Castor Phospholipid:Diacylglycerol Acyltransferase Facilitates Efficient Metabolism of Hydroxy Fatty Acids in Transgenic Arabidopsis[1][W][OA]

Harrie van Erp, Philip D. Bates, Julie Burgal, Jay Shockey[2], and John Browse*

Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164–6340

Producing unusual fatty acids (FAs) in crop plants has been a long-standing goal of green chemistry. However, expression of the enzymes that catalyze the primary synthesis of these unusual FAs in transgenic plants typically results in low levels of the desired FA. For example, seed-specific expression of castor (*Ricinus communis*) fatty acid hydroxylase (RcFAH) in Arabidopsis (*Arabidopsis thaliana*) resulted in only 17% hydroxy fatty acids (HFAs) in the seed oil. In order to increase HFA levels, we investigated castor phospholipid:diacylglycerol acyltransferase (PDAT). We cloned cDNAs encoding three putative PDAT enzymes from a castor seed cDNA library and coexpressed them with RcFAH12. One isoform, RcPDAT1A, increased HFA levels to 27%. Analysis of HFA-triacylglycerol molecular species and regiochemistry, along with analysis of the HFA content of phosphatidylcholine, indicates that RcPDAT1A functions as a PDAT in vivo. Expression of RcFAH12 alone leads to a significant decrease in FA content of seeds. Coexpression of RcPDAT1A and RcDGAT2 (for diacylglycerol acyltransferase 2) with RcFAH12 restored FA levels to nearly wild-type levels, and this was accompanied by a major increase in the mass of HFAs accumulating in the seeds. We show the usefulness of RcPDAT1A for engineering plants with high levels of HFAs and alleviating bottlenecks due to the production of unusual FAs in transgenic oilseeds.

Nature produces a wide variety of unusual fatty acids (FAs), some of which are important for industry and human health. Producing these unusual FAs in agronomically suitable plants has been a long-standing goal for companies and researchers involved in the field of oilseed engineering (Damude and Kinney, 2008; Dyer et al., 2008; Napier and Graham, 2010). One important class of unusual FAs are hydroxylated fatty acids (HFAs), which accumulate up to 90% of total FAs in the seeds of castor (*Ricinus communis*). HFAs are used in many industrial applications such as polyesters, biodiesel, and lubricants (Dyer et al., 2008). Castor is mainly grown in the tropical regions of India, China, Brazil, and Thailand and is not suitable for large-scale agriculture, due to the toxicity of the seeds. Production of HFA-containing oilseed crops that could be grown in the temperate climates of the United States would provide a less toxic and economically beneficial supply (Chan et al., 2010; Mutlu and Meier, 2010).

Castor produces the HFA ricinoleic acid (18:1-OH; 12-hydroxy-9-cis-octadecenoic acid) by hydroxylation of oleic acid (18:1). The hydroxylase likely evolved from an ancestral 18:1 fatty acid desaturase, FAD2 (Broun et al., 1998). HFAs are synthesized in the endoplasmic reticulum membrane by addition of a hydroxy group to the Δ^{12} position of oleate esterified to the sn-2 position of phosphatidylcholine (PC; Galliard and Stumpf, 1966; Moreau and Stumpf, 1981; Bafor et al., 1991; Broun et al., 1998). This is similar to FAD2, which creates a double bond at the Δ^{12} position instead of a hydroxy group. The hypothesis that the castor hydroxylase might be a FAD2 homolog led to the cloning of the cDNA encoding *FATTY ACID HYDROXYLASE12* (*RcFAH12*) from a castor endosperm cDNA library (van de Loo et al., 1995).

Attempts to produce HFAs in Arabidopsis (*Arabidopsis thaliana*) and camelina (*Camelina sativa*) have had limited success. RcFAH12 was expressed under the control of seed-specific promoters, but this resulted in maximum stable HFA levels of only 17% ± 1% in the seed oil of Arabidopsis (Broun and Somerville, 1997; Smith et al., 2000, 2003; Lu et al., 2006) and 15% in camelina (Lu and Kang, 2008). A representative example of these engineering efforts is the expression of RcFAH12 in the Arabidopsis *fatty acid elongase1* (*fae1*) mutant background (Kunst et al., 1992; Lu et al., 2006). The *fae1* mutant cannot elongate 18:1 to 20:1, resulting in an increase in 18:1. This results in an increase in the 18:1 substrate for RcFAH12, while the lack of very-long-chain FAs simplifies the gas chromatographic

[1] This work was supported by the U.S. National Science Foundation (grant no. DBI–0701919) and by the Agricultural Research Center at Washington State University.

[2] Present address: Southern Regional Research Center, U.S. Department of Agriculture-Agricultural Research Service, 1100 Robert E. Lee Blvd., New Orleans, LA 70124.

* Corresponding author; e-mail jab@wsu.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: John Browse (jab@wsu.edu).

[W] The online version of this article contains Web-only data.

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.110.167239

analysis of seed lipids, because these lines accumulate the HFAs 18:1-OH and 18:2-OH but not 20:1-OH. Despite the increase in 18:1, the *fae1* mutation did not increase HFA levels above 17%, suggesting that availability of oleic acid is not a limiting factor for HFA-triacylglycerol (TAG) synthesis in Arabidopsis (Broun and Somerville, 1997; Lu et al., 2006). Two lines, CL7 and CL37, in which a *RcFAH12* cDNA is expressed behind the seed-specific phaseolin promoter (Slightom et al., 1983) in the Arabidopsis *fae1* mutant background, were used in the studies reported here. Previous research indicates that investigation of the factors limiting the accumulation of HFAs in transgenic plants provides a valuable model system to investigate the pathways and regulation of FA metabolism and TAG accumulation in oilseeds (Lu et al., 2006; Burgal et al., 2008; Dyer et al., 2008).

Following the incorporation of 18:1 into the sn-2 position of PC and its conversion to HFA by RcFAH12, there are three mechanisms for the removal of HFAs from PC to make them available for incorporation into TAG (mechanisms A, B, and C in Fig. 1): A, removal of HFAs from PC to the acyl-CoA pool; B, removal of the PC phosphocholine head group to produce diacylglycerol (DAG) containing HFA; C, direct transfer of HFA from sn-2 of PC to the sn-3 position of DAG, producing TAG. For mechanism A, FAs esterified to PC are under a constant dynamic exchange with the acyl-CoA pool in a process termed acyl editing (Bates et al., 2007, 2009). Acyl editing allows for newly synthesized 18:1 to be rapidly incorporated into PC for modification (desaturation, hydroxylation, etc.) and for the modified FAs to reenter the acyl-CoA pool to be utilized by other acyltransferases. Removal of HFA from PC can proceed by the reverse action of acyl-CoA:lysophosphatidylcholine acyltransferase (Stymne and Stobart, 1984) or the combined action of phospholipase A_2 and long-chain acyl-CoA synthetase. It has been shown that castor microsomes contain a ricinoleate-specific phospholipase A_2 that is proposed to be involved in the removal of HFA from PC (Bafor et al., 1991). Once in the acyl-CoA pool, HFA-CoA and glycerol-3-phosphate (G3P) can be converted into TAG by the consecutive action of acyl-CoA:G3P acyltransferase (GPAT), acyl-CoA:lysophosphatidic acid acyltransferase (LPAT; Knutzon et al., 1995; Lassner et al., 1995; Brown et al., 2002), phosphatidic acid phosphatase (PAP), and acyl-CoA:diacylglycerol acyltransferase (DGAT; Katavic et al., 1995). This consecutive acylation of glycerol to form TAG is sometimes referred to as the Kennedy pathway (Weiss et al., 1960). For mechanism B, the phosphocholine head group can be removed, producing diacylglycerol containing the same FAs that were in PC. This reaction can proceed by four enzymatic mechanisms: phospholipase C, phospholipase D along with PAP, the reverse action of CDP-choline:diacylglycerol cholinephosphotransferase (Slack et al., 1983), or the recently identified phosphatidylcholine:diacyglycerol cholinephosphotransferase (Lu et al., 2009). The HFA-DAG produced by these mechanisms can then be utilized to produce TAG. For mechanism C, direct transfer of the sn-2 FA of PC to the sn-3 hydroxy of DAG produces TAG by a phospholipid:diacylglycerol acyltransferase (PDAT; Dahlqvist et al., 2000). Lyso-PC is a coproduct of the PDAT reaction, and this can be reincorporated into the acyl editing cycle to efficiently channel 18:1 into PC for hydroxylation.

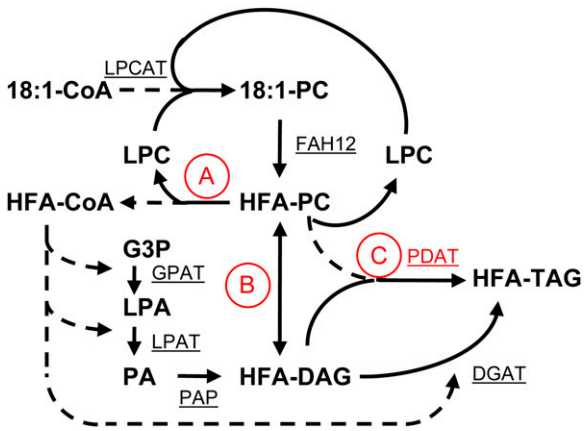

Figure 1. A simplified metabolic scheme for the production of HFAs and TAG assembly. Solid lines indicate glycerolipid flux, and dotted lines indicate transfer of acyl groups. There are three pathways for mobilizing HFAs from PC: A, removal of HFAs from PC to the acyl-CoA pool by reverse LPCAT or combined PLA_2 and LACS; B, removal of the PC phosphocholine head group to produce HFA-DAG by PDCT, reverse CPT, PLC, or PLD/PAP; C, direct transfer to DAG to TAG by PDAT. Enzyme abbreviations are as follows: CPT, CDP-choline:DAG cholinephosphotransferase; DGAT, acyl-CoA:DAG acyltransferase; FAH12, fatty acid hydroxylase 12; GPAT, acyl-CoA:G3P acyltransferase; LACS, long-chain acyl-CoA synthetase; LPAT, acyl-CoA:LPA acyltransferase; LPCAT, acyl-CoA:lysophosphatidylcholine acyltransferase; PAP, PA phosphatase; PDAT, phospholipid:diacylglycerol acyltransferase; PDCT, PC:DAG cholinephosphotransferase; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D/PAP. Substrate abbreviations are as follows: DAG, diacylglycerol; G3P, glycerol-3-phosphate; LPA, lyso-phosphatidic acid; LPC, lyso-phosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; TAG, triacylglycerol. Enzymatic reactions are underlined.

One hypothesis for the limited accumulation of HFAs in seed TAG in the CL7 and CL37 lines is that Arabidopsis enzymes may not efficiently remove HFAs from the sn-2 position of PC, whereas in castor, coevolution of the enzymes of lipid synthesis might have occurred to allow efficient use of substrates containing HFAs. One test for this hypothesis is the coexpression of RcDGAT2 with RcFAH12 in Arabidopsis seeds, which resulted in an increase in HFA levels from 17% to 28% (Burgal et al., 2008). These results suggest that RcFAH12 and RcDGAT2 coevolved to specifically accumulate HFAs in TAG.

In vitro assays of castor microsomes demonstrated a PDAT activity with specificity for HFA-PC (Banaś et al., 2000; Dahlqvist et al., 2000). Based on this observation, we decided to explore the role of castor

PDAT in HFA accumulation in Arabidopsis. A yeast (*Saccharomyces cerevisiae*) PDAT enzyme was identified as a homolog of the mammalian lecithin:cholesterol acyltransferase (LCAT; Dahlqvist et al., 2000). Based on the homology to this yeast PDAT, six LCAT family members were identified in Arabidopsis (Ståhl et al., 2004). Three genes of the Arabidopsis LCAT family have been characterized: *AtPSAT* (At1g04010) encodes a phospholipid:sterol acyltransferase (Banas et al., 2005), *AtLCAT3* (At3g03310) encodes a phospholipase A1 (Noiriel et al., 2004), and *AtPDAT1* (At5g13640) encodes a phospholipid:diacyglycerol acyltransferase (Ståhl et al., 2004). A *pdat1* knockout mutation of Arabidopsis showed no visible phenotype (Mhaske et al., 2005). However, a *pdat1 dgat1* double mutant could not be generated, because of pollen lethality (Zhang et al., 2009). RNA interference of PDAT1 in the *dgat1* background or DGAT1 RNA interference in the *pdat1* background resulted in a 70% to 80% reduction in TAG levels of seeds. These data suggest that AtPDAT1 and AtDGAT1 (At2g19450) have overlapping functions in pollen and seed development in Arabidopsis and that they are the two major enzymes involved in TAG synthesis in these organs (Zhang et al., 2009). AtPDAT1 has a close homolog, AtPDAT2 (At3g44830), but *pdat2 dgat1* double mutants were viable and showed no decrease in TAG content beyond the decrease caused by the *dgat1* mutation (Zhang et al., 2009). This suggests that AtPDAT2 has no role in TAG synthesis, although it is highly expressed during seed development (Schmid et al., 2005; Winter et al., 2007).

Three putative *RcPDAT* cDNAs were cloned from a castor cDNA library and coexpressed with RcFAH12 in Arabidopsis seeds. Expression of RcPDAT1A, but not expression of either of the other putative RcPDAT isozymes, increased HFA levels. When expressed in the CL37 line, RcPDAT1A increased HFA levels from 17% to 27%. Regiochemical analysis of TAG and analysis of HFAs in PC suggest that RcPDAT1A has PDAT activity in vivo. Expression of RcDGAT2 in plants also expressing RcFAH12 and RcPDAT1A did not significantly increase the percentage HFAs but did increase the mass of HFAs and total FAs per seed. In summary, we demonstrate the utility of RcPDAT1A in the engineering of HFA-TAG in transgenic Arabidopsis.

RESULTS

Based on homology to AtPDAT1, three putative PDAT proteins in the castor genome were identified and designated RcPDAT1A, RcPDAT1B, and RcPDAT2. RcPDAT1A and RcPDAT1B have 83.8% and 87.3% similarity with AtPDAT1 at the amino acid level, respectively. RcPDAT2 has 73.9% similarity to AtPDAT1 but 78.0% similarity to AtPDAT2 (Campanella et al., 2003; Supplemental Table S1). Based on the high homology to AtPDAT1 and the fact that AtPDAT1 is one of the major enzymes for TAG synthesis in Arabidopsis (Zhang et al., 2009), these three putative PDAT proteins were all considered candidates for being involved in HFA-TAG accumulation in castor. In order to determine which of the three putative PDATs is the most likely candidate for being involved in HFA-TAG synthesis, we analyzed the expression of *RcPDAT1A*, *RcPDAT1B*, and *RcPDAT2* in castor endosperm based on transcriptome analysis using the Roche FLX Genome Sequencer technology (http://www.ncbi.nlm.nih.gov/sra). *RcPDAT1B* was not detected in the endosperm transcriptome. Both *RcPDAT1A* and *RcPDAT2* were expressed in the developing endosperm, with *RcPDAT2* showing double the transcript levels of *RcPDAT1A*. We hypothesize that RcPDAT1A is the most likely candidate for being involved in HFA-TAG synthesis for the following reasons. *RcPDAT1A* is a close homolog of AtPDAT1 and is expressed in developing castor endosperm. The expression of *RcPDAT1B* in castor endosperm was not detected. RcPDAT2 is most closely related to AtPDAT2, which does not have an apparent function in oil synthesis (Zhang et al., 2009).

RcPDAT1A Increases HFA Accumulation When Coexpressed with RcFAH12

RcPDAT1A, *RcPDAT1B*, and *RcPDAT2* were amplified from a castor seed cDNA library and expressed in the CL37 line under control of the seed-specific phaseolin promoter (Slightom et al., 1983). The plasmid used for transformation contained a DsRed marker, and transgenic T1 seeds of all three constructs were selected by screening for red fluorescence. T1 plants were grown, and T2 seeds with a ratio of fluorescent to nonfluorescent seeds of 3:1 were selected for bulk FA analysis by gas chromatography (GC; Table I). Based on the 3:1 segregation of the marker, these plant lines are assumed to have a single insertion site. The expression level of transgenes is dependent on their position in the genome (Ahmad et al., 2010), so to obtain transgenic plant lines with the highest level of gene expression, multiple transgenic lines were generated for each transgene. T2 seed samples from 18 individual T1 plants were screened for HFA content. The HFA levels observed in these samples ranged from 17% to 25%, and two of these lines, RcPDAT1A.1 and RcPDAT1A.2 (Table I), were selected for further characterization. By contrast, analysis of T2 seeds harvested from primary transgenic plants expressing RcPDAT1B or RcPDAT2 in CL37 indicates that neither of the homologs provided any substantial increase in HFAs relative to the CL37 parent. Data from four representative lines are included in Table I. As a control, we determined the expression of *RcPDAT1A*, *RcPDAT1B*, *RcPDAT2*, and *RcDGAT2* in our transgenic plant lines by reverse transcription-PCR (Supplemental Fig. S1). All transgenic lines expressed the genes of interest. These results indicate that of these three putative enzymes, only RcPDAT1A is able to

Table I. *FA composition of transgenic Arabidopsis seeds of T1 lines expressing the castor hydroxylase (CL37 background) and RcPDAT1A, RcPDAT1B, or RcPDAT2*

The transgenic plant lines for each of these genes were obtained from independent transformation events. All data are averages of three independent measurements ± SE. n.d., Not determined.

Transgenic Line	FA Composition								Sum of HFAs
	16:0	18:0	18:1	18:2	18:3	20:1	18:1-OH	18:2-OH	
	% of total								
RcPDAT1A.1	11.5 ± 0.0	6.3 ± 0.1	33.6 ± 0.8	16.1 ± 0.1	7.3 ± 0.1	0.5 ± 0.0	19.4 ± 0.5	5.5 ± 0.1	24.9 ± 0.5
RcPDAT1A.2	11.5 ± 0.0	6.8 ± 0.3	33.3 ± 0.3	16.9 ± 0.5	7.9 ± 0.2	0.4 ± 0.2	17.6 ± 0.2	5.6 ± 0.3	23.2 ± 0.4
RcPDAT1B.1	11.0 ± 0.3	4.9 ± 0.2	43.6 ± 1.2	18.0 ± 0.3	7.3 ± 0.2	0.5 ± 0.0	11.4 ± 0.4	3.4 ± 0.2	14.8 ± 0.4
RcPDAT1B.2	12.1 ± 0.2	5.4 ± 0.1	40.9 ± 0.4	18.3 ± 0.4	7.2 ± 0.2	0.4 ± 0.0	12.5 ± 0.0	3.2 ± 0.1	15.7 ± 0.1
RcPDAT2.1	14.1 ± 1.9	5.7 ± 0.3	30.9 ± 4.7	24.4 ± 2.6	7.6 ± 0.5	n.d.	15.1 ± 0.2	2.1 ± 0.3	17.2 ± 0.3
RcPDAT2.2	17.5 ± 1.1	7.76 ± 0.3	20.8 ± 2.4	29.4 ± 1.9	8.8 ± 0.5	n.d.	15.3 ± 0.5	0.6 ± 0.3	15.8 ± 0.6
CL37	11.2 ± 0.1	4.5 ± 0.0	42.6 ± 0.0	17.7 ± 0.0	6.6 ± 0.2	0.5 ± 0.0	13.5 ± 0.2	3.5 ± 0.1	17.0 ± 0.2
CL37	14.4 ± 0.1	6.7 ± 0.3	32.5 ± 0.4	21.3 ± 0.2	7.3 ± 0.1	n.d.	14.5 ± 0.1	3.3 ± 0.0	17.8 ± 0.1

increase the percentage of HFAs in plants expressing RcFAH12. This demonstrates that RcPDAT1A, but not RcPDAT1B or RcPDAT2, is useful for HFA-TAG synthesis in CL37.

Homozygous sublines of CL37 RcPDAT1A.1 and CL37 RcPDAT1A.2 were identified by pedigree analysis. One subline of CL37 RcPDAT1A.1 was propagated through four additional generations. Analysis of seed samples from T6 plants showed that RcPDAT1A transgenics accumulated 26% to 27% HFAs in their oil compared with 16.9% in CL37 controls grown alongside (Fig. 2). Therefore, the increase in HFAs mediated by the RcPDAT1A transgene is stably inherited over multiple generations.

To determine whether overexpression of the endogenous Arabidopsis isozyme AtPDAT1 might also support increased HFA accumulation, we cloned an *AtPDAT1* cDNA into the same phaseolin vector and transformed CL37 plants. Analysis of 15 T1 lines did not identify a homozygous AtPDAT1 line with HFA levels significantly higher than the 17% found in the parental CL37 line (data not shown). Therefore, the increase in HFAs by RcPDAT1A is consistent with it having specificity for HFAs and not with overexpression of a PDAT alone. This is consistent with previous data that suggest that castor PDAT has specificity for ricinoleoyl-PC (Banaś et al., 2000; Dahlqvist et al., 2000).

Changes in HFA-TAG Molecular Species in CL37 RcPDAT1A Seeds

The HFAs containing species of TAG and their regiochemical composition were analyzed in CL37 and CL37 RcPDAT1A. This analysis was performed in order to give us insight in the biochemical mechanisms that allow, and limit, HFA accumulation in CL37 RcPDAT1A plants. There are four possible molecular species of TAG based on the number of HFAs esterified to the glycerol backbone: 0-, 1-, 2-, and 3-HFA-TAG. RcPDAT1A lowers the proportion of TAG species containing 0- and 1-HFA and increases the amount of TAG containing 2- and 3-HFAs in comparison with the parental CL37 line (Fig. 3A). A similar decrease in 0- and 1-HFA-TAG species and an increase in 2- and 3-HFA-TAG species was found between the CL7 and CL7 RcDGAT2 lines (Fig. 3B). The increases in TAG molecular species containing 2- or 3-HFAs suggest that both RcPDAT1A and RcDGAT2 efficiently utilize substrates containing HFAs. Figures 4 and 5 show the regiochemical analysis of the HFAs in 1- and 2-HFA-TAG. Figure 4 shows the HFAs as a percentage of total seed FAs at the sn-2 versus the sn-1/3 position.

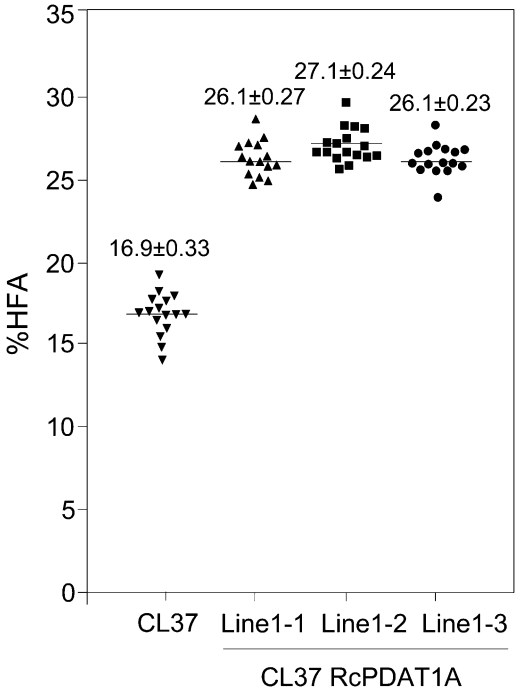

Figure 2. HFA levels in CL37 and CL37 RcPDAT1A T7 seeds. Lines 1-1, 1-2, and 1-3 are three lines derived from the same parent, which was grown for five generations. The parent had a single insertion site for RcPDAT1A. The horizontal bars represent the average of 15 to 17 individual plants. Each symbol represents an individual plant. The data represent averages ± SE.

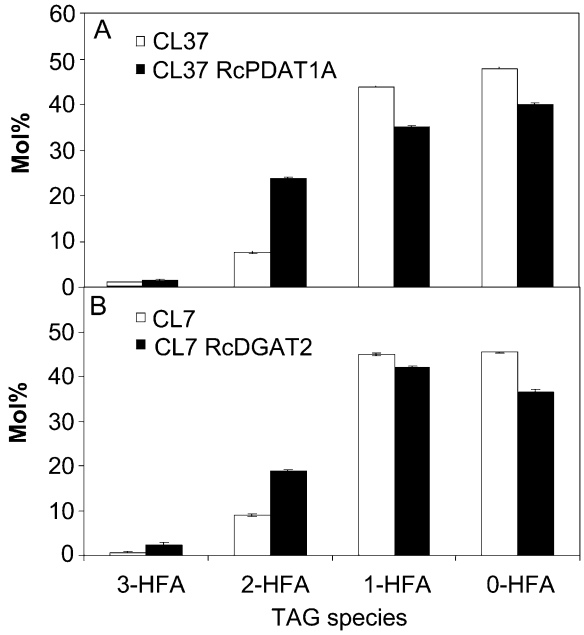

Figure 3. Molecular species composition of HFA containing TAGs of CL37, CL37 RcPDAT1A, CL7, and CL7 RcDGAT2 seeds. A, Mol % of TAG molecular species in CL37 (white bars) and CL37 RcPDAT1A (black bars) seeds. B, Mol % of TAG molecular species of CL7 (white bars) and CL7 RcDGAT2 (black bars) seeds. 0-, 1-, 2-, and 3-HFA represent TAG molecular species with zero, one, two, or three HFAs, respectively (no stereochemistry implied). The HFAs represent the sum of ricinoleate (18:1-OH) and densipolate (18:2-OH). The data represent averages of three replicates ± SE.

The percentage of total HFAs at the sn-2 position decreased from 11.6% to 10.7% and HFAs at the sn-1/3 position increased from 8.7% to 14% in CL37 RcPDAT1A compared with CL37 (Fig. 4A). Because PDAT enzymes transfer FAs to the sn-3 position of DAG, we assume that the large increase in the sn-1/3 position is due to sn-3 acylation. Figure 5 shows the HFAs as a percentage of FAs at the sn-2 versus the sn-1/3 position for individual 1- and 2-HFA-TAG molecular species. In 1-HFA-TAG, RcPDAT1A reduced the percentage HFAs at the sn-2 position (from 71.5% to 60.5%) and increased the percentage HFAs at the sn-1/3 position (from 28.5% to 39.5%) in comparison with CL37 (Fig. 5A). This suggests an increase in sn-3 HFA acylation of 0-HFA-DAG producing 1-HFA-TAG, which also corresponds with the decrease in 0-HFA-TAG (Fig. 3A). In 2-HFA-TAG, RcPDAT1A did not cause major changes in the relative proportions of HFAs at the sn-2 versus the sn-1/3 position, with all lines having approximately 40% of total HFAs at the sn-2 position (Fig. 5B). This indicates that the relative amounts of sn-1 and sn-2 1-HFA-DAG available for sn-3 HFA acylation are the same in the CL37 and RcPDAT1A lines. Together with the data in Figure 3, these results indicate that, in the RcPDAT1A line, 1-HFA-DAG is preferentially acylated with sn-3 HFA, causing an increase in 2-HFA-TAG and a reduction in 1-HFA-TAG. A similar regiochemical analysis of the CL7 and CL7 RcDGAT2 lines gave quantitatively similar results (Figs. 3B, 4B, and 5, C and D). This is expected, since both RcPDAT1A and RcDGAT2 esterify FAs to the sn-3 position of DAG.

RcPDAT1A and RcDGAT2 Lower the HFA Levels in PC

In some oilseeds, almost all FAs in TAG flux through PC (Bates et al., 2009), and oleate at the sn-2 position of PC is the substrate for the RcFAH12 hydroxylase (Bafor et al., 1991). Castor seeds can efficiently accumulate HFAs in TAG while keeping the level of HFAs in membrane lipids low and accumulating up to 90% in TAG of mature seeds; HFAs transiently accumulate to a maximum of 5% in PC during the mid stage of seed development (Thomaeus et al., 2001). In contrast, we found that PC accumulates up to 10% to 12% HFAs in the CL37 and CL7 Arabidopsis lines, which accumulate 17% HFAs in the seed oil at maturity (Fig. 6). Therefore, the high amount of HFAs in PC during TAG synthesis might cause feedback inhibition of RcFAH12. In order to investigate if the expression of RcPDAT1A influences the amount of HFAs that accumulate in PC during oil synthesis, we compared the HFA content of TAG and PC in CL37 and CL37

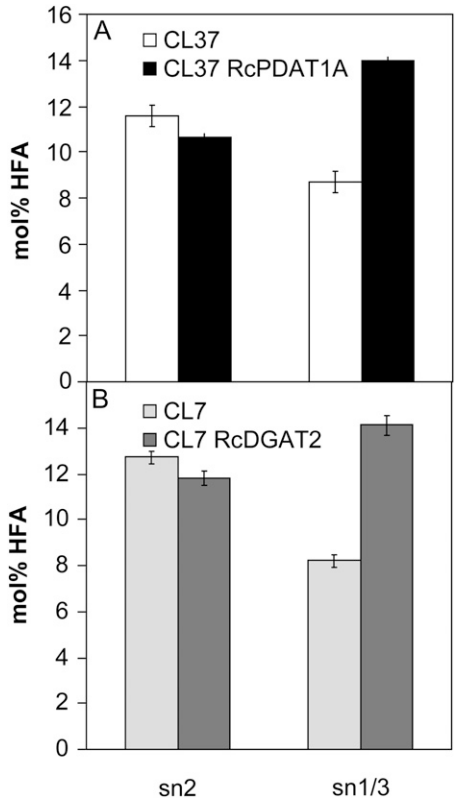

Figure 4. Percentage HFAs at the sn-2 position compared with the sn-1/3 position as a percentage of total seed TAG. A, CL37 (white bars) and CL37 RcPDAT1A (black bars). B, CL7 (light gray bars) and CL7 RcDGAT2 (dark gray bars). The data represent averages of three replicates ± SE.

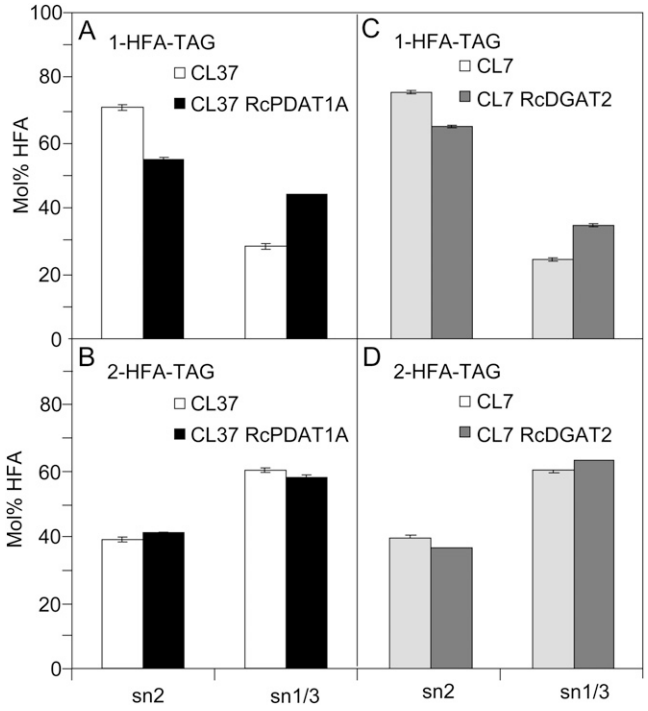

Figure 5. Regiochemical analysis of 1- and 2-HFA-TAG species in CL37, CL37 RcPDAT1A, CL7, and CL7 RcDGAT2 seeds: mol % HFA at the sn-2 position compared with the sn-1/3 position in 1- and 2-HFA-TAG. A and C, 1-HFA-TAG. B and D, 2-HFA-TAG. White bars, CL37; black bars, CL37 RcPDAT1A; light gray bars, CL7; dark gray bars, CL7 RcDGAT2. The data represent averages of three replicates ± SE.

RcPDAT1A throughout the TAG synthesis phase of seed development, 5 to 18 d after flower opening (DAF). Total lipids were extracted from seed samples and separated by thin-layer chromatography (TLC). PC and total neutral lipids (the TAG fraction) were collected, and HFAs were quantified by GC. The increase in HFA accumulation of TAG in CL37 RcPDAT1A relative to CL37 (Fig. 6A) is associated with a substantial decline in the maximum proportion of HFAs in PC during seed development (Fig. 6B). At 11 to 12 DAF, PC in CL37 seeds contained 10.6% HFAs, compared with 6.9% HFAs in PC from seeds of CL37 RcPDAT1A. An analysis of CL7 and CL7 RcDGAT2 plants gave similar results (Fig. 6, C and D), but the decrease in HFAs in PC was less pronounced.

Expression of RcDGAT2 in the CL37 RcPDAT1A Background

In order to determine if RcPDAT1A and RcDGAT2 have an additive effect on HFA levels, line CL37 RcPDAT1A.2 containing approximately 25% HFAs was transformed with *RcDGAT2* under control of the phaseolin promoter using a transformation vector that contained a glufosinate resistance marker. T1 plants were grown, and samples of bulk T2 seeds from individual plants were analyzed by GC. Single insert lines were selected with glufosinate, and seven lines with the highest levels of HFAs (27%–29%) were grown for the next generation. For each line, the genotype of each plant of the segregating T2 population was determined by pedigree analysis of T3 seeds germinated under glufosinate selection, and the percentage HFAs was measured in samples of T3 seeds. Figure 7 shows the results for the CL37 RcPDAT1A RcDGAT2 triple transgenic compared with CL37 RcPDAT1A segregants and the parental CL37 line. Expression of RcDGAT2 increased HFA levels from 25.4% ± 0.3% in CL37 RcPDAT1A to 26.7% ± 0.2% in CL37 RcPDAT1A RcDGAT2 homozygous lines (Fig. 7A; total FA compositions are shown in Supplemental Fig. S2). This relatively small increase in the proportion of HFAs in the seeds is statistically significant ($P < 0.001$). To determine if coexpression of RcPDAT1A and RcDGAT2 had an effect on the quantity of total seed lipids, the mass of total FAs and HFAs in seeds was measured. Figure 7B shows that there is an increase of 0.27 μg ($P = 0.003$) of HFAs per seed in the homozygous CL37 RcPDAT1A RcDGAT2 line in comparison with the parental CL37 RcPDAT1A line. This corresponds to a 19.6% increase in the mass of HFAs per seed. However, a large increase in the mass of total FAs concomitant with the increase in the mass of HFAs was also observed (Fig. 7C). Figure 7C shows that there is a 0.66 μg ($P = 0.038$) or 12% increase in total FAs per seed in the homozygous CL37 RcPDAT1A RcDGAT2 line in comparison with the parental CL37 RcPDAT1A line. This increase in total FAs effectively masks the increase in the mass of HFAs and highlights the problem of utilizing percentage FA content as the only metric for assessing changes in oilseed biochemistry and oil composition.

DISCUSSION

Castor oil is a high-value oil with many industrial applications such as lubricants, nylon, and biofuels (Caupin, 1997). Expression of RcFAH12 in Arabidopsis seeds resulted in HFA levels up to only 17% of total FAs, and it has been a challenge to understand this limitation in terms of the enzymology and cell biology of seed lipid metabolism in these transgenic plant lines (Broun and Somerville, 1997; Broun et al., 1998; Smith et al., 2000, 2003; Lu et al., 2006). One hypothesis for the limited accumulation of HFAs in Arabidopsis seed TAG is that Arabidopsis enzymes do not efficiently use HFA substrates. Support for the hypothesis that castor TAG synthesis enzymes coevolved with RcFAH12 to allow accumulation of HFA-TAG comes from the coexpression of RcFAH12 and RcDGAT2 in Arabidopsis. Addition of RcDGAT2 increased HFA levels from 17% to approximately 27% (Burgal et al., 2008). RcDGAT2 utilizes HFA-CoAs as a substrate and therefore still requires that Arabidopsis enzymes remove HFAs from the sn-2 position of PC and convert them to CoA esters. PDAT enzymes directly transfer

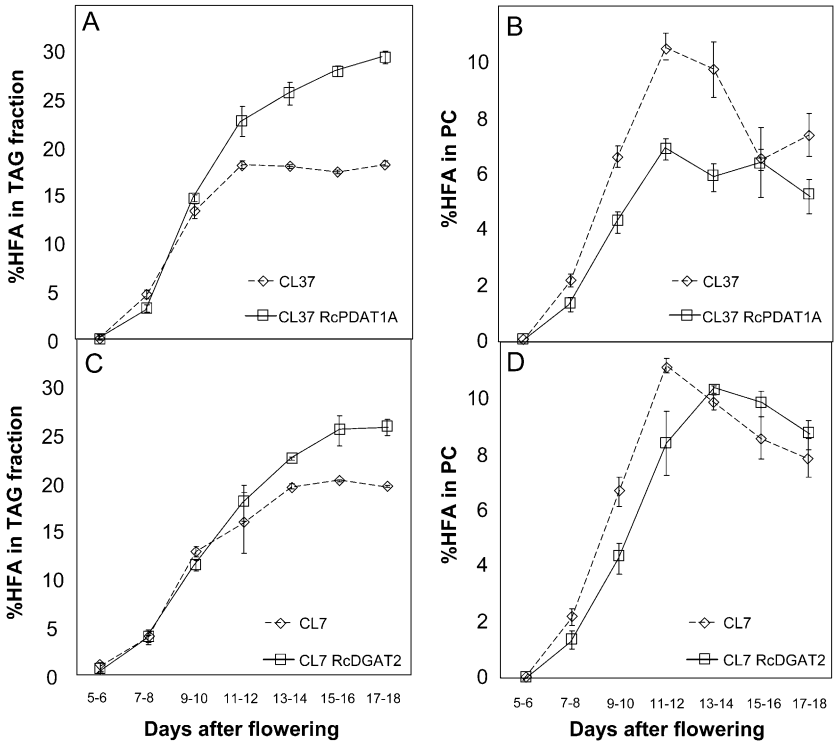

Figure 6. Changes in the percentage HFAs in PC and total neutral lipids (TAG fraction) in CL7, CL37, CL7 RcDGAT2, and CL37 RcPDAT1A during seed development. A, Percentage HFAs in neutral lipids from CL37 and CL37 RcPDAT1A. B, Percentage HFAs in PC from CL37 and CL37 RcPDAT1A. C, Percentage HFAs in neutral lipids from CL7 and CL7 RcDGAT2. D, Percentage HFAs in PC from CL7 and CL7 RcDGAT2. The data represent averages of three replicates ± SE.

FAs from the sn-2 position of PC to the sn-3 position of DAG, producing TAG. Therefore, *RcPDAT* genes appear to represent a more direct engineering strategy to enhance the production of HFAs in transgenic oilseeds. RcPDAT1A is a homolog of AtPDAT1 (Ståhl et al., 2004; Supplemental Table S1). Coexpression of RcPDAT1A in the CL37 background increased the percentage HFAs in seed oil from 17% to 27%. However, coexpression of two closely related proteins, RcPDAT1B and RcPDAT2, in the CL37 background did not lead to an increase in HFA levels, nor did overexpression of the Arabidopsis homolog AtPDAT1. The observation that RcPDAT1B does not increase HFA-TAG accumulation in CL37 is consistent with the observation that its expression was not detected during castor endosperm development. The observation that RcPDAT2 does not increase HFA-TAG accumulation in CL37 is supported by the fact that it is the closest homolog of AtPDAT2, which has no apparent role in TAG synthesis (Zhang et al., 2009). In CL37 RcPDAT1A seeds, the amount of 1-HFA-TAG was reduced and the amounts of 2- and 3-HFA-TAG were increased in comparison with the parental CL37 line (Fig. 3A). These data indicate that RcPDAT1A has higher specificity for HFA-DAG and/or acyl substrates containing HFAs than the homologous Arabidopsis isozyme. Similar observations were made for the CL7 RcDGAT2 line (Fig. 3B). The changes in HFA-TAG species in CL7 RcDGAT2 in comparison with CL7 are similar to the observations made previously with different TAG analysis techniques (Burgal et al., 2008).

The TAG molecular species analysis (Fig. 3) and regiochemical analysis (Figs. 4 and 5) allow us to estimate the amount of HFA-DAG for TAG synthesis that is provided by endogenous Arabidopsis metabolic enzymes in CL37 RcPDAT1A. The regiochemistry of the 2-HFA-TAG species (Fig. 5) suggests that stereochemistry of the 1-HFA-DAG pool is approximately 20:80 sn-1:sn-2. Assuming that the increase in sn-1/3 HFAs of 1-HFA-TAG is due to the sn-3 activity of PDAT, then together with quantities of the individual HFA-TAG molecular species, we can estimate the composition of the DAG pool as approximately 52% 0-HFA, approximately 47% 1-HFA, and approximately 1% 2-HFA. Together with the stereochemistry estimate, only about 10% of total DAG has sn-1 HFA and approximately 40% has sn-2 HFA. Arabidopsis enzymes may limit the amount of HFA at the sn-1 position by two mechanisms. If HFA-DAG is mostly produced by the removal of HFA to the CoA pool (mechanism A in Fig. 1) and the consecutive action of Kennedy pathway enzymes, this implies that GPAT has a higher selectivity against HFA than does LPAT. Alternatively, mostly sn-2 HFA-DAG may also be produced by removal of the phosphocholine head group of PC after sn-2 hydroxylation (mechanism B in Fig. 1). Therefore, further increases in the accumulation in 3-HFA-TAG may require engineering of DAG synthesis pathways to produce higher amounts of DAG containing both sn-1 and sn-2 HFAs for sn-3 acylation by RcPDAT1A or RcDGAT2. RcFAH12 hydroxylates oleate at the sn-2 position of PC. RcFAH12 lines CL37 and CL7 contain 10% to 12% HFAs in PC.

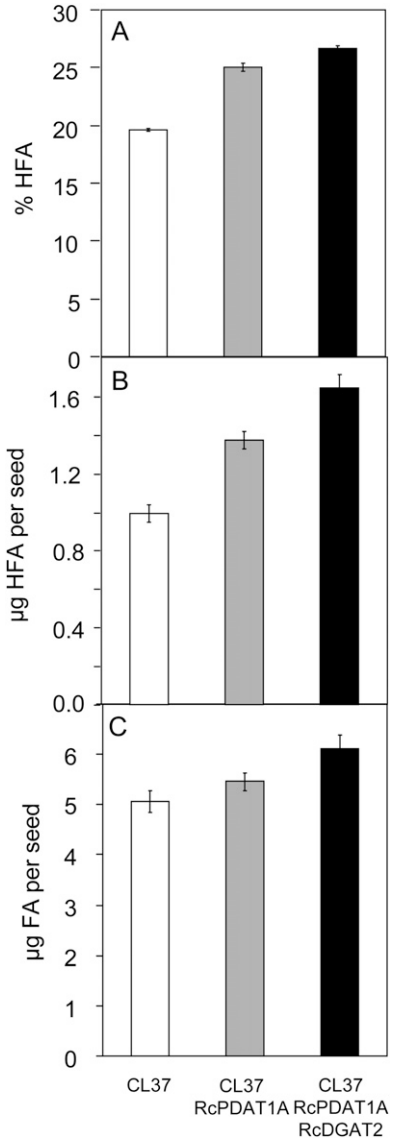

Figure 7. Summary of the analysis of HFA accumulation in CL37, CL37 RcPDAT1A, and CL37 RcPDAT1A RcDGAT2 T3 seeds. A, Percentage HFAs (total FA composition is in Supplemental Fig. S2). B, HFAs per seed (μg). C, Total FAs per seed (μg). Numbers are as follows: CL37 (n = 4), CL37 RcPDAT1A (n = 17), and CL37 RcPDAT1A RcDGAT2 (n = 14). The data represent averages ± SE.

This appears to reflect an inefficient transfer of HFAs from sn-2 PC into TAG and may be one reason for the low HFA accumulation in TAG in the CL37 and CL7 lines. The high levels of HFAs in PC could also lead to feedback inhibition of RcFAH12. Our results show that RcPDAT1A significantly decreases the accumulation of HFAs in PC during the phase of rapid TAG accumulation (7–12 DAF; Fig. 6B). RcDGAT2 has a similar, but less pronounced, effect on reducing HFAs in PC at 7 to 12 DAF when expressed in the CL7 line (Fig. 6D). RcPDAT1A and RcDGAT2 probably reduce HFA accumulation in PC by different but complementary mechanisms. RcPDAT1A likely catalyzes the direct transfer of FAs from the sn-2 position of PC to the sn-3 position of DAG, producing TAG (mechanism C in Fig. 1). The reduction of HFAs in PC by RcDGAT2 presumably occurs indirectly as a result of the removal of HFAs from the acyl-CoA pool. FAs esterified to PC are under a constant dynamic exchange with the acyl-CoA pool, involving a cycle of deacylation and reacylation of PC (Bates et al., 2007, 2009), so HFA-CoAs that are not rapidly utilized by Arabidopsis acyltransferases may be reincorporated into PC through the acyl editing cycle (mechanism A in Fig. 1). Removal of HFA-CoAs from the acyl-CoA pool may cause a net flux of HFAs out of PC into TAG.

For engineering of seed oil, the desired FAs need to be a high proportion of the oil without any reduction in total oil yield. The parental Arabidopsis ecotype used in this study, Columbia, produces seeds containing approximately 6.4 μg of FAs per seed (Li et al., 2006), and this is comparable to the oil content of *fae1* seeds determined in our laboratory. Our analysis indicates that production of HFAs in Arabidopsis seeds through the expression of RcFAH12 in the CL37 line reduces the quantity of seed oil to 5.1 μg per seed (Fig. 7C). This is a 20% reduction in comparison with wild-type Arabidopsis and *fae1*. Previously, it has been proposed that HFAs produced in Arabidopsis are targeted for degradation by β-oxidation (Moire et al., 2004). This might cause a futile cycle of FA synthesis and degradation and cause a reduction in oil content. Coexpression of RcPDAT1A and RcDGAT2 in CL37 recovered a substantial part of the reduction in seed oil, with total content rising to 6.1 μg of FAs per seed (Fig. 7C). The increase in total oil was due to increases in both common FAs and HFAs, suggesting that increased rates of β-oxidation may break down both common FAs and HFAs. A previous report demonstrated increased rates of nonselective FA β-oxidation in mutant plants deficient in the accumulation of palmitic acid (Bonaventure et al., 2004). We hypothesize that an increased rate of HFA sequestration in TAG by RcPDAT1A and RcDGAT2 activity may prevent increased rates of total FA β-oxidation. This may reduce futile cycling of FA synthesis and degradation, which could recover the total oil content. Alternatively, feedback inhibition of FA synthesis might also be involved in reducing FA accumulation. Buildup of acyl-CoA has been shown to down-regulate FA synthesis (Shintani and Ohlrogge, 1995). When Arabidopsis acyltransferases do not efficiently utilize HFA-CoA, then their buildup may cause feedback inhibition of FA synthesis. RcDGAT2 efficiently utilizes HFA-CoA to produce TAG, and direct incorporation of HFAs from PC into TAG by RcPDAT1A may limit the production of HFA-CoA. Either mechanism may lower the amount of HFA-CoA and alleviate the reduced TAG accumulation, as shown in Figure 7. Further analysis of this phenomenon may identify new strategies for the engineering of unusual FAs in oilseeds. In conclusion, this study describes the clon-

ing of a gene encoding a PDAT isozyme from castor and its utilization in engineering HFAs in Arabidopsis seeds. Our results support the hypothesis that TAG biosynthetic enzymes that efficiently utilize the unusual FAs produced in transgenic oilseeds are required to optimize the accumulation of novel oils. Additionally, we demonstrate that adverse side effects of the production of unusual FAs in transgenic systems, such as reduced oil, may be alleviated by sequestrating the FAs in TAG. These contributions to understanding the bottlenecks in unusual FA accumulation will benefit future engineering efforts and bring the goal of creating crop plants producing high levels of HFAs a step closer.

MATERIALS AND METHODS

Identification of Castor Genes and Cloning Procedures

Cloning of castor (*Ricinus communis*) *RcPDAT1A* and *RcPDAT1B* (National Center for Biotechnology Information [NCBI] accession no. XM_002521304) was performed as described previously (Burgal et al., 2008). One cloned version of *RcPDAT1A* has a mutation near its C terminus causing a frame shift, altering the last two amino acids (Pro→Ala, Leu→Ala); three amino acids are added (Ile, Asp, and Ser) in comparison with the NCBI version of RcPDAT1A (XM_002514026). This same clone has a nucleotide change from A to T at position 1,041 that does not result in an amino acid change. All experiments utilize transgenic plants created with the mutant version of *RcPDAT1A*. *AtPDAT1*, *RcPDAT1A*, and *RcPDAT1B* were cloned in the pOEA vector (Lu et al., 2006) with DsRed as a marker (Stuitje et al., 2003). *RcPDAT2* was identified by BLAST analysis of the castor genome (http://castorbean.jcvi.org/; Chan et al., 2010), and its amino acid sequence was compared with AtPDAT2 using the ClustalX2 algorithm (Larkin et al., 2007). *RcPDAT2* was amplified from castor seed cDNA using gene-specific primers and cloned into the pENTR-D-TOPO vector (Invitrogen), followed by cloning into a Gateway-compatible pOEA vector under control of the phaseolin promoter (Slightom et al., 1983). Based on our sequence data, the amino acid and nucleotide sequences of *RcPDAT2* (XM_002527387) in the NCBI database are incorrect. The correct start codon is 291 bp upstream of the start codon of the NCBI version of *RcPDAT2* (Supplemental Fig. S3), resulting in a protein that is 97 amino acids longer. In this corrected sequence, amino acid 110 is changed from Leu to Phe, followed by insertion of an additional Leu not included in NCBI XM_002527387. This is due to an insertion in the genome sequence at bp 329 of the corrected open reading frame that is not present in the cDNA we amplified (Supplemental Fig. S3). There are also two nucleotide changes in the cDNA we amplified when compared with the NCBI sequence: one change from C to G at nucleotide 377 leads to an amino acid change from Pro to Arg at position 126, and a second change of G to C at nucleotide 791 leads to an amino acid change from Ser to Thr at position 264. The nucleotide and amino acid changes in RcPDAT1A and RcPDAT2 could be due either to cultivar differences or errors in sequencing of the castor genome (http://castorbean.jcvi.org/). *RcPDAT1A* and *RcPDAT2* were amplified from castor endosperm mRNA twice in order to confirm that the sequence differences observed were real.

Growing of Arabidopsis and Transformation

For all experiments, Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia *fae1* mutant AC56 (Kunst et al., 1992) expressing *RcFAH12* (lines CL7 and CL37; Lu et al., 2006) was used as a parental line. Seeds were sterilized by 5 min of incubation in sterilization solution (0.01% NaClO, 27% ethanol, and 1 g L^{-1} SDS), followed by five washes with sterile water and 3 d of incubation at 4°C. Seeds that germinated on half-strength Murashige and Skoog, 1% agar plates containing 1% Suc were transplanted to soil. Herbicide selection was performed using glufosinate (Finale, Farnam Companies). Plants for the experiments in Figures 2 to 6 were grown in growth chambers under continuous fluorescent light (100–200 μmol m^{-2} s^{-1}) at 22°C. Plants for the experiments in Figure 7 were grown in randomized order in an air-conditioned greenhouse under natural light supplemented with lamps to provide a 16-h/8-h light/dark cycle. Arabidopsis transformation was performed by floral dip (Clough and Bent, 1998).

Gene Expression Analysis

In order to confirm that the transgenic plant lines expressed the genes of interest, we performed reverse transcription-PCR using total RNA isolated from developing siliques (Supplemental Fig. S1). Total RNA was isolated using the TRIzol reagent (Invitrogen). The RNA was treated with DNase I and purified using the DNA-free RNA kit (Zymo Research). RNA was reverse transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen). Primers were designed in order to amplify full-length transcripts of the genes of interest (Supplemental Table S2), and PCR was performed. Supplemental Figure S1 shows that all transgenic plant lines express the genes of interest. Full-length transcripts could be amplified using the primers for *RcDGAT2*, *RcPDAT1B*, and *RcPDAT2*. Primers for the 3′ 500 bp of the *RcPDAT1A* cDNA were designed and used for PCR. As a control, PCR was performed on cDNA obtained from the parental CL37 line using primers for each of the transgenes. Supplemental Figure S1 shows that RNA from CL37 siliques did not yield a band corresponding *RcPDAT1A*, *RcDGAT2*, *RcPDAT1B*, or *RcPDAT2*, indicating that the primers used are gene specific. The presence of the *RcDGAT2* cDNA in the CL7 RcDGAT2 transgenic line was determined by Burgal et al. (2008).

GC Analysis

The FAs of between 10 and 100 whole seed were derivatized to FA methyl esters in 1 mL of 2.5% (v/v) sulfuric acid in methanol for 1.5 h at 80°C (Miquel and Browse, 1992). FA methyl esters were quantified by GC with flame ionization detection on a wax column (EC Wax; 30 m × 0.53 mm i.d. × 1.20 μm; Alltech). GC parameters were as follows: 220°C for 2 min followed by a ramp to 245°C at 10°C min^{-1}, with a 6-min final temperature hold.

Determination of Seed FA Content

The FA content of seeds was determined according to Li et al. (2006), except that 20 seeds were used per measurement instead of 50. In order to determine if there was a significant difference in percentage HFAs and mass of HFAs and total seed FAs between the CL37 RcPDAT1A and CL37 RcPDAT1A RcDGAT2 lines, a *t* test was performed (Sigmastat; Systat Software). Supplemental Figure S2 shows the seed FA composition of the CL37, CL37 RcPDAT1A, and CL37 RcPDAT1A RcDGAT2 lines.

Lipid Extraction

Lipid extraction was based on a modified protocol (Bligh and Dyer, 1959) from the Kansas Lipidomics Center (http://www.k-state.edu/lipid/lipidomics/AT-seed-extraction.html). Between 10 and 50 mg of seeds was added to 1 mL of 85°C isopropanol containing 0.01% butylated hydroxy toluene (BHT) and heated for 15 min. After heat-quenched samples were homogenized, the homogenizer was rinsed with 2 mL of chloroform and 3 mL of methanol to recover all the seed parts and lipids. The rinses combined with the samples were mixed with 1 mL of isopropanol. A phase separation was produced by adding 1.6 mL of water, 2 mL of $CHCl_3$, and 2 mL of 0.88% KCl. The chloroform layer was collected, and the aqueous phase was back extracted twice more with chloroform. The chloroform and lipid mixture was dried under N_2 and resuspended in 0.5 mL of toluene containing 0.005% BHT before TLC analysis.

Characterization of TAG Species

Total extracted lipids were separated by TLC (Silica gel 60; 20 × 20 cm; EMD Chemicals). For analytical quantitation of TAG species, each was separated based on the number of hydroxy groups by using a double development: first develop 12 cm in $CHCl_3$:methanol:HOAc (93:3:0.5, v/v/v) in a vacuum for 15 min, followed by a full development in $CHCl_3$:methanol:HOAc (99:0.5:0.5, v/v/v). All TLC solvents contained approximately 0.005% BHT antioxidant. Lipid bands were visualized under UV light after staining with 0.005% primulin in 80% acetone. The TAG bands corresponding to standards produced from castor oil were collected, transmethylated, and analyzed by GC as described above. The 3-HFA-TAG fraction was calculated from the HFAs recovered in this fraction to allow for contamination from a

minor lipid band. The 1- and 2-HFA-TAG fractions did not require correction, because they had HFA levels very close to the expected 33% and 66%, respectively (Supplemental Fig. S4).

Regiochemical Analysis of 1- and 2-HFA-TAG

For bulk collection of 1- and 2-HFA-TAG, 9 mg of total TAG was separated by TLC with one development in $CHCl_3$:acetone:acetic acid (96:3.5:0.5, v/v/v). TAGs were eluted from the TLC silica twice by washing with 5 mL of $CHCl_3$:methanol (4:1, v/v). A phase separation was induced by the addition of 2 mL of methanol and 4 mL of 0.88% KCl. The $CHCl_3$ phase was collected, and the aqueous phase was back extracted with 5 mL of $CHCl_3$. The $CHCl_3$ was dried under N_2 and resuspended in 0.5 mL of toluene plus 0.005% BHT. Lipase digestion was modified from a described protocol (Cahoon et al., 2006). One-half to 1 mg of 1- or 2-HFA-TAG was resuspended in 1 mL of diethyl ether and 0.8 mL of buffer containing 50 mM NaBr, pH 7.6, and 5 mM $CaCl_2$. A total of 200 μL of lipase (*Rhizomucor miehei* lipase; Sigma-Aldrich) was added, and the tubes were vortexed for 40 min, allowing approximately 50% TAG digestion. The reaction was stopped by adding 2 mL of methanol:$CHCl_3$ (1:1, v/v). The $CHCl_3$ layer was collected, and the TAGs, DAGs, monoacylglycerols (MAGs), and free FAs were separated by TLC in $CHCl_3$:methanol:acetic acid (98:2:0.5, v/v/v) and then stained with primulin as above. The MAG and TAG bands were collected and quantified by GC analysis. The mol % HFAs in the MAG fraction represents the percentage HFAs at the sn-2 position. The percentage HFAs at the sn-1 and sn-3 positions was calculated in the following way: 1-HFA-TAG (100% – % HFA at sn-2 position) = % HFA at the sn-1 and sn-3 positions; 2-HFA-TAG (100% – % HFA at sn-2 position)/2 = % HFA at the sn-1 and sn-3 positions. The percentage HFAs at the sn-2 position compared with the sn-1/3 position as a percentage of total seed FAs was calculated based on the results of the above-mentioned regiochemical analysis of individual TAG species.

PC Analysis

Transgenic plant lines were grown under a day/night cycle of 16/8 h. After the first flowers opened, the number of opened flowers and siliques were counted daily for a period of 18 d to determine the age of each silique. Lipids of developing seeds were extracted as described above. Seeds from 10 siliques were pooled for each replicate, and three replicates were analyzed per time point. PC and total neutral lipids were separated by TLC in $CHCl_3$:methanol:acetic acid (75:25:8, v/v/v), and lipid bands were collected and analyzed by GC as before.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At1g04010 (*AtPSAT*), At3g03310 (*AtLCAT3*), At5g13640 (*AtPDAT1*), At2g19450 (*AtDGAT1*), At3g44830 (*AtPDAT2*), XM_002514026 (*RcPDAT1A*), XM_002521304 (*RcPDAT1B*), and XM_002527387 (*RcPDAT2*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Gene expression analysis of the CL37, CL37 RcPDAT1A, CL37 RcPDAT1A RcDGAT2, CL37 RcPDAT1B, and CL37 RcPDAT2 lines.

Supplemental Figure S2. Seed FA composition of CL37, CL37 RcPDAT1A, and CL37 RcPDAT1A RcDGAT2.

Supplemental Figure S3. Genomic structure of the *RcPDAT2* gene.

Supplemental Figure S4. Percentage HFAs in different TAG fractions of CL37, CL37 RcPDAT1A, CL7, and CL7 RcDGAT2 seeds.

Supplemental Table S1. Percentage amino acid identity and similarity between Arabidopsis and castor PDAT proteins.

Supplemental Table S2. Primers used for reverse transcription-PCR analysis.

ACKNOWLEDGMENTS

We thank present and past members of the Browse laboratory for their help with this paper.

Received October 9, 2010; accepted December 17, 2010; published December 20, 2010.

LITERATURE CITED

Ahmad A, Zhang Y, Cao XF (2010) Decoding the epigenetic language of plant development. Mol Plant **3:** 719–728

Bafor M, Smith MA, Jonsson L, Stobart K, Stymne S (1991) Ricinoleic acid biosynthesis and triacylglycerol assembly in microsomal preparations from developing castor-bean (*Ricinus communis*) endosperm. Biochem J **280:** 507–514

Banas A, Carlsson AS, Huang B, Lenman M, Banas W, Lee M, Noiriel A, Benveniste P, Schaller H, Bouvier-Navé P, et al (2005) Cellular sterol ester synthesis in plants is performed by an enzyme (phospholipid: sterol acyltransferase) different from the yeast and mammalian acyl-CoA:sterol acyltransferases. J Biol Chem **280:** 34626–34634

Banaś A, Dahlqvist A, Ståhl U, Lenman M, Stymne S (2000) The involvement of phospholipid:diacylglycerol acyltransferases in triacylglycerol production. Biochem Soc Trans **28:** 703–705

Bates PD, Durrett TP, Ohlrogge JB, Pollard M (2009) Analysis of acyl fluxes through multiple pathways of triacylglycerol synthesis in developing soybean embryos. Plant Physiol **150:** 55–72

Bates PD, Ohlrogge JB, Pollard M (2007) Incorporation of newly synthesized fatty acids into cytosolic glycerolipids in pea leaves occurs via acyl editing. J Biol Chem **282:** 31206–31216

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol **37:** 911–917

Bonaventure G, Bao X, Ohlrogge J, Pollard M (2004) Metabolic responses to the reduction in palmitate caused by disruption of the *FATB* gene in Arabidopsis. Plant Physiol **135:** 1269–1279

Broun P, Shanklin J, Whittle E, Somerville C (1998) Catalytic plasticity of fatty acid modification enzymes underlying chemical diversity of plant lipids. Science **282:** 1315–1317

Broun P, Somerville C (1997) Accumulation of ricinoleic, lesquerolic, and densipolic acids in seeds of transgenic Arabidopsis plants that express a fatty acyl hydroxylase cDNA from castor bean. Plant Physiol **113:** 933–942

Brown AP, Carnaby S, Brough C, Brazier M, Slabas AR (2002) Limnanthes douglasii lysophosphatidic acid acyltransferases: immunological quantification, acyl selectivity and functional replacement of the Escherichia coli plsC gene. Biochem J **364:** 795–805

Burgal J, Shockey J, Lu CF, Dyer J, Larson T, Graham I, Browse J (2008) Metabolic engineering of hydroxy fatty acid production in plants: RcDGAT2 drives dramatic increases in ricinoleate levels in seed oil. Plant Biotechnol J **6:** 819–831

Cahoon EB, Dietrich CR, Meyer K, Damude HG, Dyer JM, Kinney AJ (2006) Conjugated fatty acids accumulate to high levels in phospholipids of metabolically engineered soybean and Arabidopsis seeds. Phytochemistry **67:** 1166–1176

Campanella JJ, Bitincka L, Smalley J (2003) MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences. BMC Bioinformatics **4:** 29

Caupin HJ (1997) Products from castor oil: past, present and future. *In* FD Gunstone, FB Padley, eds, Lipid Technologies and Applications. Marcel Dekker, New York, pp 787–795

Chan AP, Crabtree J, Zhao Q, Lorenzi H, Orvis J, Puiu D, Melake-Berhan A, Jones KM, Redman J, Chen G, et al (2010) Draft genome sequence of the oilseed species *Ricinus communis*. Nat Biotechnol **28:** 951–956

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743

Dahlqvist A, Stahl U, Lenman M, Banas A, Lee M, Sandager L, Ronne H, Stymne S (2000) Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proc Natl Acad Sci USA **97:** 6487–6492

Damude HG, Kinney AJ (2008) Enhancing plant seed oils for human nutrition. Plant Physiol **147:** 962–968

Dyer JM, Stymne S, Green AG, Carlsson AS (2008) High-value oils from plants. Plant J **54:** 640–655

Galliard T, Stumpf PK (1966) Fat metabolism in higher plants. 30. Enzymatic synthesis of ricinoleic acid by a microsomal preparation from developing Ricinus communis seeds. J Biol Chem **241:** 5806–5812

Katavic V, Reed DW, Taylor DC, Giblin EM, Barton DL, Zou JT,

Mackenzie SL, Covello PS, Kunst L (1995) Alteration of seed fatty acid composition by an ethyl methanesulfonate-induced mutation in *Arabidopsis thaliana* affecting diacylglycerol acyltransferase activity. Plant Physiol **108:** 399–409

Knutzon DS, Lardizabal KD, Nelsen JS, Bleibaum JL, Davies HM, Metz JG (1995) Cloning of a coconut endosperm cDNA encoding a 1-acyl-sn-glycerol-3-phosphate acyltransferase that accepts medium-chain-length substrates. Plant Physiol **109:** 999–1006

Kunst L, Taylor DC, Underhill EW (1992) Fatty-acid elongation in developing seeds of *Arabidopsis thaliana*. Plant Physiol Biochem **30:** 425–434

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al (2007) Clustal W and Clustal X version 2.0. Bioinformatics **23:** 2947–2948

Lassner MW, Levering CK, Davies HM, Knutzon DS (1995) Lysophosphatidic acid acyltransferase from meadowfoam mediates insertion of erucic acid at the sn-2 position of triacylglycerol in transgenic rapeseed oil. Plant Physiol **109:** 1389–1394

Li YH, Beisson F, Pollard M, Ohlrogge J (2006) Oil content of Arabidopsis seeds: the influence of seed anatomy, light and plant-to-plant variation. Phytochemistry **67:** 904–915

Lu C, Xin Z, Ren Z, Miquel M, Browse J (2009) An enzyme regulating triacylglycerol composition is encoded by the *ROD1* gene of Arabidopsis. Proc Natl Acad Sci USA **106:** 18837–18842

Lu CF, Fulda M, Wallis JG, Browse J (2006) A high-throughput screen for genes from castor that boost hydroxy fatty acid accumulation in seed oils of transgenic Arabidopsis. Plant J **45:** 847–856

Lu CF, Kang JL (2008) Generation of transgenic plants of a potential oilseed crop *Camelina sativa* by *Agrobacterium*-mediated transformation. Plant Cell Rep **27:** 273–278

Mhaske V, Beldjilali K, Ohlrogge J, Pollard M (2005) Isolation and characterization of an Arabidopsis thaliana knockout line for phospholipid:diacylglycerol transacylase gene (At5g13640). Plant Physiol Biochem **43:** 413–417

Miquel M, Browse J (1992) Arabidopsis mutants deficient in polyunsaturated fatty acid synthesis: biochemical and genetic characterization of a plant oleoyl-phosphatidylcholine desaturase. J Biol Chem **267:** 1502–1509

Moire L, Rezzonico E, Goepfert S, Poirier Y (2004) Impact of unusual fatty acid synthesis on futile cycling through beta-oxidation and on gene expression in transgenic plants. Plant Physiol **134:** 432–442

Moreau RA, Stumpf PK (1981) Recent studies of the enzymic synthesis of ricinoleic acid by developing castor beans. Plant Physiol **67:** 672–676

Mutlu H, Meier MAR (2010) Castor oil as a renewable resource for the chemical industry. Eur J Lipid Sci Technol **112:** 10–30

Napier JA, Graham IA (2010) Tailoring plant lipid composition: designer oilseeds come of age. Curr Opin Plant Biol **13:** 330–337

Noiriel A, Benveniste P, Banas A, Stymne S, Bouvier-Navé P (2004) Expression in yeast of a novel phospholipase A1 cDNA from *Arabidopsis thaliana*. Eur J Biochem **271:** 3752–3764

Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, Schölkopf B, Weigel D, Lohmann JU (2005) A gene expression map of *Arabidopsis thaliana* development. Nat Genet **37:** 501–506

Shintani DK, Ohlrogge JB (1995) Feedback inhibition of fatty acid synthesis in tobacco suspension cells. Plant J **7:** 577–587

Slack CR, Campbell LC, Browse JA, Roughan PG (1983) Some evidence for the reversibility of the cholinephosphotransferase-catalysed reaction in developing linseed cotyledons in vivo. Biochim Biophys Acta **754:** 10–20

Slightom JL, Sun SM, Hall TC (1983) Complete nucleotide sequence of a French bean storage protein gene: phaseolin. Proc Natl Acad Sci USA **80:** 1897–1901

Smith M, Moon H, Kunst L (2000) Production of hydroxy fatty acids in the seeds of *Arabidopsis thaliana*. Biochem Soc Trans **28:** 947–950

Smith MA, Moon H, Chowrira G, Kunst L (2003) Heterologous expression of a fatty acid hydroxylase gene in developing seeds of *Arabidopsis thaliana*. Planta **217:** 507–516

Ståhl U, Carlsson AS, Lenman M, Dahlqvist A, Huang BQ, Banas W, Banas A, Stymne S (2004) Cloning and functional characterization of a phospholipid:diacylglycerol acyltransferase from Arabidopsis. Plant Physiol **135:** 1324–1335

Stuitje AR, Verbree EC, van der Linden KH, Mietkiewska EM, Nap JP, Kneppers TJA (2003) Seed-expressed fluorescent proteins as versatile tools for easy (co)transformation and high-throughput functional genomics in Arabidopsis. Plant Biotechnol J **1:** 301–309

Stymne S, Stobart AK (1984) Evidence for the reversibility of the acyl-CoA: lysophosphatidylcholine acyltransferase in microsomal preparations from developing safflower (*Carthamus tinctorius* L.) cotyledons and rat liver. Biochem J **223:** 305–314

Thomaeus S, Carlsson AS, Stymne S (2001) Distribution of fatty acids in polar and neutral lipids during seed development in *Arabidopsis thaliana* genetically engineered to produce acetylenic, epoxy and hydroxy fatty acids. Plant Sci **161:** 997–1003

van de Loo FJ, Broun P, Turner S, Somerville C (1995) An oleate 12-hydroxylase from *Ricinus communis* L. is a fatty acyl desaturase homolog. Proc Natl Acad Sci USA **92:** 6743–6747

Weiss SB, Kennedy EP, Kiyasu JY (1960) The enzymatic synthesis of triglycerides. J Biol Chem **235:** 40–44

Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ (2007) An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS ONE **2:** e718

Zhang M, Fan JL, Taylor DC, Ohlrogge JB (2009) DGAT1 and PDAT1 acyltransferases have overlapping functions in *Arabidopsis* triacylglycerol biosynthesis and are essential for normal pollen and seed development. Plant Cell **21:** 3885–3901