

Current progress towards the metabolic engineering of plant seed oil for hydroxy fatty acids production

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Abstract

Key message Hydroxy fatty acids produced in plant seed oil are important industrial material. This review focuses on the use of metabolic engineering approaches for the production of hydroxy fatty acids in transgenic plants.

Abstract Vegetable oil is not only edible but can also be used for industrial purposes. The industrial demand for vegetable oil will increase with the continued depletion of fossil fuels and ensuing environmental issues such as climate change, caused by increased carbon dioxide in the air. Some plants accumulate high levels of unusual fatty acids in their seeds, and these fatty acids (FAs) have properties that make them suitable for industrial applications. Hydroxy fatty acids (HFAs) are some of the most important of these industrial FAs. Castor oil is the conventional source of HFA. However, due to the presence of toxin ricin in its seeds, castor is not cultivated on a large scale. Lesquerella is another HFA accumulator and is currently being developed as a new crop for a safe source of HFAs. The mechanisms of HFA synthesis and accumulation have been extensively studied using castor genes and the model plant *Arabidopsis*. HFAs accumulated to 17 % in the seed

oil of *Arabidopsis* expressing a FA hydroxylase gene from castor (*RcFAH12*), but its seed oil content and plant growth decreased. When *RcFAH12* gene was coexpressed with additional castor gene(s) in *Arabidopsis*, ~30 % HFAs were accumulated and the seed oil content and plant growth was almost restored to the wild-type level. Further advancement of our understanding of pathways, genes and regulatory mechanisms underlying synthesis and accumulation of HFAs is essential to developing and implementing effective genetic approaches for enhancing HFA production in oilseeds.

Keywords Hydroxy fatty acid · Industrial fatty acid · Castor · Lesquerella · *Arabidopsis* · Transgenic oilseed crop

Introduction

Vegetable oil is stored in plants as triacylglycerol (TAG), which can be obtained from most seeds and some fruits (mesocarp), including the olive (*Olea europaea*), avocado (*Persea americana*), and oil palm (*Syagrus cocoides*) (Huang et al. 2009). The perennial shrub jojoba (*Simmondsia chinensis*) is one of the few plants that stores wax esters in its seeds (Ohlrogge et al. 1978). Fatty acids (FAs) from vegetable oils provide humans and animals with both chemical energy and essential nutrients, such as linoleic acid (18:2) and linolenic acid (18:3). Vegetable oils are mainly used as food, but some are used for other purposes, including livestock feed and industrial applications. The ratio between food, feed, and industrial uses of global vegetable oils and animal fats was 80:6:14, but has recently changed to 74:6:20, mainly due to higher biodiesel production (Gunstone 2008), which is predicted to increase.

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The global production of vegetable-oil-based biodiesel dramatically increased 125 % in 4 years, from 10.4 billion liters in 2007 and 23.4 billion liters in 2011 (<http://www.eia.gov/cfapps/ipdbproject/iedindex3.cfm?tid=79&pid=81&aid=1&cid=ww,&syid=2007&eyid=2011&unit=TBPD>). The global production of vegetable oils and animal fats was 100–110 megatonne (Mt) in 2000 (Gunstone and Hamilton 2001), whereas the production of major vegetable oils alone was 169.23 Mt in 2013/14 (United States Department of Agriculture 2014).

The industrial uses of vegetable oils can be classified into two groups, biofuels and industrial chemical feedstocks. Biofuels, obtained by the methylation of the FAs from vegetable oils, are used for vehicle transportation and for aviation fuels (Sorda et al. 2010; Fairley 2011). Due to the chemical structures and physical properties of some vegetable FAs similar to those of petroleum (Metzger 2009), theoretically vegetable oil-derived FAs can replace all petrochemical feedstocks. Vegetable-oil-based biodiesel is a renewable and environmentally friendly alternative fuel (Demirbas 2009; Fairley 2011). Chemical feedstock FAs are used as additives or raw materials for numerous industrial products, including lubricants, inks, plastics, soaps, cosmetics, paints, coatings, and adhesives.

Most of the FAs in vegetable oils are composed of palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1),

18:2, and 18:3, the so-called “common” FAs. Some plants produce uncommon FAs with unique physical and chemical properties suitable for specific industrial applications, they are often called “unusual FAs” (UFAs; Smith 1971). UFAs can be classified based on the following criteria: (1) carbon chain length; (2) functional groups; (3) numbers and positions of unsaturated bonds; and (4) the types of unsaturated bonds. Information on the UFAs found in plants are given in Table 1. For example, lauric acid (12:0) is a medium-chain FA, shorter than the common FAs (C16–18). Erucic acid (22:1 Δ^{13}) is a very-long-chain FA. Ricinoleic acid (18:1-OH) and lesquerolic acid (20:1-OH) contain a hydroxyl group, and vernolic acid (12,13-epoxy-18:1 Δ^9) has an epoxy group. Petroselinic acid (18:1 Δ^6) is a monounsaturated FA but the position of the double bond differs from that of oleic acid. α -Eleostearic acid (18:3 $\Delta^{9,11t,13t}$) and calendic acid (18:3 $\Delta^{8t,10t,12}$) are conjugated FAs that contain conjugated double bonds, rather than methylene-interrupted double bonds. Crepenynic acid (18:2 $\Delta^{9,12a}$) contains a triple bond and sterculic acid (9,10-methylene-18:1 Δ^9) is a cyclic FA that contains a cyclopropane ring. Hydroxy FAs (HFAs) are among the most important industrially useful FAs and can be widely used in industry as chemical feedstock.

To produce industrial oils at lower cost, vegetable oils should contain a uniformed FA at high concentration for

Table 1 Unusual fatty acids that accumulate to high levels in plant seed oils

Unusual FA (abbreviation)	Producing plants	References
Lauric acid (12:0)	California bay (<i>Umbellularia californica</i>), <i>Cuphea</i> spp., Oil palm-kernel (<i>Elaeis guineensis</i>), Coconut (<i>Cocos nucifera</i>)	Kleiman (1990) and Amri (2011)
Erucic acid (22:1 Δ^{13})	<i>Brassica</i> spp., Crambe (<i>Crambe abyssinica</i>)	Princen and Rothfus (1984)
Petroselinic acid (18:1 Δ^6)	Coriander (<i>Coriandrum sativum</i>)	Ramadan and Morsel (2002)
Ricinoleic acid (12-hydroxy-18:1 Δ^9)	Castor (<i>Ricinus communis</i>)	Badami and Patil (1980)
Vernolic acid (12,13-epoxy-18:1 Δ^9)	Iron weed (<i>Vernonia galamensis</i>), Euphorbia (<i>Euphorbia lagascae</i>), Stokesia (<i>Stokesia laevis</i>)	Kleiman (1990)
Crepenynic acid (18:2 $\Delta^{9,12a}$)	Crepis (<i>Crepis alpina</i>), Parsley (<i>Petroselinum crispum</i>), English ivy (<i>Hedera helix</i>)	Lee et al. (1998) and Cahoon et al. (2003)
α -Eleostearic acid (18:3 $\Delta^{9,11t,13t}$)	Tung tree (<i>Vernicia fordii</i>), Bitter melon (<i>Momordica charantia</i>), Chinese cucumber (<i>Trichosanthes kirilowii</i>)	Armougom et al. (1998)
Calendic acid (18:3 $\Delta^{8t,10t,12}$)	Pot marigold (<i>Calendula officinalis</i>)	Cahoon et al. (2001)
Santalbic acid (18:2 $\Delta^{9a,11t}$)	Sandalwood (<i>Santalum</i> spp.)	Hettiarachchi et al. (2010)
Dimorphecolic acid (9-hydroxy-18:2 $\Delta^{10t,12t}$)	African daisy (<i>Dimorphotheca sinuata</i>)	Smith et al. (1960)
Lesquerolic acid (13-hydroxy-20:1 Δ^{11})	Lesquerella (<i>Physaria</i> spp.)	Jenderek et al. (2009)
cis-5-eicosenoic acid (20:1 Δ^5)	Meadowfoam (<i>Limnanthes</i> spp.)	Kleiman (1990)
Sterculic acid (9,10-methylene-18:1 Δ^9)	Java olive (<i>Sterculia foetida</i>)	Bao et al. (2002)

a indicates a triple bond (acetylene bond), *t* indicates a *trans* double bond

ease of purification and processing. However, most vegetable oils are mixtures of TAGs containing at least three of these five common FAs. Moreover, most unusual and novel FAs are produced in species not yet developed as crops. Thus, additional knowledge and tools are required to engineer desirable FAs in existing oilseeds. Recent research efforts have been focused on production of industrial FAs in transgenic plants using biotechnology. In this review, we discuss mostly the current metabolic engineering of plant oils for the production of HFAs as well as future perspectives.

HFA-producing plants: castor and lesquerella

Castor oil contains up to 90 % 18:1-OH (Table 1), so it is a valuable commercial source of HFAs. Castor oil and its derivatives are widely used in industrial products such as anticorrosion coatings, nylon, plastics, paints, cosmetics, soaps, detergents, and lubricants, and as an additive in aircraft fuel (Caupin 1997). Castor is mainly cultivated in tropical–subtropical regions. India is currently the foremost producers of castor seed, followed by China. Although castor oil is useful for industry, several factors limit castor oil production. First, castor seeds contain the toxin ricin and potent allergenic 2S albumins, which entail safety concerns. Second, only a few cultivars suitable for mechanical harvesting have been developed, limiting large-scale of commercial production. Third, the supply of castor oil fluctuates because of the economic and political instability of the countries where castor oil is produced (Holic et al. 2012). Improving agronomic traits of castor cultivar using biotechnology has been investigated, but due to the low transformation efficiency in castor, limited successes have been reported (Malathi et al. 2006; Sujatha and Sailaja 2005; Sailaja et al. 2008; Sujatha et al. 2009).

Lesquerella (*Physaria fendleri*), which belongs to the mustard family (Brassicaceae), produces 60 % 20:1-OH (Table 1) in its seed oil and is a potential crop for the semi-arid regions of North America (Dierig et al. 2011). Lesquerella does not contain the toxin ricin, so it is considered a safe source of HFAs. Considerable efforts have been made to improve the agronomic traits of lesquerella through breeding (Isbell et al. 2008; Dierig et al. 1993, 2011). Some lesquerella lines with 33 % have been developed (Dierig et al. 2006), which is comparable to most Brassicaceae oilseed crops. However, there are still several agronomic traits can be improved to make lesquerella a commercially viable crop, such as seed yield, herbicide tolerance and auto-fertility (Dierig et al. 2011). Recently, a highly efficient and stable transformation system was established (Chen 2011), which provides means to improve this crop through genetic engineering.

Biosynthesis pathways of TAG and HFA

Biosynthesis of TAG follows de novo synthesis of FA in the plastid and TAG assembly in the endoplasmic reticulum (ER) (Chapman and Ohlrogge 2012; Li-Beisson et al. 2013). Newly synthesized common FAs such as 18:1, 16:0 and 18:0 are released from the plastid and then converted to acyl-coenzyme As (CoAs). The acyl-CoAs can be incorporated into TAG through glycerol-3-phosphate (G3P) pathway also known as the Kennedy pathway (Weiss and Kennedy 1956; Weiss et al. 1960; Somerville et al. 2000). As exemplified in Fig. 1, the reactions in Kennedy pathway are catalyzed sequentially by several enzymes, including glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAT), and diacylglycerol acyltransferase (DGAT). First, acyl-CoA is incorporated into the *sn*-1 position of G3P by GPAT, and the subsequent product, lysophosphatidic acid (LPA), is converted into phosphatidic acid (PA) by LPAT. PA is dephosphorylated and converted to diacylglycerol (DAG) by PA phosphatase (PAP). Finally, TAG is synthesized through the acylation of the *sn*-3 position of DAG by DGAT. Since PA and DAG are also precursors for synthesis of membrane glycerolipids, GPAT and LPAT overlap the pathway for membrane lipids in all cells. In addition to Kennedy pathway, the newly synthesized acyl-CoAs can also be incorporated directly into membrane lipid phosphatidylcholine (PC) by the acyl editing reactions (Lands 1965; Li-Beisson et al. 2013). These acyl editing reactions can be catalyzed either by forward and reverse reactions of lyso-PC acyltransferase (LPCAT) to yield acyl-CoA, or by a phospholipase A-type activity to yield a free FA that then is activated to acyl-CoA (Fig. 1). Since PC is the substrate for many FA-modifying enzymes (desaturase, hydroxylase, etc.), rapid de-acylation and re-acylation of PC results in an acyl-CoA pool enriched with modified FAs (mFA), which are then utilized for TAG synthesis. In fact, TAG assembly may not be as simple as the sequential acylation of glycerol with GPAT, LPAT, and DGAT by the Kennedy pathway. Phospholipid:DAG acyltransferase (PDAT) has been identified that synthesizes TAG by transacylation of the *sn*-2 FA from PC onto *sn*-3 position of DAG, with lyso-PC as a co-product (Dahlqvist et al. 2000) (Fig. 1). Besides, metabolic labeling experiments indicate that there are at least two pools of DAG, de novo DAG and PC-derived DAG, and the relative proportion of each DAG utilized for TAG synthesis is likely to be species-dependent (Bates et al. 2009; Bates and Browse 2011). The de novo synthesis of DAG involves Kennedy pathway as described above. The PC-derived DAG utilizes de novo DAG to first synthesize PC. Removal of the headgroup of PC by PC:DAG phosphocholine transferase (PDCT) generates PC-derived DAG (Hu et al. 2012) (Fig. 1). Alternatively, PC-derived DAG

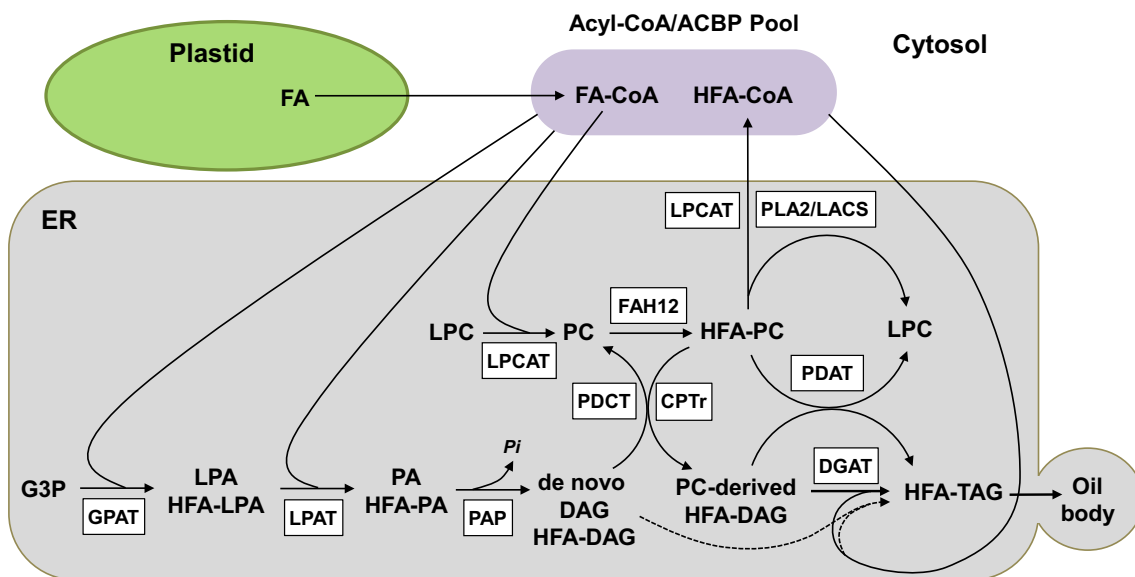


Fig. 1 Schematic diagram of the predicted pathways in the synthesis and accumulation of HFAs in the castor oil endosperm. FAs are synthesized in the plastid and C16–18 FAs are released into the cytosol. The biosynthesis of TAG and PC is initiated from G3P in the ER, but little TAG is synthesized directly from de novo DAG. PC and PC-derived HFA-DAG are converted simultaneously from de novo DAG and HFA-PC, respectively, by PDCT. HFA is synthesized from 18:1 incorporated in the *sn*-2 position of PC by FAH12. HFAs are removed from HFA-PC by LPCAT or PLA2/LACS and released into the cytosol as HFA-CoA, which is used to synthesize HFA-TAG. HFAs in HFA-PC are used directly for the synthesis of HFA-TAG without acyl editing by PDAT. LPC, a byproduct of acyl editing or TAG synthesis by PDAT, is reconverted into PC with acyl-CoA (mainly 18:1-CoA) by LPCAT. The HFA-TAG synthesized by PDAT or DGAT is stored in oil bodies budded from the ER. The white boxes

near the arrows indicate the enzymes that catalyze the conversion between the molecules connected by the arrow. Dashed arrows demonstrate the reactions that are least likely to occur. ACBP acyl-CoA-binding protein, DAG diacylglycerol, DGAT diacylglycerol acyltransferase, FAH12 oleate Δ 12-hydroxylase, G3P glycerol-3-phosphate, GPAT glycerol-3-phosphate acyltransferase, LACS long-chain acyl-CoA synthetase, LPA lysophosphatidic acid, LPAT lysophosphatidic acid acyltransferase, LPC lysophosphatidylcholine, LPCAT lysophosphatidylcholine acyltransferase, PA phosphatidic acid, PAP phosphatidic acid phosphatase, PC phosphatidyl choline, PDAT phosphatidylcholinediacylglycerol acyltransferase, PDCT phosphatidylcholinediacylglycerol cholinephosphotransferase, CPT_r CDP-choline:DAG cholinephosphotransferase reverse reaction, PLA₂ phospholipase A₂, TAG triacylglycerol

can be produced by the reverse action of CDP-choline:DAG cholinephosphotransferase (CPT) (Slack et al. 1983). As FA in PC can be modified, PC-derived DAG also provides a means to increase the presence of mFA in TAG.

Castor oil contains 90 % 18:1-OH (Table 1), over 70 % of the TAGs have all three *sn* positions esterified with 18:1-OH (Lin et al. 2003). The gene responsible for the synthesis of 18:1-OH in castor is oleate Δ 12-hydroxylase (RcFAH12; van de Loo et al. 1995). RcFAH12 and the gene encoding microsomal oleate Δ 12-desaturase (FA desaturase 2; FAD2) from Arabidopsis share about 67 % amino acid identity (van de Loo et al. 1995). FAH12 is known to be one of the various divergent forms of FAD2, together with the genes encoding FA conjugase, FA acetylase, and FA epoxygenase (Shanklin and Cahoon 1998; Lee et al. 1998; Cahoon et al. 1999; Dyer et al. 2002; Cahoon and Kinney 2004). FAD2 catalyzes the synthesis of 18:2 by forming a double bond between the 12th and 13th carbons of 18:1 esterified on the *sn*-2 position of phosphatidylcholine (PC; Okuley et al. 1994). Likewise, FAH12 catalyzes the synthesis of 18:1-OH by adding a hydroxyl group to the 12th carbon of 18:1, also esterified

on the *sn*-2 position of PC (Galliard and Stumpf 1966; Bafor et al. 1991). In castor, 18:1-OH is released from 18:1-OH-PC and activated to 18:1-OH-coenzyme A (CoA) (Moreau and Stumpf 1981). Since 18:1-OH-PC only accumulates to about 5 % of FA in PC (Ståhl et al. 1995), this requires that at least 90 % of seed 18:1 fluxes into the *sn*-2 position of PC is efficiently removed from PC and redistributed into the *sn*-1, *sn*-2, and *sn*-3 positions of TAG. Acyl editing and Kennedy pathway were suggested to be major routes of TAG synthesis in castor (Bafor et al. 1991), but other routes mediated through PC, such as PDAT (Dahlqvist et al. 2000), PDCT (Hu et al. 2012) cannot be excluded (Fig. 1).

Lesquerella oil contains ~60 % of 20:1-OH, almost all of the 20:1-OH are esterified on the *sn*-1 and *sn*-3 positions of TAG (Hayes and Kleiman 1996). Biochemical studies indicated that in lesquerella, 18:1-OH was initially formed by hydroxylation of 18:1 at *sn*-2-PC, then the 18:1-OH was released and activated to 18:1-OH-CoA; the newly formed 18:1-OH-CoA was rapidly elongated to 20:1-OH-CoA which is further utilized for TAG assembly (Engeseth and Szymne 1996; Reed et al. 1997). Kennedy pathway could

be a major mechanism to incorporate 20:1-OH into lesquerella TAG. The lack of HFA at the *sn*-2 of TAG could be explained by LPATs in lesquerella discriminating 20:1-OH substrate. The lesquerella *FAH12* gene responsible for hydroxylation of 18:1-PC to form 18:1-OH-PC was isolated and characterized (Broun et al. 1998). The gene encoding the condensing enzyme from lesquerella, *PfKCS3*, has also been isolated, and its activity has been shown to specifically catalyze elongation of 18:1-OH-CoA (Moon et al. 2001). In addition to 18:1-OH and 20:1-OH, lesquerella seed accumulates a low level of densipolic acid (12-hydroxy-octadec-cis-9,15-enoic acid: 18:2-OH) and auricolic acid (14-hydroxy-eicos-cis-11,17-enoic acid: 20:2-OH), which are formed by involving a microsomal Δ 15 desaturase (Engeseth and Stymne 1996; Reed et al. 1997).

Expression of various *FAH12* genes in *Arabidopsis* and other Brassicaceae plants

In a transgenic *Arabidopsis* (Col-0) expressing seed-specific *RcFAH12*, four types of HFAs accumulated, 18:1-OH (7.8 %), 18:2-OH (6.6 %), 20:1-OH (2.5 %), and 20:2-OH (0.4 %). These HFAs comprised a total 17 % of the seed oil (Broun and Somerville 1997). *Arabidopsis* seed oils contain large proportions of 18:3 and 11-eicosenoic acid (20:1 Δ ¹¹), each at ~15 % in seed oil (Rossak et al. 2001; Guo et al. 2009). To generate seed oil with uniform HFA, such as 18:1-OH, various *Arabidopsis* mutants defecting at FA desaturation (*fad3*), elongation (*fae1*), or both steps (*fad2lfae1*, *fad3lfae1*) were used as a background plants for expressing *RcFAH12*. When *RcFAH12* was expressed in *fad3*, 18:1-OH accumulated at 16.2 % (total 18.7 %), in *fae1* at 13–15 % (total 17 %), in *fad2lfae1* at 8.7 % (total 19.2 %), and in *fad3lfae1* at 7.0 % (total 7.0 %) (Smith et al. 2003; Lu et al. 2006).

According to Bursal et al. (2008), the *RcFAH12*-transgenic *Arabidopsis* lines that accumulated >20 % HFAs in their seed oil were invariably unstable, and the progeny seeds did not germinate well. These results suggest that high levels of HFAs may damage the metabolism and physiology of seeds which leads to the loss of oil content, seed weight and seed vigor. When *RcFAH12* is expressed alone, the highest and inheritable level of HFA in seed oil is 17 % observed by multiple groups (Broun and Somerville 1997; Smith et al. 2003; Kumar et al. 2006; Lu et al. 2006). Two transgenic *Arabidopsis* lines expressing *RcFAH12* in *fae1*, CL37 and CL7, showing both stable and the highest level of 17 ± 1 % HFAs in seed oil were selected from over 50 lines (Lu et al. 2006; Bursal et al. 2008). These lines have been used as a background plants for testing additional genes contributing to HFAs

accumulation in the seed oils (Bursal et al. 2008; van Erp et al. 2011; Kim et al. 2011; Hu et al. 2012).

RcFAH12 were also introduced into Brassicaceae oil-seeds *Brassica napus* and *Camelina*. The resulting transgenic plants showed 15.6 or 15 % HFA content in seed oil of *B. napus* (Broun et al. 1998) or *Camelina* (Lu and Kang 2008), respectively. These levels are similar to those of transgenic *Arabidopsis* seeds expressing *RcFAH12*.

Based on *RcFAH12* sequence similarity, a lesquerella 12-hydroxylase gene, *PfFAH12*, was isolated and characterized (Broun et al. 1998). Unlike *RcFAH12* which is a strict hydroxylase, *PfFAH12* is a bifunctional oleate 12-hydroxylase/desaturase (Broun et al. 1998). Transgenic *Arabidopsis* or *B. napus* expressing *PfFAH12* showed accumulation of HFA at 16.5 or 9.9 %, respectively (Broun et al. 1998). To examine whether the hydroxylases from other *Physaria* species were also bifunctional, a gene encoding oleate 12-hydroxylase was amplified by PCR from *Physaria lindheimeri*, a species that accumulates over 80 % 20:1-OH in its seed oil (Dauk et al. 2007). Molecular and biochemical analysis revealed that *PIFAH12* was primarily a fatty acid hydroxylase and should not be considered as bifunctional (Dauk et al. 2007). Transgenic *Arabidopsis* expressing *PIFAH12* accumulated 18 % HFA in seed oil (Dauk et al. 2007).

Besides *RcFAH12*, *PfFAH12*, and *PIFAH12*, *Arabidopsis* has also been transformed with the *FAH12* gene from fungi. The fungal pathogen *Claviceps purpurea*, which causes ergot disease in wheat and barley, forms a specialized structure called the “sclerotium”, which accumulates high levels of HFA (Mantle and Nisbet 1976). When the *FAH12* gene from *C. purpurea* was introduced in *Arabidopsis* Col-0 and *fad2lfae1* mutant, HFAs accounted for 19.65 and 25 % of the seed oils, respectively (Mee-sapyodsuk and Qiu 2008).

Barriers of producing HFAs in transgenic plants

Inhibition of HFA synthesis in developing seeds

As described, transgenic experiments with various *FAH12* have consistently failed to achieve high levels of HFAs. Besides, transgenic *Arabidopsis* expressing *FAH12* alone was often accompanied by reduced oil content, seed weight, and seed vigor (Dauk et al. 2007; Bursal et al. 2008; Bates and Browse 2011; van Erp et al. 2011). It was hypothesized that *Arabidopsis* may not have an efficient system to incorporate HFA into TAG, the buildup HFA might be targeted for β -oxidation or a futile cycle of synthesis and degradation, leading to limited accumulation of HFA in seed oil and reduction of total oil per seed (van de Loo et al. 1995; Broun and Somerville 1997; Moire et al.

2004; Bates and Browse 2011). Similar phenomenon were reported for transgenic plants expressing other UFA such as laurate-FA (Eccleston and Ohlrogge 1998) and epoxy-FA (Li et al. 2012). However, there were evidences suggested that acetyl-CoA carboxylase (ACCase) activity could be down-regulated by FA feedback inhibition and consequently, the rate of fatty acid synthesis decreased (Shintani and Ohlrogge 1995; Andre et al. 2012). Recent experiments demonstrated that the major factor limiting oil content in transgenic *Arabidopsis* expressing *RcFAH12* was due to inefficient utilization of HFAs during TAG assembly on the ER, which may induce an unknown pathway that activates post-translational down-regulation of ACCase in plastid leading to reduced FA and TAG synthesis (Bates et al. 2014). Therefore, any mechanism that helps on efficient channeling of HFAs into TAG may limit the buildup of HFA and increase HFA-TAG content in seed oil.

Metabolic bottlenecks in HFA flux

Since PC is the site for production of UFA, introduction of transgenes that produce UFA into non-UFA accumulators typically cause accumulation of higher UFA on PC in the host plants than in the native source (Singh et al. 2001; Cahoon et al., 2006; van Erp et al. 2011; Bates and Browse 2012; Snapp et al. 2014; Bates et al. 2014). When host transgenic plants fail to efficiently utilize the PC-UFA, bottlenecks could occur in any pathways that direct UFA into TAG assembly, such as removal of UFA from PC, channeling UFA into acyl-CoA pool, synthesis of de novo DAG and PC-derived DAG, and final acylation by DGAT and/or PDAT (Bates and Browse 2012). Metabolic labeling experiments have demonstrated one such bottleneck in transgenic *Arabidopsis* expressing *RcFAH12* that was at HFA flux through PC-derived DAG (Bates and Browse 2011). Although HFA-containing de novo DAG was synthesized, it was not efficiently utilized for TAG synthesis, or incorporated to PC and appeared to be turned over (Bates and Browse 2011). Even only 17 % HFA accumulated in transgenic *Arabidopsis* expressing *RcFAH12*, large proportion of HFA were found at the *sn*-2 position of TAG (van Erp et al. 2011). It was suggested that *Arabidopsis* may mostly utilize PC-derived DAG for TAG synthesis, the accumulation of HFA mostly at the *sn*-2 position of TAG was attributed to the substantial flux of HFA-containing PC-derived DAG out of PC after production of HFA by *RcFAH12* (Bates and Browse 2012). Therefore, the bottleneck in conversion of HFA-containing de novo DAG to PC may limit the accumulation of HFA at the *sn*-1 position of TAG, and potentially lead to reduction in oil accumulation (Bates and Browse 2011).

Overcoming the barriers

Increase of HFAs in seed oils using additional genes

DGAT DGAT catalyzes the final and rate-limiting step of TAG biosynthesis (Ichihara et al. 1988; Fig. 1). It is generally accepted that depending on the plant species, DGAT1 or DGAT2 is the major enzyme responsible for the accumulation of seed TAG. DGAT1 and DGAT2 are localized in the endoplasmic reticulum (ER; Zou et al. 1999; Lardizabal et al. 2001; Shockey et al. 2006). *Arabidopsis* DGAT1 is strongly involved in regulating the seed oil content in plants that do not produce UFAs (Zou et al. 1999; Jako et al. 2001; Zheng et al. 2008). DGAT2 from some UFA-producing plants plays an important role in the accumulation of UFAs in TAG (Kroon et al. 2006). Consistent with these results, when castor *DGAT2* was expressed in CL37, HFAs accumulation boosted from 17 to 30 % of the seed oil (Burgal et al. 2008). In addition, *Arabidopsis* double-transgenic lines expressing *RcFAH12/RcDGAT2* increased seed weight and oil content. These seeds germinated well and their subsequent plant growth and seed production were also normal (Burgal et al. 2008).

PDAT PDAT catalyzes the transfer of the FA at the *sn*-2 position of PC to the *sn*-3 position of DAG (Dahlqvist et al. 2000). Since the FA at the *sn*-2 position of PC can be hydroxylated, the resulting HFA can be removed and incorporated into TAG by PDAT (Banas et al. 2000; Fig. 1). The role of castor PDAT in HFA-containing TAG synthesis has been well studied by two groups (van Erp et al. 2011; Kim et al. 2011), both of whom found that castor *PDATs* were comprised of three members, and one of them, *RcPDAT1A* (van Erp et al. 2011), the same as *RcPDAT1-2* (Kim et al. 2011), was highly expressed in developing castor seeds. Since castor accumulates 90 % HFA in seed TAG, it has been proposed that the enzyme encoded by *RcPDAT1A* (or *RcPDAT1-2*) could be important for HFA-TAG synthesis. Indeed, when *RcPDAT1A* or *RcPDAT1-2* was expressed in CL37, the HFAs content increased from 17 to 25–28 % in the seed oil (van Erp et al. 2011; Kim et al. 2011). Besides, CL37 seeds expressing *RcPDAT1A* (or *RcPDAT1-2*) showed increased seed weight and had normal seed germination and plant growth phenotypes (van Erp et al. 2011; Kim et al. 2011). To determine if *RcPDAT1A* (or *RcPDAT1-2*) and *RcDGAT2* have an additive effect on HFA levels, both groups introduced *RcDGAT2* to CL37 expressing *RcPDAT1A* (or *RcPDAT1-2*). A small increase of HFA content was observed in the seed oil of triple transgenic CL37 *RcPDAT1A RcDGAT2* homozygous lines (van Erp et al. 2011). In addition, the triple transgenic seeds showed a 19.6 % increase in the mass of HFAs per seed (van Erp et al. 2011).

PDCT PDCT catalyzes PC and DAG conversion by headgroup exchange between PC and DAG (Lu et al. 2009; Hu et al. 2012; Fig. 1). *Arabidopsis* PDCT (AtPDCT) plays a major role in regulating the degree of unsaturation in seed oils (Lu et al. 2009). AtPDCT can convert *sn*-2-PUFA-PC and *sn*-2-18:1-DAG to *sn*-2-18:1-PC and *sn*-2-PUFA-DAG, respectively (Bates et al. 2012), i.e., PDCT catalyzes the reaction that exchanges the head group of unsaturated PC and de novo DAG, thus producing PC-derived DAG. It was assumed that PDCT from seed of HFA-producing plant can convert HFA-PC into HFA-DAG, subsequently HFA-DAG can be converted to HFA-TAG (Fig. 1). A castor *PDCT* gene was isolated and coexpressed in CL37. The resulted transgenic *Arabidopsis* had increased HFA content from 17 to 23 % in seed oil (Hu et al. 2012). Additional expression of RcDGAT2 further enhanced the HFA content to 28 % (Hu et al. 2012). The authors noted that coexpression of AtPDCT did not increase HFA in CL37, indicating that RcPDCT had evolved to effectively convert HFA-PC to HFA-DAG.

3-Ketoacyl-CoA synthase (KCS) The *KCS3* from *P. fendleri* (*PfKCS3*) encodes the enzyme responsible for the specific elongation of C18-HFAs to C20-HFAs in lesquerella (Moon et al. 2001). Interestingly, when the *PfKCS3* gene was coexpressed with the *RcFAH12* gene in *Camelina*, the proportion of HFAs, especially C20-HFAs, in the transgenic *Camelina* seed oil increased 1.4-fold (from 13.8 to 19.4 %), and the proportion of HFAs in the polar lipids decreased from 5 to 1.5 % (Snapp et al. 2014). Furthermore, the oil content and germination rate of *Camelina* seeds expressing *PfKCS3/RcFAH12* were restored to levels similar to those of nontransgenic *Camelina*. Hence, the HFA content per seed of *Camelina* expressing *PfKCS3-RcFAH12* was 150 % greater than that of *Camelina* expressing *RcFAH12* alone. It was speculated that C20-HFAs elongated by *PfKCS3* may not be effectively incorporated into the *sn*-2 position of PC, because no C20-HFAs were detected in the polar lipids. Therefore C20-HFAs would be more readily incorporated into TAG through Kennedy pathway (Snapp et al. 2014).

As described above, at least 4 different genes, *RcDGAT2*, *RcPDAT1A* (or *RcPDAT1-2*), *RcPDCT*, and *PfKCS3*, have been demonstrated to be effective in channeling of HFAs into TAG which may limit the buildup of HFA and restore HFA-TAG content in seed oil.

On-going or under-explored candidate genes involved in HFA-TAG synthesis

LPCAT This enzyme plays a role in regulating the degree of unsaturation in seed oils. Hence, the proportion of PUFAs in the seed oil was reduced in the *Arabidopsis lpcat1/2* double mutant (Bates et al. 2012), and the *LPCAT*-

overexpressing line contained a higher proportion of PUFAs in the seed oil than the wild type (Wang et al. 2012). The constant deacylation and reacylation is called acyl editing or the Lands cycle (Stymne and Stobart 1984; Lager et al. 2013; Lands 1965). In *Arabidopsis*, two *LPCAT* genes, *LPCAT1* and *LPCAT2*, are involved in acyl editing (Bates et al. 2012). In the developing silique of the *Arabidopsis dgat1* mutant, PDAT1 was upregulated to compensate for the lack of DGAT activity and was mainly responsible for the synthesis of TAG. The expression of *LPCAT2*, but not *LPCAT1*, was also upregulated and was chiefly responsible for the reacylation of the LPC produced by PDAT1 (Xu et al. 2012). Recent studies have revealed the role of LPCATs in transferring polyunsaturated acyl and ricinoleoyl groups from PC directly into the acyl-CoA pool by acyl exchange. (Xu et al. 2012; Bates et al. 2012; Wang et al. 2012; Lager et al. 2013). They suggest that LPCATs are responsible for incorporation of newly synthesized FA into PC (forward reaction), and transferring polyunsaturated FA and HFAs produced on PC back to the acyl-CoA pool (reverse reaction). When reverse reactions were measured, a ricinoleoyl group at the *sn*-2 position of PC was removed three- to sixfold faster than an oleoyl group by seven LPCATs from five species tested, including *Arabidopsis*, castor and lesquerella. These results suggest an important role for LPCATs in removing ricinoleoyl groups from the *sn*-2 position of PC to an acyl-CoA pool (Fig. 1). The acyl-CoA pool enriched with HFA can facilitate production of HFA-TAG through the Kennedy pathway. The annotated castor *LPCAT* genes 30170.m014002 and 30174.m008937 show broadly equivalent expression in the whole-plant tissues, rather than seed-specific or seed-abundant expression (Brown et al. 2012). The *Arabidopsis* genes corresponding to these genes were confirmed to be lysophosphatidyl ethanolamine acyltransferase (*LPEAT*) genes (Stålberg et al. 2009). 30190.m01126, annotated as the membrane-bound O-acyltransferase (*MBOAT*) gene of castor, is significantly expressed in the endosperm (Brown et al. 2012), and was confirmed to have an LPCAT function (Arroyo-Caro et al. 2013a; Lager et al. 2013).

Phospholipase A₂ (PLA₂) PLA₂ hydrolyzes phospholipids at the *sn*-2 position and releases free FAs. One of the routes of the released FAs is to enter the acyl-CoA pool for TAG assembly (Fig. 1). Thus, PLA₂ together with LPCAT serve as FA editing enzymes for modifying the composition of acyl-CoA pool. The role of PLA₂ in castor oil biosynthesis has been long proposed (Bafar et al. 1991), but the gene encoding PLA₂ has been elusive. Recently, a lecithin:cholesterol acyltransferase (LCAT)-like PLA with high PLA₂ activity was identified in *Arabidopsis* (Chen et al. 2012). Its ortholog in lesquerella was found highly expressed in developing seeds. Coexpression of *C.*

purpurea FAH12 and the *PfLCAT-PLA* gene in yeast revealed that *PfLCAT-PLA* facilitates the channeling of HFA from PC to TAG (Chen et al. 2014). Identification of *PLA₂* and more *LCAT-PLA* genes and overexpression of them in transgenic oilseeds would help to elucidate the role of *PLA₂* or *LCAT-PLA* in HFA-TAG synthesis in plants.

CPT CDP-choline:DAG cholinephosphotransferase (CPT) catalyzes the condensation reaction of CDP-choline with DAG to generate PC. However, the reaction was found to be reversible (Slack et al. 1983; 1985; Dewey et al. 1994). Since PC is the substrate for FA modification, such as hydroxylation and desaturation. The reverse reaction of CPT provides a mechanism for supplying mFA-DAG, which can then be utilized for mFA-TAG synthesis (Fig. 1). Thus, CPT could contribute to PC-derived DAG formation, in addition to PDCT, for TAG assembly. A castor CPT gene (*RcCPT*) has been identified, ubiquitously expressed in all organs examined (Brown et al. 2012). Compared with *RcCPT*, the expression of *RcPDCT* was two-fold higher in the endosperm than in the leaves or male flowers, and was higher than the expression of *RcCPT* (Brown et al. 2012). The role *RcCPT* in castor oil biosynthesis remains to be investigated. Identification of CPTs favoring production of PC-derived HFA-DAG would provide as new target for increased HFA in transgenic plants.

Long-chain Acyl-CoA synthetase (LACS) Nine *LACS* genes have been identified in *Arabidopsis* (Shockey et al. 2002). *AtLACS1* and *AtLACS2* are involved in the wax and cutin synthesis (Schnurr et al. 2004; Lü et al. 2009). *AtLACS6* and *AtLACS7* are involved in the β -oxidation of fatty acid (Fulda et al. 2002). *AtLACS9* is localized on the outer plastidial envelope and activates free fatty acid released from acyl-ACP by fatty acyl-ACP thioesterase. *AtLACS8* was predicted to encode the plastidial LACS like *AtLACS9* (Schnurr et al. 2002). Due to functionally redundancy of these family members, research has not identified a single member responsible for seed oil synthesis (Schnurr et al. 2002; Zhao et al. 2010). Surprisingly, *AtLACS9* and *AtLACS8*, which were found to be involved in seed oil synthesis together with *AtLACS1*, which is also associated with cuticular lipid synthesis (Zhao et al. 2010). Even though both genes are expressed seed-abundantly, *AtLACS8* localizes in the ER, unlike *AtLACS9* (Zhao et al. 2010). Putative seven *LACS* genes are found in castor (Brown et al. 2012). *LACS* localized in the ER from castor seed is predicted to contribute to convert HFA free fatty acid into HFA-CoA form, which is used as a constituent of HFA-TAG (Fig. 1). Functional studies need to be carried out to determine its role in castor TAG synthesis.

Acyl-CoA binding protein (ACBP) ACBP binds acyl-CoA and is associated with the stability of acyl-CoA in the cytosol, the transport of acyl-CoA, and the prevention of acyl-CoA hydrolysis by acyl-CoA hydrolase (Xiao and

Chye 2009). Overexpression of ACBP in plants resulted in modifications of FA composition in seed oil (Enikeev and Mishutina 2005; Yurchenko et al. 2009). Overexpression of a *B. napus* ACBP6 gene (*BnACBP6*) increased 18:2 and 18:3 at expense of 20:1 and saturated FA in transgenic *Arabidopsis* seed oil (Yurchenko et al. 2009). The results of in vitro LPCAT assays suggested that ACBP may enhance the incorporation of 18:1 into PC by promoting acyl exchange between PC and acyl-CoA catalysed by LPCAT (Yurchenko et al. 2009). An increased rate of 18:1 esterification to PC creates more substrate for FAD2 desaturase, which may result in a greater production of 18:2, which may be further desaturated to 18:3 by FAD3. A castor *ACBP* gene (*RcACBP6*), 29827.m002594, corresponding to *AtACBP6* and *BnACBP6* has been identified and found to be highly expressed in the endosperm (Brown et al. 2012). Whether *RcACBP6* plays a similar role to *BnACBP6* remains to be investigated. Overexpression of *RcACBP6* would induce HFA group of HFA-PC to release into cytosol and bind HFA-CoA, and consequently increasing the level of HFAs in seed oil.

LPAT LPAT acylates the *sn*-2 position of the LPA with acyl-CoA and yields PA. The LPAT enzyme belongs to a multigene family and is considered one of the most stringent acyltransferases with respect to substrate discrimination (Frentzen 1998). Thus, identification of a LPAT that participates in the channeling of HFA-CoA to TAG is a key to the successful genetic engineering of HFA-producing crops using this enzyme. Five isoforms of LPAT exist in *Arabidopsis* and *AtLPAT2* is known to be involved in TAG synthesis in developing seeds (Kim et al. 2005). Castor has nine *LPATs*, *RcLPAT2* and *RcLPATB* are expressed in developing seeds (Arroyo-Caro et al. 2013b). In vitro enzyme assays indicate that *RcLPAT2* had a preference for acylating 18:1-OH-CoA when 18:1-OH-LPA was used as an acyl acceptor, however when 18:1-LPA was used as the acyl-acceptor, *RcLPAT2* preferred 18:1-CoA as a substrate (Arroyo-Caro et al. 2013b). The results indicate that *RcLPAT2* might participate in both TAG and membrane glycerolipid synthesis in castor. *RcLPATB*, having no orthologue in *Arabidopsis*, is highly homologous to the *LPAT* genes from meadowfoam (*Limnanthes* spp.) or coconut (*Cocos nucifera*), whose oils are enriched with UFAs, 20:1 Δ^5 or 12:0, at the *sn*-2 positions of their TAGs (Table 1). Enzymes encoded by these genes exhibited a strong preference for the utilization of 20:1 Δ^5 or 12:0, respectively, in agreement to microsomal activities recorded in their seeds (Frentzen 1998). However, enzyme assays indicated that *RcLPATB* prefers saturated fatty acids (12:0–16:0) as substrates independently of the acyl group in the acceptor molecule, and has a broad specificity on other acyl-CoAs including 18:1-OH and 18:1 (Arroyo-Caro et al. 2013b). Although these in vitro data cannot infer

definitely whether RcLPAT2 and/or RcPLATB play important roles in castor TAG assembly in vivo, it is possible to test their functions by seed specific expression of these genes in transgenic oilseeds.

GPAT catalyzes the first reaction in the Kennedy pathway by acylation of FA into the *sn*-1 position of G3P. One plastidial GPAT and nine nonplastidial GPATs have been identified in *Arabidopsis* (Nishida et al. 1993; Zheng et al. 2003; Li et al. 2007; Gidda et al. 2009). Most GPATs are involved in the synthesis of cutin and suberin (Li et al. 2007; Beisson et al. 2007; Yang et al. 2010). AtGPAT9 is located in the ER and is predicted to be associated with TAG synthesis in seeds (Gidda et al. 2009). A tung tree (*Vernicia fordii*) GPAT9 (VfGPAT9) was found colocalized in the same ER subdomain with VfDGAT2 and interacted with VfDGAT2 (Gidda et al. 2011). Castor GPAT9 (30122.m000357), which is highly homologous to VfGPAT9, also localizes in the ER (Brown et al. 2012). Therefore, the RcGPAT9 may potentially contribute to HFA-TAG synthesis.

Conclusion and future perspectives

It has been nearly two decades since the identification of the key gene *RcFAH12*. Research efforts have made significant progress toward production of HFAs in transgenic oilseeds through discovery and overexpression of additional genes. The maximum percentage of HFA in transgenic seed oil has progressed up to 30 % (Burgal et al. 2008). However, this increase is not yet sufficient for economically sustainable production of HFAs from transgenic oilseed crops. Obviously, there are additional candidate genes waiting to be discovered. These include underexplored genes, or isoforms from known gene families that have high selectivity to HFAs and specifically favors channeling and incorporation them into TAG. Genomics has provided tools for generating genome-wide database useful for uncovering castor genes (Brown et al. 2012; Troncoso-Ponce et al. 2011). Establishment of new seed transcriptomes from other HFA accumulators, such as *lesquerella* and *Physaria lindheimeri*, which accumulates 60 and 80 % of 20:1-OH, respectively, should provide additional valuable resources for discovering new target genes. Choice of host crop that has a high rate of acyl editing to remove HFA from PC and also primarily utilizes Kennedy pathway for TAG assembly has been suggested to be ideal for transgenic production of HFA (Bates and Browse 2012). Identification of such crops requires determination of the relative fluxes through various TAG assembly pathways. *Lesquerella* is under development as an industrial crop for production of a safe source of HFA. Its seed oil already contains 55–60 % 20:1-OH, mostly at

the *sn*-1 and the *sn*-3 positions of TAG (Hayes and Kleiman 1996). Identification of a LPAT highly selective to 20:1-OH-CoA is the key to increase HFA level over 80 % of seed oil. Knowledge and information obtained from studies of HFA synthesis may improve our understanding of the mechanism underlying other UFA production in oilseed crops, which will advance the development of plant-based petrochemical feedstocks.

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Conflict of interest The authors declare that they have no conflicts of interest.

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