Running head: Engineering of hydroxy fatty acids

Corresponding author: John Browse,
Institute of Biological Chemistry,
Clark Hall, Washington State University,
Pullman, WA 99164-6340, USA
tel.: 509-335-2293
e-mail: jab@wsu.edu

Appropriate category: Biochemical Processes and Macromolecular structures
Castor phospholipid:diacylglycerol acyltransferase facilitates efficient metabolism of hydroxy fatty acids in transgenic Arabidopsis.

Harrie van Erp, Philip D. Bates, Julie Burgal, Jay Shockey, John Browse*
Institute of Biological Chemistry, Clark Hall, Washington State University, Pullman, WA 99164-6340, USA
This work was supported by grant DBI-0701919 from the U.S. National Science Foundation and by the Agricultural Research Center at Washington State University.

Present address: Southern Regional Research Center, United States Department of Agriculture-Agricultural Research Service, 1100 Robert E. Lee Blvd., New Orleans, LA 70124, USA

*Corresponding author; email: jab@wsu.edu
Abstract

Producing unusual fatty acids (FA) 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 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-TAG molecular species and regiochemistry, along with the analysis of the HFA content of PC, 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 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 production of unusual FAs in transgenic oilseeds.
Introduction

Nature produces a wide variety of unusual fatty acids (FA), 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 endoplasmatic reticulum (ER) membrane by addition of a hydroxy group to the Δ12 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 Δ12 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 HYDROXYLASE 12* (*RcFAH12*) from a castor endosperm cDNA library (Van de Loo., 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; Smith et al., 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 elongase 1 (*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 fatty acids simplifies the gas chromatographic (GC) 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 (A, B, C in Fig. 1). A: Removal of HFAs from PC to the acyl-CoA pool. B: Removal of the PC phosphocholine headgroup to produce DAG containing HFA. C: Direct transfer of HFA from sn-2 of PC to the sn-3 position of DAG producing TAG. **Mechanism A:** FA esterified to PC are under a constant dynamic exchange with the acyl-CoA pool in a process termed acyl editing (Bates et al., 2007; Bates et al., 2009). Acyl editing allows for newly synthesized 18:1 to be rapidly incorporated into PC for modification (desaturation, hydroxylation, etc.) and the modified FA 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 (LPCAT) (Stymne and Stobart, 1984), or the combined action of phospholipase A2 (PLA2) and long chain acyl-CoA synthetase (LACS). It has been shown that castor microsomes contain a ricinoleate specific phospholipase A2 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). **Mechanism B:** The phosphocholine headgroup 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 (CPT) (Slack et al., 1983), or the recently identified phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) (Lu et al., 2009). The HFA-DAG produced by these mechanisms can then be utilized to produce TAG. **Mechanism C:** Direct transfer of the sn-2 FA of PC to the sn-3 hydroxy of DAG producing TAG by a phospholipid:diacylglycerol acyltransferase (PDAT) (Dahlqvist et al., 2000). Lyso-PC is a co-product of the PDAT reaction and this can be reincorporated into the acyl editing cycle to efficiently channel 18:1 into PC for hydroxylation.

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 (Banas 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 PDAT enzyme was identified as a homologue 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 (Stahl 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:diacylglycerol acyltransferase (Stahl 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). RNAi of PDAT1 in the **dgat1** or DGAT1 RNAi 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 \textit{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 \textit{RcPDAT} cDNAs were cloned from a castor cDNA library and coexpressed with \textit{RcFAH12} in Arabidopsis seeds. Expression of \textit{RcPDAT1A}, but not expression of either of the other putative \textit{RcPDAT} isozymes, increased HFA levels. When expressed in the CL37 line, \textit{RcPDAT1A} increased HFA levels from 17\% to 27\%. Regiochemical analysis of TAG and analysis of HFAs in PC suggests that \textit{RcPDAT1A} has PDAT activity in vivo. Expression of \textit{RcDGAT2} in plants also expressing \textit{RcFAH12} and \textit{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 \textit{RcPDAT1A} in the engineering of HFA-TAG in transgenic Arabidopsis.

\textbf{Results}

Based on homology to AtPDAT1, three putative PDAT proteins in the castor genome were identified and designated \textit{RcPDAT1A}, \textit{RcPDAT1B} and \textit{RcPDAT2}. \textit{RcPDAT1A} and \textit{RcPDAT1B} have 83.8\% and 87.3\% similarity with AtPDAT1 at the amino acid level respectively. \textit{RcPDAT2} has 73.9\% similarity to AtPDAT1 but 78.0\% similarity to AtPDAT2 (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 \textit{RcPDAT1A}, \textit{RcPDAT1B} and \textit{RcPDAT2}, in castor endosperm based on transcriptome analysis using the Roche FLX Genome Sequencer technology (\texttt{http://www.ncbi.nlm.nih.gov/sra}). \textit{RcPDAT1B} was not detected in the endosperm transcriptome. Both \textit{RcPDAT1A} and \textit{RcPDAT2} were expressed in the developing endosperm, with \textit{RcPDAT2} showing double the transcript levels of \textit{RcPDAT1A}. We hypothesize that \textit{RcPDAT1A} is the most likely candidate for being involved in HFA-TAG synthesis for the following reasons. \textit{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 T\textsubscript{1} seeds of all three constructs were selected by screening for red fluorescence. T\textsubscript{1} plants were grown and T\textsubscript{2} seeds with a ratio of fluorescent to non-fluorescent seeds of 3:1 were selected for bulk FA analysis by gas chromatography (GC) (Table 1). 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. T\textsubscript{2} seed samples from 18 individual T\textsubscript{1} 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 1) were selected for further characterization. By contrast, analysis of T\textsubscript{2} seeds harvested from primary transgenic plants expressing *RcPDAT1B* or *RcPDAT2* in CL37 indicates that neither of the homologues provided any substantial increase in HFAs relative to the CL37 parent. Data from four representative lines are included in Table 1. As a control we determined the expression of *RcPDAT1A*, *RcPDAT1B*, *RcPDAT2* and *RcDGAT2* in our transgenic plant lines by reverse transcriptase (RT)-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 increase the percentage of HFAs in plants expressing RcFAH12. This demonstrates that *RcPDAT1A* but not *RcPDAT1B* or *RcPDAT2* are 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 T\textsubscript{6} 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 over-expression of a PDAT alone. This is consistent with previous data which suggest that castor PDAT has specificity for ricinoleoyl-PC (Banas et al., 2000; Dahlqvist et al., 2000).

Changes in HFA-TAG molecular species in CL37 RcPDAT1A seeds

The HFA 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 to 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. 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 to 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 to 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 ~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 (Fig. 3B; Fig. 4B; Fig. 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 oil seeds 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, while 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 10-12% 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 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 RcPDAT1A throughout the TAG synthesis phase of seed development, 5 to 18 days 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 11-12 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 ~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 to 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 increase of 0.27 µg (P = 0.003) HFAs per seed in the homozygous CL37 RcPDAT1A RcDGAT2 line in comparison to 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 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 to the parental CL37 RcPDAT1A line. This increase in total FAs effectively masks the increase in 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.
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; Smith et al., 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 ~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 FAs from the sn-2 position of PC to sn-3 position of DAG producing TAG. Therefore RcPDAT genes appear to represent a more direct engineering strategy to enhance production of HFAs in transgenic oilseeds.

RcPDAT1A is a homologue of AtPDAT1 (Stahl 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 homologue 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 is 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 amount of 2- and 3-HFA-TAG was 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
to CL7 are similar to observations made previously with different TAG analysis techniques (Burgal et al., 2008).

The TAG molecular species analysis (Fig. 3) and regiochemical analysis (Fig. 4; Fig. 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 ~20/80 sn-1/sn-2. Assuming that the increase in sn-1/sn-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 ~52% 0-HFA, ~47% 1-HFA, and ~1% 2-HFA. Together with the stereochemistry estimate only about 10% of total DAG has sn-1 HFA and ~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 removal of HFA to the CoA pool (mechanism A in Fig. 1) and the consecutive action of Kennedy pathway enzymes, then 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 headgroup of PC after sn-2 hydroxylation (mechanism B in Fig. 1). Therefore further increases in 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. 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 feed back 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 7-12 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 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 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; Bates et al., 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, Col-0, produces seeds containing approximately 6.4 μg 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 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 to 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. Co-expression of RcPDAT1A and RcDGAT2 in CL37 recovered a substantial part of the reduction in seed oil, with total content rising to 6.1 μg 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 breakdown both common and HFAs. A previous report demonstrated increased rates of non-selective FA β-oxidation in mutant plants deficient in 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. Build up 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 build up 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 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 engineering strategies for the engineering of unusual FAs in oilseeds.

In conclusion, this study describes the cloning 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 production of unusual FAs in transgenic systems, such as reduced oil, may be alleviated by sequestering 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 \textit{RcPDAT1A} and \textit{RcPDAT1B} (NCBI accession number: XM\_002521304) was performed as described previously (Burgal et al., 2008). One cloned version of \textit{RcPDAT1A} has a mutation near its C-terminus causing a frame shift altering the last two amino acid (P \rightarrow A, L \rightarrow A); three amino acids are added (I, D and S) in comparison to the NCBI version of \textit{RcPDAT1A} (XM\_002514026). This same clone has a nucleotide change from A to T at position 1041 that does not result in an amino acid change. All experiments utilize transgenic plants created with the mutant version of \textit{RcPDAT1A}. \textit{AtPDAT1}, \textit{RcPDAT1A} and \textit{RcPDAT1B} were cloned in the pOEA vector (Lu et al., 2006) with DsRed as a marker (Stuitje et al., 2003). \textit{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 \textit{AtPDAT2} using the ClustalX2 algorithm (Larkin et al., 2007). \textit{RcPDAT2} was amplified from castor seed cDNA using gene-specific primers and cloned into the pENTR-D-TOPO vector (Invitrogen, Carlsbad, CA), 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 \textit{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 \textit{RcPDAT2} (Supplemental Fig. S3), resulting in a protein that is 97 amino acids longer. In this corrected sequence, amino acid 110 is changed from L to F followed by insertion of an additional L 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 to the NCBI sequence: One change from C to G at nucleotide 377 leads to an amino acid change from P to R at position 126, and a second change of G to C at nucleotide 791 leads to an amino acid change from S to T 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/index.php). 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 thaliana* ecotype Columbia fatty acid elongase 1 (*fae1*) mutant AC56 (Kunst et al., 1992) expressing *RcFAH12* (lines called CL7 and CL37) (Lu et al., 2006) was used as a parental line. Seeds were sterilized by five min incubation in sterilization solution (0.01% NaClO, 27% EtOH, 1 g L⁻¹ SDS), followed by five washes with sterile water and three days incubation at 4º C. Seeds that germinated on ½ MS, 1% agar plates containing 1% sucrose were transplanted to soil. Herbicide selection was performed using glufosinate (Finale, Farnam Companies Inc., Phoenix, AZ). 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 22º C. Plants for experiments in figure 7 were grown in randomized order in an air-conditioned greenhouse under natural light supplemented with lamps to provide at 16/8 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 RT-PCR using total RNA isolated from developing siliques (Supplemental Fig. S1). Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA). The RNA was treated with DNAse I and purified using the DNA-free RNA kit (Zymo research, Irvine, CA). RNA was reverse transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen,
Carlsbad, CA). 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 the 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 FA of between 10 and 100 whole seed were derivatized to FA methyl esters in one 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 x 0.53 mm i.d. x 1.20 µm, Alltech, Dierfield, IL). GC method parameters: 220°C for two min followed by a ramp to 245°C at 10°C min⁻¹, with six 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, San Jose, CA). 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 were added to one 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 two ml chloroform and three ml methanol to recover all the seed parts and lipids. The rinses combined with the samples were mixed with the one ml of isopropanol. A phase separation was produced by adding 1.6 ml of H₂O, two ml of CHCl₃ and two 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 N₂ and resuspended in 0.5 ml toluene containing 0.005% BHT before TLC analysis.

**Characterization of TAG species**

Total extracted lipids were separated by TLC (Silica gel 60, 20 x 20 cm, EMD Chemicals Inc., Gibbstown, NJ). For analytical quantization of TAG species each was separated based on the number of hydroxy groups by using a double development: first develop 12 cm in CHCl₃/MeOH/HOAc (93/3/0.5, v/v/v), in a vacuum for 15 min, followed by a full development in CHCl₃/MeOH/HOAc (99/0.5/0.5, v/v/v). All TLC solvents contained ~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, nine mg of total TAG was separated by TLC with one development in CHCl₃/acetone/acetic acid (96/3.5/0.5, v/v/v). TAGs were eluted from the TLC silica twice by washing with five ml of CHCl₃/MeOH (4:1, v/v). A phase separation was induced by addition of two ml of MeOH and four ml of 0.88% KCl. The CHCl₃ phase was collected and the aqueous phase was back-extracted with five ml CHCl₃. The CHCl₃ was dried.
under N₂ and resuspended in 0.5 ml toluene plus 0.005% BHT. Lipase digestion was modified from an already described protocol (Cahoon et al., 2006). Half to one mg of 1- or 2-HFA-TAG was resuspended in one ml of diethyl ether and 0.8 ml of buffer containing 50 mM NaBr pH 7.6 and 5 mM CaCl₂. 200 µl of lipase (Rhizomucor miehei lipase, Sigma-Aldrich Co, St Louis, MO) was added and the tubes were vortexed for 40 min allowing ~50% TAG digestion. The reaction was stopped by adding two ml of MeOH:CHCl₃ (1:1, v/v). The CHCl₃ layer was collected and the TAGs, DAGs, monoacylglycerol (MAGs) and free FAs were separated by TLC in CHCl₃/MeOH/acetic acid (98/2/0.5, v/v/v) 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 position 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 to 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. Phosphatidylcholine and total neutral lipids were separated by TLC in CHCl₃/MeOH/acetic acid (75/25/8, v/v/v) and lipid bands were collected and analyzed by GC as before.

**Supplemental Data**

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

**Supplemental Table S2.** Primers used for reverse transcriptase PCR analysis
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.

Acknowledgments

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

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-Nave P, Sty\ymne S (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

Banas A, Dahlqvist A, Stahl U, Lenman M, Sty\ymne S (2000) The involvement of phospholipid : diaclylglycerol 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, Ba XM, 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) Limanthes 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 Biotech 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, Cahoon EB, Gedil M, Stanke M, Haas BJ, Wortman JR, Fraser-Liggett CM, Ravel J, Rabinowicz PD (2010) Draft genome sequence of the oilseed species Ricinus communis. Nat Biotechnol 28: 951-953

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 H (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 XXX. 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 JC (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, Valetin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (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-Nave 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, Scholkopf B, Weigel D, Lohmann JU (2005) A gene expression map of Arabidopsis thaliana development. Nat Genet 37: 501-506

Shitani 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
Stahl 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 Biotech 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 Science 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) 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: Article No.: 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
Figure legends

Figure 1. A simplified metabolic scheme for the production of HFAs and TAG assembly. Solid lines indicate glycerolipid flux; 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 headgroup to produce HFA-DAG by PDCT, reverse CPT, PLC or PLD / PAP. C, Direct transfer to DAG to TAG by PDAT. Enzyme abbreviations: FAH12, fatty acid hydroxylase 12; LPCAT, acyl-CoA:lyso phosphatidylcholine acyltransferase; PLA$_2$, phospholipase A$_2$; LACS, long chain acyl-CoA synthetase; GPAT, acyl-CoA:G3P acyltransferase; LPAT, acyl-CoA:LPA acyltransferase; PAP, PA phosphatase; PDCT, PC:DAG cholinephosphotransferase; CPT, CDP-choline:DAG cholinephosphotransferase; PLC, phospholipase C; PLD, phospholipase D / PAP; DGAT, acyl-CoA:DAG acyltransferase; PDAT, phospholipid:diacylglycerol acyltransferase. Substrate abbreviations: PC, phosphatidylcholine; LPC, lyso-phosphatidylcholine; G3P, glycerol-3-phosphate; LPA, lyso-phosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol. Enzymatic reactions are underlined.

Figure 2. HFA levels in CL37 and CL37 RcPDAT1A T$_7$ 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 15-17 individual plants. Each symbol represents an individual plant. The data represent the average ± 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) and CL37 RcPDAT1A seeds (black), B, mol% TAG molecular species of CL7 (white) and CL7 RcDGAT2 seeds (black). 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 the average of three replicates ± SE.
**Figure 4.** Percentage HFAs at the sn-2 position compared to 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 the average 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 to 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 the average of three replicates ± SE.

**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, % HFAs in neutral lipids from CL37 and CL37 RcPDAT1A. B, % HFAs in PC from CL37 and CL37 RcPDAT1A. C, % HFAs in neutral lipids from CL7 and CL7 RcDGAT2. D, % HFAs in PC from CL7 and CL7 RcDGAT2. The data represent the average of three replicates ± SE.

**Figure 7.** Summary of analysis of HFA accumulation in CL37, CL37 RcPDAT1A and CL37 RcPDAT1A RcDGAT2 T₃ seeds. A, percentage HFAs (total FA composition is in Supplemental Figure S2). B, µg HFAs per seed. C, µg total FAs per seed. CL37 (n = 4), CL37 RcPDAT1A (n = 17) and CL37 RcPDAT1A RcDGAT2 (n =14). The data represent the average ± SE.
Table 1. Fatty acid 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 are obtained from independent transformation events. All data are the average of three independent measurements ±SE

| Transgenic line | Fatty acid composition (% of total) | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:1 | 18:1-OH | 18:2-OH | Sum of HFAs |
|-----------------|-----------------------------------|------|------|------|------|------|------|---------|---------|-------------|
| 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 | 29.8±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     |
Figure 1. A simplified metabolic scheme for the production of HFAs and TAG assembly. Solid lines indicate glycerolipid flux; 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 PLA2 and LACS. B, Removal of the PC phosphocholine headgroup to produce HFA-DAG by PDCT, reverse CPT, PLC or PLD / PAP. C, Direct transfer to DAG to TAG by PDAT. Enzyme abbreviations: FAH12, fatty acid hydroxylase 12; LPCAT, acyl-CoA:lysophosphatidylcholine acyltransferase; PLA2, phospholipase A2; LACS, long chain acyl-CoA synthetase; GPAT, acyl-CoA:G3P acyltransferase; LPAT, acyl-CoALPA acyltransferase; PAP, PA phosphatase; PDCT, PC:DAG cholinephosphotransferase; CPT, CDP-choline:DAG cholinephosphotransferase; PLC, phospholipase C; PLD, phospholipase D / PAP; DGAT, acyl-CoA:DAG acyltransferase; PDAT, phospholipid:diacylglycerol acyltransferase. Substrate abbreviations: PC, phosphatidylcholine; LPC, lyso-phosphatidylcholine; G3P, glycerol-3-phosphate; LPA, lyso-phosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol. Enzymatic reactions are underlined.
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 15-17 individual plants. Each symbol represents an individual plant. The data represent the average ± 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) and CL37 RcPDAT1A seeds (black), B, mol% TAG molecular species of CL7 (white) and CL7 RcDGAT2 seeds (black). 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 the average of three replicates ± SE.
Figure 4. Percentage HFAs at the sn-2 position compared to 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 the average 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 to 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 the average of three replicates ± SE.
**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**, % HFAs in neutral lipids from CL37 and CL37 RcPDAT1A. **B**, % HFAs in PC from CL37 and CL37 RcPDAT1A. **C**, % HFAs in neutral lipids from CL7 and CL7 RcDGAT2. **D**, % HFAs in PC from CL7 and CL7 RcDGAT2. The data represent the average of three replicates ± SE.
Figure 7. Summary of analysis of HFA accumulation in CL37, CL37 RcPDAT1A and CL37 RcPDAT1A RcDGAT2 T3 seeds. A, percentage HFAs (total FA composition is in Supplemental Figure S2). B, µg HFAs per seed. C, µg total FAs per seed. CL37 (n = 4), CL37 RcPDAT1A (n = 17) and CL37 RcPDAT1A RcDGAT2 (n =14). The data represent the average ± SE.