PCP
PLANT & CELL PHYSIOLOGY

Regular Paper

# Endoplasmic Reticulum-Located PDAT1-2 from Castor Bean Enhances Hydroxy Fatty Acid Accumulation in Transgenic Plants

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(Received October 24, 2010; Accepted April 13, 2011)

**Ricinoleic acid (12-hydroxy-octadeca-9-enoic acid) is a major unusual fatty acid in castor oil. This hydroxy fatty acid is useful in industrial materials. This unusual fatty acid accumulates in triacylglycerol (TAG) in the seeds of the castor bean (*Ricinus communis* L.), even though it is synthesized in phospholipids, which indicates that the castor plant has an editing enzyme, which functions as a phospholipid:diacylglycerol acyltransferase (PDAT) that is specific to ricinoleic acid. Transgenic plants containing fatty acid Δ12-hydroxylase encoded by the castor bean *FAH12* gene produce a limited amount of hydroxy fatty acid, a maximum of around 17% of TAGs present in Arabidopsis seeds, and this unusual fatty acid remains in phospholipids of cell membranes in seeds. Identification of ricinoleate-specific PDAT from castor bean and manipulation of the phospholipid editing system in transgenic plants will enhance accumulation of the hydroxy fatty acid in transgenic seeds. The castor plant has three *PDAT* genes; *PDAT1-1* and *PDAT2* are homologs of *PDAT*, which are commonly found in plants; however, *PDAT1-2* is newly grouped as a castor bean-specific gene. PDAT1-2 is expressed in developing seeds and localized in the endoplasmic reticulum, similar to FAH12, indicating its involvement in conversion of ricinoleic acid into TAG. PDAT1-2 significantly enhances accumulation of total hydroxy fatty acid up to 25%, with a significant increase in castor-like oil, 2-OH TAG, in seeds of transgenic Arabidopsis, which is an identification of the key gene for oilseed engineering in production of unusual fatty acids.**

**Keywords:** Castor bean • Endoplasmic reticulum • Hydroxy fatty acid • Phospholipids • Ricinoleic acid • Triacylglycerol • Unusual fatty acid.

**Abbreviations:** CaMV, cauliflower mosaic virus; CBR, cytochrome b5 reductase; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; FAH12, fatty acid hydroxylase 12; GC, gas chromatography; LACS, long-chain acyl-CoA synthetase; LCAT, lecithin:cholesterol acyltransferase; PDAT, phospholipid:diacylglycerol acyltransferase; RT–PCR, reverse transcription–PCR; TAG, triacylglycerol; TLC, thin-layer chromatography; YFP, yellow fluorescent protein.

The nucleotide sequence data for the genes described in this study have been deposited in the GenBank/EMBL data libraries with the following accession number: *AtPDAT1* (At5g13640); *AtPDAT2* (At3g44830); *HsLCAT* (NM_000229); *RcPDAT1-1* (HM807520); *RcPDAT1-2* (HM807521); *RcPDAT2* (HM807522); *ScPDAT* (NM_001183185).

## Introduction

The castor bean plant (*Ricinus communis* L.) is a perennial shrub cultivated in tropical and subtropical areas of India, China and Brazil as an oilseed crop, and its seed accumulates 60% of oil in the form of triacylglycerol (TAG), which serves as a major energy source for seed germination and seedling establishment. TAG in seeds contains 80–90% ricinoleic acid (12-hydroxy-octadeca-9-enoic acid), an unusual fatty acid, which has numerous industrial applications, including high quality lubricants, paints, coatings, plastics, soaps, medications for treatment of skin infections, and cosmetics (Caupin 1997). Castor oil can be used as a lubricity additive for replacement of sulfur-based lubricity components in petroleum diesel, helping to reduce sulfur emissions (Goodrum and Geller 2005).

Due to the presence of the toxic ricin and hyperallergenic 2S albumins in the seed, development of transgenic oilseed crops for production of ricinoleic acid in high amounts by genetic engineering is desirable. In the past decade, scientists have sought to identify genes responsible for ricinoleic acid synthesis, and a castor plant gene, *FAH12*, for Δ12-hydroxylase, which is directly responsible for synthesis of ricinoleic acid, was

*Plant Cell Physiol.* 52(6): 983–993 (2011) doi:10.1093/pcp/pcr051, available online at www.pcp.oxfordjournals.org
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identified in the endosperm of the castor bean (van de Loo et al. 1995). Several independent experiments on transgenic expression of the *FAH12* gene under the control of different promoters in the model oilseed Arabidopsis produced only 17% hydroxy fatty acids as a percentage of the total fatty acids of seed, which was an undesirable amount for industrial purposes (Broun and Somerville 1997, Smith et al. 2003, Kumar et al. 2006, Lu et al. 2006). These results suggest that expression of a single key gene, *FAH12*, for ricinoleic acid biosynthesis in transgenic plants is insufficient for production of a large amount of ricinoleic acid in TAG. Limited synthesis of ricinoleic acid in Arabidopsis suggests that transgenic plants might not have an efficient mechanism for removal of unusual fatty acids synthesized in phospholipids and for transfer to TAGs in seed, in contrast to the original wild-type plants that accumulate high amounts of unusual fatty acids in their seeds (Millar et al. 2000, Jaworski and Cahoon 2003, Cahoon et al. 2007, Snyder et al. 2009, Napier and Graham 2010).

The regulatory genes encoding enzymes that influence TAG accumulation in castor bean seeds are largely unknown (Ohlrogge and Jaworski 1997). However, metabolic pathways and genes encoding enzymes involved in TAG synthesis in Arabidopsis, a model oilseed plant, have been well characterized by mutant (Routaboul et al. 1999, Zou et al. 1999, Zheng et al. 2003, Kim et al. 2005, Mhaske et al. 2005) and genomic analyses (Beisson et al. 2003). Based on information established in Arabidopsis, the TAG synthesis pathway can be investigated in castor bean for the identification of key regulatory genes for ricinoleic acid accumulation in seeds. Acyl-CoA-dependent diacylglycerol acyltransferase (DGAT) is thought to be a key enzyme in control of the biosynthetic rate of TAG in most oilseeds (Kennedy, 1961). DGAT is responsible for acylation at the *sn*-3 position of 1,2-diacylglycerol (DAG) using an acyl-CoA substrate (Zou et al. 1999, Lardizabal et al. 2001, Lu et al. 2003). Two types of enzyme, DGAT1 and DGAT2, have been reported in castor plants (He et al. 2004, Kroon et al. 2006, Shockey et al. 2006, Chen et al. 2007). A key role of castor bean DGAT2 in enhancement of hydroxy fatty acid accumulation in seeds of *FAH12* transgenic plants and in enhancement of synthesis of castor oil-like TAG species has recently been demonstrated (Burgal et al. 2008).

Phospholipid:diacylglycerol acyltransferase (PDAT) is an enzyme involved in synthesis of TAG during seed maturation by independent mediation of acyl-CoA in plants. This enzyme catalyzes formation of TAG by an acyl transfer from the *sn*-2 position of phospholipids at the *sn*-3 position of DAG. The *LOR1* gene encoding PDAT, first identified from yeast, and, subsequently, its homologous *PDAT1* (At5g13640) and *PDAT2* (At3g44830) from Arabidopsis have been characterized (Dahlqvist et al. 2000, Stahl et al. 2004). In Arabidopsis, PDAT1 was not a major determinant of TAG composition or content in developing seeds (Stahl et al. 2004, Mhaske et al. 2005) and it has overlapping function with DGAT1 in TAG biosynthesis in seeds of Arabidopsis (Zhang et al. 2010). PDAT substrate specificity has only been characterized in a few plant species (Stahl et al. 2004). In some plants that produce unusual fatty acids, PDAT has been shown to play a major role in removal of these unusual acyl groups from membrane phospholipids (Banas et al. 2000). In the development of castor bean seeds, PDAT activity with high specificity for ricinoleic acid was demonstrated in microsomal preparations (Dahlqvist et al. 2000). Ricinoleic acid is restricted only to TAGs in seeds rather than to phospholipids in cell membranes, even though this is synthesized at the *sn*-2 position of fatty acid in phospholipids. This indicates that castor plants have an editing mechanism for transfer of ricinoleic acid from phospholipid to TAG through PDAT. If PDAT having ricinoleate specificity is identified, this gene will be very useful for enhancement of production of hydroxy fatty acids in transgenic plants.

In this paper, we identified a ricinoleate-specific PDAT in seed development among three *PDAT* genes, *PDAT1-1*, *PDAT1-2* and *PDAT2*, isolated from castor bean. Castor bean *PDAT1-1* and *PDAT2* have similar gene structures and expression patterns to *PDAT1* and *PDAT2* of Arabidopsis (Stahl et al. 2004), respectively. However, castor bean-unique PDAT1-2, evolved from PDAT1, showed the same endoplasmic reticulum (ER) localization and similar seed-dominant expression to the FAH12 enzyme synthesizing ricinoleic acid. Transformation of castor bean *PDAT1-2* significantly enhanced the production of hydroxy fatty acids in FAH12 plants. Castor bean PDAT1-2 acts positively to increase the desired hydroxy fatty acids and decrease the oleic acids that are substrates for FAH12, and additionally to increase the TAG content in seeds without growth defects for transgenic plants. Ricinoleate-specific castor bean PDAT1-2 during TAG synthesis in seeds is a key regulator gene for production of hydroxy fatty acid in transgenic plants.

## Results

### The existence of three PDATs and PDAT1-2 is specific to the castor bean plant

Using the BLASTP algorithm with amino acid sequences of Arabidopsis PDAT1 (At5g13640) and PDAT2 (At3g44830), corresponding castor bean *PDAT* genes were searched in the castor bean genomic database (http://castorbean.jcvi.org/index.php). Three castor bean *PDAT* genes were identified with a low *P*-value (<2.3E-204) from the database. These castor bean *PDAT1-1*, *PDAT1-2* and *PDAT2* cDNAs were amplified and cloned by reverse transcriptase using gene-specific primers for each gene from RNA of the flower and developing seeds. Three castor bean *PDAT* cDNAs were sequenced and their deduced amino acids were aligned with amino acids of Arabidopsis and yeast PDATs, and human lecithin:cholesterol acyltransferase (LCAT) (**Fig. 1**, **Supplementary Fig. S1**, **Supplementary Table S1**). Castor bean PDAT1-1, the one closest to Arabidopsis PDAT1, with 79% identity, showed 76 and 61% identity in amino acids with castor bean PDAT1-2 and PDAT2, respectively. Castor bean PDAT2 is more similar to Arabidopsis PDAT2, showing 70% identity, than among

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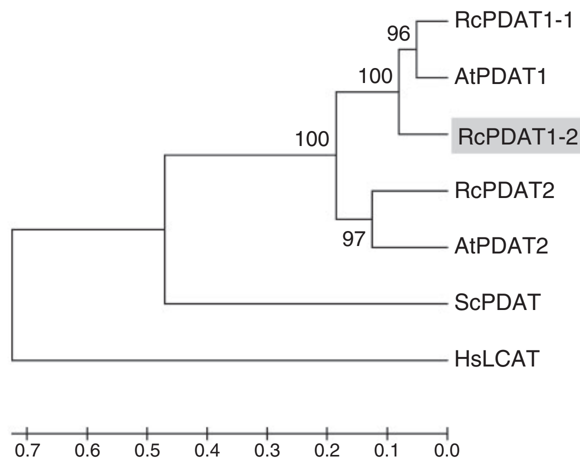


**Fig. 1** Phylogenic tree of castor bean PDAT1-1, PDAT1-2 and PDAT2 with known Arabidopsis and yeast PDATs, and human LCAT. The tree was calculated from aligned protein sequences using the CLUSTALW multiple alignment program with the Neighbor–Joining method (Saitou and Nei, 1987). The bootstrap consensus was analyzed by 500 replicates, and <50% bootstrap replicates were collapsed in branches.

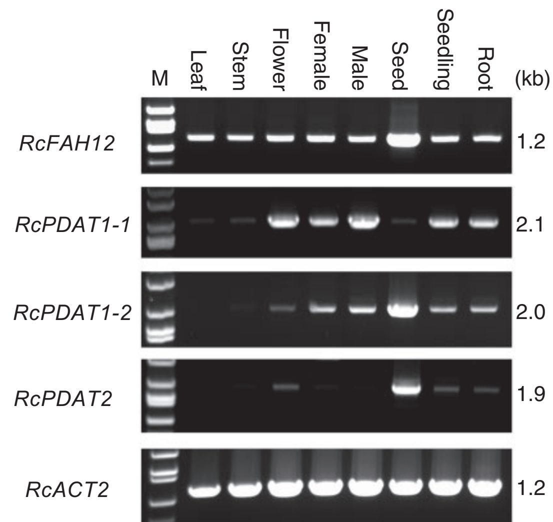


**Fig. 2** Expression of *PDAT1-1*, *PDAT1-2* and *PDAT2* genes in different tissues of castor bean by RT–PCR. Eight different tissues indicated in the figure were used as samples. Numbers on the right indicate DNA fragment size. The castor bean *ACTIN2* gene, *RcACT2*, was used as a control.

identified castor bean PDATs. Castor bean *PDAT1-2* has evolved from the *PDAT1* gene; however, this corresponding gene was not found in Arabidopsis (**Fig. 1**). The newly identified castor bean-specific isoform, PDAT1-2, might be a major PDAT for channeling ricinoleic acid in TAG synthesis in seeds.

## Expression of castor bean PDAT

Ricinoleic acids in castor bean are synthesized at the *sn*-2 position of phospholipids in the ER and excluded from phospholipids and then mainly accumulated in TAGs in seeds. Expression profiling of genes encoding PDAT-like enzyme can provide a clue to identification of the PDAT whose role excludes ricinoleic acids from phospholipids and transfers them to TAGs during seed development. Reverse transcription–PCR (RT–PCR) of the three putative castor bean *PDAT* genes shows that *PDAT1-1* is strongly expressed in reproductive tissues in flower and non-vegetative tissues, such as seedlings and roots, except for developing seeds. *PDAT2* is predominant in development of seeds and rare in other tissues. *PDAT1-1* and *PDAT2* have very similar expression patterns to those of Arabidopsis *PDAT1* and *PDAT2*, respectively (Stahl et al. 2004). Transcripts of the new isoform, *PDAT1-2*, are expressed mainly in developing seeds and weakly in all other tissues, indicating a unique function in the castor plant, and show similar expression patterns to those of the *FAH12*-encoded enzyme for synthesis of ricinoleic acids in castor bean seeds (**Fig. 2**).

## ER localization of castor bean PDAT1-1 and PDAT1-2

Synthesis of phospholipid-Δ12 hydroxy fatty acids by FAH12 and formation of TAGs containing ricinoleic acids occur in the ER. In order to act as a lipase for removal of ricinoleic acids from the *sn*-2 position of phospholipids and as an acyltransferase for direct transfer of these fatty acids to TAGs during seed development, ricinoleate-specific PDAT should be localized in the ER. Three *PDAT* cDNAs were fused in front of the *yellow fluorescent protein* (*YFP*) gene in-frame under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Each *Agrobacterium* containing the construct was infiltrated into epidermal cells of tobacco leaf and the fluorescence signal of YFP was detected by confocal microscopy. Fluorescence signals from cells transformed with PDAT1-1::YFP and PDAT1-2::YFP represent ER localization. The signal is visualized around the nucleus and thread-like ER networks, which indicates typical ER localization, whereas PDAT2::YFP shows a fluorescence signal indicating localization in the plasma membrane. As a control for ER localization, FAH12::YFP was tested using the same procedure and showed the same fluorescence signal pattern as those of PDAT1-1::YFP and PDAT1-2::YFP (**Fig. 3**). Taking these results together, PDAT1-1 and PDAT1-2 are localized in the ER, whereas PDAT2 is in the plasma membrane.

## Castor bean PDAT1-2 boosts accumulation of hydroxy fatty acids in seeds

The *PDAT1-1*, *PDAT1-2* and *PDAT2* genes driven by the *FAE1* seed-specific promoter (Rossak et al. 2001) were separately transformed into a CL37 line, transgenic Arabidopsis synthesizing 17% of hydroxy fatty acids in TAGs of seeds. A total of 12, 15 and 20 $T_1$ transgenic lines for the *PDAT1-1*, *PDAT1-2* and *PDAT2* genes, respectively, were selected by hygromycin resistance and PCR screening using gene-specific primers for detection of the castor bean *PDAT* transgene. Fatty acids were

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analyzed in $T_2$ seeds (the bulk of the segregant) from individual $T_1$ transgenic plants. Introduction of *PDAT1-1* and *PDAT2* into CL37 transgenic plants did not increase hydroxy fatty acids in seeds, recording an average of 17.3 ± 0.96 and 17.4 ± 0.68%, respectively, compared with 17.1 ± 0.48% (average ± SD) in CL37, whereas PDAT1-2 significantly increased the amount of hydroxy fatty acids, with an average of 19.8 ± 1.43% (average ± SD) in the bulk of segregant $T_2$ seeds compared with the parental CL37 line. The increase in hydroxy fatty acids ranged from 17.5 to 21.6% in PDAT1-2 transgenic plants toward 17% hydroxy fatty acids in CL37 (**Supplementary Fig. S2**).

Individual segregants in $T_2$ plants showing high accumulation of hydroxy fatty acids in seeds were grown and the harvested $T_3$ seeds were analyzed for their fatty acid composition. Average hydroxy fatty acids in segregant $T_3$ seeds of $T_2$ plants, three independent (1-1, 1-3, 1-4 lines) for PDAT1-1 and one (3-1 line) for PDAT2 transgenic plants, were less than that of the CL37 parent. Even in segregant $T_3$ seeds from $T_2$ plants, PDAT1-2 showed a significant effect on the increase in hydroxy fatty acids in transgenic plants (2-1 and 2-2 lines), indicating a high copy number or homozygous transgenes. The $T_2$ trangenic plant 2-4 line showed a heterozygote containing one copy of the transgene based on a $T_3$ segregation ratio of 3 : 1 ($\chi^2 = 0.77$, $P > 0.95$) for hydroxy fatty acid, indicating that 10 out of 12 plants produced >18.1%, a higher level than the CL37 parent. One line of $T_2$ homozygous plants from the 2-1 line for PDAT1-2 produced 25.1% hydroxy fatty acids in Arabidopsis seeds, which is a 41% further increase in production of hydroxy fatty acid compared with CL37 (**Fig. 4**). Individual $T_4$ seeds from two $T_3$ lines, 2-1-8 and 2-1-16, were germinated and grown. Fatty acids were analyzed in $T_5$ seeds harvested from six $T_4$ independent plants and compared with those of CL37 parent plants. Hydroxy fatty acids (18:1-OH+18:2-OH) are produced at ∼22.1% in seed from $T_4$ plants of two CL37 + RcPDAT1-2 lines (**Table 1**). The increase in hydroxy fatty acids in *PDAT1-2* transgenic seeds is not a dose effect of the transgene. In developing seeds of *PDAT1-1* (17.6% hydroxy fatty acids) and *PDAT2* (17.8% hydroxy fatty acids) transgenic lines producing a similar amount of hydroxy fatty acid to the CL37 parent (17.5% hydroxy fatty acids), their transgene expression is detected at almost the same levels compared with that of *PDAT1-2* (22.1%) (**Supplementary Fig. S3**). Altogether, seed-predominant expression, subcellular localization to the ER and enhanced accumulation of hydroxy fatty acids in seeds indicate that PDAT1-2 has specific activity for hydroxy fatty acids.

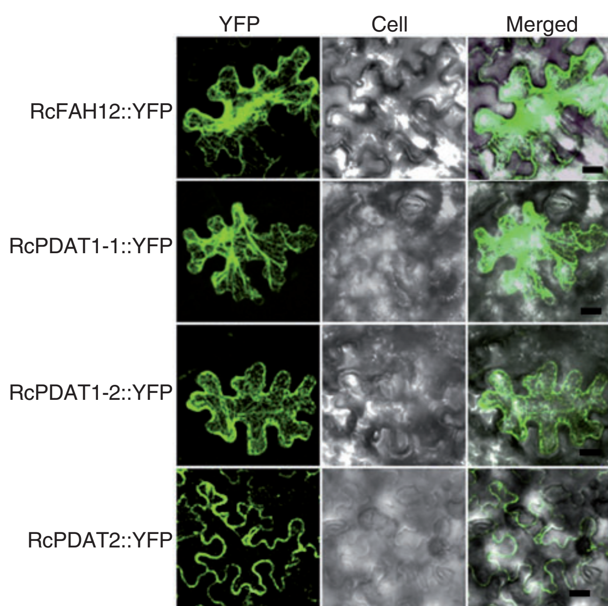


**Fig. 3** Subcellular localization of castor bean PDAT1-1, PDAT1-2 and PDAT2. A sequence encoding YFP was fused to the 3′ end of three full-length *PDAT* cDNAs without a stop codon under the control of the CaMV 35S promoter. The RcFAH12::YFP, RcPDAT1-1::YFP, RcPDAT1-2::YFP and RcPDAT2::YFP constructs were transformed into tobacco epidermal cells by infiltration of leaves with *Agrobacterium* cultures. RcFAH12::YFP was used as an ER control marker. Scale bars represent 20 µm.

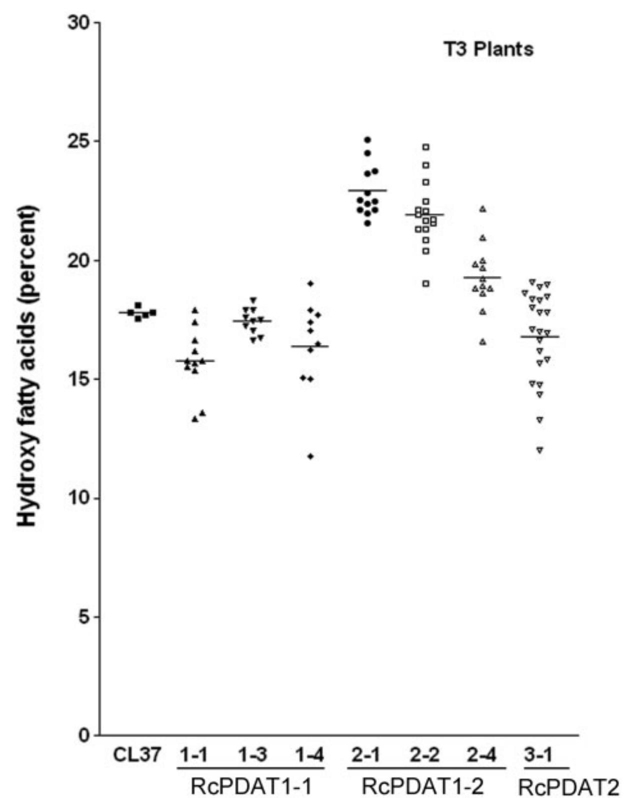


**Fig. 4** Hydroxy fatty acid content of seeds from Arabidopsis lines co-expressing castor bean fatty acid hydroxylase 12 (*RcFAH12*) and phospholipid:diacylglycerol acyltransferase, *RcPDAT1-1*, *RcPDAT1-2* and *RcPDAT2*. Each data point represents the hydroxy fatty acid content in a $T_3$ plant of a $T_2$ individual progeny plant derived from $T_1$ transgenic plants. Horizontal bars indicate the mean for each data set.

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**Table 1** Fatty acid composition from seeds of two individual $T_4$ plants for CL37 + RcPDAT1-2

| Transgenic line ($n=6$) | Fatty acid composition (% of total) | | | | | | | | | Sum of HFAs[a] (±SD) | Increase (%) in HFAs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:0 | 20:1 | 18:1-OH | 18:2-OH | | |
| CL37 | 15.5 | 7.3 | 31.1 | 21.4 | 5.7 | 1.0 | 0.4 | 14.8 | 2.9 | 17.8 ± 0.41 | 0.0 |
| CL37 + RcPDAT1-2 ($T_4$ of 2-1-8) | 14.0 | 6.7 | 28.4 | 20.0 | 7.6 | 0.8 | 0.4 | 18.3 | 3.8 | 22.1 ± 0.33 | 24.7 |
| CL37 + RcPDAT1-2 ($T_4$ of 2-1-16) | 14.3 | 7.6 | 28.5 | 19.3 | 6.8 | 1.0 | 0.4 | 18.8 | 3.3 | 22.1 ± 0.35 | 24.3 |

The value of fatty acids was the average of six independent plants.
[a] HFAs, hydroxy fatty acids.

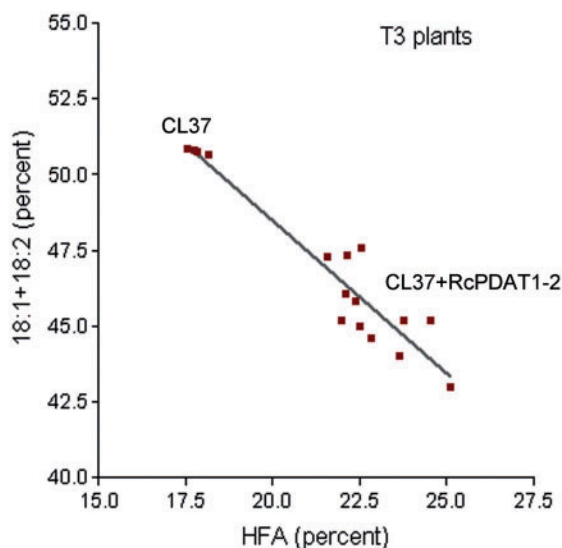


**Fig. 5** Correlation between the increase in hydroxy fatty acids and decrease in the sum of their substrates oleic acid (18:1) and linoleic acid (18:2) in seeds of CL37 + RcPDAT1-2 transgenic plants. CL37 and CL37 + RcPDAT1-2 transgenic seed lines were analyzed. The fatty acid composition of individual $T_3$ seeds derived from the $T_2$ (2-1) transgenic line was used to test correlation.

## Castor bean PDAT1-2 is ricinoleate specific and enhances castor-like oil content

Castor bean PDAT1-2 enhances flux to TAGs of hydroxy fatty acids synthesized by FAH12 in transgenic seeds. PDAT1-2 acts specifically on hydroxy fatty acids. Individual transgenic plants of the 2-1 line containing *PDAT1-2* in a CL37 background exhibited reverse linear correlation ($r^2 = 0.8981$) between an increase of hydroxy fatty acids and a decrease in the contents of their substrates oleic acid and linoleic acid in seeds, which shows castor bean PDAT1-2 to be a hydroxy fatty acid-specific acyltransferase (**Fig. 5**).

TAG containing hydroxy fatty acids in seeds between CL37 and CL37 + PDAT1-2 was analyzed by thin-layer chromatography (TLC). Total lipids from 30 seeds from CL37 and three independent CL37 + PDAT1-2 $T_3$ lines, 2-1-8, 2-2-7 and 2-4-10, were extracted and analyzed by TLC. In CL37, TAG1 having the same mobility as TAGs from wild-type Arabidopsis seeds, and TAG2 and TAG3, having the same mobility as the 1OH-TAG and 2OH-TAG of castor bean seeds, respectively, were detected, which is comparable only with existing normal TAG in wild-type Arabidopsis (data not shown). All three types of TAG showed a greater increase in CL37 + PDAT1-2 than in CL37, especially the 2OH-TAG, which was significantly increased by about 3.4- to 6-fold (**Fig. 6A, B**).

PDAT1-2 increased the amount of total TAGs per seed. The relative TAG amount was calculated by fatty acid analysis based on the spot area of the TAG standard as 100% in CL37. Total TAGs from seeds of the CL37 + PDAT1-2 lines 2-1-8, 2-2-7 and 2-4-10 were increased to 124, 159 and 132%, respectively (**Fig. 6B**). Actually, PDAT1-2 could recover the amount of TAGs from 64% of the wild-type level in CL37 to 89% the level of the wild type (data not shown). Castor bean PDAT1-2 showed a positive effect on the increase in both the molar ratio of hydroxy fatty acids (**Fig. 4**) and the amount of total TAGs in transgenic seeds (**Fig. 6A, B**).

The fatty acid composition of each TAG molecule from CL37 and CL37 + PDAT1-2 lines was determined from a scraped TAG spot. TAG1 represents normal TAG, which does not contain hydroxy fatty acid. TAG1 of CL37 + PDAT1-2 contains a relatively higher percentage of polyunsaturated fatty acids (18:2 and 18:3) than that of CL37. The fatty acid composition of TAG2 and TAG3 did not show a significant change between CL37 and CL37 + PDAT1-2; however, a reverse relationship between the amount of ricinoleic acid and its substrate, oleic acid, was noticed. The fatty acid composition indicates that TAG2 and TAG3 are monoricinoleoyl-TAG (1OH-TAG) and diricinoleoyl-TAG (2OH-TAG), respectively (**Table 2**).

A TLC spot containing total polar lipids of seeds from CL37 + PDAT1-2 and CL37 plants was analyzed for fatty acid composition. Ricinoleic acid in polar lipids of CL37 + PDAT1-2 was significantly reduced compared with the CL37 parent line. The CL37 + PDAT2 lines 2-1-8, 2-2-7 and 2-4-10 contained only ricinoleic acid (18:1-OH), about 1.4, 1.1 and 1.5% of polar lipids, respectively, compared with 5.4% in CL37 (**Fig. 7**). Densipolic acid, 18:2-OH, found in total fatty acids in seeds, was not detected in the above three types of TAG molecules and polar lipids in seeds (**Table 2**, **Fig. 7**).

## Increased hydroxy fatty acid content does not affect seed physiology

Transgenic plants producing seeds containing a high percentage of hydroxy fatty acids of about 20–22% may affect growth

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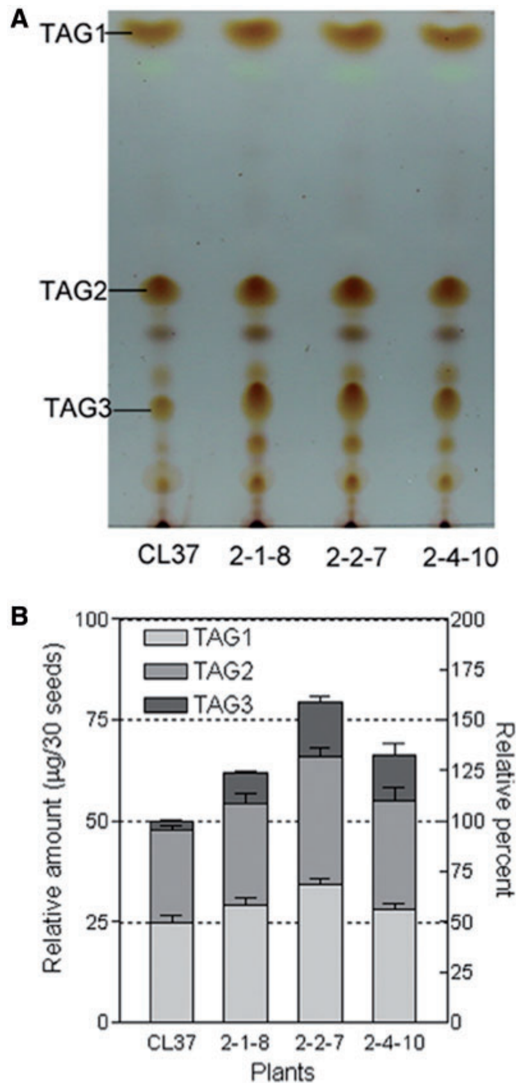


**Fig. 6** TLC analysis of TAGs isolated from seeds of CL37 and CL37 + PDAT1-2 transformed lines. (A) TAG separation in TLC. TAG1, normal TAG; TAG2, 1OH-TAG, TAG3, 2OH-TAG. (B) Relative amount of TAGs in seeds from CL37 and CL37 + PDAT1-2 lines (three individual $T_3$ lines, 2-1-8, 2-2-7 and 2-4-10, were analyzed). Three separate experiments (A) were repeated and showed similar results. Each value (B) is the mean ± SE of three independent measurements.

in the life cycle and germination of the seed of their progeny. In order to determine whether a high amount of hydroxy fatty acids in seeds driven by castor bean PDAT1-2 damages plant growth and physiology of seeds, CL37 parental plants (~17% hydroxy fatty acids) and three independent $T_3$ homozygous CL37 + PDAT1-2 (range from 20 to 22% hydroxyl fatty acids) were compared. The high level of hydroxy fatty acids in CL37 + PDAT1-2 transgenic plants was clearly heritable. Furthermore, seeds of CL37 + PDAT1-2 transgenic lines were germinated and grew as well as the parental CL37, and subsequent plant growth, development and seed production were normal. Seed weight and total seed lipid content of parental CL37 and six different $T_3$ lines of CL37 + PDAT1-2 were compared. The 100 seed weight averaged across six CL37 + PDAT1-2 lines was 1.47 ± 0.084 mg, compared with 1.34 ± 0.061 mg (average ± SE) for CL37 parental lines (**Supplementary Fig. S4A**). The average total fatty acid content of seeds was 228 ± 4.56 and 207 ± 5.6 µg $mg^{-1}$ for six different $T_3$ lines of CL37 + PDAT1-2 and CL37, respectively (**Supplementary Fig. S4B**). The increase in the total fatty acid content in CL37 + PDAT1-2 may be correlated with enhancement of the amount of TAG (**Figs. 6A, B**). These results indicate that the increase in total fatty acids due to efficient hydroxy fatty acid accumulation in TAG by the action of castor bean PDAT1-2 also increased seed weight, which had no detrimental effect on germination and growth, compared with CL37 parental controls. Castor bean PDAT1-2 does not significantly alter the fatty acid composition of leaves (**Supplementary Fig. S5**).

### Co-expression of castor bean *PDAT1-2* and *DGAT2* does not further increase the accumulation of hydroxy fatty acids in transgenic seeds

Castor bean DGAT2 is known as a hydroxy fatty acid booster (Burgal et al. 2008), so we tested if co-expression of *DGAT2* and *PDAT1-2* in developing seed has an additive effect on the accumulation of hydroxy fatty acids. CL37 + RcPDA1-2 homozygous plants producing 22.1% hydroxy fatty acids were transformed with *DGAT2* under the control of a seed-specific napin promoter. Even though five individual $T_1$ transgenic plants that were resistant to glufosinate expressed all three transgenes, *FAH12*, *PDAT1-2* and *DGAT2*, in their developing seed, the level of hydroxy fatty acids did not increase compared with the parental CL37 + PDAT1-2 line. Co-expression of *PDAT1-2* and *DGAT2* leads to 20–22% hydroxy fatty acids compared with the 22% of the CL37 + PDAT1-2 line (**Supplementary Fig. S6**). This indicated no significant additive effect of DGAT2 in the CL37 + PDAT1-2 background, probably due to competition in transferring hydroxy fatty acids to the same target position in *sn*-3 of DAG to produce TAG. van Erp et al. (2011) also conducted this same experiment and the results showed an increase of about 1.3% in hydroxy fatty acids in Arabidopsis seeds when using the phaseolin promoter. This difference may be caused by the different strengths and times needed by the napin and phaseolin promoters.

## Discussion

Castor bean accumulates an unusual fatty acid, ricinoleic acid, comprising >80–90% of TAG during seed development. This fatty acid is synthesized from oleic acid by a Δ12- hydroxylase, FAH12, to add a hydroxy group to the 12th position carbon in the castor plant instead of forming a double bond in the same position by FAD2 in other plants (van de Loo et al. 1995). The presence of the hydroxy group in fatty acids gives the advantage of availability to chemical reaction for production of diverse,

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**Table 2** Fatty acid composition (mol %) of seed TAGs from transgenic plants

| Fatty acid | TAG1 | | | | TAG2 (1OH-TAG) | | | | TAG3 (2OH-TAG) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CL37 | 2-1-8 | 2-2-7 | 2-4-10 | CL37 | 2-1-8 | 2-2-7 | 2-4-10 | CL37 | 2-1-8 | 2-2-7 | 2-4-10 |
| 16:0 | 36.5 | 23.0 | 27.2 | 27.2 | 24.8 | 15.9 | 16.2 | 16.8 | 8.1 | 7.4 | 9.1 | 9.4 |
| 18:0 | 14.7 | 17.0 | 20.0 | 13.9 | 10.1 | 11.9 | 11.6 | 10.2 | 7.0 | 5.8 | 6.0 | 6.1 |
| 18:1 | 41.6 | 36.6 | 33.5 | 31.2 | 32.1 | 34.5 | 26.3 | 30.7 | 17.2 | 17.2 | 16.0 | 20.2 |
| 18:2 | 5.6 | 16.8 | 13.2 | 20.1 | 6.6 | 11.5 | 9.7 | 10.1 | 8.3 | 6.5 | 7.4 | 7.4 |
| 18::3 | 0.0 | 4.0 | 2.8 | 5.2 | 1.2 | 2.8 | 2.0 | 2.4 | 0.9 | 2.1 | 2.2 | 2.9 |
| 20:0 | 1.6 | 2.3 | 2.6 | 1.8 | 1.0 | 1.2 | 1.1 | 0.7 | 0.0 | 0.3 | 0.5 | 0.7 |
| 20:1 | 0.0 | 0.3 | 0.7 | 0.6 | 0.0 | 0.5 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 18:1-OH | 0.0 | 0.0 | 0.0 | 0.0 | 24.2 | 21.7 | 32.8 | 29.1 | 58.5 | 60.7 | 58.8 | 53.3 |

Data are representative of one of three separate experiments that gave similar results.
Densipolic acid, 18:2-OH, was not detected in TAGs of seeds.

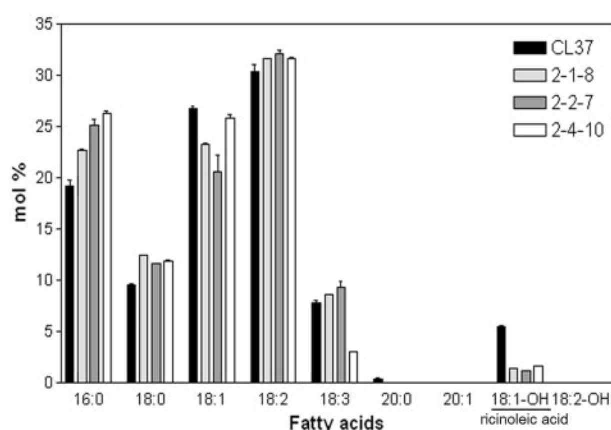


**Fig. 7** Fatty acid composition of the total polar lipid fraction in seeds of CL37 and CL37 + PDAT1-2 lines (2-1-8, 2-2-7 and 2-4-10). The lipid spot in the origin line on TLC developed with hexane : diethylether : acetic acid (140 : 6 : 2) was extracted and analyzed by GC. Each value is the mean ± SE of three independent measurements.

useful industrial raw materials (Caupin 1997). Due to the nature of the poor agricultural traits in the castor plant, such as the presence of the toxin ricin, hyperallergenic proteins in seeds, irregular ripening of seeds and the high height of the plant for harvesting using a machine (Atsmon 1989), research to produce hydroxy fatty acids in oilseed crops is desired and focused. However, expression of the oleate $\Delta$12-hydroxylase gene, *FAH12*, isolated from the castor bean, in Arabidopsis as a model system has produced no more than 17% hydroxy fatty acids in transgenic lines (Broun and Somervile 1997, Smith et al. 2003, Kumar et al. 2006, Lu et al. 2006). The main reason for the low yield of the hydroxy fatty acids in seeds of transgenic plants was predicted by the absence of the acyltransferase for catalysis specifically of hydroxy fatty acids. In this study, castor bean *PDAT1-2* has been identified as a gene that is an ER-located ricinoleate-specific PDAT in seeds (**Figs. 2**, **3**) and is able to significantly increase the hydroxy fatty acid content in castor-like TAG from 17% to 25% in transgenic Arabidopsis (**Fig. 4**, **Supplementary Fig. S2**).

In order to find genes that boost hydroxy fatty acid accumulation in seed oils of transgenic Arabidopsis, a high-throughput screening method was applied by shotgun transformation with the entire cDNA of developing castor bean seed endosperm into Arabidopsis expressing a castor bean FAH12; however, the result did not demonstrate great success (Lu et al. 2006). Conversely, random mutation in the genome of Arabidopsis accumulating 17% hydroxy fatty acids pointed out the necessary enzymes for the hydroxylase activity. A decrease in hydroxy fatty acids by mutation of a gene revealed that the FAH12 catalytic reaction requires an electron from NADH:cytochrome b5 reductase (CBR). This study indicates that the original castor bean electron donor system, cyt *b*5 and CBR, may be important in order to boost hydroxy fatty acid accumulation in transgenic plants (Kumar et al. 2006). Regarding the low production rate of hydroxy fatty acids in transgenic plants, it was estimated that the host crop plant is lacking acyltransferases involved in efficient conversion of these unusual fatty acids to TAG; however, castor plants might present acyltransferases, co-evolved with FAH12 that favor hydroxy fatty acids. For example, castor bean DGAT2, a rate-limiting acyltransferase for the final stage of TAG synthesis, effectively improves the hydroxy fatty acid content from 17 to 30% in Arabidopsis transgenic seeds (Burgal et al. 2008).

Enzymes encoding TAG synthesis genes in castor bean seeds are largely unknown. However, Arabidopsis genes involved in TAG synthesis have been well studied. A project for sequencing of the castor bean genome was recently completed (Chan et al. 2010) and is currently available for investigation of putative genes for TAG synthesis. Since ricinoleic acids are synthesized from oleic acid by FAH12 at the *sn*-2 position of phospholipids in the ER of developing seeds (Bafor et al. 1991), ricinoleic acids should be edited to ricinoleoyl-CoA by an unidentified phospholipase $A_2$ (Stahl et al. 1998) and long-chain acyl-CoA synthetase (LACS) (Shocky et al. 2002), in order to accumulate in TAG by acyl-CoA-dependent DGAT2. Another known TAG

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synthesis mechanism is the acyl-CoA-independent pathway involving PDAT, which directly transfers an acyl group from the *sn*-2 position of phospholipids to DAG for TAG synthesis. In the castor plant, a preference of ricinoleic acid for PDAT activity in the microsomal fraction of the seeds was detected; however, the genes encoding an enzyme for this activity have not yet been isolated (Bafor et al. 1991, Dahlqvist et al. 2000). The PDAT gene was identified by mutant analysis in yeast. The yeast *LOR1* gene encodes PDAT activity and subsequently predicted Arabidopsis *PDAT* genes (Dahlqvist et al. 2000). In vitro testing of Arabidopsis PDAT1 showed high catalytic activity toward unusual fatty acids, including ricinoleic acid, relative to common fatty acids, oleic and linolenic acids (Stahl et al. 2004). In this study, we identified the ER-located PDAT1-2 in castor bean seeds and demonstrated that PDAT1-2 catalyzes specifically the reaction to produce ricinoleic acid in vivo and generates castor-like TAGs in transgenic seeds. This authentic function of PDAT1-2 towards ricinoleic acid was supported by the following traits. Among members of the PDAT gene family, the PDAT1-2 gene is uniquely present in the castor plant. This PDAT1-2 was evolved from PDAT1 present in all plants (**Fig. 1**). This indicates that PDAT1-2 is co-evolved from PDAT1, having activity for the common acyl moiety, simultaneously with the appearance of FAH12 derived from FAD2. PDAT1-2 shows co-expression of transcript and co-localization of enzyme with FAH12 which is predominantly expressed in seeds and present in the ER (**Figs. 2**, **3**). PDAT1-2 showed a similar effect to DGAT2 for enhancement of the hydroxy fatty acid in seeds of transgenic plants through different mechanisms (**Figs. 4**, **5**, **Table 1**). Identification of castor bean PDAT1-2 facilitates selection of genes encoding PDAT1-2 from a variety of plants producing unusual fatty acids and provides an important gene for overcoming the bottleneck that limits production of unusual fatty acids in transgenic plants.

Introduction of castor bean PDAT1-2 into Arabidopsis CL37 produced an increase of 38% in TAG (**Fig. 6**) and the amount of total fatty acids compared with host CL37 lines (**Supplementary Fig. S4B**) as well as an increase of hydroxy fatty acids in seeds (**Fig. 4**, **Table I**). Castor bean PDAT1-2 might positively flux hydroxy fatty acid to TAG and generate recovery of the amount of TAG compared with inefficient transition of TAG in CL37. PDAT1-2 does not affect gene expression involved in the fatty acid composition in transgenic seeds. Transcript levels of *FAH12*, *FAD2* and *FAD3* genes in seeds of CL37 and CL37 + PDAT1-2 were not altered (**Supplementary Fig. S7**). These results suggest that PDAT1-2 increased the flux of synthesized hydroxy fatty acids in phospholipids (**Fig. 7**) to TAG without affecting the expression of synthesis and desaturation enzymes for hydroxy fatty acid.

Identification of castor bean PDAT genes can help to propose a model editing mechanism between phospholipids and TAG for unusual fatty acids in lipid biosynthesis. In this study, we found that castor bean PDAT1-1 is located in the ER (**Fig. 3**) and expressed in other tissues rather than seeds (**Fig. 2**). Since FAH12 shows leaky expression in all tissues (van de Loo et al. 1995), perhaps PDAT1-1 could be involved in removal of ricinoleic acids in phospholipids of the cell membrane. Castor bean PDAT2 is expressed specifically in the seeds (**Fig. 2**); however, it is located in the plasma membrane rather than in the membrane of the ER (**Fig. 3**). In seed development of the castor bean, ricinoleic acids are synthesized actively in phospholipids in the membrane of the ER, and PDAT2 may be the final editor for removal of this hydroxy fatty acid of the cell membrane in tissue where massive synthesis occurs. PDAT2, unlike PDAT1-2 and PDAT1-1, does not have a transmembrane domain in the N-terminus of the protein (**Supplementary Fig. S1**). The question of how PDAT2 can be destined to the plasma membrane and work for unusual fatty acids needs to be investigated.

To evaluate the physiological roles of three PDATs in castor plants, further study is still necessary to obtain information relating to enzymatic activity and substrate specificity.

## Materials and Methods

### Plant materials and transformation

Castor bean (*R. communis* L.) seeds, IT196881, were obtained from the Germplasm Resources Center in the Rural Development Administration, Republic of Korea. Castor plants were grown in the greenhouse at temperatures between 18 and 28°C. The CL37 line, which stably expresses the Δ12-hydroxylase gene, *FAH12*, and produces approximately 17% of hydroxy fatty acids, was used as a parental plant to test PDAT activity by gene transformation. Plant transformation was accomplished by the floral dipping method using *Agrobacterium* GV3101 containing each *PDAT* gene under a seed-specific promoter (Clough and Bent 1998). Arabidopsis transgenic plants were grown in controlled growth chambers at 22°C under a 16 h photoperiod.

### Identification of three PDAT genes from castor bean

Three castor bean *PDAT* genes were identified by BLAST search in the castor bean genomic database (http://castorbean.jcvi.org/index.php) using two previously identified Arabidopsis PDAT1 and PDAT2 protein sequences (Stahl et al. 2004). For isolation of cDNA corresponding to the identified three castor bean *PDAT* genes, specific primers were designed, as mentioned below, and used to amplify genes from seeds and flower RNAs by RT–PCR.

### Cloning of castor bean PDAT genes

Full-length cDNAs of castor bean *PDAT1-1*, *PDAT1-2* and *PDAT2* were amplified by RT–PCR from RNA of flowers or seeds and inserted into pENTR/D-TOPO (Invitrogen). Primers representing sequences at the 5′ and 3′ termini of the open reading frame from PDAT1-1, PDAT1-2 and PDAT2 cDNA, respectively, were as follows: 5′-CACCATGCCTGTAATTCGGAGG

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AAA-3′ and 5′-TTACAGTGGTAATTTGATCTT-3 (for *PDAT1-1*); 5′-CACCATGTCGATTTTGAGACGGAGA-3′ and 5′-CTATAG CGGCAAGTTGATCTT-3′ (for *PDAT1-2*); and 5′-CACCATGAT TGGGTATTTATGCACT-3′ and 5′-TTAGAGTTGAATGTTTAT TTT-3′ (for *PDAT2*).

## RT–PCR

PCR (30 cycles) was conducted on cDNA prepared from 1 μg of RNA from various tissues, using the *PDAT1-1-*, *PDAT1-2-* and *PDAT2*-specific primers mentioned above. The castor bean *ACTIN2* cDNA, accession No. AY360221, was used as an internal control, and was amplified using the following primers: 5′-GAG GATATTCAGCCCCTTG-3′ (forward) and 5′-TAGAAGCACTT CCTGTGGACA-3′ (reverse). *FAH12* was used as a control and amplified using the following primers: 5′-CACCATGGGAGGTG GTGGTCGCATG-3′ (forward) and 5′-TTAATACTTGTTCCGGT ACCA-3′ (reverse).

## Vector construction

A seed-specific Gateway vector was constructed by replacement of the 35S promoter with the Arabidopsis *FAE1* promoter (Rossak et al. 2001) and named pAtFAE1P-gate. The entry clones pENTR-PDAT1-1, pENTR-PDAT1-2 and pENTR-PDAT2 were reacted with the destination vector, pAtFAE1P-gate, by LR clonase (Invitrogen), and the corresponding PDAT gene was inserted under the *AtFAE1* promoter.

A full-length cDNA of *DGAT2* (Burgal et al. 2008) was amplified from the total RNA of developing seeds of castor bean plants using RT–PCR with primers 5′-CACCATGGGGG AAGAAGCGAATCAT-3′ and 5′-TCAAAGAATTCAAGTGTA AG-3′. This amplified fragment was cloned into pENTR/ D-TOPO and this generated an entry clone, pENTR-DGAT2. Using the LR clonase reaction, the *DGAT2* cDNA in the entry vector was inserted under a napin promoter in a Gateway vector, pNapin-gate, which was made by replacing the 35S promoter in pB2GW7 with a seed-specific napin promoter (Karimi et al. 2002). This plant expression vector, Napin-RcDGAT2, was used for additional transformation into Arabidopsis plants containing castor bean *FAH12* and *PDAT1-2* transgenes.

## Subcellular localization

Full-length genes of the three castor bean *PDAT* genes and *FAH12* minus the stop codon were amplified from cDNA and inserted by ligation with restriction endonucleases in front of the *eYFP* gene in-frame in the pFAST vector. The primers contained a *Sac*I site in the 5′ terminus and a *Sma*I site in the 3′ terminus; the primers 5′-TGGAGCTCATGCCTGTAATTCGGA GGAAA-3′ and 5′-TCCCCCGGGCAGTGGTAATTTGATCTT CTG-3′ for PDAT1-1; 5′-TGGAGCTCATGTCGATTTTGAGAC GGAGA-3′ and 5′-TCCCCCGGGTAGCGGCAAGTTGATCTT CTC-3′ for PDAT1-2; and 5′-TGGAGCTCATGATTGGGTATTT ATGCACT-3′ and 5′-TCCCCCGGGGAGTTGAATGTTTATTTT CTC-3′ for PDAT2 were used for amplification of cDNAs, respectively. Full-length FAH12 minus the stop codon was amplified from cDNA with the forward primer 5′-TCCCCCGG GATGGGAGGTGGTGGTCGCATG-3′ and the reverse primer 5′-TCCCCCGGGATACTTGTTCCGGTACCAGAA-3′. Amplicates digested with *Sma*I were ligated into the pFAST vector with infusion with eYFP under the CaMV 35S promoter.

The constructs PDAT1-1::YFP, PDAT1-2::YFP, PDAT2::YFP and FAH12::YFP were used for transformation of tobacco leaves. The *Agrobacterium* GV3101 cell-containing constructs were infiltrated into the abaxial side of leaves of 4- to 5-week-old *Nicotiana benthamiana* plants (Sparkes et al. 2006). A 40 h after infiltration, tobacco leaves were imaged with a TCS SP5 AOBS/Tandem laser confocal scanning microscope (Leica, Germany).

## Fatty acid analysis

The fatty acid composition of seeds and leaves was determined by gas chromatography (GC) after transmethylation at 90°C for 1 h in 0.5 ml of toluene and 1 ml of methanol containing 5% $H_2SO_4$ (v/v). Heptadecanoic acid (17:0) was added to each sample as an internal standard. Following transmethylation, 1 ml of aqueous 0.9% NaCl was added, and fatty acid methyl esters were recovered by three sequential extractions with 1 ml of hexane. Fatty acid methyl esters were then analyzed by GC (Shimadzu, Japan) on a 30 m × 0.32 mm (inner diameter) DB-23 column (Agilent Technologies), while increasing the oven temperature from 160 to 220°C at 2.5°C $min^{-1}$.

## TLC analysis

Seeds were homogenized in 1 ml of 2 : 1 (v/v) chloroform : methanol and then centrifuged at 20,000 × *g* for 5 min. Supernatants were lyophilized with $N_2$ gas and the remaining materials were dissolved in 20 μl of chloroform. Lipid was spotted on TLC plates (silica gel G60 plates; EM Separations Technology) and spots were developed with hexane : diethylether : acetic acid [140 : 60 : 2 (by vol.)]. Lipid was visualized by spraying the plate with 20% sulfuric acid and heating at 120°C for 15 min. TAG bands separated by TLC, visualized by lightly staining with iodine and scraped from the TLC plates were converted to fatty acid methyl esters and analyzed by GC, as described above.

## Supplementary data

Supplementary data are available at PCP online.

## Funding

This study was carried out with the support of the Research Program for Agricultural Science & Technology Development (Project No. PJ006715), National Academy of Agricultural Science, Rural Development Administration, Republic of Korea and the World Class University Project (R31-2009-000-20025-0) from the National Research Foundation of Korea.

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## Acknowledgments

We thank Dr. John Browse (Institute of Biological Chemistry, Washington State University) for providing the CL37 line, and Ju Young Kim (Chonnam National University) for providing technical assistance.

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