TITLE

Castor Patatin-like Phospholipase A IIIβ Facilitates Removal of Hydroxy Fatty Acids from Phosphatidylcholine in Transgenic Arabidopsis Seeds

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KEY MESSAGE

Castor patatin-like phospholipase A IIIβ facilitates the exclusion of hydroxy fatty acids from phosphatidylcholine in developing transgenic Arabidopsis seeds.

ABSTRACT

Hydroxy fatty acids (HFAs) are industrial useful, but their major natural source castor contains toxic components. Although expressing a castor *OLEATE 12-HYDROXYLASE* in *Arabidopsis thaliana* leads to the synthesis of HFAs in seeds, a high proportion of the HFAs are retained in phosphatidylcholine (PC). Thus, the liberation of HFA from PC seems to be critical for obtaining HFA-enriched seed oils. Plant phospholipase A (PLA) catalyzes the hydrolysis of PC to release fatty acyl chains that can be subsequently channeled into triacylglycerol (TAG) synthesis or other metabolic pathways. To further our knowledge regarding the function of PLAs from HFA-producing plant species, two class III patatin-like PLA cDNAs (*pPLAIIIβ* or *pPLAIIIδ)* from castor or *Physaria fendleri* were overexpressed in a transgenic line of *A. thaliana* producing C18-HFA, respectively. Only the overexpression of *RcpPLAIIIβ* resulted in a significant reduction in seed HFA content with concomitant changes in fatty acid composition. Reductions in HFA content occurred in both PC and TAG indicating that HFAs released from PC were not incorporated into TAG. These results suggest that RcpPLAIIIβ may catalyze the removal of HFAs from PC in the developing seeds synthesizing these unusual fatty acids.

KEYWORDS

castor, hydroxy fatty acid, phosphatidylcholine, phospholipase A, *Physaria fendleri*, *Ricinus communis*

INTRODUCTION

Hydroxy fatty acids (HFAs), such as ricinoleic acid (12-OH 18:1Δ^{9cis}; hereafter 18:1-OH), contain a hydroxyl group (-OH) in their carbon chains, which provides unique properties for oleochemical applications. HFAs are used as feedstocks for production of high performance polymers, coatings, varnishes lubricants, cosmetics and surfactants. The major natural source of HFA is castor (*Ricinus communis*) seed oil, which contains 90% (w/w) of its fatty acids as ricinoleic acid and is an established renewable feedstock for the oleochemical industry (McKeon, 2016; Mutlu and Meier, 2010). The corresponding supply of castor oil, however, is limited by the presence of the toxin ricin and the allergenic 2S albumins in castor seeds, which are undesirable agronomic features. There are also difficulties in mechanical harvesting, and economic and political instability in castor-producing regions of the world (Lee et al., 2015).

An emerging oilseed crop producing high amount of HFAs is *Physaria* (synonym *Lesquerella*) *fendleri*. *P. fendleri* produces lesquerolic acid (13-OH 20:1Δ^{11cis}; hereafter 20:1-OH) at levels of 55 -60% in its seed oil, contains no toxic components and can grow in semi-arid regions (Chen, 2016; Chen et al., 2011a; Dierig et al., 2001). Although this plant is considered an emerging source of HFA, a number of agronomic issues relating to potential pests or diseases, suitable soil types, irrigation requirements and herbicide options make it challenging to adopt this plant as a commercial oilseed crop (Dierig et al., 2011). In addition, the major HFA of *Physaria* seed oil is two carbons longer than ricinoleic acid, impacting its potential applications. For instance, ricinoleic acid from castor oil is a unique renewable feedstock for the synthesis of the 10-carbon (C10) and C11 monomers sebacic acid and 11-aminoundecanoic acid, used in the synthesis of high performance polymers, plasticizers and lubricants (Ogunniyi 2006). Similar processing of 20:1-OH from physaria seed oil could produce C12 and C13 monomers. Although not able to directly substitute for castor oil derivatives, these may have value as novel bio-based monomers for industry.

The metabolic engineering of established temperate oil crops for HFA production therefore has the potential to be a feasible strategy to overcome the limitations associated with natural sources of HFA. Numerous studies, however, have shown that it is challenging to obtain a substantial level of HFAs in the seed oils of these engineered crops (for reviews, see Jaworski and Cahoon, 2003; Vanhercke et al., 2013; Lee et al., 2015; Singer and Weselake, 2018). A deeper understanding of HFA biosynthesis and regulation is required to overcome this obstacle.

In developing seeds of castor and *P. fendleri*, 18:1-OH is synthesized from oleic acid (18:1Δ^{9cis}; hereafter 18:1) on the *sn*-2 position of phosphatidylcholine (PC) in the endoplasmic reticulum (ER) catalyzed by Δ-12 fatty acid hydroxylase (FAH12) (van de Loo et al., 1995; Lee et al., 2015). *FAH12* genes have been isolated from a number of sources including castor (van de Loo et al., 1995), *P. fendleri* (Broun et al., 1998), *P. lindheimeri* (Dauk et al., 2007) and the fungus *Claviceps purpurea* (Meesapyodsuk and Qiu, 2008). The *P. fendleri* FAH12 has been shown to be a bifunctional FAH12 also possessing desaturase activity (Broun et al., 1998) whereas *P. lindheimeri* FAH12 has been shown to mainly exhibit FAH12 activity (Dauk et al., 2007). Heterologous expression of the various *FAH12* cDNAs in *Arabidopsis thaliana* (hereafter Arabidopsis) and/or mutants of Arabidopsis defective in ER fatty acid elongation activity and/or fatty acid desaturase activity resulted in a HFA content no higher than about 25% (Broun and Sommerville, 1997; Broun et al., 1998; Smith et al., 2003, Kumar et al., 2006, Lu et al., 2006; Meesapyodsuk and Qiu, 2008). Ongoing studies are now suggesting that castor and *Physaria* spp. have evolved efficient mechanisms for routing of HFAs from their site of synthesis on PC to triacylglycerol (TAG), which do not operate efficiently in plants engineered for HFA production (Millar et al., 2000; Jaworski and Cahoon, 2003; Brown et al., 2012; Vanhercke et al., 2013; Kim and Chen, 2015; Lee et al., 2015; Horn et al., 2016). Thus, additional metabolic engineering interventions, beyond the introduction of FAH12 from an HFA-producing species, are required to promote further increases in the HFA content of Arabidopsis and oil crops that do not naturally produce HFA.

Within the last two decades, metabolic engineering interventions have achieved only modest gains in HFA content beyond 25% (Burgal et al., 2008; van Erp et al., 2011; Kim et al., 2011; Hu et al., 2012; van Erp et al., 2015; Adhikari et al., 2016; Lunn et al., 2018a). These studies have included the introduction of castor diacylglycerol acyltransferase (DGAT) 2 and/or castor phospholipid:diacylglycerol acyltransferase (PDAT)1 into the *fae1* mutant line of Arabidopsis (Kunst et al., 1992) coexpressing *RcFAH12* to achieve levels of HFA as high as 30% (Burgal et al., 2008; van Erp et al., 2011; Kim et al., 2011). DGAT catalyzes the acylation of *sn*-1,2-diacylglycerol (DAG) to form TAG using acyl-CoA as an acyl donor whereas PDAT catalyzes the transfer of an acyl chain from the *sn*-2 position of PC to DAG to form TAG (for reviews, see Liu et al., 2012; Xu et al., 2018). Metabolic engineering is also being explored to overcome the

poor seedling development observed for Arabidopsis engineered to produce HFA-containing seed oil (Lunn et al., 2018b).

During seed development in oleaginous plants, acyl-exchange between the *sn*-2 position of modified PC and plastidially derived acyl-CoA (mainly 18:1-CoA) is catalyzed by the combined forward and reverse reactions catalyzed by lysophosphatidylcholine acyltransferase (LPCAT) (Yurchenko et al., 2009; Bates et al., 2012; Lager et al., 2013; for reviews, see Bates et al., 2013, Chen et al., 2015). In the forward reaction, LPCAT catalyzes the acyl-CoA-dependent acylation of *sn*-1 lysophosphatidylcholine (LPC) to generate PC whereas in the reverse reaction, the enzyme catalyzes deacylation at the *sn*-2 position of PC to generate acyl-CoA and LPC. Thus, LPCAT-mediated acyl-exchange represents a mechanism for enriching the acyl-CoA pool in modified fatty acids (such as HFA) produced on PC. Ricinoleoyl-CoA would then be available for use in TAG biosynthesis by the acyltransferases of the Kennedy (1961) pathway (Bafor et al., 1991). Furthermore, LPC can also result from the deacylation of PC catalyzed by PDAT (Dahlqvist et al., 2000; Xu et al., 2018).

In contrast, a traditional Lands (1960) cycle involves the deacylation of PC catalyzed by phospholipase A_2 (PLA_2) acting at the *sn*-2 position of PC followed by activation of released fatty acid to acyl-CoA via the action of long chain acyl-CoA synthetase (LACS) and reacylation of LPC catalyzed by the forward reaction of LPCAT (for a review, see Chen et al., 2015). There are three families of PLA, categorized bythe position of the cleaved fatty acid. PLA_1 and PLA_2 attack the *sn*-1 and *sn*-2 positions of phospholipids, respectively, whereas patatin-like PLAs display activity at both positions (for reviews, see Chen et al., 2011b; Chen et al., 2013). Metabolic studies with the Arabidopsis double mutant *lpcat1 lpcat2-2* have suggested that PLA_2 action may also play a role in the deacylation of PC during TAG accumulation, especially where LPCAT activity has become compromised (Wang et al., 2012). Early biochemical studies with microsomes prepared from endosperm of developing castor seed have indicated the presence of PLA_2 activity which is selective for releasing ricinoleic acid from the *sn*-2 position of PC (Bafor et al., 1991; Ståhl et al., 1995; Lin et al., 1998). Recently, Bayon et al. (2015) isolated the most highly expressed *PLA*, *RcPLA$_2\alpha$*, from the developing castor endosperm and demonstrated that the recombinant enzyme selectively released HFA from PC *in vitro*. Interestingly, they found a dramatic decrease in HFA accumulation in both PC and TAG in seed oils of *fae1* Arabidopsis coexpressing *RcFAH12* and *RcPLA$_2\alpha$*. These results suggested that RcPLA$_2\alpha$ may specifically catalyze the release of HFA

from HFA-PC but the released HFAs may have undergone β-oxidation in the peroxisome and thus were not incorporated into TAG (Bayon et al., 2015; Moire et al 2004).

As of yet, little is known concerning the function of class III patatin-like PLAs (pPLAIII) in the release of modified fatty acids from PC, although the Arabidopsis *pPLAIIIβ* gene has been shown to be involved in glycerolipid biosynthesis in vegetative tissues (Li et al., 2013). Similarly, the seed-specific overexpression of the Arabidopsis *pPLAIIIδ* cDNA has been found to increase seed oil content in both Arabidopsis and *Camelina sativa* (Li et al., 2013; Li et al., 2015), and shown to alter the levels of C20 and C22 fatty acids in seed oil (Li et al., 2013). The homologs of *AtpPLAIIIβ* and *AtpPLAIIIδ* have been identified in castor and *P. fendleri*. In order to elucidate the function of *pPLAIII* genes in the context of their role in the release of HFA from PC, we assessed the effect of over-expressing two castor cDNAs (*RcpPLAIIIβ* and *RcpPLAIIIδ*) and the two *P. fendleri* homologs (*PfpPLAIIIβ* and *PfpPLAIIIδ*) in transgenic Arabidopsis co-expressing *RcFAH12*. Intriguingly, our results showed that only RcpPLAIIIβ was effective in facilitating the *in vivo* release of HFA from PC in seeds.

MATERIALS AND METHODS

Plant growth conditions

Arabidopsis seeds were planted in soil, cold-treated at 4 ºC in the dark for 3 d, and then placed in a growth chamber at 22 ºC. Arabidopsis plants were cultivated with a photoperiod of 18h day/6h night cycle and a light intensity of 250 μmol M^{-2} S^{-1} with regular watering and fertilization.

Isolation of *pPLAIIIs* from Arabidopsis, castor and *P. fendleri*

Arabidopsis total RNA was extracted from developing siliques (Col-0 background) harvested 18 days after flowering using the RNeasy Mini Kit (Qiagen Canada Inc, Toronto, Ontario). First-strand cDNA synthesis was carried out using the QuantiTect Reverse Transcription Kit according to the manufacturer's instructions (Qiagen). The same methods were used to extract total RNA from *P. fendleri* developing seeds and synthesize the first-strand cDNA. Full-length *pPLAIII* coding sequences were isolated using *Phusion* polymerase (Invitrogen) with the primers listed in Table S1. Arabidopsis and *P. fendleri* cDNA isolated in this study were used as template, whereas an endosperm cDNA library generated previously (Chen et al., 2004) was used as the

template in the case of the castor *pPLAIII* coding sequences. Thermal cycling parameters were as follows: 98 ºC for 30 s, 35 cycles of 98 ºC for 8 s, 63 ºC (*AtpPLAIIIδ* and *RcpPLAIIIδ*)/65 ºC (*AtpPLAIIIα*)/66 ºC (*RcpPLAIIIβ*) for 20 s, and 72 ºC for 1 min; and 72 ºC for 10min. The resulting amplicons were cloned into pCR^{TM}2.1-TOPO vectors (Invitrogen) and sequenced to confirm their identities.

pPLAIII sequence alignment and phylogenetic analysis

Amino acid sequences of the Arabidopsis, castor and *P. fendleri* pPLAIIIs were deduced from their corresponding cDNA sequences and aligned using the progressive pairwise alignment method in Geneious Pro 5.4.6 (Biomatters Inc, Auckland New Zealand). Phylogenetic analysis was carried out using the Geneious Tree Builder program, where clustering analysis was performed using the Unweighted Pair Group Method with Arithmetic Mean and the branch length was calculated using the "Jukes Cantor" genetic distance model.

Generation of transgenic Arabidopsis lines

Six binary vectors including seed-specific expression cassettes bearing the coding sequences of *AtpPLAIIIα*, *AtpPLAIIIδ*, *RcpPLAIIIβ*, *RcpPLAIIIδ*, *PfpPLAIIIβ* and *PfpPLAIIIδ,* respectively, were generated. The vectors were produced by first amplifying the full-length coding sequence of each gene using primers containing restriction sites near their 5` ends (Table S1), and inserting the resulting fragments between the seed-specific napin promoter and NOS terminator within a modified version of the pPZP-RCS1 binary vector (Goderis et al., 2002) which contains an *NPTII* selection cassette (Mietkiewska et al., 2014).

Vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 via electroporation and the resulting recombinant bacteria were used for the transformation of Arabidopsis CL7 using the floral dip method (Clough and Bent, 1998). The presence of target constructs in transgenic plants was confirmed by PCR (data not shown). Transgenic homozygous lines containing a single copy of the *pPLAIII* cassette were identified using segregation analysis (Bent 2006; Mietkiewska et al., 2014). For every experiment, transgenic experimental lines were grown in the same growth chamber at the same time as the CL7 line (control).

Analysis of gene expression in developing seeds of transgenic Arabidopsis using quantitative real-time PCR

Developing siliques were harvested from transgenic and untransformed CL7 plants 14 days after flowering. Quantitative real-time (RT)-PCR analysis was performed as reported previously (Chen et al., 2012).Briefly, total RNA extraction and first-strand cDNA synthesis were carried out as described in a previous section. Quantitative RT-PCR assays were performed using SYBR green PCR master mix (Invitrogen) on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) with the primers listed in Table S1. The relative expression ($2^{-\Delta CT}$) of the *pPLAIII* transcripts were calculated based on the expression level of the constitutively expressed 18s rRNA cDNA with the SDS v2.4 software (Applied Biosystems).

Determination of the total acyl lipid content and fatty acid composition of mature seeds

Total lipid extractions were carried out as described previously (Mietkiewska et al., 2014). Briefly, approximately 10 mg of dried T_2, T_3 or CL7 seeds were weighed and placed in Teflon-lined screw capped glass tubes, and 100 ng of internal standard (C17:0 TAG) was added to each tube and dried under nitrogen gas. For transmethylation, 2 ml of 3N methanolic HCl was added to the mixture, followed by incubation for 16 h at 80℃. After cooling, 2 mL of 0.9% NaCl was added to the reactions and fatty acid methyl esters (FAMEs) were extracted twice with hexane. Pooled extracts were dried completely under nitrogen gas at 37℃ and dissolved in 1 mL of iso-octane. The samples were analyzed by gas chromatography -mass spectrometry equipped with a DB-23 capillary column (Agilent Technologies: 30 m × 250 μm × 0.25 μm) and calculated from FAME peak areas and the established response factor justification form (Mietkiewska et al., 2014). The temperature program was 165℃ for 4 min, 165-180℃ for 5 min, and 180-230℃ for 5 min.

Determination of fatty acid composition of triacylglycerol and phosphatidylcholine isolated from mature seeds

Approximately 50 mg of dried T_2, T_3 or CL7 seeds were weighed and place into hexane-rinsed teflon-lined screw cap glass tubes. Following the addition of 3 mL of chloroform/methanol (2:1, v/v), the seeds were homogenized in a blender and an additional 3 mL of chloroform/methanol (2:1, v/v) were added. The mixture was vortexed vigorously for 1 min, and 2 mL of 0.9% NaCl were added and vortexed for 20 s to extract lipids. The chloroform phase (lower layer) was transferred to a fresh glass tube and 4 mL of chloroform were added to the original

mixture for a second extraction. Lipids were recovered from the chloroform by drying under nitrogen and dissolving in 80 μL of chloroform (Mietkiewska et al., 2014).

Total extracted lipids were then separated on TLC plate in two different solvent systems. The TLC plate was developed in chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:2, v/v/v/v/v) until the solvent front was halfway up the plate. After drying, the plate was further developed using hexane/ether/acetic acid (65:35:2, v/v/v) until the solvent line was approximately 1 cm from the top of the plate (Mietkiewska et al., 2014). TAG and phospholipid (PL) bands were visualized by spraying with 0.05% primuline (w/v) in acetone/water (80:20, v/v). Direct methylation was performed on TAG and PC that had been scraped from the TLC plate by incubating in 2 mL of 3N methanolic HCl at 80ºC for 1 h. Subsequently, 2 mL of 0.9% NaCl were added to the reaction and the FAMEs were extracted with 4 mL of hexane twice and dried under nitrogen. FAMEs produced from TAG or PC were dissolved in iso-octane and analyzed as described above.

RESULTS

Phylogenetic characterization of pPLAIIIs and selection of cDNAs for overexpression in transgenic Arabidopsis

While four *pPLAIII* (α, β, γ, δ) genes have been identified in Arabidopsis (Li et al., 2011), only three candidate *pPLAIIIs* (β, γ, δ) have been found to be expressed in the developing endosperm of castor (Brown et al., 2012; Bayon et al., 2015). Similarly, only two *P. fendleri pPLAIII* transcripts (*PfpPLAIIIβ* and *PfpPLAIIIδ*) were identified in a cDNA library derived from developing seeds (unpublished data). Since only β and δ *pPLAIII* transcripts affected lipid biosynthesis in Arabidopsis and were identified in *P. fendleri* developing seeds, we focused on homologs of these two genes throughout the remainder of the study.

The deduced amino acid sequences of the pPLAIIIs were compared. As shown in Fig. 1A, RcpPLAIIIβ is closely related to both AtpPLAIIIα and AtpPLAIIIβ, sharing 71% identity with both Arabidopsis counterparts; PfpPLAIIIβ exhibits 87% identity with AtpPLAIIIβ and 74% with AtpPLAIIIα (Fig. 1A). The identity of RcpPLAIIIβ and PfpPLAIIIβ is 72%. On the other hand, RcpPLAIIIδ and PfpPLAIIIδ display 73% and 89% identity with AtpPLAIIIδ, respectively, whereas the identity between RcpPLAIIIδ and PfpPLAIIIδ is 73% (Fig 1B). The subsequent phylogenetic analysis confirmed that RcpPLAIIIβ, RcpPLAIIIδ, PfpPLAIIIβ and PfpPLAIIIδ are

members of the pPLAIII family. In addition, AtpPLAIIIα, AtpPLAIIIβ, RcpPLAIIIβ and PfpPLAIIIβ are closely related, and RcpPLAIIIβ is equally related to both AtpPLAIIIα and AtpPLAIIIβ (Fig. 1A, Fig. 2).

Generation of transgenic Arabidopsis CL7 lines over-expressing *pPLAIII* cDNAs

The coding sequences representing the pPLAIIIs from castor and *P. fendleri* were overexpressed using the seed-specific napin promoter in the Arabidopsis CL7 line (van Erp et al., 2011; Lu et al., 2006), which expresses *RcFAH12* in a *fae1* background. Since AtpPLAIIIα, AtpPLAIIIβ, RcpPLAIIIβ and PfpPLAIIIβ are in the same group and RcpPLAIIIβ is equally related to both AtpPLAIIIα and AtpPLAIIIβ (Fig. 1A, Fig. 2), *AtpPLAIIIα* and *AtpPLAIIIδ* were selected at the control in the generation of transgenic Arabidopsis CL7 lines over-expressing *pPLAIIIs*. The resulting transgenic plants were designated as AtpPLAIIIα-CL7, AtpPLAIIIδ-CL7, RcpPLAIIIβ-CL7, RcpPLAIIIδ-CL7, PfpPLAIIIβ-CL7 and PfpPLAIIIδ-CL7, respectively. Quantitative qRT-PCR was utilized to assess the relative expression levels of each transgene in developing T_2 seeds from two independent lines of each transformant compared with two CL7 control lines (Fig. 3). Variable levels of gene expression were observed for each transformant among the three independent lines evaluated. As expected, both *AtpPLAIIIα* and *AtpPLAIIIδ* were found to be expressed in the developing seeds of CL7 lines because of endogenous gene expression (Fig. 3).

Expression of *RcpPLAIIIβ* Decreases Hydroxy Fatty Acid Content in Arabidopsis CL7

The HFA content of oil extracted from the mature T_2 seeds of 20 independent lines of RcpPLAIIIβ-CL7 and RcpPLAIIIδ-CL7 is shown in Fig. 4A. *RcpPLAIIIβ* expression resulted in an average HFA content of about 12.9%, which was 17% lower than the average HFA content of CL7 alone. In contrast, overexpression of *RcpPLAIIIδ* in CL7 had no effect on HFA content of the seed oil. The oil contents of RcpPLAIIIβ-CL7 seed and RcpPLAIIIδ-CL7 seed were not significantly different from that of CL7 seed (Fig. 4B).

The fatty acid compositions of the oil extracted from T_2 seeds of CL7, RcpPLAIIIβ-CL7 and RcpPLAIIIδ-CL7 are shown in Table 1. Although the fatty acid composition of the oils from CL7 and RcpPLAIIIδ were similar, there were substantial differences in the fatty acid composition of the oil from RcpPLAIIIβ-CL7. Significant changes were noted for 18:0 (stearic acid), 18:1, 18:2

(linoleic acid, 18:2$\Delta^{9cis,12cis}$; hereafter 18:2), 18:3 (α-linolenic acid, 18:3$\Delta^{9cis,12cis,15cis}$), 18:1-OH and 18:2-OH (densipolic acid, 12-OH 18:2$\Delta^{9cis,15cis}$) when compared to CL7.

To obtain further evidence that the decrease in HFA content was indeed caused by the expression of *RcpPLAIIIβ*, T_3 seeds from five homozygous lines containing a single *RcpPLAIIIβ* transgene insert and exhibiting reduced HFA content were subjected to further seed oil analysis. In line with the results obtained using T_2 seeds, the mean HFA content in the total seed oil of T_3 RcpPLAIIIβ-CL7 lines was 10.2%, which was significantly reduced compared to 13.9% seen in CL7 plants (26.6% relative reduction; Fig. 5). All RcpPLAIIIβ-CL7 lines tested displayed significant reductions in at least one HFA compared to CL7, and lines 1-4 exhibited a significant decrease in total HFA levels (Table S2). Lines 2 and 3 displayed the most substantial reduction in average HFA content among the five lines tested relative to CL7 (47% and 38% relative reductions, respectively), with a few plants possessing only 5 - 6% HFA in their seeds. Significant variations in the proportions of other fatty acids, such as 18:0, 18:2 and 18:3 were also observed in the seed oil of T_3 RcpPLAIIIβ-CL7 lines compared to CL7, and generally corresponded to changes noted in T_2 lines (Table S2).

Over-expression of *RcpPLAIIIβ* in CL7 Arabidopsis reduces HFA levels in both PC and TAG

In order to elucidate the basis for the reduction in HFA levels seen in the total seed oil of RcpPLAIIIβ-CL7 lines, TAG and PC were separated and isolated from the seed oil of four T_3 RcpPLAIIIβ-CL7 line 2 plants (exhibiting the most substantial reduction in HFA content in total seed oil), as well as untransformed CL7 controls. TAG from RcpPLAIIIβ-CL7-line 2 plants contained an average HFA content of 4.5% compared to 14.6% in CL7 lines, which represents a relative decrease of 69.2% (Fig, 6). Similarly, the average HFA content of PC in RcpPLAIIIβ-CL7-line 2 plants was 3.0% compared to 8.0% in CL7 lines, which represents a 62.5% relative decrease in HFA content (Fig. 6). Significant decrease of HFA of 46.7% was also observed in the whole seeds of RcpPLAIIIβ-CL7 line 2 plants, which was 7.4% compared to 13.9% in the CL7 line (Table S2).

Overexpression of *AtpPLAIIIα*, *AtpPLAIIIδ*, *PfpPLAIIIβ* or *PfpPLAIIIδ* in Arabidopsis CL7 does not change hydroxy fatty acid content

Overexpression of either *AtpPLAIIIα* or AtpPLAIIIδ in Arabidopsis CL7 did not lead to significant changes in total HFA contents compared with the CL7 control (Fig. 7A; Table 1). AtpPLAIIIα-CL7, however, exhibited a significant decrease in seed oil content compared to CL7 (27.5% for AtpPLAIIIα-CL7 versus 29.5% for CL7), whereas the seed oil content of AtpPLAIIIδ-CL7 was not significantly different from CL7(Fig. 7B). In general, the overall fatty acid composition of oil from AtpPLAIIIα-CL7 or AtpPLAIIIδ-CL7 was similar to CL7.

PfpPLAIIIβ-CL7 and PfpPLAIIIδ-CL7 contained, on average, about 16.3% and 16.7% HFA in the seed oil, respectively, which was not significantly different from the average of 15.6% HFA content observed for CL7 (Fig. 8A). In addition, all PfpPLAIIIβ-CL7 seed samples had HFA contents within the range of CL7 HFA content (mostly from 12.9% to 18.6%), except for a single seed sample which displayed nearly 24% HFA content in the seed oil (Fig. 8A). Similarly, the seed oil content of either PfpPLAIIIβ-CL7 or PfpPLAIIIδ-CL7 was not significantly different from CL7 lines (Fig. 8B). As for fatty acid composition in seed oil, either PfpPLAIIIβ-CL7 or PfpPLAIIIδ-CL7 was similar to CL7, though there was a statistically significant but slight decrease in the proportion of 18:1 for PfpPLAIIIβ-CL7 and 18:2 for PfpPLAIIIδ-CL7 (Table 1).

DISCUSSION

Due to the value of HFAs and the difficulties associated with the large-scale agronomic production of plants that produce HFAs in a native context, many attempts have been carried out to elicit the production of high levels of HFAs in plants that do not normally produce them. RcFAH12 catalyzes the hydroxylation of oleoyl moieties esterified to the *sn*-2 position of PC to generate ricinoleoyl PC (Lee et al., 2015). Transgenic Arabidopsis expressing *RcFAH12* (line CL7) contains twice as much HFA in PC as castor, suggesting that removal of HFA from PC represents a bottleneck for production of HFAs in Arabidopsis CL7 (van Erp et al., 2011). Plant pPLAs catalyze the liberation of fatty acids from both the *sn*-1 and *sn*-2 positions of phospholipids and other glycerolipids (Chen et al., 2011b; Chen et al., 2013). When *RcpPLAIIIβ* was expressed in CL7, the HFA content of PC was reduced to 3% from 8% in CL7 (Fig. 6). In addition, 18:2 and 18:3 levels were elevated while the level of 18:1 tended to decrease. It has been proposed that the buildup of HFAs in PC might inhibit the activity of endogenous FAD2 enzymes in Arabidopsis, causing decreases in 18:2 and 18:3 and an increase in 18:1 (Thomæus et al., 2001; Smith et al.,

2003). Therefore, *RcpPLAIIIβ* might represent a PLA that selectively catalyzes the liberation of HFAs from the site of synthesis and partially alleviates the putative inhibition of FAD2 activity. We cannot rule out the possibility, however, that *RcpPLAIIIβ* is very effective in catalyzing the removal of all fatty acids including regular and hydroxyl ones from PC but the transgenic plants lack the ability to efficiently use HFA in TAG assembly. Detailed enzymatic analysis of the recombinant PLAIIIs, especially their specificity and selectivity towards HFA-PC, and the change of related FAD2 activities would provide additional insight. *RcPLA$_2$α*, another *PLA* expressed in the developing castor bean endosperm, has recently been reported to cause comparable results in transgenic *fae1* Arabidopsis plants overexpressing *RcFAH12* (Bayon et al., 2015). Collectively, the results of the current study and that of the study conducted by Bayon et al. (2015) suggest that castor may utilize a PLA-mediated acyl editing process to help remove HFAs from PC followed by activation of the released HFA to hydroxy acyl-CoA via the action of LACS in support of hydroxy-TAG synthesis, and more than one PLA appears to be involved in this pathway. In addition, ricinoleoyl-CoA may also be generated by the reverse reaction catalyzed by LPCAT in castor, which would then make HFA-CoA available for TAG biosynthesis via the Kennedy pathway (Lager et al., 2013).

Castor seed produces a high level of HFAs in the seed oil and nearly 70% of TAG molecular species are in the form of tririncinoleoylglycerol (Lin et al., 2003). This requires not only the efficient release of HFAs from PC but also the efficient incorporation of HFAs into the three positions of the glycerol backbone of TAG (Bates and Browse, 2012). Arabidopsis, however, appears to lack the processes required for efficient incorporation of HFAs into TAG (Bayon et al., 2015). After being removed from PC through PLA action, released HFAs might have been degraded via β-oxidation in the peroxisome (Bayon et al., 2015). The possibility that HFA-CoA is hydrolyzed via the catalytic action of RcpPLAIIIβ and thus unavailable for TAG synthesis cannot be ruled out. Indeed, Arabidopsis pPLAIIIs have been shown to exhibit thioesterase activity (Li et al., 2011; Li et al., 2013). In this scenario, free HFAs may have been channeled into peroxisomes for degradation via the ABCD1-independent pathway (de Marcos Lousa et al., 2013).

Arabidopsis uses PC-derived DAG as the major source of acyl chains for TAG production (Bates and Browse, 2011) with phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) playing a major role in catalyzing the removal of the phosphocholine head group from polyunsaturated-enriched DAG and transferring the polar head group to 18:1-enriched DAG produced *de novo* in the Kennedy pathway (Lu et al., 2009). Similarly, RcPDCT was shown to be

necessary for effective HFA accumulation in TAG in *fae1* Arabidopsis overexpressing *RcFAH12* or *RcFAH12* combined with *RcDGAT2* overexpression (Hu et al., 2012). In Arabidopsis overexpressing *RcFAH12*, however, half of the TAG molecules were mono-HFA-TAG, with 70% of HFAs located in the *sn*-2 position (Burgal et al. 2008). Most of these TAG molecules were synthesized from the PC-derived mono(*sn*-2)-HFA-DAG molecules (van Erp et al., 2011; Bates and Browse, 2011). The selective removal of HFAs from the *sn*-2 position of PC may result in a decrease in mono (*sn*-2)-HFA-DAG for TAG synthesis. The released HFAs, however, were likely not incorporated efficiently into TAG. Thus, both the inability to channel released free HFA into TAG and the possibly reduced level of HFA-enriched DAG may explain why HFA content was substantially reduced in the RcpPLAIIIβ-CL7 lines compared with Arabidopsis CL7 (see Fig. 5).

A possible approach to increase the HFA content of TAG in RcpPLAIIIβ-CL7 might involve coexpressing a cDNA encoding a LACS enzyme that specifically activates HFA to HFA-CoA. Coexpression of three different castor *LACSs* (orthologs of *AtLACS1*, *AtLACS4* or *AtLACS8*) with *RcPLA$_2\alpha$* in transgenic *fae1* Arabidopsis plants overexpressing *RcFAH12*, however, failed to promote the HFA accumulation in TAG (Bayon et al., 2015). *RcLACS9* has been shown to be the mostly highly expressed *LACS* in the developing castor endosperm (Brown et al., 2012), while its ortholog appeared to be involved in TAG metabolism in Arabidopsis (Jessen et al., 2015). Therefore, it may be worthwhile expressing *RcLACS9* in RcpPLAIIIβ-CL7. Interestingly, recombinant microsomal RcLACS2, produced in yeast, exhibited an enhanced specificity for 18:1-OH compared to several other fatty acids (He et al., 2007). In addition, the highest expression of *RcLACS2* occurred during seed germination. Sequence analysis of RcLACS2 indicated similarity to the peroxisomal AtLACS6 and the presence of a type 1 peroxisomal targeting sequence. Thus, it might be useful to implement a metabolic engineering strategy that results in the substantial production of extra-peroxisomal RcLACS2 during seed development in RcpPLAIIIβ-CL7.

Another potential approach would be to overexpress cDNAs encoding acyltransferases of Kennedy pathway specific for HFA-CoA in RcpPLAIIIβ-CL7. In developing castor seed, TAG formation involves the catalytic action of RcDGAT1 and RcDGAT2 (Kroon et al., 2006; McKeon and He, 2015). It has been shown that RcDGAT2 effectively uses diricinoleoylgylcerols or ricinoleoyl-CoAs as substrates (Kroon et al., 2006; Burgal et al., 2008) while RcDGAT1 utilizes dricinoleoylglycerol more effectively than AtDGAT1 (McKeon and He, 2015). When castor *LYSOPHOSPHATIDIC ACID ACYLTRANSFERASE* (*LPAAT*) *2* was overexpressed in a seed-specific fashion in *P. fendleri*, ricinoleic acid incorporation at the *sn*-2 position of TAG increased

from 2% to 17%, and tri-HFA-TAG levels increased from 5% to almost 14% (Chen et al., 2016). Operating within the Kennedy pathway, LPAAT catalyzes the acyl-CoA-dependent acylation of lysophosphatidic acid to produce phosphatidic acid (Kennedy, 1961; Jayawardhane et al., 2018). The absence of *de novo* di-HFA-DAG in Arabidopsis hosting RcFAH12 may be due to the activity of endogenous LPAAT which excludes HFA-LPA as an HFA acceptor (Bates and Browse, 2011). Recently, GPAT9, which catalyzes the production of lysophosphatidic acid from *sn*-glycerol-3-phosphate, was shown to be involved in the Kennedy pathway leading to intracellular TAG in Arabidopsis (Shockey et al., 2016; Singer et al., 2016). A recent genome wide analysis of the *GPAT* gene family identified one *GPAT9* ortholog in castor (Waschburger et al., 2018). A recent genome wide analysis of the *GPAT* gene family identified one *GPAT9* ortholog in castor (Waschburger et al., 2018). A recent study by Shockey et al. (2019) demonstrated that the overexpression of *RcLPAAT2* and *RcDGAT2* in Arabidopsis CL37 seeds (which express RcFAH12) results in a significant increase of HFA in T_2 populations. Although the overexpression of *RcGPAT9* with *RcLPAAT2* and *RcDGAT2* did not increase HFA content in seeds, coexpression of these cDNAs significantly increased the HFA content of TAG (Shockey et al., 2019). Another recent study indicated that the overexpression of *RcGPAT9* with *RcLPAAT2* and *RcPDAT* significantly increased the HFA content in Arabidopsis CL37 seeds when compared to overexpression of *RcLPAAT2* and *RcPDAT* (Lunn et al., 2019). Collectively, these recent studies indicated that RcGPAT9 and other acyltransferases play important roles in channeling HFA into TAG. Multiple interventions may be required to bring about an elevated level of HFA content in the TAG of RcpPLAIIIβ-CL7. This may require the combined heterologous introduction of forms of LACS, GPAT9*,* LPAAT2, phosphatidic acid phosphatase (PAP) and/or DGAT with demonstrated enhanced specificity and selectivity for substrates containing HFA. Within the Kennedy pathway, PAP catalyzes the removal of inorganic phosphate from phosphatidic acid prior to the final acylation catalyzed by DGAT (Kennedy 1961, Chen et al., 2015). DGATs from castor and/or *C. purpurea* (Burgal et al., 2008; Mavraganis et al., 2010; McKeon and He, 2015) may prove useful in these types of metabolic engineering proof-of-concept studies.

In the current study, six *pPLAIII* cDNAs were screened for their impact on HFA accumulation in Arabidopsis expressing *RcFAH12*. *RcpPLAIIIβ* was the only *pPLAIII* cDNA that resulted in a reduction of HFAs in PC when expressed in Arabidopsis CL7. In contrast to RcpPLAIIIβ, which could efficiently catalyze the hydrolysis of PC to release HFAs, two pPLAIII enzymes from the *P. fendleri*, PfpPLAIIIβ and PfpPLAIIIδ, did not affect HFA accumulation

when the encoding cDNAs were expressed in Arabidopsis CL7. It is possible that pPLAIIIs do not play a role in the selective release of HFAs from *sn*-2 position of PC in *P. fendleri*. The slight decrease in C18 normally occurring fatty acids in PfpPLAIIIβ-CL7 or PfpPLAIIIδ-CL7 lines may simply be due to the increased pPLAIII activities, since the AtpPLAIIIs have been reported to promote the accumulation of C20 fatty acids at the expense of C18 fatty acids in Arabidopsis (Li et al., 2011; 2013).

A notable difference from castor, which only produces ricinoleic acid, is that *P. fendleri* is known to produce C20 HFAs through elongation of C18 HFAs in the form of acyl-CoA catalyzed by a fatty acid condensing enzyme 3-ketoacyl-CoA synthase 3 (LfKCS3) (Moon et al., 2001). The expression of *LfKCS3* in *C. sativa* led to both an increase in total HFA content and accumulation of C20 HFAs, together with less HFAs retained in PC (Snapp et al., 2014). Interestingly, even though no PLAs specific to HFAs were introduced into the transgenic host, HFAs were removed from PC with high efficiency. The phenomenon implied that efficient routing of HFAs from PC to the acyl-CoA pool in *P. fendleri* may be associated with acyl editing acting in concert with acyl-CoA modification. Thus, it cannot be ruled out that PfpPLAIIIs are responsible for the selective removal of HFAs from the site of synthesis in the natural source plant where cooperation with LfKCS3 might also be required. It may be useful to conduct substrate specificity and selectivity studies with recombinant PfpPLAIIIs to gain more insight into the enzyme's ability to utilize *sn*-2 HFA-PC. In addition, seed-specific overexpression of *LfKCS3* in RcpPLAIIIβ-CL7 may promote HFA accumulation in seed TAG. This intervention, however, would potentially result in the seed oil containing C18 C20 HFAs, limiting its industrial usefulness compared to an oil containing a single type of HFA.

AtpPLAIIIα, the ortholog of *RcpPLAIIIβ*, also did not affect the HFA content of the seed oil when overexpressed in CL7. The HFA content did not change despite a slight decrease in 18:2-OH content (from 3.1% to 2.6%). The seed oil content of CL7 overexpressing *AtpPLAIIIα*, however, was somewhat compromised (see Fig. 7B). This observation suggested that AtpPLAIIIα may play a role in oil synthesis in Arabidopsis which may be associated with the release of other fatty acids from PC.

Expression of *RcFAH12* has been shown to result in seed oil content reduction in Arabidopsis that may be due to the post-translational down-regulation of *de novo* fatty acid synthesis induced by the inefficient glycerolipid synthesis (Bates et al., 2014). The overexpression of *AtpPLAIIIδ* elevated seed oil accumulation in wild-type Arabidopsis or *C. sativa* (Li et al.,

2013; 2015). In the current study, however, overexpression of *RcpPLAIIIδ, AtpPLAIIIδ* or *PfpPLAIIIδ* did not result in increases in seed oil content (see Figs. 4, 7 and 8). In addition, the expression of *pPLAIII*s in the HFA-producing Arabidopsis CL7 line led to differences in seed lipid profiles and other phenotypes compared with previous studies of *pPLAIII* overexpression in plants (Table S3). The expression of *RcpPLAIIIβ* decreased the contents of HFA and 18:0 but increased the contents of 18:2 and 18:3 compared with Arabidopsis CL7. The overexpression of *AtpPLAIIIα* caused a significant decrease in seed oil content and a slight decrease in 18:2-OH content compared to CL7. The contents of other fatty acids were not significantly affected and no obvious change in seed phenotypes were observed. On the contrary, the overexpression of *AtpPLAIIIδ* in wild-type Arabidopsis and camelina resulted in increased seed oil content, decreased C18 fatty acids and increased C20 and C22 fatty acids. In addition, the overexpression of *pPLAIIIα,β,δs* in Arabidopsis and other plants generally resulted in shorter tissues and shorter but wider seeds (Dong et al. 2014; Li et al. 2011; 2013; 2015; Liu et al. 2015; Table S3).

It is possible that these pPLAIIIδ may not be selective for HFA-containing substrates and were not able to relieve the turnover of HFA-containing DAG that may be responsible for impaired *de novo* fatty acid synthesis (Bates et al., 2014). Nevertheless, the difference in seed lipid profile and plant morphology phenotype may also due to other factors such as differences in the abundance of recombinant pPLAIII proteins or their limited activities. In this study, the coding sequences of all six *pPLAIII*s were individually cloned into the same binary vector backbone using the same seed-specific promoter, and the expression of the *pPLAIII* genes in transgenic Arabidopsis plants was confirmed using qRT-PCR (Fig. 3). The different lipid profiles in the Arabidopsis lines hosting *RcpPLAIIIβ* and *AtpPLAIIIα*, respectively, indicate that these two PLAIIIs were produced and active in the transgenic plants. On the contrary, the overexpression of the other four *PLAIII*s did not significantly affect seed oil content or fatty acid composition. Although gene expression analyses and the same experimental procedure used to generate the transgenic plants might indicate the production of active forms of these other PLAIIIs, the current study did not establish whether these pPLAIII proteins were indeed produced and active in the transgenic plants. Further analysis is therefore needed to provide direct evidence for the presence of active pPLAIII proteins in our transgenic Arabidopsis lines and to elucidate the precise cause(s) of the phenotypes.

The underlying structural reasons for the enhanced selectivity of RcpPLAIIIβ for *sn*-2 HFA-PC, when compared to the other PLAIIIβs, could be associated with segments 1-51, 81-117, 201-240 and/or 508-529 where major differences in amino acid sequence are observed (see Fig. 1A). In this regard, it would be useful to use site directed mutagenesis to make amino acid residue substitutions in these regions. Thus, the recombinant enzymes and their variants produced in a suitable host micro-organism could be enzymatically assayed so as to probe the relationship between amino acid sequence and substrate specificity and selectivity.

In conclusion, RcpPLAIIIβ from castor was shown to reduce HFA content in PC in transgenic *fae1* Arabidopsis plants overexpressing *RcFAH12*. Thus, both *pPLAIIIβ* and *RcPLA$_2$α* (Bayon et al., 2015) may cooperate in catalyzing the release of HFA from PC in developing castor seeds. The highest possible levels of HFA accumulation in the seed oil of Arabidopsis RcpPLAIIIβ-CL7 may come from the additional combined heterologous expression of *LACS*, *GPAT9*, *LPAAT2*, *PAP* and *DGAT* encoding enzymes with demonstrated high selectivity for substrates containing HFAs.

SUPPLEMENTARY MATERIAL

The following supplemental materials are available.

Table S1. Primers used in this study.

Table S2. Fatty acid composition of the oil of T3 seeds of RcpPLAIIIβ-CL7 homozygous lines.

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AUTHOR CONTRIBUTIONS

R.J.W. oversaw the project; R.J.W. and G.C. conceived the project; Y.L., G.C., E.M. and R.J.W. designed the experiments; R.J.W., G.C. and S.D.S. supervised the experiments; Y.L. performed

most of the experiments and data analysis; G.C. conducted some of the experiments; Z.S. performed qRT-PCR, K.C. and Y.L. made constructs for gene transformation; J.D., M.S., and T.M. generated important plant materials, genes, and gene libraries. Y.L. and G.C. wrote the initial draft of the article. All authors participated in interpretation of the data and were instrumental in the preparation of the final article.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: The authors declare that they have no conflict of interest.

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Table 1. *Fatty acid composition of the oil of CL7 seed and T_2 seeds* overexpressing *pPLAIII*

Transgenic	Fatty acid composition (%)											Sum of HFA
	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	18:1-OH	18:2-OH	
CL7	10.4 ± 0.6	0.3 ± 0.1	5.1 ± 0.3	36.7 ± 1.4	20.5 ± 0.9	9.8 ± 1.3	1.0 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	12.5 ± 2.2	3.1 ± 0.6	15.6 ± 2.7
RcpPLAIIIβ-CL7	10.4 ± 0.6	0.3 ± 0.1	4.8 ± 0.3**	37.8 ± 1.4*	21.2 ± 1.2*	10.9 ± 1.2**	1.0 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	10.6 ± 2.4*	2.4 ± 0.6**	12.9 ± 2.9**
RcpPLAIIIδ-CL7	10.5 ± 0.6	0.3 ± 0.1	5.1 ± 0.2	36.7 ± 1.4	20.7 ± 1.2	9.6 ± 1.0	1.0 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	12.5 ± 2.0	2.9 ± 0.8	15.4 ± 2.6
AtpPLAIIIα-CL7	10.6 ± 0.6	0.3 ± 0.1	5.0 ± 0.3	37.1 ± 1.5	20.6± 1.2	10.0 ± 1.3	1.1 ± 0.0	0.3± 0.0	0.3 ± 0.0	12.1 ± 2.3	2.6 ± 0.6	14.6 ± 2.7
AtpPLAIIIδ-CL7	10.5 ± 0.6	0.3 ± 0.1	5.2 ± 0.2	36.1 ± 1.7	20.8 ± 1.4	9.8 ± 1.2	1.0 ± 0.1	0.3 ± 0.0	0.3 ± 0.2	12.8 ± 2.4	2.9± 0.6	15.7 ± 2.9
PfpPLAIIIβ-CL7	10.5 ± 0.5	0.3± 0.1	5.2 ± 0.3	35.7 ± 1.4	20.6± 1.3	9.7 ± 1.2	1.1± 0.0	0.32 ± 0.0	0.31± 0.0	13.2 ± 2.6	3.1 ± 0.6	16.3 ± 3.2
PfpPLAIIIδ-CL7	10.4 ± 0.6	0.3 ± 0.1	5.1 ± 0.3	36.5± 1.2	19.9 ±0.7	9.5 ± 0.7	1.0 ± 0.1	0.32 ± 0.0	0.30 ± 0.0	13.5 ± 1.3	3.2 ± 0.3	16.7 ± 1.4

* and ** denote significant differences ($p<0.05$ and $p<0.01$, respectively) between transgenic and CL7 plants by t-test. The data are mean values ± standard deviation corresponding to 20 independent lines (n=2) expressing each transgene. Abbreviation: HFA, hydroxy fatty acid.

FIGURE LEGENDS

Fig. 1 Alignment of deduced amino acid sequences of *pPLAIIIs* from Arabidopsis (At), *P. fendleri* (Pf) and castor (Rc).(A) Deduced amino acid sequences of AtpPLAIIIα, AtpPLAIIIβ, PfpPLAIIIβ and RcpPLAIIIβ; (B) Deduced amino acid sequence of AtpPLAIIIδ, PfpPLAIIIδ and RcpPLAIIIδ. The grey and black shadings represent consensus of two or more sequences. The catalytic center, including the phosphate or anion binding element (DGGGxxG), esterase box (GxGxG) and the catalytic dyad-containing motif (DGG or DGA or GGG), is indicated

Fig. 2 Phylogenetic relationship of the *pPLAIII* family from Arabidopsis (At), *P. fendleri* (Pf) and castor (Rc). In the tree, the tips represent the sampled sequences, the internal nodes represent the putative ancestors and the horizontal branch lengths are proportional to divergence (i.e., substitutions per site)

Fig. 3 Relative gene expression of *pPLAIIIs* from Arabidopsis (At), *P. fendleri* (Pf) and castor (Rc) in transgenic Arabidopsis CL7 lines. Total RNA was obtained from T_2 RcpPLAIIIβ-CL7, RcpPLAIIIδ-CL7, AtpPLAIIIα-CL7, AtpPLAIIIδ-CL7, PfpPLAIIIβ-CL7, PfpPLAIIIδ-CL7 and CL7 developing seeds. Data are presented as the relative expression levels ($2^{-\Delta CT}$) of target genes compared with the reference gene 18S rRNA

Fig. 4 Hydroxy fatty acid (HFA) content of the oil (A) and total oil content (B) in T_2 seeds of RcpPLAIIIβ-CL7 and RcpPLAIIIβ-CL7. (A) HFA content as a percentage of total fatty acids based on weight. Each point represents the HFA content in T_2 seed samples from an individual T_1 transgenic plant (n = 2). Horizontal bars represent the mean of HFA content of 20 independent lines expressing the same transgene. (B) Oil content is percentage of dry seed weight. Each column represents the mean of 20 independent lines (two technical replications for each line) expressing the same transgene. Error bars represent standard deviation. Significant differences were identified using a two-tailed *t*-test

Fig. 5 Hydroxy fatty acid (HFA) content of the oil in T_3 seeds of RcpPLAIIIβ-CL7 lines. HFA content represents the percentage of total fatty acids on a weight basis. Each point represents the

HFA content in seed samples of an individual T_2 transgenic plant (*n*=3); horizontal bars represent the mean HFA content of homozygous plants derived from the same parent T_1 line with a single transgenic insertion. Significant differences were identified using a two-tailed *t*-test

Fig. 6 Hydroxy fatty acid (HFA) content of triacylglycerol (TAG) and phosphatidylcholine (PC) from T_3 seeds of RcpPLAIIIβ-CL7 lines. Horizontal bars represent mean (±SD) HFA content of total fatty acids (w/w) in four independent RcpPLAIIIβ-CL7 Line 2 or CL7 plants, respectively (three technical replications for each plant). Data were analyzed using a two-tailed *t*-test. */** indicates significant differences at the α=0.05/α=0.01 level.

Fig. 7 Hydroxy fatty acid (HFA) content of the oil (A) and total oil content (B) from T_2 seeds of AtpPLAIIIα-CL7 and AtpPLAIIIδ-CL7. (A) HFA content is the percentage of total fatty acids based on weight. Each data point represents the HFA content in seed samples of an individual T_1 transgenic plant (n=2); horizontal bars represent the mean of HFA content of 20 independent lines expressing the same transgene. (B) Oil content is percentage of dry seed weight. Each column represents the mean of 20 independent lines (technical two replications each line) expressing the same transgene. Error bars represent standard deviation of samples (SD). Data were analyzed by two-tailed *t* test

Fig. 8 Hydroxy fatt acid (HFA) content of the seed oil (A) and total seed oil content (B) in T_2 seeds of PfpPLAIIIβ-CL7 and PfpPLAIIIδ-CL7. (A) HFA content as a percentage of total fatty acids based on weight. Each point represents the HFA content in seed samples of an individual T_1 transgenic plant (n=2); horizontal bars represent the mean of HFA content of 20 independent lines expressing the same transgene. (B) Oil content is percentage of seed weight. Each column represents the mean of 20 independent lines (technical two replications each line) expressing the same transgene. Error bars represent standard deviation of samples (SD). Data were analyzed by two-tailed *t* test

Table S1 *Sequences of primers used in this study*

Name	Direction	Restriction site	Sequence (5`→ 3`)
primers used in the isolation of full-length *pPLAIII* cDNAs			
AtpPLAIIIα	F		ATGTTAACTACGATGCAAAGAGTACAC
	R		TCAAAACATACAATCAATATCCTTGAA
AtpPLAIIIδ	F		ATGGAGATGGATCTCAGCAAG
	R		TTAACGGCCGTCAGCG
RcpPLAIIIβ	F		ATGGCTAGCGATCAATCTTTAGA
	R		CTAGGTGGGTTTAGAAGCAGCT
RcpPLAIIIδ	F		ATGGAGCTTAGTAAGGTAACACTTGAG
	R		CTAACGGCCGTTGGATAGTG
PfpPLAIIIβ	F		ATGGCTAGCGATCAATCTTTAGA
	R		CTAGGTGGGTTTAGAAGCAGCT
PfpPLAIIIδ	F		ATGGAGCTTAGTAAGGTAACACTTGAG
	R		CTAGGTGGGTTTAGAAGCAGCT
primers used in the generation of pPLAIII over-expression vectors			
Napin	F	EcoRI	TATAGAATTCAAGCTTTCTTCATCGGTGATTG
	R	BamHI	ATATGGATCCGTCCGTGTATGTTTTTAATCTTGTTTG
AtpPLAIIIα	F	SalI	TATAGTCGACATGTTAACTACGATGCAAAGAGTACAC
	R	Acc65I	ATATGGTACCTCAAAACATACAATCAATATCCTTGAA
AtpPLAIIIδ	F	BamHI	TATAGGATCCATGGAGATGGATCTCAGCAAG
	R	NcoI	ATATCCATGGTTAACGGCCGTCAGCGA
RcpPLAIIIβ	F	SalI	TATAGTCGACATGGCTAGCGATCAATCTTTAGA
	R	Acc65I	ATATGGTACCCTAGGTGGGTTTAGAAGCAGCT
RcpPLAIIIδ	F	SalI	TATAGTCGACATGGAGCTTAGTAAGGTAACACTTGAG
	R	NcoI	ATATCCATGGCTAACGGCCGTTGGATAGTG
LfpPLAIIIβ	F	SalI	TATAGTCGACATGCATAGAGTACGCAATAAG
	R	NcoI	ATATCCATGGTCATCGTTCTCTTGCAGTAAC
LfpPLAIIIδ	F	XhoI	ATATCTCGAGATGGATATTGATCTCAGTAAGG
	R	XbaI	TATATCTAGATTAACGGCCGTCAGCTAGT
primers used in the analysis of gene expression in developing seed of transgenic Arabidopsis with t-qRT-PCR			
At18sRNA	F		GCCAAAACGGCTCCGAAACA
	R		ACTGGCAGTCCCTCGTGAG

PfpPLAIIIβ	F	GAAGAGGAGCCCAAAGTCAAT
	R	CCTCCATCGATGCTCAAGATAC
AtpPLAIIIα	F	CGGGTACAGTTAAGAACCAGAG
	R	GCATGTTCCAGATAAGCCAAAG
RcpPLAIIIβ	F	CCACAGATACCAATAATGGCCTC
	R	CAGGATTGCCTGATTTTGTCTTG
PfpPLAIIIδ	F	GACATTGTGCTTGACGGAGTTT
	R	TCTTCCGCCGTTCTCGG
AtpPLAIIIδ	F	CGGAGAGTAACGGAGAAAGAATAG
	R	CAAGGACTTGGAGGTAGACTTG
RcpPLAIIIδ	F	TTCTCTTGTTCACCTCGAAGAC
	R	GCAGAGAGTAGAGCACCAATAC
PfpPLAIIIβ	F	GAAGAGGAGCCCAAAGTCAAT
	R	CCTCCATCGATGCTCAAGATAC

F, forward; R, reverse; restriction sites are underlined.

Table S2 *Fatty acid composition of the oil of T_3 seeds of RcpPLAIIIβ-CL7 homozygous lines*

Line	Fatty acid composition (%)											Sum of HFA
	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	18:1-OH	18:2-OH	
CL7	11.3 ± 0.2	0.4 ± 0.0	5.5 ± 0.3	35.8 ± 0.6	20.0 ± 1.2	11.2 ± 1.2	1.2 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	10.6 ± 1.7	3.3 ± 0.6	13.9 ± 2.2
Line 1	11.2 ± 0.2	0.4 ± 0.1	5.4 ± 0.3	35.9 ± 0.7)	21.2 ± 1.1*	11.8 ± 1.1	1.2 ± 0.1	0.4 ± 0.0*	0.4 ± 0.2	9.4 ± 1.3*	2.6 ± 0.5**	12.0± 1.8*
Line 2	11.2 ± 0.2	0.4 ± 0.0	5.3 ± 0.1*	35.0 ± 1.0*	24.4 ± 1.3*	14.5 ± 0.8*	1.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	5.9 ± 1.0*	1.5± 0.3*	7.4 ± 1.3*
Line 3	11.3 ± 0.1	0.4 ± 0.0	5.4 ± 0.1*	35.7 ± 0.7	23.3 ± 0.6*	13.4 ± 0.6*	1.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	6.9 ± 0.7*	1.7 ± 0.3*	8.6 ± 1.0*
Line 4	11.1 ± 0.1*	0.4 ± 0.1	5.3 ± 0.2*	35.5 ± 0.6	21.6 ± 0.5*	12.6 ± 0.7*	1.2 ± 0.0	0.4 ± 0.0*	0.4 ± 0.0	9.0 ± 0.7*	2.5± 0.3*	11.5 ± 0.9*
Line 5	11.3 ± 0.2	0.4 ± 0.0	5.4 ± 0.3	35.9 ± 1.0	20.9 ± 1.1*	12.0 ± 1.0*	1.2 ± 0.0*	0.4 ± 0.0*	0.4 ± 0.0*	9.3± 1.6	2.8± 0.5*	12.1 ± 2.1

* and ** denotes significant difference ($p<0.05$ and $p<0.01$, correspondingly) between transgenic and CL7 plants by t-test. The data are mean values ± standard deviation corresponding to 26 independent lines (n=3) expressing each transgene. HFA, hydroxy fatty acid.

Table S3 Comparison of the morphology phenotype and seed lipid profile of *pPLAIII* overexpression plants

gene	host plant	promoter	growth condition	seed oil content	seed HFA	other seed FA profile	typical plant morphology	Ref
AtpPLAIIIα	At-CL7 (HFA-producing)	seed-specific Bn napin	growth chamber, 22 ºC, 18h day/ 6h night cycle, 250 µmol M^{-2} S^{-1}	↓	18:2-OH↓	—	N/A	this study
AtpPLAIIIδ	At-CL7	napin	growth chamber, 22 ºC, 18h day/ 6h night cycle, 250 µmol M^{-2} S^{-1}	—	—	—	N/A	this study
RcpPLAIIIβ	At-CL7	napin	growth chamber, 22 ºC, 18h day/ 6h night cycle, 250 µmol M^{-2} S^{-1}	—	18:1-OH↓ 18:2-OH↓	18:0 ↓ 18:2 & 18:3↑	N/A	this study
RcpPLAIIIδ	At-CL7	napin	growth chamber, 22 ºC, 18h day/ 6h night cycle, 250 µmol M^{-2} S^{-1}	—	—	—	N/A	this study
PfpPLAIIIβ	At-CL7	napin	growth chamber, 22 ºC, 18h day/ 6h night cycle, 250 µmol M^{-2} S^{-1}	—	—	—	N/A	this study
PfpPLAIIIδ	At-CL7	napin	growth chamber, 22 ºC, 18h day/ 6h night cycle, 250 µmol M^{-2} S^{-1}	—	—	—	N/A	this study
AtpPLAIIIδ	At-Col-0; Bn-J572	constitutive 35S	growth chambers, 12h day/ 12h night cycle, at 23/21 ºC, 50% humidity, 200 µmol M^{-2} S^{-1}	At:↑ Bn:N/A	no HFA	At: 20-FA & 22-FA↑ 18-FA ↓ Bn: N/A	decrease in plant height and seed yield, broadened radial cell growth of hypocotyl and reduced leaf pavement cell polarity	(Dong et al. 2014; Li et al. 2013)
AtpPLAIIIδ	At-Col-0	seed-specific Gm β-conglycinin	growth chambers, 12h day/ 12h night cycle, at 23/21 ºC, 50% humidity, 200 µmol M^{-2} S^{-1}	↑	no HFA	20- and 22-FA↑ 18-FA↓	no change in seed yield	(Li et al. 2013)
AtpPLAIIIβ	At-Col-0	constitutive 35S	growth chambers, 12h day/ 12h night cycle, at 23/21 ºC, 50% humidity, 200 µmol M^{-2} S^{-1}	N/A	no HFA	20:1↑	shorter organs, lower cellulose content and mechanical strength	(Li et al. 2011)
AtpPLAIIIδ	Cs-Suneson	constitutive 35S	greenhouse, 16h day/8h night cycle; 20/21 °C; 50% minimum humidity; 566 µmol M^{-2} S^{-1}	↑	no HFA	20:1↑ 18:1↓	shorter vegetative and reproductive tissues, repressed cell elongation and cellulose accumulation, shorter but wider seeds, decreased seed production	(Li et al. 2015)
AtpPLAIIIδ	Cs-Suneson	seed-specific Gm β-onglycinin	greenhouse, 16h day/ 8h night cycle; 20/21 °C; 50% minimum humidity; 566 µmol M^{-2} S^{-1}	↑	no HFA	18:0 & 18:1↓ 20:1↑	no observable impact on plant vegetative growth, shorter and wider seeds, no difference in seed weight and yield	(Li et al. 2015)
OspPLAIIIα	Os-Dongjin	constitutive maize ubiquitin	grown in the field, greenhouse, or liquid medium	N/A	N/A	N/A	decreased height and cell elongation, wider and shorter seeds, lower seed weight, reduced mechanical strength and cellulose content,	(Liu et al. 2015)

At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Cs, *Camelina sativa*; GM, *Glycine max*; Pf, *Physaria fendleri*; Rc, *Ricinus communis*; OS, *Oryza sativa*; FA, fatty acid; HFA, hydroxy fatty acid; FA, fatty acid; N/A, not available; ↓, decrease; ↑, increase; —, no change.