta 12$ -hydroxylated ricinoleic acid, structurally and functionally variant LPATs have been identified that enable accumulation of these unusual fatty acids at the TAG *sn*-2 position (Kim, Silva, Iskandarov, et al., 2015; Lunn et al., 2019). Diacylglycerol acyltransferases (DGATs) of the DGAT1

and DGAT2 classes, which catalyze the introduction of the third fatty acid onto glycerol-containing DAG, have also evolved variant specificities in selected species to promote the efficient accumulation of unusual fatty acids in TAGs (Burgal et al., 2008; Iskandarov et al., 2017; Li et al., 2010). The evolution of other acyltransferases with altered substrate specificities, including phosphatidylcholine:diacylglycerol acyltransferase1, has also been implicated in the production of TAGs that are highly enriched in unusual fatty acids (Lunn et al., 2019). These downstream metabolic enzymes, along with variant biosynthetic enzymes, have proved to be vital components of successful engineering strategies for oilseeds (e.g., Lunn et al., 2019) and the eventual extension of these strategies to biomass crops.

After assembly, TAGs with unusual fatty acids are packaged into oil bodies, storage organelles that arise from the ER (Chapman et al., 2012; Olzmann & Carvalho, 2019). TAGs are deposited between the leaflets of the ER bilayer. The lens-shaped structures are enlarged by accumulation of TAGs and subsequently bud off from the ER to form oil bodies. After budding from the ER, nascent oil bodies grow and expand by fusion and local TAG synthesis. Several studies reported that oil body-associated proteins such as oleosin, SEIPIN, CGI-58, and SRPs/LDAPs play

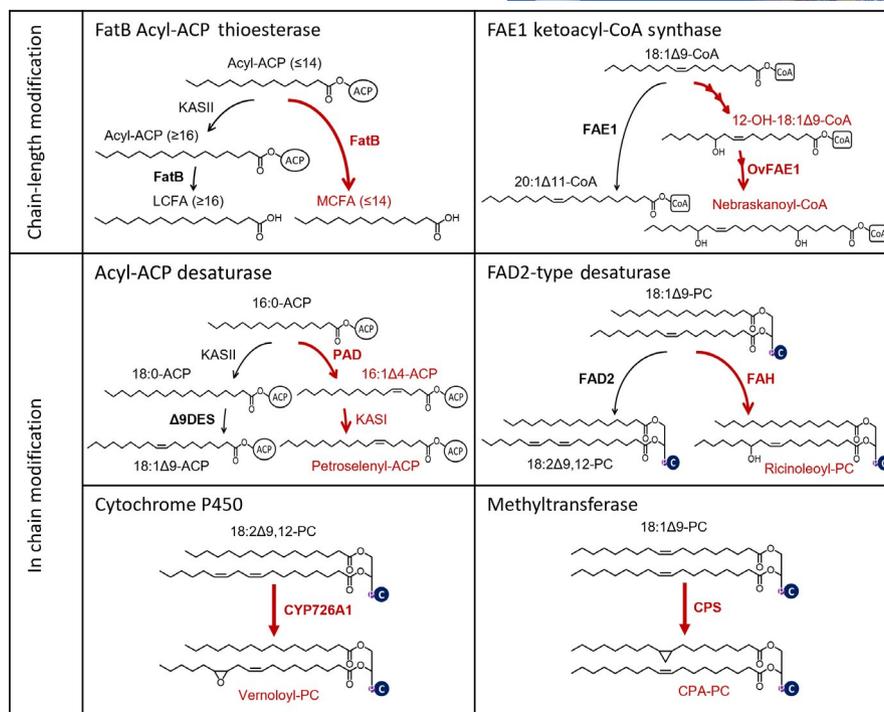


FIGURE 2 Major classes of specialized enzymes for unusual fatty acid biosynthesis. As shown, these enzymes are typically associated with variant fatty acid chain lengths and/or modifications of the carbon chain (e.g., hydroxylation). These enzymes are typically divergent forms of widely occurring acid fatty modification enzymes (shown in black arrows). Modifications of primary structures of these enzymes results in specialized activities (shown in red arrows) that are associated with altered catalytic outcomes. ACP, acyl carrier protein; CoA, coenzyme A; PAD, palmitoyl-ACP desaturase; CPA, cyclopropane fatty acid; CPS, CPA synthase; DES, desaturase; FAD2, fatty acid desaturase 2; FAE1, fatty acid elongase 1; FAH, fatty acid hydroxylase; FatB, fatty acid thioesterase B; KASI, 3-keto-acyl-ACP synthase I; KASII, 3-keto-acyl-ACP synthase II; LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; PC, phosphatidylcholine

key roles in oil body biogenesis (Cai et al., 2015; Frandsen et al., 2001; Gidda et al., 2016; James et al., 2010; Kim et al., 2016; Shimada et al., 2018; Siloto et al., 2006). *SEIPIN* and *CGI-58* were originally reported as defective genes in the human lipid storage diseases and studies of plant homologs suggested conserved function of the genes for cellular lipid homeostasis in eukaryotes (Cai et al., 2015; James et al., 2010). Oleosin and SRPs/LDAPs are plant-specific oil body components and major oil body proteins in seeds and leaves, respectively. Their molecular function is yet fully characterized but they are regarded as determinators for the attachment of other proteins to oil bodies (Huang, 2018; Pyc et al., 2017). TAGs are highly accumulated during seed maturation, a process that is regulated by a network of transcription factors including *WRINKLED1* (*WRI1*) and *LEAFY COTYLEDON2* (*LEC2*) (Focks & Benning, 1998; Stone et al., 2001). *LEC2* is one of four master regulators for seed maturation along with *LEAFY COTYLEDON1* (*LEC1*), *ABSCISIC ACID INSENSITIVE3* (*ABI3*), and *FUSCA3* (*FUS3*; Giraudat et al., 1992; Keith et al., 1994; West et al., 1994). *LEC1* has the highest hierarchy in the signaling and regulates downstream plant-specific *ABI3*, *FUS3*, and *LEC2* (collectively *AFL*) networks. *AFL* regulators control the synthesis of storage molecules during

seed maturation by activating secondary transcription factors such as *WRI1* (Kong et al., 2020; Kong, Yang, Low, et al., 2020). *WRI1* is a tissue-specific positive regulator of fatty acid synthesis. Not only transcriptional regulation, but *WRI1* is also post-translationally regulated by phosphorylation and ubiquitination as well as by *WRI1*-interacting proteins such as 14-3-3 and mediator complex *MED15* subunit (Kim et al., 2016; Kong, Yang, Guo, et al., 2020; Ma et al., 2016; Zhai et al., 2017). *WRI1* has not only proven useful for oil enhancement in oilseed crops but is also widely used to enhance carbon flux for fatty acid biosynthetic flux in metabolic engineering of vegetative tissues (Van Erp et al., 2014; Xu & Shanklin, 2016).

2.3 | Vegetative oil production

While plant oils as fuel have several advantages over other fuels, the current supply of these energy-rich compounds is constrained by oilseed crop yields, limited availability of arable land, and the conflict arising from the need to use arable land for food versus biofuel production (Durrett et al., 2008; Dyer et al., 2008; Ohlrogge & Chapman, 2011). Given these limitations for oilseed

production and the growing global demand for vegetable oils, vegetative organs (e.g., leaves, stems, and roots) of biomass crops offer an attractive system for expansion of vegetable oil production and to overlay unusual fatty acid biosynthetic pathways to increase the utility and value of oils. The environmental resilience of biomass crops such as sorghum, switchgrass, and miscanthus allows for vegetable oil production in climates that are not well suited to cultivate conventional oilseeds (Ohlrogge & Chapman, 2011). While all plant cells have the capacity to produce TAGs, this is typically a temporary storage repository to presumably reduce the lipotoxicity of free fatty acids that may arise from stress-induced membrane damage (Xu & Shanklin, 2016). The introduction of three key steps for oil biosynthesis confers seed-specific pathways for TAG production and storage to vegetative organs of biomass crops such as sugar cane and sorghum (Napier et al., 2014; Vanhercke et al., 2014; Zale et al., 2016). These three steps, referred to as “push–pull–protect” or “3P,” include: (1) “Push” or enhance glycolytic and fatty acid biosynthetic flux by use, transcription factors, such as WRI1, or by redirection of carbon from competing pathways such as starch biosynthesis (Sanjaya et al., 2011; Vanhercke et al., 2014); (2) “Pull” or enhance the sequestration of fatty acids into TAGs by enhanced catalysis by enzymes such as DGAT (Vanhercke et al., 2014); and (3) “Protect” or mitigate the catabolism of TAGs by lipases and subsequent fatty acid β -oxidation by downregulation of these metabolic processes and by the introduction of oil body coat proteins, such as oleosins, seipins, and SRPs/LDAPs (Cai et al., 2015; Eastmond, 2006; Gidda et al., 2016; Kim, Park, et al., 2016). This “3P” strategy has been widely adopted to enhance the accumulation of oil and oil with unusual fatty acids in seeds and vegetative organs (Napier et al., 2014; Song et al., 2017; Vanhercke et al., 2014). To reduce yield penalties, the implementation of this strategy has to balance the needs of photosynthetic carbon and redox state to support growth and carbon stored in vegetative organs (Horn, 2021). The challenges of combining the 3P strategy with the introduction of unusual fatty acid biosynthetic and metabolic pathways are described below.

3 | RECENT EXAMPLES OF UNUSUAL FATTY ACID METABOLIC ENGINEERING IN OILSEEDS

Extensive efforts have been directed toward discovering genes for biosynthetic and specialized metabolic enzymes for metabolic engineering of unusual fatty acid-rich oil production in oilseed models (e.g., *Arabidopsis*) and established crops (e.g., rapeseed, soybean, and camelina).

Technical advances in transcriptomics and genomics have accelerated the identification of novel enzymes for these metabolic pathways (Kim, Silva, Vu, et al., 2015; Li et al., 2018; Nguyen et al., 2013). Examples are provided below of efforts directed at transferring pathways from seeds of plants with the limited agronomic potential to produce medium-chain and hydroxy fatty acids. These examples illustrate the necessity of multigene engineering to produce unusual fatty acids and efficiently sequester the fatty acid onto the three stereospecific carbon atoms of the TAG glycerol backbone. The example of hydroxy fatty acid production shows that the efficient assembly of unusual fatty acids for TAG formation maintains a high rate of total oil production. The strategies described for oilseeds will also guide efforts to produce unusual fatty acids in vegetative organs of biomass crops.

3.1 | Medium-chain fatty acids

Medium-chain fatty acids are composed of 8–14 carbon atoms rather than the C16 and C18 fatty acids typically found in seed oils. These fatty acids are important substrates for various industrial applications such as cosmetics, detergents, emulsifiers, and soaps. (Dyer et al., 2008; Knaut & Richtler, 1985). Studies of MCFAs production in living organisms have received more commercial attention as renewable sources for the hydrocarbon component of transportation fuels (e.g., Jet A fuel: C8–C16; gasoline: C4–C12; diesel: C10–15; Kallio et al., 2014; Knothe et al., 2009). Palm kernel (*Elaeis guineensis* Jacq.) and coconut (*Cocos nucifera* L.) oils have been used as major sources of plant-derived MCFAs. Other plant-derived sources of MCFAs are seeds from *Cuphea* species native to temperate regions (Graham, 1989; Graham et al., 2016). High levels of MCFAs are accumulated in seeds of *Cuphea* species. For example, *Cuphea palustris* seeds contain ~64 mol% myristic acid (C14:0) and ~20 mol% caprylic acid (C8:0), *Cuphea pulcherrima* seeds contain ~95 mol% C8:0, and *Cuphea viscosissima* seeds contain ~64 mol% C8:0 and ~25 mol% C10:0. *Cuphea* species have been the subject of genetic improvement for production as an oilseed crop with limited success (Berti & Johnson, 2008). Given its genetic diversity for variations in MCFAs content and composition, the *Cuphea* genus has been genetically “mined” to identify genes for specialized enzymes controlling MCFAs biosynthetic and metabolic pathways. Several studies have revealed that variant FatBs from *Cuphea* species have a catalytic activity to release fatty acids from ACP during de novo fatty acid synthesis in plastids to generate fatty acids such as C8:0, C10:0, C12:0, and C14:0 (Dehesh et al., 1996; Jones et al., 1995; Kim, Silva, Iskandarov, et al., 2015; Kim, Silva, Vu, et al., 2015). Co-expression studies using

combinations of variant FatBs showed that transgenic *Camelina* could synthesize oil with mixtures of MCFAs, suggesting that the application of tailored oil production for biofuels is feasible. Notably, MCFA-containing oil production increases in *Camelina* seeds when MCFA-specific acyltransferases are co-expressed (Iskandarov et al., 2017; Kim, Silva, Iskandarov, et al., 2015; Kim, Silva, Vu, et al., 2015). The results indicate that the substrate specificity of acyltransferases is one of the key determinants for the accumulation of unusual fatty acids. Moreover, introducing MCFA-specific LPAT and diacylglycerol acyltransferase 1 (DGAT1) from *Cuphea* species in combination with MCFA-specific FatB into oilseed crops provided direct evidence for metabolic cooperation of these two acyltransferases in channeling MCFAs into TAGs enriched in fatty acids such as 10:0 at each of its three stereospecific positions (Iskandarov et al., 2017). Notably, these studies showed that the transgenic co-expression of a specialized LPAT and DGAT1 from *Cuphea* species in camelina seeds resulted in C10:0 accumulation in TAGs and excluded this fatty acid from membrane phospholipids, the desired outcome for agronomic fitness of engineered seeds and other plant organs.

3.2 | Hydroxy fatty acids

Hydroxy fatty acids increase TAG functionality for lubricant applications (Durrett et al., 2008; Dyer et al., 2008). The main commercial source of hydroxy fatty acids is the seed oil of castor (*Ricinus communis*), which contains ~90 mol% ricinoleic acid (C18:1-OH). Ricinoleic acid contains Δ^9 unsaturation and a hydroxyl group at its C-12 position. Commercial production of castor is limited due to the toxic protein ricin found in its seeds (Patel et al., 2016; Severino et al., 2012). Seeds from certain *Physaria* and *Paysonia* species of the Brassicaceae family also synthesize and accumulate diverse mono-hydroxy fatty acids (e.g., C18:1-OH, C18:2-OH, C20:1-OH, and C20:2-OH; Chen et al., 2011; Dierig et al., 2011; Hayes et al., 1995; Mikolajczak et al., 1962). Hydroxylation of fatty acids is mediated by ER-localized variant form of the Δ^{12} oleoyl-PC desaturase encoded by *FAD2* (Vandeloos et al., 1995). A structure–function study between the hydroxylase variant FAD2 (FAH) and a typical FAD2 showed that swapping as few as six amino acid residues is sufficient to switch enzyme activities between desaturation to hydroxylation (Broun et al., 1998). Introducing FAH cDNA into model plants exhibited low total oil content, and impaired germination and seedling establishment were observed in transgenic plants (Adhikari et al., 2016; Bates et al., 2014). Although this mechanism is not fully understood, there is a strong correlation between the

amount of hydroxy-containing PCs and downregulated fatty acid synthesis/reduced oil content. Given that the amount of hydroxy-PC does not exceed 4% of total PC in castor seeds (Thomaeus et al., 2001), a radioisotope labeling study suggested that insufficient accumulation of hydroxy fatty acid in TAGs is caused by the inefficient conversion of hydroxy fatty acid-containing DAG or PC to TAGs in non-host plants (Bates & Browse, 2011). Recent studies showed that co-expression of FAH12 with three specialized acyltransferases—GPAT, LPAT, and PDAT—from castor targeting incorporation of hydroxy fatty acid into TAGs with each stereochemical position (*sn*-1, *sn*-2, and *sn*-3, respectively) successfully rescued both low oil content and impaired seedling establishment phenotype of transgenic plants which FAH12 is introduced individually (Lunn et al., 2019). Moreover, it was notable that increasing the capacity of unusual fatty acid incorporation into TAGs by co-expression of specialized acyltransferases also affects the general utilization of TAGs. Similar strategies of combining biosynthetic enzymes for unusual fatty acid biosynthesis with specialized fatty acid metabolic enzymes (e.g., acyltransferases) has also been applied to the engineering of other unusual fatty acids, including epoxy and cyclopropane fatty acids (Li, Yu, Hatanaka, et al., 2010; Li et al., 2010; Yu et al., 2018, 2019).

Research into the engineering of ricinoleic acid biosynthesis has also uncovered the effects of inefficient metabolism of unusual fatty acids on the regulation of de novo fatty acid biosynthetic flux. In this regard, Arabidopsis seeds engineered for ricinoleic acid production have $\leq 50\%$ reduction in total oil content (Bates et al., 2014). Reductions in oil content were rescued mainly by approaches to overcome fatty acid flux regulation or to increase the efficiency of hydroxy fatty acid incorporation into TAGs (Adhikari et al., 2016; Lunn et al., 2019; Yu et al., 2021).

In addition to ricinoleic acid and its elongated forms, a seed oil was recently reported containing two C24 fatty acids with hydroxyl groups at their C-7 and C-18 positions. Fatty acid hydroxylation is also catalyzed during “discontinuous” elongation steps in the ER (Li et al., 2018). Thin-layer chromatography analysis has shown that the seed oil of Chinese violet cress (*Orychophragmus violaceus*) contains very long-chain dihydroxy fatty acids nebraskanic [7,18-(OH)2-24:1 Δ^{15}] and wuhanic [7,18-(OH)2-24:2 $\Delta^{15,21}$] acids. In these two novel dihydroxy fatty acids, hydroxylation in the C-18 position is mediated by variant FAD2 type hydroxylase (OvFAD2-2) as a similar homolog from previously reported FAHs. In contrast, hydroxylation in the C-7 position is catalyzed by a divergent 3-ketoacyl-CoA synthase (OvFAE1-1) that uses a 3-OH intermediate at the C20 stage of fatty acid elongation (Li et al., 2018). This process, which bypasses the subsequent stages of typical fatty acid elongation,

is referred to as “discontinuous” elongation. Oil from Chinese violet cress showed an exceptional lubricating property compared with conventional castor oil, which arises from the natural accumulation of TAG estolides rather than typical TAG species (Li et al., 2018; Romsdahl et al., 2019). TAG estolides are high molecular TAG species produced by polymerizing fatty acids through ester linkages between hydroxy fatty acids. In Chinese violet cress oil, identified TAG estolides can contain 132 FA carbon atoms, 10 total double bonds, and eight substituted or free hydroxyl groups (Romsdahl et al., 2019). Understanding more about the biosynthetic pathway of TAG estolides in Chinese violet cress could provide new insights into the diversity of plant metabolic pathways.

Overall, the development of engineered crops producing unusual fatty acid-enriched oils is highly dependent on understanding the metabolic bottleneck of unusual fatty acids in the target tissue and/or plants may give insights for enhanced production of beneficial vegetative oil without abnormal growth phenotype. These efforts will likely be advanced by functional genomics efforts that link biochemical characterizations with emerging genomic information, such as that from the recent elucidation of genome of jojoba (*Simmondsia chinensis*) that accumulates wax esters, variant fatty acid storage forms, in its seeds (Sturtevant et al., 2020).

4 | TOWARD PRODUCTION OF UNUSUAL FATTY ACIDS IN BIOMASS CROPS

Although seeds are the predominant oil storage organ in plants, there are several exceptions, such as oil accumulation in mesocarp cells of avocado, olive, and palm (Dabbou et al., 2011; Horn et al., 2013; Tranbarger et al., 2011). The capacity of non-seed tissues such as fruit mesocarps for TAG production and storage points to the feasibility of engineering oil production in vegetative tissues. Building on this, current research has focused mainly on the 3P strategy to elevate oil accumulation in leaves and stems (Parajuli et al., 2020; Vanhercke et al., 2019; Zale et al., 2016). By contrast, only limited research has explored the production of unusual fatty acids in vegetative organs, and research to date has largely been limited to model systems such as *Arabidopsis* and *Nicotiana benthamiana* (Okada et al., 2020; Reynolds et al., 2015; Yurchenko et al., 2017). Early studies on the production of unusual fatty acids in vegetative organs included research into the constitutive expression of divergent FAD2 hydroxylases in *N. tabacum* and *Arabidopsis* (Broun et al., 1998; Vandeloos et al., 1995). In these studies, production of the hydroxylated fatty acid ricinoleic acid was observed in seeds but

not in leaves or roots. In the case of the expression of the lesquerella FAD2-type hydroxylase in *Arabidopsis*, the transcript of this gene from CaM35SV-mediated expression was detectable in roots. Still, amounts of the corresponding protein were low or below detection, although hydroxylase activity was measurable in root microsomes (Broun, Boddupalli, et al., 1998). These findings suggested that post-transcriptional regulation limits hydroxylase levels and/or catabolic reactions prevent hydroxy fatty acid accumulation in roots (Broun, Boddupalli, et al., 1998). More recently, transgenic and transient expression results showed that combinatorial expression of unusual fatty acid biosynthetic enzymes successfully allows the accumulation of unusual fatty acid-containing oil in plant leaves. The 3P strategy was employed with specialized enzymes to engineer unusual fatty acid-containing oil production in vegetative tissues because the accumulation of free unusual fatty acids or unusual fatty acids in membrane lipids is toxic to cellular tissues (Iskandarov et al., 2017; Kim, Silva, Iskandarov, et al., 2015; Kim, Silva, Vu, et al., 2015; Lunn et al., 2019; Reynolds et al., 2015; Yurchenko et al., 2017). For example, impaired growth phenotypes of eleostearic acid (18:3- $\Delta^{9,11,13}$)-producing transgenic *Arabidopsis* such as yellow leaves and low conductivity were partially restored by co-expression of DGAT2 (Yurchenko et al., 2017). In a transient expression assay, co-expression of an MCFA-specific LPAT led to the formation of tri-MCFA TAG species, suggesting that the 3P strategy is helpful in vegetative tissues for accumulation of unusual fatty acids while avoiding undesired plant growth defects (Reynolds et al., 2015).

However, there are still possibilities that the metabolic context of vegetative oil production could differ from that in seeds. Thus, understanding the oil accumulation process in vegetative tissues and developing the 3P strategy are required to optimize unusual fatty acid production in high biomass-yielding crops, without losing their environmental resilience and high biomass. Leaf oil accumulation by blocking fatty acid breakdown and/or lipid trafficking to chloroplast causes the developmental defect of plants by altering photosynthetic capacity, phytohormone homeostasis, and stress responses (Kunz et al., 2009; Li et al., 2012; Yurchenko et al., 2017). Since constitutive expression of transgenes affects entire developmental stages of plants, application of inducible or stage-/tissue-specific promoter is one potential strategy for unusual fatty acid accumulation in vegetative tissues (Kim, Lee, et al., 2015). In addition, TAGs are naturally synthesized in vegetative tissues, but this process is transitory (Ischebeck et al., 2020). One possibility is that TAG synthesis is increased by the release of free fatty acids from damaged membranes. Sequestration of the released fatty acids in an inert form in TAGs possibly mitigates fatty acid-linked lipotoxicity

(Listenberger et al., 2003; Zhang et al., 2003). The 3P strategy is directed, in part, to overcome the natural transitory occurrence of oil bodies in vegetative tissues. In this regard, major oil body coat proteins in vegetative tissues are SRPs/LDAPs, rather than oleosins (Brocard et al., 2017; Horn et al., 2013; Zhi et al., 2017). The composition of oil body coat proteins determines the molecular and physiological functions of oil bodies (Gidda et al., 2016; Kim, Park, et al., 2016; Kim et al., 2010; Shimada et al., 2018). Exploring signaling and the metabolic context in non-seed tissues through studies from transitory oil bodies and their surrounded protein pools will provide insights into the complex network of carbon flux in plants and strategies for unusual fatty acid production in vegetative tissues that avoid adverse effects on primary metabolism and growth.

5 | EMERGING STRATEGIES FOR METABOLIC ENGINEERING OF UNUSUAL FATTY ACID BIOSYNTHESIS IN OILSEEDS AND BIOMASS CROPS

As described above, extensive research has focused on the reconstitution of unusual fatty acid biosynthesis and increased TAG assembly in oilseed crops and model plants. However, with only a few exceptions (e.g., γ -linolenic acid, omega-7 monounsaturated fatty acids; Clemente et al., 2003; Nguyen et al., 2015; Qin et al., 2012), the accumulation of unusual fatty acids in transgenic oilseeds has yet to reach levels found in seeds of plants that “naturally” produce unusual fatty acids (Broun & Somerville, 1997; Cahoon et al., 2006). Non-desirable plant fitness phenotypes, such as poor seed germination, impaired seedling establishment, and low total seed oil content, have also been reported to accompany the introduction of at least several of these pathways into plants (Cahoon et al., 2006; van Erp et al., 2011; Jaworski & Cahoon, 2003). Optimizing the production of oils rich in unusual fatty acids in engineered oilseed crops will likely require strategies involving the introduction of multiple transgenes that target biosynthetic pathways and downstream metabolic pathways to sequester these fatty acids in TAGs effectively. The efficacy of this strategy has been demonstrated, as described above, for rescuing low seed oil content and reduced seed germination for production of hydroxyl fatty acid-enriched TAGs in Arabidopsis seeds by co-expression of the castor FAH12 with multiple castor specialized acyltransferases (Lunn et al., 2019). Comparisons of hydroxy fatty acid-producing *Physaria fendleri* seeds with those of hydroxy fatty acid-null *Camelina sativa* seeds also pointed to the likelihood that differences in the expression of multiple genes for fatty acid metabolic enzymes that lack specialized function contributed to the evolution of hydroxy

fatty acid TAG production capacity in the Brassicaceae family (Horn et al., 2016). Collectively, these findings suggest that the success of transferring pathways for unusual fatty acid biosynthesis and accumulation to existing oilseeds will require the introduction of numerous transgenes with more rigorous control of the level and developmental timing of their expression.

Additionally, it may be necessary to downregulate native biosynthetic or metabolic pathways in engineered oilseeds and vegetative tissues to mitigate those that compete with or otherwise constrain unusual fatty acid production. These may include, for example, native acyltransferase that lack specificity for a given unusual fatty acid. Implementation of these complex metabolic engineering strategies will necessitate large/multiple DNA assembly methods such as Gibson assembly, integrase, and GoldenBraid systems to reconstitute enzymatic biosynthetic and metabolic pathways through a single transformation of one large vector containing multigene cassettes (Casini et al., 2015; Gibson et al., 2009). More careful selection of promoters to achieve the desired expression strength and timing of transgenes will also likely be needed. Moreover, using strategies such as artificial miRNAs and gene editing (e.g., clustered regularly interspaced short palindromic repeats) will allow selective suppression or knockout of non-desired pathways to tailor the metabolic background in the target oilseed or biomass crop for maximal unusual fatty acid production (Shockey, 2020).

Protein engineering targeted to functionally divergent enzymes associated with unusual fatty acid biosynthesis and accumulation has also contributed to the enhanced production of oils with novel fatty acid compositions. Historically, the most effective approaches have been directed at soluble enzymes such as acyl-ACP desaturases with guidance from crystal structure data to generate novel reaction outcomes that have been subsequently used to generate modified oil compositions in oilseed crops (Cahoon et al., 1997; Cahoon & Shanklin, 2000; Nguyen et al., 2015; Whittle & Shanklin, 2001; Yuan et al., 1995). However, beyond those that catalyze reactions using ACP substrates, most fatty acid modification and metabolic enzymes are membrane-associated and more challenging for structure-based engineering. An abundance of primary structural data from related enzymes of diverse function has enabled domain swapping experiments to identify domains or specific amino acid residues that are critical for divergent functional properties of enzymes such as FAD2- and FAE1-related enzymes (e.g., Blacklock & Jaworski, 2002; Broun, Shanklin, et al., 1998; Rawat et al., 2012). The use of primary sequence diversity coupled with directed evolution using a yeast-based selection system has also been effective for developing DGATs with enhanced activity (Chen et al., 2017;

Hernandez Lozada et al., 2018; Roesler et al., 2016; Siloto et al., 2009; Xu et al., 2017). The utility of these designed enzymes to increase oil content has been demonstrated in transient plant expression systems or oilseeds (Chen et al., 2017; Roesler et al., 2016). Beyond membrane-associated enzymes, directed evolution using error-prone PCR to generate sequence diversity and a microbial selection system was used to understand amino acid residues that mediate acyl-ACP substrate specificities of FatB thioesterases and to develop variants with high specificity for 8:0-ACP that has potential application in plant metabolic engineering research (Hernandez Lozada et al., 2018). Likely, structural design based on emerging sequence data from plants and other organisms with divergent fatty acid and oil compositions will yield a wealth of new information for understanding determinants of enzyme functional outcomes and substrate specificities. This knowledge will guide the development of modified enzymes for advanced metabolic engineering of oil content and compositions in crop plants and ultimately lead to desired oil compositions that are not currently found in nature.

6 | SUMMARY

Bio-prospecting in the plant kingdom has led to the identification and biochemical characterization of specialized enzymes involved in the synthesis and metabolism of unusual fatty acids. These enzymes provide the structural basis for understanding variant substrate specificities, regiospecificities, and catalytic outcomes, as well as tools for engineering novel and high-value oil compositions. This research has also highlighted the need for specialized enzymes, including functionally divergent acyltransferases, in engineered oilseeds. In addition, recent studies have led to new strategies to limit feedback inhibition of fatty acid and TAG biosynthesis caused by unusual fatty acid metabolism in engineered oilseed hosts. Additional challenges remain for engineering biomass crops for unusual fatty acid-enriched TAG production. While vegetative organs have the capacity for TAG production, overcoming the natural transitory accumulation of TAGs in these organs through an emphasis on the “protect” component of the “3P” strategy is an important target. Additional challenges for the production of unusual fatty acid-containing TAGs in vegetative organs of biomass crops is the assembly of specialized metabolic pathways to promote the biosynthesis of these variant TAG molecules and maintain the activity and stability of unusual fatty acid biosynthetic enzymes in a non-seed cellular environment. Overall, identifying differences in metabolic context and signaling networks between seeds and vegetative organs is paramount to the

development of a renewable and sustainable oleochemical industry built around the use of biomass feedstocks.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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