Chapter

Biotechnology for Improving Hydroxy Fatty Acids Production in Lesquerella (*Physaria fendleri*)

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Abstract

Hydroxy fatty acid (HFA) is a vital raw material for numerous industrial products, such as lubricants, plasticizers and surfactants. Castor oil is the current commercial source of HFA which contains 90% ricinoleic acid (18,1OH). Castor seeds contain the toxin ricin and hyperallergic 2S albumins; it is detrimental to castor oil production. Lesquerella is a potential industrial oilseed crop for a safe source of HFA, because lesquerella seeds contain a valuable HFA, lesquerolic acid (20,1OH), at 55–60% in seed oil. This chapter describes current progress on improving HFA production in lesquerella through metabolic engineering.

Keywords: hydroxy fatty acid, ricinoleic acid, lesquerolic acid, triacylglycerol, *Physaria fendleri*, lesquerella, seed oil, genetic transformation

1. Introduction

Lesquerella seed oil (triacylglycerol, TAG) biosynthesis follows common *de novo* fatty acid (FA) biosynthesis in plastid (**Figure 1**). Once oleic acid (18,1) is synthesized and exported to cytosol, it is activated to 18:1-Coenzyme A (CoA) for endoplasmic reticulum (ER)-mediated fatty acid modification and TAG assembly [1]. The 18:1-CoA can be esterified directly into membrane lipid phosphatidylcholine (PC) in the ER by the forwarding reaction of lyso-PC acyltransferase (LPCAT) [2–4] resulting in 18:1-PC (**Figure 1**). An oleate 12-hydroxylase (FAH12) [5–8] hydroxylates 18:1-PC to form 18:1OH-PC (**Figure 1**). Lesquerella PfFAH12, however, converts 18:1-PC to both 18:1OH-PC and linoleic acid (18,2)-PC, because PfFAH12 posesses bi-functional FAD2-related oleate Δ12 - hydroxylase: desaturase activities [8].

Through the reverse reaction of LPCAT (**Figure 1**), or phospholipase A (PLA2)–type activity [9], the 18:1OH can be removed from PC and transferred back to cytosol to be activated as 18:1OH-CoA. A lesquerella fatty acid condensing enzyme (PfKCS18) (also called KCS3 or FAE1) elongates 18:1OH-CoA to 20:1OH-CoA [10] (**Figure 1**). Rapid acylation of 18:1 and de-acylation of 18:1OH by LPCAT (or by PLA2), and together with elongation of 18:1OH-CoA by PfKCS18 leads to enrichment of 20:1OH-CoA in cytosol. FA desaturase 2 (FAD2) [11] and FA desaturase 3 (FAD3) [12] converts 18:1-PC to 18:2-PC and 18:2-PC to linolenic acids (18:3)-PC,

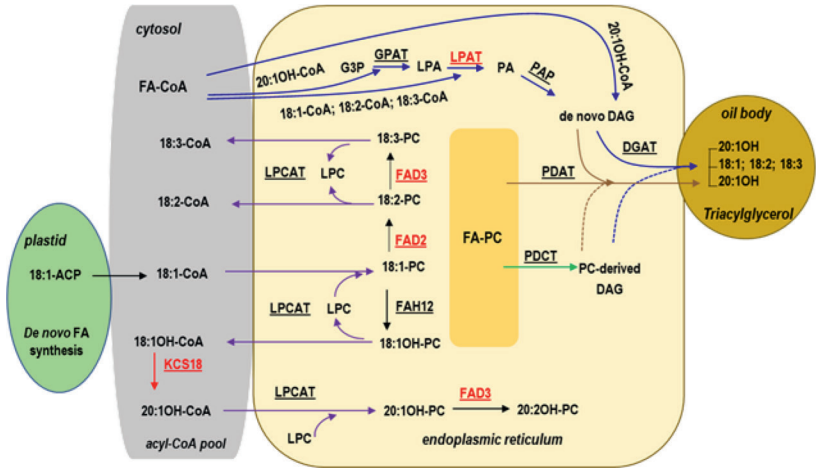


Figure 1.
Proposed pathways for TAG biosynthesis in lesquerella seed. Kennedy pathway is indicated by blue arrows. Acyl editing reactions are indicated by purple arrows. PC-derived DAG formation is indicated by a green arrow. Enzymes catalyzing these reactions are underlined. Fatty acid numerical symbols and abbreviations are described in introduction.

respectively (**Figure 1**). The FAD3 is also resposible for converting 20-1OH to auricolic acid (20,2OH) [13, 14] in lesquerella seeds. A functional lesquerella PfFAD3–1 isoform is a key enzyme producing 18:3 and 20:2OH [15]. FA-CoA or FA-PC are assembled to TAG through multiple mechanisms [1, 2]. First, FA-CoAs are acylated to a glycerol-3-phosphate (G3P) backbone by enzymes in Kennedy pathway [16]. Glycerol-3-phosphate acyltransferase (GPAT) acylates FA-CoA to the *sn*-1 position of G3P backbone forming lysophosphatidic acid (LPA). LPA acyltransferase (LPAT) is responsible to add another FA-CoA to the *sn*-2 of LPA forming phosphatidic acid (PA). Through PA phosphatase (PAP), PA is changed to 1,2-*sn*-diacylglycerol (DAG). The DAG produced in Kennedy pathway is referred to de novo DAG. The last FA-CoA can be added to the *sn*-3 of DAG by 1,2-*sn*-diacylglycerol acyltransferase (DGAT), creating TAG. In lesquerella, almost all seed TAG having 20:1OH at the *sn*-1 and *sn*-3 positions, and the *sn*-2 positions are occupied by unsaturated FAs, ie., 18:1, 18:2 and 18:3 [17–20]. The reason of lack of HFA at the *sn*-2 position of TAG could be due to the selectivity of lesquerella LPAT (PfLPAT2) for unsaturated FA [21], which is a typical characteristic for most plant LPAT2 [22]. Second, PC can be converted to DAG (PC-derived DAG). PC:DAG cholinephosphotransferase (PDCT) [23–25] is a major enzyme to produce PC-derived DGA through exchange of the head group between PC and DAG. Alternatively, PC-derived DGA can be produced by other enzymatic reactions catalyzed by CDP-choline: DAG cholinephosphotransferase (CPT) [26], or phospholipases (PLC, or PLD) [2, 27]. The conversion of PC into DAG also provides a mechanism to increase the amount of 18:1, 18:2, 18:3 in *sn*-2-TAG. Third, FA on the *sn*-2 PC can be transferred to the *sn*-3 position of DAG by phospholipid:DAG acyltransferase (PDAT) (**Figure 1**) [28–30].

To develop lesquerella that produces 18:1OH-rich seed oils like castor, we have over-expressed castor RcLPAT2 [21]. The resulted transgenic lesquerella seeds increase 18:1OH content at the *sn*-2 position of TAG from 2–17%, and consequently, oil accumulates more TAGs with all three *sn* positions occupied by HFA [20, 21]. RNA interference sequences targeting KCS18, FAD2 and FAD3 have been introduced

to lesquerella. Seeds from the transgenic lines had increased 18:1OH up to 26.6% compared with that of 0.4–0.6% in wild type (WT) seeds. Our studies enhance our understanding of plant lipid metabolism.

2. Castor LPAT2 increases castor oil-like triacylglycerols in lesquerella seed

We have produced 17 transgenic lesquerella lines expressing *RcLPAT2* under the control of a seed specific promoter [21]. Our results indicate that RcLPAT2 enables the incorporation of 18:1OH at *sn*-2 position of LPA which increases the accumulation of 18:1OH and also tri-HFA-TAGs in lesquerella (**Figures 2** and **3**).

In transgenic lesquerella expressing *RcLPAT2*, we observed an increase in 0-, 1-, and 3-HFA-TAG levels and a reduction in 2-HFA-TAG species (**Figure 2**). Regiochemical analysis showed that *sn*-2 18:1OH increased 6–7-fold or 1.5-fold, respectively (**Figure 3**). Further analysis of regioisomer of the transgenic seed oil reveals that RcLPAT2 increased 3-HFA-TAG content by acylating mostly 18:1OH at the *sn*-2 position of 20:1OH-LPA forming tri-HFA-TAG (20,1OH at *sn*-1, 3 and 18:1OH at *sn*-2) [18–20, 31]. This indicates that *RcLPAT2* allows for a more efficient acylation of 18:1OH than 20:1OH to the *sn*-2 position of TAG *in vivo*. We have demonstrated that castor LPAT2 increases 18:1OH level through exclusively acylating 18:1OH at the *sn*-2 position of tri-HFAs-TAGs in lesquerella. RcLPAT2 holds a valuable property for the engineering of a new castor oil-producing crop, such as lesquerella.

3. Ricinoleic acid content can be increased in lesquerella seeds through suppressing an elongase and fatty acid desaturases

To develop lesquerella that produces 18:1OH-rich seed oils like castor, silencing PfKCS18 would result in accumulation of 18:1OH; silencing FAD2 and FAD3 would block 18:1 flux to 18:2 and 18:3, respectively. To test the hypothesis, we generated transgenic lines expressing RNAi of camelina CsFAD2, CsFAD3, and Arabidopsis

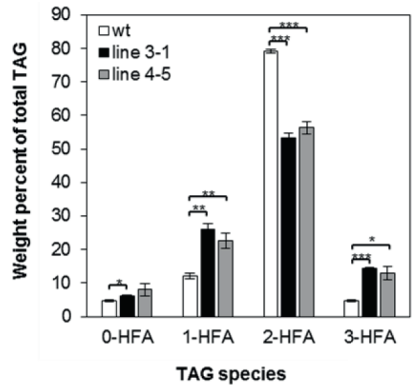


Figure 2.
*TAG species composition. Triplicates of 50-seed sample were measured for wild type (wt) and transgenic lines (line 3–1, line 4–5). The data represent averages of three replicates ± SE. Two-tailed Student's t test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. 0-HFA, 1-HFA, 2-HFA, and 3-HFA indicate TAG molecular with zero, one, two, or three HFAs, respectively.*

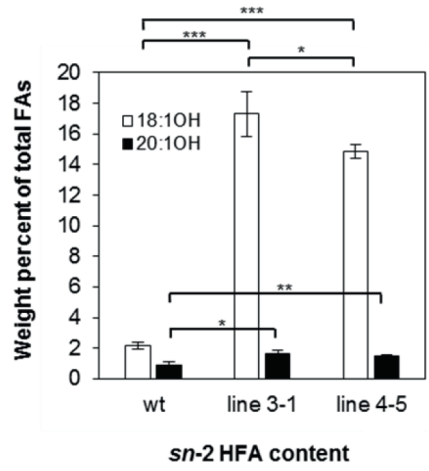


Figure 3.
*HFA content at the sn-2 position of TAG. Triplicates of 50-seed sample were measured for wild type (wt) and transgenic lines (line 3–1, line 4–5). The data represent averages of three replicates ± SE. Two-tailed Student's t test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. 18:1OH and 20:1OH represent ricinoleic acid and lesquerolic acid, respectively.*

AtKCS18, which have 82–95% sequence homology with corresponding lesquerella genes [18]. RNAi constructs, *CsFAD2 RNAi*, *CsFAD3 RNAi* and *AtFEA1 RNAi,* are effective in silencing corresponding gene expression in camelina [32–34]. We therefore generated 16 transgenic lesquerella lines expressing *AtFAD3 RNAi* + *CsFAE1 RNAi* (2-dsRNA) (**Table 1**) [35], and 15 lines expressing *CsFAD2 RNAi* + *AtFAD3 RNAi* + *CsFAE1 RNAi* (**Table 2**) [35].

As shown in **Table 1**, when the 2-dsRNAs (*AtFAD3 RNAi* and *CsFAE1 RNAi*) was introduced to lesquerella, we observed significant increases in 18:1OH from 0.6% of WT to 26.6% in line 1 (**Table 1**) and decreases in 20:1OH from 51.2% of WT to lowest 19% in line 1 (**Table 1**). 18:1 content was increased in 50% of transgenic population with the highest level of 32.1% in line 2 compared with 17% of WT (**Table 1**). Correlation analysis was performed to show the relationships between FA accumulation for 2-dsRNA group. Among the 16 T_1 transgenic lesquerella lines, 15 lines shifted the accumulation of 18:3 to 18:2, showing a strong negative correlation between 18:2 and 18:3 ($r = 0.93$); 13 lines shifted 20:1OH to 18:1OH, which also displayed a strong negative correlation ($r = -0.99$). These results indicate that *AtFAD3 RNAi* and *CsFAE1 RNAi* are effective in silencing *PfFDA3–1* and *PfKCS18*, respectively.

As shown in **Table 2**, 15 independent transgenic lines expressing the 3-dsRNAs, *CsFAD2 RNAi* + *AtFAD3 RNAi* + *CsFAE1 RNAi* were generated and their T_1 seeds were analyzed for FA composition. Compared with 2-dsRNAs (**Table 1**), similar average contents in 18:2, 18:3 and 20:1 in lines expressing 3-dsRNA (**Table 2**) were observed. With the addition of *CsFAD2 RNAi* in the 3-dsRNA group, the average of 18:1 was higher at 27.8% (**Table 2**) compared with the average of 20.5% in the 2-dsRNA group (**Table 1**). Besides, less dynamic changes between average increase of 18:1OH and average decrease of total HFA were observed in the 3-dsRNA group, showing averages of 4.7% and 48.9%, respectively, (**Table 2**), compared with that of 7.7% and 53% in lines expressing 2-dsRNA, respectively (**Table 1**). FA composition in WT seeds (**Tables 1** and **2**) were similar to described [21, 36]. There was no change of growth phenotype for transgenic lesquerella expressing *CsFAD2 RNAi*, *CsFAD3 RNAi* and *AtFEA1 RNAi.*

Line	Total minor fatty acid[a]	18:1	18:2	18:3	18:1OH	20:1OH	20:2OH	Total hydroxy fatty acid
wild-type	4.7 ± 0.4	17.0 ± 0.4	7.6 ± 0.4	13.3 ± 0.6	0.6 ± 0.2	51.2 ± 1.0	4.3 ± 0.6	56.0 ± 0.5
line 1	4.3 ± 0.2	30.5 ± 2.8 ***	16.2 ± 1.7 ***	2.2 ± 0.7 ***	26.6 ± 0.2 ***	19.0 ± 2.0 ***	0.2 ± 0.2 ***	45.8 ± 1.9 ***
line 2	5.1 ± 0.2	32.1 ± 1.3 **	17.0 ± 0.5 ***	1.7 ± 0.5 ***	16.8 ± 0.5 ***	26.1 ± 1.0 ***	0.0 ± 0.0 ***	42.9 ± 0.5 ***
line 3	5.0 ± 0.2	25.5 ± 1.9 **	17.6 ± 1.2 ***	2.6 ± 1.4 ***	16.6 ± 1.0 ***	31.4 ± 2.1 ***	0.5 ± 0.4 ***	48.4 ± 1.5 ***
line 4	4.7 ± 0.4	22.7 ± 2.7 *	16.8 ± 0.6 ***	3.3 ± 0.8 ***	11.7 ± 2.7 **	39.3 ± 5.0 **	0.4 ± 0.2 ***	51.4 ± 2.6 *
line 5	4.7 ± 0.2	23.4 ± 2.0 **	18.7 ± 0.2 ***	1.8 ± 0.6 ***	10.2 ± 0.4 ***	40.2 ± 1.4 ***	0.1 ± 0.1 ***	50.5 ± 1.5 ***
line 6	4.4 ± 0.1	20.5 ± 1.8 *	14.4 ± 1.0 ***	6.2 ± 0.5 ***	8.5 ± 0.4 ***	43.7 ± 1.4 **	1.2 ± 0.1 ***	53.4 ± 1.5 *
line 7	4.1 ± 0.1	20.5 ± 0.9 **	16.7 ± 2.0 ***	4.9 ± 1.7 ***	8.0 ± 1.3 ***	45.5 ± 2.0 **	0.3 ± 0.2 ***	53.8 ± 1.2 *
line 8	4.5 ± 0.0	17.6 ± 0.5	16.1 ± 0.5 ***	7.6 ± 0.4 ***	7.5 ± 0.5 ***	45.0 ± 0.5 ***	1.3 ± 0.2 ***	53.8 ± 0.2 **
line 9	4.4 ± 0.1	17.9 ± 0.5	17.2 ± 0.7 ***	4.3 ± 0.7 ***	4.9 ± 0.4 ***	49.3 ± 0.6 *	0.7 ± 0.2 ***	54.9 ± 0.5
line 10	4.8 ± 0.0	19.0 ± 1.0 *	13.0 ± 0.5 ***	9.6 ± 0.5 ***	4.7 ± 1.2 **	46.9 ± 0.6 *	1.2 ± 0.1 ***	52.8 ± 1.4 *
line 11	4.3 ± 0.0	16.5 ± 0.4	16.3 ± 0.2 ***	5.4 ± 0.3 ***	3.6 ± 0.3 ***	51.9 ± 0.4	0.8 ± 0.2 ***	56.2 ± 0.0
line 12	4.1 ± 0.2	16.3 ± 0.5	14.1 ± 0.9 ***	7.7 ± 1.2 **	1.8 ± 0.3 **	53.2 ± 1.0	1.8 ± 0.2 **	56.7 ± 1.1
line 13	3.9 ± 0.3	17.1 ± 2.1	9.5 ± 0.2 **	13.3 ± 0.4	1.2 ± 0.1 **	51.1 ± 2.7	2.2 ± 0.1 **	54.5 ± 2.7
line 14	4.3 ± 0.3	16.2 ± 0.5	20.0 ± 0.7 ***	1.5 ± 0.1 ***	0.5 ± 0.1	56.2 ± 1.2 **	0.1 ± 0.0 ***	56.8 ± 1.3
line 15	4.0 ± 0.4	16.1 ± 0.5	14.2 ± 1.2 ***	6.6 ± 0.9 ***	0.5 ± 0.1	55.5 ± 0.7 **	1.8 ± 0.4 **	57.7 ± 0.8 *
line 16	4.1 ± 0.1	15.4 ± 0.4 **	13.4 ± 1.5 **	8.3 ± 1.3 **	0.5 ± 0.1	55.6 ± 0.2 **	1.7 ± 0.3 **	57.8 ± 0.5 *
average of transgenics	4.4 ± 0.1	20.5 ± 0.9	15.7 ± 0.5	5.4 ± 0.4	7.7 ± 0.7	44.4 ± 1.2	0.9 ± 0.1	53.0 ± 0.8

Three or four replicates of 30-seed sample were measured for wild-type and each transgenic line. All data are averages of measurements ±SD. Fatty acid legend: 18:1 is oleic; 18:2 is linoleic; 18:3 is linolenic; 18:1OH is ricinoleic; 20:1OH is lesquerolic; and 20:2OH is auricolic acid. a, total content of five common fatty acids: palmitic (16:0), palmitoleic (16:1), stearic (18:0), arachidic (20:0), and eicosenoic acids (20:1). Two-tailed Student's t-test. $p < 0.05$.*
*** $p < 0.01$.*
**** $p < 0.001$.*

Table 1.
Fatty acid composition (mole %) in T_1 seeds expressing AtFAD3 RNAi + CsFAE1 RNAi.

Line	Total minor fatty acid[a]	18:1	18:2	18:3	18:1OH	20:1OH	20:2OH	Total hydroxy fatty acid
wild-type	4.2 ± 0.2	16.7 ± 0.2	8.0 ± 0.2	13.7 ± 0.2	0.40 ± 0.0	53.0 ± 0.9	3.1 ± 0.5	56.5 ± 0.7
line 1	4.2 ± 0.1	27.7 ± 0.3 ***	13.8 ± 0.4 ***	4.8 ± 0.5 ***	15.4 ± 0.7 ***	33.3 ± 0.8 ***	0.9 ± 0.1 ***	49.6 ± 0.1 ***
line 2	4.3 ± 0.1	26.4 ± 2.4 **	13.6 ± 0.3 ***	5.5 ± 0.8 ***	10.3 ± 0.9 ***	38.8 ± 2.5 ***	1.1 ± 0.1 **	50.1 ± 1.9 **
line 3	5.2 ± 0.0 ***	35.7 ± 1.6 **	15.1 ± 0.4 ***	3.1 ± 1.0 ***	8.2 ± 1.1 ***	32.4 ± 1.8 ***	0.5 ± 0.3 **	40.9 ± 1.1 ***
line 4	4.1 ± 0.3	22.6 ± 0.6 ***	15.8 ± 1.5 ***	4.3 ± 2.5 **	7.5 ± 1.0 ***	44.9 ± 0.7 ***	0.7 ± 0.6 **	53.2 ± 1.3 **
line 5	5.4 ± 0.1 ***	30.2 ± 1.0 ***	13.2 ± 1.1 ***	3.8 ± 0.3 ***	6.5 ± 0.8 ***	37.3 ± 1.0 ***	0.7 ± 0.1 ***	44.5 ± 1.8 ***
line 6	4.5 ± 0.0 *	35.8 ± 4.1 ***	15.1 ± 0.2 ***	5.0 ± 0.7 ***	6.3 ± 0.4 ***	35.6 ± 3.7 ***	0.7 ± 0.3 ***	42.6 ± 4.1 **
line 7	4.1 ± 0.1	17.5 ± 0.8	17.3 ± 1.2 ***	3.9 ± 0.9 ***	6.3 ± 0.5 ***	50.2 ± 0.5 **	0.7 ± 0.0 ***	57.2 ± 0.6
line 8	4.3 ± 0.2 *	38.8 ± 3.5 ***	14.4 ± 0.9 ***	1.7 ± 0.3 ***	4.6 ± 0.3 ***	36.1 ± 2.8 ***	0 ± 0.3 ***	40.7 ± 2.6 ***
line 9	3.9 ± 0.2	24.6 ± 2.1 **	13.3 ± 0.5 ***	6.0 ± 0.5 ***	1.9 ± 0.2 ***	48.9 ± 1.4 **	1.2 ± 0.2 **	52.1 ± 1.4 **
line 10	4.5 ± 0.8	32.3 ± 2.8 ***	10.7 ± 1.3 ***	7.1 ± 1.1 ***	1.1 ± 0.2 ***	43.1 ± 2.2 **	1.5 ± 0.3 **	45.7 ± 1.8 ***
line 11	4.8 ± 0.3 *	22.9 ± 0.8 ***	13.9 ± 0.1 ***	7.0 ± 0.7 ***	0.7 ± 0.1 **	49.7 ± 0.7 **	0.9 ± 0.1 **	51.3 ± 0.6 ***
line 12	4.3 ± 0.2	28.4 ± 1.4 ***	16.0 ± 0.7 ***	3.0 ± 0.2 ***	0.4 ± 0.0	47.9 ± 0.7 **	0.0 ± 0.0 ***	48.3 ± 0.7 ***
line 13	4.4 ± 0.1	22.7 ± 2.8 *	17.4 ± 0.6 ***	1.9 ± 0.4 ***	0.4 ± 0.1	53.2 ± 2.0	0.0 ± 0.0 ***	53.6 ± 2.0
line 14	4.3 ± 0.3	24.3 ± 2.0 **	14.6 ± 1.1 ***	4.5 ± 0.9 ***	0.4 ± 0.0	50.9 ± 1.1*	1.1 ± 0.5 **	52.4 ± 1.6 **
line 15	4.0 ± 0.0 ***	26.3 ± 1.6 ***	14.5 ± 0.4 ***	4.0 ± 0.6 ***	0.4 ± 0.0	50.1 ± 0.9 *	0.7 ± 0.2 ***	51.2 ± 1.0 **
average of transgenic line	4.4 ± 0.4	27.8 ± 5.9	14.6 ± 1.7	4.4 ± 1.6	4.7 ± 4.5	43.5 ± 7.2	0.7 ± 0.4	48.9 ± 5.0

Three or four replicates of 30-seed sample were measured for wild-type and each transgenic line. All data are averages of three measurements ±SD. Fatty acid legend: 18:1 is oleic; 18:2 is linoleic; 18:3 is linolenic; 18:1OH is ricinoleic; 20:1OH is lesquerolic; and 20:2OH is auricolic acid. a, total content of five common fatty acids: palmitic (16:0), palmitoleic (16:1), stearic (18:0), arachidic (20:0), and eicosenoic acids (20:1). Two-tailed Student's t-test.$p < 0.05$.*
***$p < 0.01$.*
****$p < 0.001$.*

Table 2.
Fatty acid composition (mole %) in T_1 seeds expressing CsFAD2 RNAi + AtFAD3 RNAi + CsFAE1 RNAi.

We have demonstrated that high levels of 18:1OH can be achieved by blocking the elongation of 18:1OH to 20:1OH. Also, high levels of 18:1 and 18:2 were accumulated through suppression of desaturation steps. However, the accumulated 18:1 was not converted to 18:1OH and instead, 18:1 was largely channeled to seed TAG.

4. Bottlenecks and potential for production of a high 18:1OH-containing oil in lesquerella

Based on the data presented in **Tables 1** and **2**, significant amount of 18:1 was not utilized for 18:1OH production. One of the factors could be due to the bifunctional activity of PfFAH12 [8], which hydroxylates and desaturases 18:1 to produce 18:1OH and 18:2, respectively, thus diverting 18:1 flux to 18:2 production. Seeds contain 90% 18:1OH from castor [37] or 85% 20:1OH from *Physaria lindheimeri* [38, 39]. These species have strict *FAH12*s, *RcFAH12* [7] and *PlFAH12* [38]. Deleting *PfFHA12* and at the same time introducing *RcFAH12* or *PlFAH12* in lesquerella should allow increased 18:1OH accumulation. On the other hand, the accumulation of 18:1 could also be due to a lesquerella LPAT that has substrate preference for 18:1-CoA, resulting in efficient incorporation of 18:1-CoA into TAG through Kennedy pathway. We already showed that castor *RcLPAT2* increased 18:1OH in lesquerella [20, 21]. Besides, castor *RcLPAT3B* and *RcLPATB* also showed substrate preference to 18:1OH in Arabidopsis [40]. To enhance 18:1OH to a higher level in lesquerella, further engineering design should include knocking out a lesquerella *PfLPAT2* and overexpressing *RcLPAT2*, *RcLPATB*, and *RcLPAT3B*. In addition to Kennedy pathway, some of the 18:1-PC could be converted by PDCT to 18:1-DAG for TAG assembly in lesquerella (**Figure 1**). Lesquerella seed TAGs contain about 21% 18:2 and 18:3 (**Tables 1** and **2**). There is strong evidence that seeds enriched with 18:2 or 18:3 may use the PC-derived pathway [2]. Therefore, it is likely that PC-derived DAGs are utilized in TAG assembly in lesquerella. To enhance flux from 18:1OH-PC to 18:1OH-DAG, it is advantageous to replace a lesquerella *PfPDCT* with a castor *RcPDCT* which was demonstrated effective in converting 18:1OH-PC to 18:1OH-DAG in Arabidopsis [25].

We observed increase in 18:1OH and decrease in 20:1OH in transgenic lesquerella seeds expressing *CsFAE1 RNAi*, which are expected. We, however, found that total HFAs are reduced (**Tables 1** and **2**). Considering lesquerella PfKCS18 is evolved to specifically elongate 18:1OH-CoA to 20:1OH-CoA [10] (**Figure 1**), it is possible that some enzymes in Kennedy pathway are co-evolved to adapt and utilize 20:1OH efficiently. Introducing additional enzymes with 18:1OH substrate selectivity may enhance total HFA level in lesquerella seeds. Although most plant GPATs select a wide range of acyl-CoA substrates [2, 41], castor RcGPAT9 was able to incorporate HFAs including 18:1OH at the *sn*-1 position of G3P, thus playing a critical role for *sn*-2 and *sn*-3 HFA acylation by LPAT and DGAT [42, 43]. Castor RcDGAT2 prefers 18:1OH to common FAs for esterifying 18:1OH to the s*n*-3 position of DAG [44, 45]. Thus future engineering design may target *RcGPAT9* and *RcDGAT2*. Lesquerella seed transcriptome analysis reveals one PfGPAT9 and three PfDGATs [46]. Evaluation on substrate preference by these genes will provide insights for enzyme characteristics in HFA-rich species.

One of the engineering examples demonstrates the interplay between Kennedy pathway and PC-mediated pathway for acylating HFA into *PfKCS18* was found to efficiently elongate 18:1OH to 20:1OH [47] in transgenic camelina expressing *RcFAH12* [32]. The transgenic camelina seeds expressing both *RcFAH12* with *PfKCS18* increased HFA content to 21% compared with the background line

expressing single *RcFAH12* [47]. 18:1OH-PC generated by RcFAH12 in camelina may be subjected to β-oxidation [48], or represents a bottleneck [24], because camelina is not equipped with enzymes and pathways for channeling 18:1OH-PC into storage TAG. PfKCS18 may ease the 18:1OH flux from PC to cytosol by converting 18:1OH to 20:1OH, thus relieving the bottleneck and facilitating the incorporation of HFA into TAG by Kennedy pathway (**Figure 1**) [16].

FA at the *sn*-2 position of PC can be transferred to the *sn*-3 position of DAG, by PDAT [28, 49] (**Figure 1**). Castor *RcPDAT1–2* (or *RcPDAT1A*) transfers 18:1OH from its PC to DAG for HFA-TAG synthesis [29, 30]. To further enhance 18:1OH accumulation in lesquerella TAGs, *RcPDAT1–2* is another candidate target for genetic engineering.

5. Summary

Significant increases in 18:1OH content are achieved through over expressing *RcLPAT2* and silencing *FAD2*, *FAD3* and *KCS18*. Intriguingly, the accumulated 18:1 was not efficiently utilized to produce 18:1OH and instead, 18:1 was largely channeled to seed TAG. Future research efforts may focus on implementing genetic approach that targets not only enhancement of 18:1OH synthesis, but also on increased 18:1OH acylation to TAG. These genes include *RcFAH12* or *PlFAH12*, *RcGPAT9*, *RcLPAT2*, *RcDGTAT2*, *RcPDCT*, *RcLPAT1–2*. Nevertheless, we have demonstrated that lesquerella can be engineered for large increases in 18:1OH levels from 0.4–0.5% in WT to a stable high level of 15–20% in transgenic seed oils.

Conflicts of interest

The authors declare no conflict of interest.

Notes/thanks/other declarations

Authors note that **Figure 1** was revised from the original publication listed in ref. [36]; **Figures 2** and **3** are cited from the original publication listed in ref. [21]; **Tables 1** and **2** are cited from the original publication listed in ref. [36].

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