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Molecular and biochemical characterization of the OLE-1 high-oleic castor seed (*Ricinus communis* L.) mutant

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Running title: hydroxylase from a high-oleic castor bean

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Main conclusion: The natural OLE-1 high oleic castor mutant has been characterized, demonstrating that point mutations in the FAH12 gene are responsible for the high oleic phenotype. The contribution of each mutation was evaluated by heterologous expression in yeast and lipid studies in developing OLE-1 seeds provided new evidence of unusual fatty acids channelling into TAGs.

Abstract

Ricinus communis L. is a plant of the *Euphorbiaceae* family well known for producing seeds whose oil has a very high ricinoleic (12-hydroxy-octadecenoic) acid content. Castor oil is considered the only commercially renewable source of hydroxylated fatty acids, which have many applications as chemical reactants. Accordingly, there has been great interest in the field of plant lipid biotechnology to define how ricinoleic acid is synthesised, which could also provide information that might serve to increase the content of other unusual fatty acids in oil crops. Accordingly, we set out to study the biochemistry of castor oil synthesis by characterizing a natural castor bean mutant deficient in ricinoleic acid synthesis (OLE-1). This mutant accumulates high levels of oleic acid and displays remarkable alterations in its seed lipid composition. To identify enzymes that are critical for this phenotype in castor oil, we cloned and sequenced the oleate desaturase (FAD2) and hydroxylase (FAH12) genes from wild type and OLE-1 castor bean plants, and we analyzed their expression in different tissues. Heterologous expression in yeast confirmed that three modifications to the OLE-1 FAH12 protein were responsible for its weaker hydroxylase activity. In addition, we studied the expression of the genes involved in this biosynthetic pathway at different developmental stages, as well as that of other genes involved in lipid biosynthesis, both in wild-type and mutant seeds.

Keywords: Castor Bean, Fatty Acids, Lipid Synthesis, OLE-1 mutant, Oleate 12-hydroxylase, Oleate desaturase, *Ricinus communis*, Triacylglycerol.

Abbreviations:

FAD2 oleate Δ12-desaturase

FAH12 oleate Δ12-hydroxylase

OLE-1 High oleic castor mutant

PDAT Phospholipid:Diacylglycerol Acyltransferase

PDCT phosphatidyl choline:diacylglycerol choline phosphotransferase

ROD Phosphatidylcholine:diacylglycerol cholinephosphotransferase

TAG triacylglycerol

TM transmembrane helices

Introduction

The Castor bean (*Ricinus communis* L.) is an oilseed crop that produces oil containing around 85% ricinoleic acid (12-hydroxy-octadec-*cis*-9-enoic acid; 18:1-OH), conferring it with a higher viscosity, polarity and chemical reactivity than can not be found in common seed oils. Such properties are of particular value for many industrial fields, including the manufacturing of soaps, lubricants, hydraulic and brake fluids, paints, dyes, coatings, inks, cold resistant plastics, waxes and polishes, nylon, pharmaceuticals and perfumes. Despite being one of the most important industrial oils on the market worldwide, the development of castor bean oils that do not have a ricinoleic acid rich profile would help cover a wider range of market niches. As such, the isolation of the OLE-1 castor bean mutant that has a high-oleic and low-ricinoleic acid profile has given rise to new potential applications for castor oil (Rojas-Barros et al. 2004). Genetic studies on this line revealed that two genes (*Ol*, *Ml*) that display epistatic interactions are involved in this trait (Rojas-Barros et al. 2005). Interestingly, the increase in the proportion of oleic acid is not paralleled by an increase in linoleic acid, its most immediate derivative that is generated by the action of reticular desaturases. Thus, the low concentrations of polyunsaturated fatty acids in castor bean seeds is not a direct consequence of the competition between the hydroxylation and desaturation branches of the fatty acid synthesis pathway, but to lower levels of oleoyl-desaturase activity as was shown by McKeon et al. (1997).

The two enzymes responsible for the final oleic acid content of castor bean seeds, are oleate Δ12-hydroxylase (FAH12, EC 1.14.13.26) and oleate Δ12-desaturase (FAD2, EC 1.3.1.35), which are involved in ricinoleic and linoleic acid synthesis, respectively. Both these enzymes compete for oleate esterified at the *sn*-2 position of phosphatidylcholine (PC), which is their main substrate (Schmidt et al. 1993), and the

resultant acyl products are subsequently modified or transferred to triacylglycerol (TAGs; Bafor et al. 1991; Richards et al. 1993). Plant FAD2 desaturase catalyzes the O_2-dependent insertion of a cis-Δ12 double bond into oleic acid (18:1 Δ9) to produce linoleic acid (18:2 Δ9,12), using cytochrome b5 and NADH as co-factors (Kearns et al. 1991; Smith et al. 1992). In castor beans, 18:1-OH is synthesized by oleate Δ12-hydroxylase (RcFAH12) which catalyzes the reaction from 2-oleoyl-PC to 2-ricinoleoyl-PC (Moreau and Stumpf 1981; Bafor et al. 1991), using the same co-factors as FAD2. Both the FAD2 and FAH12 enzymes are integral membrane-bound desaturases found in the endoplasmic reticulum (ER; Sperling et al. 2003) and they share a common phylogenetic origin. These non-heme iron containing enzymes use a di-iron cluster for catalysis, and they contain three histidine boxes in their sequence ($H(X)_{3-4}H$, $H(X)_{2-3}HH$ and $H/Q(X)_{2-3}HH$) that are implicated in iron binding and that are essential for catalysis (Shanklin and Cahoon 1998). Moreover, both enzymes are closely related in terms of their overall amino acid identity (Van de Loo et al. 1995), unsurprisingly since oleate Δ12-hydroxylase is a divergent member of the FAD2 enzyme family, as are epoxygenases, acetylenases, conjugases and other desaturases (Lee et al. 1998). In fact the genes for the oleate 12-hydroxylase from castor (*RcFAH12*; Van de Loo et al. 1995), *Hiptage benghalensis* (Zhou et al. 2012) or *Claviceps purpurea* (Meesapyodsuk and Qiu 2008) have been cloned through their predicted homologies to the oleate Δ12-desaturase. Another hydroxylase from the unrelated *Lesquerella fendleri* was subsequently isolated using the castor hydroxylase sequence, which is involved in the first step of lesquerolic acid (14-OH-20:1-11c) biosynthesis (Broun et al. 1998a).

Functional analysis of *Rc*FAH12 in yeast (*Saccharomyces cerevisiae*), transgenic tobacco and Arabidopsis plants demonstrated that this enzyme predominantly acts as a hydroxylase, introducing hydroxyl groups at the 12-position of oleic acid (Van

de Loo et al. 1995; Broun and Somerville 1997; Broadwater et al. 2002; Smith et al. 2003a;). Expression of the *L. fendleri* hydroxylase gene in yeast (Broun et al. 1998b) and Arabidopsis (Broun et al. 1998a) results in the synthesis of either ricinoleic or linoleic acid (18:2) and their derivatives, displaying bifunctional oleate 12-hydroxylase:desaturase activity. By contrast, *Rc*FAH12 should not be considered a bifunctional enzyme even though close examination of its activity in the Arabidopsis *fad2* mutant revealed trace desaturase activity (Smith et al. 2003b). Nevertheless, *Rc*FAH12 and plant FAD2 sequences have been used to identify key amino acids that are important in determining the catalytic outcome of the reaction (Broun et al. 1998b; Mayer et al. 2005). Indeed, the substitution of six amino acids located adjacent to the histidine boxes with their hydroxylase equivalents is sufficient to convert a strict desaturase into a bifunctional desaturase:hydroxylase, and vice versa (Broun et al. 1998b).

In order to better understand the fatty acid biosynthesis in developing high-oleic castor bean seeds, we focused on the molecular characterization of the Δ12-hydroxylase and Δ12-desaturase enzymes in this mutant. In this study, we clarified the role of these genes in the high-oleic OLE-1 castor bean mutant, defining their sequences and their expression during seed development. Furthermore, the role that the differences found in the OLE-1 hydroxylase gene sequence play in the activity of each enzyme was studied through their heterologous expression in yeast. Finally, a parallel study on the fatty acid composition of the mutant glycerolipids and the implications of additional genes was also carried out.

Materials and methods

Biological material and growth conditions

Castor bean (*R. communis* L.) plants from the OLE-1 high oleic mutant (Rojas-Barros et al. 2004) and the near-isogenic wild type (WT), IN15 line, were obtained from the Instituto de Agricultura Sostenible (CSIC, Córdoba, Spain). Castor bean plants were cultivated in growth chambers on a 25/15 ºC (day/night) cycle, with a 16 h photoperiod and a photon flux density of 200 µmol m^{-2} s^{-1}. Mature female flowers were pollinated individually and tagged, and the tagging dates were recorded as 0 days after pollination (0 DAP). Capsules were harvested at 7-day intervals from 14 to 63 DAP, and the stages of development were classified (Greenwood and Bewey 1982). Dissected seeds were frozen immediately in liquid nitrogen and stored at –80ºC.

The *Saccharomyces cerevisiae* strain W303-1A MATa {leu2-3112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15} was used for the functional characterization of the genes. Yeast cells were grown at 30 ºC on a rotary shaker (200 rpm) in synthetic defined (SD) medium containing raffinose as a carbon source, and mixtures of amino acids and nucleoside precursors were used for marker selection.

The *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, CA, USA) was used as the host to clone the *RcFAH12* and *RcFAD2* genes. The bacteria were grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl [pH 7]) and the liquid cultures were shaken vigorously at 37 ºC, selecting the plasmid with ampicillin (100 µg ml^{-1}).

RNA preparation and cDNA synthesis

Developing castor bean seeds (approximately 0.25 g) were ground in liquid nitrogen using a precooled sterile mortar and pestle. Total RNA was isolated and first strand cDNA was synthesized as described previously (Sánchez-García et al. 2010).

Nucleic acid manipulation and PCR-based cloning

Single-stranded cDNAs were amplified with iProof™ High-Fidelity DNA Polymerase (Bio-Rad, Hercules, CA, USA) using specific primer pairs to synthesize full-length copies of each gene: *RcFAH*-F/*RcFAH*-R for *RcFAH12*; and *RcFAD2*-F/*RcFAD2*-R for *RcFAD2* (Table 1). The PCR reactions were first heated at 98 ºC for 30 s and they were then subjected to 30 cycles: 98 ºC for 10s, 50 ºC for 30s and 72 ºC for 30s. The PCR amplification products were cloned into the pMBL-T vector (Dominon North Kingstown, RI, USA) and their identities verified by sequencing.

Gene-specific primers were designed complementary to the 5´and 3´ends of the *FAH12* coding region, adding a *Hind*III (Hind*RcFAH12*F) and *Xba*I (Xba*RcFAH12*R) restriction site at the 5´ and 3´ end, respectively, for directional cloning into the yeast pYES2 expression vector (Invitrogen, Carlsbad, CA, USA; Table 1). To improve gene translation initiation in yeast cells, all the constructs contained a Kozak sequence (ACCATGG) (Table 1).

Castor bean FAH12 site-directed mutagenesis

Wild type amino acid residues were restored into the OLE1-*FAH12* gene by mutagenesis using the QuikChange Site-directed Mutagenesis Kit (Stratagene) and following the manufacture's protocol, and the construct was then cloned into the pYES2 vector (ThermoFisher Scientific, Waltham, MA, USA; pYES2:HO-FAH12). The S49F and Q319H mutations were introduced using the primers S49FMutHOFAH12F/S49FMutHOFAH12R and Q319HMutHOFAH12F/Q319HMutHOFAH12R, respectively (Table 1), generating the

constructs pYES2:Q319H-HO-FAH12 (m_1OLE1), pYES2:S49F-HO-FAH12 (m_2OLE1) and pYES2:S49F_Q319H-HO-FAH12 (m_3OLE1).

Functional expression in yeast

The plasmids encoding the wild type OLE-1 and the mutated hydroxylase activities were introduced into the *S. cerevisiae* strain W303-1A using the lithium acetate transformation protocol (Elble 1992). The cultures were grown at 22 ºC in the presence of raffinose (2%, v/v) and transgene expression was induced by adding galactose (2%, w/v). The cells were collected 3 days after this induction, washed with water and dried. *S. cerevisiae* cells containing the empty pYES2 vector were used as a control.

Quantitative real time PCR

First strand cDNAs obtained from developing seeds at different stages were amplified by quantitative real time PCR (qRT-PCR) using the following gene-specific primer pairs: q*RcFAH*-F and q*RcFAH*-R for *RcFAH12* (155bp fragment), q*RcFAD2*-F and q*RcFAD2*-R for *RcFAD2* (200bp fragment), q*RcPDAT1.1*-F and q*RcPDAT1.1*-R for *RcPDAT1.1* (193 bp fragment), q*RcPDAT1.2*-F and q*RcPDAT1.2*-R for *RcPDAT1.2* (115 bp fragment), q*RcPDAT2*-F and q*RcPDAT2*-R for *RcPDAT2* (82 bp fragment), q*RcROD*-F and q*RcROD*-R for *RcROD* (147 bp fragment), q*Rc*Actin-F and q*Rc*Actin-R (133 bp fragment) for the *R. communis* actin gene (Table 1). The reactions were carried out with SYBR Green I according to the manufacturer's instructions (QuantiTect™ SYBR® Green PCR Kit, Quiagen, Hilden, Germany) using a MiniOpticon system (Bio-Rad). Calibration curves were drawn up using sequential dilutions of cDNA. The Livak method (Livak and Schmittgen 2001) was applied to calculate the expression relative to

the *R. communis* actin gene Rc*ACT* (GenBank Accession number AY360221). Three biological and two technical replicates were obtained for each sample.

Castor bean lipid analysis

Developing and mature castor bean seeds were harvested, hulled and ground with sand in a glass tube. Seed samples were weighed and placed in an oven at 100 ºC for two hours.. Total lipids from approximately 0.25 g of seeds were extracted by the method described by Hara and Radin (1978), and the lipid weight was determined gravimetrically. The total lipid extract was loaded onto a Sep-pack column (Thermo Fisher Scientific) and fractionated into neutral lipids, glycolipids and polar lipids, which were eluted with chloroform/acetic acid (100/1, v/v), acetone/acetic acid (100/1, v/v) and methanol, respectively. These fractions were further resolved by thin-layer chromatography on 0.25 mm thick silica gel plates (Thermo Fisher Scientific). Neutral lipids were developed with hexane/ethyl ether/formic acid (75/25/1, v/v/v) and polar lipids with chloroform/methanol/ammonia/water (70/30/4/1, by vol.). The individual lipid classes were identified under UV light after applying a primuline spray (0.05% [w/v] in acetone/water 80/20, v/v), scraped from the plate, and used directly for methylation/sylanization or extracted for further analysis.

Fatty acid methyl esters (FAMEs) were obtained directly from castor bean seeds by maintaining the samples for 1.5 h at 80 ºC in 1.8 ml of a solution of methanol/toluene/dimethoxypropane/sulphuric acid (33/14/20/10, by vol.). Heptadecanoic acid (50 µg) was added to each sample (50 mg) as an internal standard (IS) and after cooling, 1 ml heptane and 1 ml of a saturated aqueous NaCl solution were added and mixed. The FAMEs were then recovered from the upper phase, transferred to a fresh tube and evaporated to dryness under nitrogen. The residue that contained

hydroxyl-FAMEs was silanized with 100 µl of a hexamethyldisilazane/trimethylchlorosilane (TMS)/pyridine mixture (3/1/5, by vol.). After incubation for 1 min at room temperature, the methyl ester derivatives were extracted with 1.8 ml heptane and analysed by GC (Hewlett–Packard 6890 gas chromatography apparatus; Palo Alto, CA, USA) using a Supelco SP-2380 fused-silica capillary column (30 m length, 0.25 mm i.d., 0.20 µm film thickness; Supelco, Bellefonte, PA, USA). Hydrogen was used as the carrier gas at 28 cm s^{-1}, the temperature of the flame ionization detector and injector was 200 ºC, the oven temperature was 170 ºC and the split ratio was 1:50. Peaks were identified by comparing their retention times with those of the corresponding commercial standards.

Thetriacylglycerol species in approximately 5 mg of the TAG fraction obtained previously were analysed by TLC in 1.8 ml of heptane according to Fernández-Moya et al. (2000). The analysis of TAGs was carried out by injecting 1 µl aliquots into the GC system (Agilent 6890 gas chromatography apparatus; Palo Alto, CA, USA) and using hydrogen as the carrier gas. The injector and detector temperatures were both 370 °C and the oven temperature was 335 °C, applying a head pressure gradient from 70 to 120 kPa. The gas chromatography column was a Quadrex Aluminium-Clad 400-65HT (30 m length, 0.25 mm i.d., 0.1 µm film thickness; Woodbridge, CT, USA), and a linear gas rate of 50 cm s^{-1}, a split ratio 1:80 and a flame ionisation detector (FID) were used. The TAG species were identified according to Fernández-Moya et al. (2000) and quantified by applying the correction factors reported in Carelli and Cert (1993).

Lipase hydrolysis

For the positional analysis of TAG *sn*-2 fatty acids, 10 mg of purified TAGs were hydrolyzed with 2 mg of pancreatic lipase in 1 ml of 1 M Tris-HCl buffer [pH 8],

0.1 ml CaCl2 (22%) and 0.25 ml deoxycholate (0.1%). The reaction was stopped when approximately 60% of the TAGs were hydrolyzed (1-2 min) by adding 0.5 ml of 6N HCl (Martínez-Force et al. 2009). The lipids were extracted three times with 1.5 ml aliquots of ethyl ether and the reaction products were separated by TLC (see above). Free fatty acids and *sn*-2-monoacylglycerol bands, representing the positions *sn*-1,3 and *sn*-2 of TAGs, respectively, as well as the remaining TAGs, were scraped off the plate and transmethylated (see above).

Yeast lipid extraction and fatty acid analysis

The total lipids were extracted from yeast cultures and analysed by GC as methyl ester derivatives, as described previously (Venegas-Calerón et al. 2010). As above, fatty acids were identified by comparing their retention times with those of the corresponding commercial standards.

Topology prediction

Topology predictions for the castor Δ12-hydroxylase were based on the SOSUI (Hirokawa et al. 1998) and TMHMM (version 2.0: Krogh et al. 2001) algorithms. Both models were in close agreement with the previously predicted and validated topologies of desaturases (Stukey et al. 1990; Man et al. 2006).

Results

High oleic mutant lipid composition

Common castor bean seed lipids contain high levels of palmitic and linoleic acid in the initial stages of development. As development proceeds, the ricinoleic acid content constitutes up to 80% of the total fatty acids in mature seeds (Fig. 1a). In the high oleic mutant seed, embryos mostly accumulate palmitic, oleic and linoleic in the first stages of development, which is followed by an important accumulation of oleic acid during seed maturation. The ricinoleic acid content remains low in these seeds throughout development (Fig.1b).

Studies of gene expression

The expression of *RcFAH12* transcripts and that of other genes involved in fatty acid and TAG synthesis was studied in common and OLE-1 high oleic castor lines by qRT-PCR, including the *RcFAD2*, phosphatidyl choline-diacylglycerol acyl transferase (PDAT) and phosphatidyl choline-diacylglycerol phosphocholine transferase (ROD1 or PDCT) genes. Of all the genes studied the RcFAH12 gene was expressed most strongly (Fig. 2). Moreover, no important difference in the expression of the desaturase and hydroxylase genes was detected between the common and mutant lines. By contrast, the expression of the TAG assembly genes was generally higher in the high oleic mutant, especially that of the *RcROD1*, *RcPDAT1.1* and *RcPDAT2* genes in the later stages of seed development.

Mutations in fatty acid hydroxylase from OLE-1

The hydroxylase gene from OLE1 mutant was sequenced and aligned with the hydroxylases available from the sequenced castor bean genome (GenBank

XM_002528081). The nucleotide sequences obtained for WT and OLE-1 hydroxylase genes displayed 99% identity, differing in only 4 positions: nucleotides 24, 146, 725 and 957. These mutations were responsible for the modification of 3 amino acids: F49S, V242A and H319Q (Fig. 3).

Modelling the structure of castor bean fatty acid hydroxylase

The secondary structure of the castor bean hydroxylase was modelled on the basis of studies on membrane-bound desaturases (Hoffman et al. 2007; Vanhercke et al. 2011). The RcFAH12 sequence was divided into nine consecutive domains from I through to IX (Fig. 4): domain I, the cytosolic N-terminal region; II, two transmembrane helices (TM1 and TM2) connected by a short ER luminal loop; III, a short cytosolic loop containing the first histidine box that is involved in the catalytic site; IV, the first peripheral membrane-associated segment (PMS1); V, the second short cytosolic loop containing the second histidine box; VI, the second peripheral membrane-associated region (PMS2); VII, the third short cytosolic loop; VIII, the second group of transmembrane helices (TM3 and TM4) that are connected by a short ER-luminal loop; and domain IX, the cytosolic C terminus containing the third histidine motif.

Expression of WT and mutated OLE-1 hydroxylases in yeast

The genes encoding *RcFAH12* and the different mutated hydroxylases related to *OLE1FAH12* were expressed in yeast using the pYES2 system. The expression of the castor bean hydroxylase RcFAH12 altered the fatty acid composition of the culture and its induction provoked the appearance of ricinoleic acid in a proportion close to 2% (Table 2). There was no change in the fatty acid composition of cultures expressing the

mutated OLE1FAH12 hydroxylase that contains the 3 amino acid changes (F49S, V242A, H319Q), displaying a phenotype similar to the control cells that were transformed with the empty pYES2 plasmid. The m1OLE1FAH12 mutant that contained the F49S and V242A mutations only produced traces of ricinoleic acid after induction. Indeed, similar results were obtained with the m2OLE1FAH12 mutant that carried the V242A and H319Q modifications. Finally, induction of the m3 OLE1FAH12 mutant that carried the single V242A mutation produced ricinoleic acid, but significantly less (0.8%) than that produced by the unaltered RcFAH12 gene.

Fatty acid composition of different lipid fractions and species from OLE1

The fatty acid composition of the total, neutral and polar lipids was determined in the OLE1 castor mutant and compared with that in the WT, as was the PC, PE and PI content (Table 3). In the WT, ricinoleic acid accounted for 88.6% of the total lipids present in the TAGs, while linoleic acid accounted for 4.9% and oleic acid for 3.1%. By contrast, the OLE1 mutant accumulated large amounts of oleic acid (76.8%) and shown lower levels of ricinoleic acid (16.4%) in the total lipids. Nevertheless, the levels of linoleic acid remained low (4.1%) as in the WT plant. While the saturated palmitic acid was similar in OLE1 to WT (1.7% vs. 1.3%), more stearic acid was accumulated in the WT than in the OLE1 mutant (2.1% vs. 1.0%). There was a similar neutral lipid composition as that of total lipids, yet with a slightly lower linoleic acid content (4.2%) in the OLE1 mutant. The lower neutral lipid content in the OLE1 mutant was also noteworthy. The most remarkable difference in the fatty acid composition between WT and OLE1 remained in the linoleic acid content of polar lipids, which reached 34% linoleic acid in the WT seeds and only 4.4% in the OLE1 mutant seeds, almost 8-fold

less. This difference was evident in the three species of polar lipids studied: PC, PE and PI.

Fatty acid distribution in the TAGs from OLE1

The fatty acid distribution in the WT and OLE1 mutant was studied by analysing the TAG composition of their oils. The main component of WT castor oil is triricinoleyl glycerol (RRR, 75.4 %), with other species containing two molecules of ricinoleic plus a non-hydroxylated fatty acid (Table 4). The TAG composition of OLE1 is more complex and the main species found were trioleyl glycerol (OOO, 41.3%) and dioleyl ricinoleyl glycerol (31.4%). To apply the random sn-1,3 non-random sn-2 distribution in order to establish the expected TAG composition of this oil (Martínez-Force et al. 2009), it is necessary to find the fatty acid composition in sn-2 of the TAGs in the oil. This was achieved by pancreatic lipase digestion of TAGs and the subsequent determination of the fatty acid products (Table 5). Thus, the main difference between the real and predicted TAG composition in OLE1 remains the species containing linoleic acid (Table 4: dioleyl-linoleyl glycerol, OOL; dilinoleyl-oleyl glycerol, LLO; and trilinoleyl glycerol, LLL). The LLL, LLO and SOL TAGs were more abundant in OLE1 than was predicted by the model, whereas OOL was quantitatively more important in the prediction than in the final oil.

Discussion

The high oleic castor bean mutant OLE-1 has been characterized here and in contrast to the WT castor bean, it has a low ricinoleic acid content in its seeds at all developmental stages, while its oleic acid precursor accumulates in large amounts. The

enzyme directly involved in the synthesis of ricinoleic acid is delta-12 hydroxylase, which is encoded by the *RcFAH12* gene. Thus, the phenotype of the OLE-1 mutant should be explained by weaker expression of this gene or a mutation affecting its activity. To assess whether the mutant's phenotype was due to an alteration in the *RcFAH12* gene promoter region affecting its expression, the expression of the hydroxylase gene was studied at different stages of seed development by qRT-PCR. The expression of the hydroxylase gene did not appear to be affected at any developmental stage and moreover, RcFAH12 was expressed more strongly than other genes related to fatty acid synthesis, such as the RcFAD2 gene (Chen et al. 2007; Brown et al. 2012; Hu et al. 2012). Since the expression of the hydroxylase gene was not affected, the sequence of the hydroxylase gene in the OLE-1 mutant was analyzed.

We compared the sequence of the OLE-1 hydroxylase gene with that of the WT castor bean hydroxylase available from the sequenced castor bean genome (GenBank XM_002528081). The nucleotide sequences obtained for the WT and OLE-1 hydroxylase genes differed at only 4 positions (24, 146, 725 and 957), which modified 3 amino acids (F49S, V242A and H319Q) and that could be responsible for the weaker hydroxylase activity in the mutant line. When the structure of the castor bean hydroxylase was examined, based on previous studies of membrane-bound desaturases (Hoffman et al. 2007; Vanhercke et al. 2011), this shown large hydrophobic regions (TMs) that cross or are anchored to the membrane bilayer. These domains can be identified by applying the SOSUI (Hirokawa et al. 1998) and TMHMM (Krogh et al. 2001) algorithms, allowing the secondary structure of the enzyme to be modelled. As such, the transmembrane and cytosolic domains of *Rc*FAH12 coincide with those proposed for membrane bound desaturases (Stukey et al. 1990; van Beilen et al. 1992; Díaz et al. 2002; Man et al. 2006).

In the light of the data available on desaturases and hydroxylases (Broadwater et al. 2002), the amino acid change that might have the strongest impact on the activity of the hydroxylase enzyme would be the exchange of a histidine residue for a glutamine in the third histidine box of this protein, H319Q. This amino acid is involved in the formation of the di-iron centre of these enzymes that is essential for catalysis (Shanklin et al. 1994). Furthermore, the F49S mutation at the N-terminal extreme of the protein, preceding the first TM domain, is a non-conservative change that might also affect the enzyme's activity. Finally the V242A mutation is a semi-conservative change within the third transmembrane helix and it should not influence the functionality of the protein.

To evaluate the effect of the three mutations on the activity of the FAH12 enzyme, we expressed the *Rc*FAH12 and OLE1FAH12 proteins in yeast. As expected, transgenic yeast expressing the *RcFAH12* gene accumulated detectable hydroxylated fatty acids, confirming that the castor hydroxylase protein expressed in yeast was active and capable of altering the lipid composition of the host. By contrast, expression of the OLE1FAH12 protein did not generate any hydroxylated fatty acids in the yeast lipids, indicating that the mutations present in its sequence caused a loss of functionality. To assess the contribution of each mutation to this phenotype, constructs containing combinations of the OLE1FAH12 mutations were expressed in yeast cells. Thus, the m1OLE1FAH12 mutant containing the substitutions F49S and V242A displayed very low, yet detectable, amounts of hydroxylated fatty acids, indicating that the enzyme retains some residual activity. The m2OLE1FAH12 mutant, with the H319Q and V242A substitutions that affect the important histidine box in the enzyme, displayed similar activity. Finally, yeast with the m3OLE1FAH12 mutant, which only contains the semi-conservative V242A substitution accumulated more hydroxylated fatty acids than the double mutants, although this mutation alone caused an important decrease

(58%) in the total ricinoleic acid in the yeast lipids compared to those found when the wt *RcFAH12* gene was expressed. Hence, all the 3 substitutions contributed to the loss of OLE-1 hydroxylase activity. The contribution of the V242A mutation was analysed by modelling the secondary structure of this protein, particularly with reference to the third and fourth TM domains obtained by the SOSUI algorithm for both *Rc*FAH12 and OLE1FAH12 (Hirokawa et al. 1998). The V242A change modifies the fourth helix, which was displaced by one amino acid position with respect to the WT hydroxylase. This could produce torsion in the final domain of the protein that affects the third histidine box involved in the active site of the enzyme, altering the enzyme's activity. The effect of point mutations in these regions within related membrane desaturases have been recently highlighted in new high oleic acid sunflower lines obtained by mutagenesis (León et al. 2013), where amino acid changes in sunflower FAD2 drastically reduce its activity.

Another interesting aspect of the OLE-1 phenotype was related to castor bean fatty acid metabolism. Thus, the weaker hydroxylase activity provoked by the mutations affecting this enzyme diminished the hydroxylated fatty acids at the expense of an increase in oleic acid and a small reduction in the proportion of linoleic acid in the storage oils accumulated in the mutant seeds, although these seeds had a higher polar lipid content (Table 3).

The fatty acid composition of the different lipid species in the seeds of the different castor bean lines could help better understand the metabolism addressed here. The decrease in ricinoleic acid content in the OLE-1 mutant coincides with a decrease in the oil content from 60.5% to 53.6%, and a decrease in the linoleic acid content that was moderate in neutral lipids but more drastic in polar ones. To clarify the destiny of the linoleic acid, we analyzed the TAG species present in the OLE-1 mutant and the wild

type oils, and the fatty acid composition at the *sn*-2 position of OLE1 TAGs. As clearly seen, the accumulation of oleic acid in the OLE1 mutant produces a drastic change in the TAG species when compared to those present in the wild type castor bean seeds. The TAG profile changed from an oil rich in triricinolein and TAG species with two ricinoleic molecules, to one more similar to that described for high-oleic varieties of oilseeds (Fernández-Moya et al. 2000), despite contrasting some less abundant species with one ricinoleic moiety. When the linoleic distribution in TAGs was further investigated by determining the fatty acid composition at the *sn*-2 position, and the expected TAG species in OLE1 for a random *sn*-1,3 non-random *sn*-2 distribution were calculated, the observed values reflected the higher proportion of linoleic acid allocated to OLL and LLL species in OLE1 TAGs (44.52% versus 9.31% in wild type TAGs). These data indicate that concomitant with the reduction in ricinoleic acid and the increase in oleic acid, most of the linoleic acid produced (around 98.4%) is incorporated directly into TAGs through the activity of PDAT (*sn*-3) and PDCT (*sn*-2)/DAGAT (*sn*-3). Accordingly, more TAG species are produced with two or three molecules of linoleic acid than would be expected for a random distribution, and the incorporation of this fatty acid into the acyl-CoA pool and its use in polar lipid biosynthesis is diminished.

To further investigate this phenomenon, we characterized the expression of the castor bean *ROD1* gene that is responsible for PDCT activity, and the three genes encoding PDAT activity (*PDAT1.1*, *PDAT1.2* and *PDAT2*). In both castor bean lines there is an increase in the expression of *Rc*ROD1, *Rc*PDAT1.2 and *Rc*PDAT2 during seed development, together with a decrease in *Rc*PDAT1.1 expression. In addition, all these genes appeared to be expressed more strongly in the OLE1 mutant than in the WT, although only the expression of *Rc*PDAT2 was significantly different. It has been

proposed that the enzyme encoded by *RcPDAT1.2* could determine the amount of hydroxyl fatty acids in the TAGs synthesized by developing seeds (van Erp et al. 2011; Kim et al. 2011). By contrast, RcPDAT2 is thought not to play an important role in TAG synthesis, even though it is strongly expressed during seed development. Our assays suggest that PDAT2 could contribute significantly to TAG synthesis in OLE1 seeds, facilitating the transfer of fatty acids from PC to TAGs in this castor bean mutant. Therefore, the role of RcPDAT2 remains to be determined and its study will provide crucial information for future studies into enzymatic activity and substrate specificity in castor bean seed acyl-lipid metabolism.

Author contribution statement

MVC, EMF and RG conceived and designed the research. MVC carried out the experiments and elaborated the data. RS contributed to the qRT-PCR analyses. MVC, RS, JJS and EMF analyzed data. MVC, RS, JJS, RG and EMF wrote the manuscript, with all authors having read and approved the manuscript prior to its submission.

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Figure legends

Fig. 1 Fatty acid profile during the development of wild-type castor bean seeds (**a**) and OLE-1 mutant (**b**). Cross-sections of developing seeds are shown from stage I to stage VII according to Greenwood and Bewley (1982). The data represent the mean values and standard deviations of five independent experiments

Fig. 2 Quantitative real-time PCR analysis of (**a**) *RcFAD2*, *RcFAH12* and *RcROD1* gene expression, and that of (**b**) the PDAT family of genes (*RcPDAT1.1*, *RcPDTA1.2* and *RcPDAT2*), in developing seeds from the castor OLE-1 mutant (grey bars) and wt (white bars). The castor bean seed stages are as shown in Fig. 1 and the gene expression is expressed relative to *RcActin*. The values represent the mean ± SD of two replicates of three independent biological samples

Fig. 3 Comparison of the oleate hydroxylase (*Rc*FAH12) and oleate desaturase (*Rc*FAD2) amino acid sequence from wild type and OLE-1 castor bean lines. The conserved histidine clusters are in boxes and the changes in the OLE-1 FAH12 sequence are in shaded. The symbols under the sequences indicate the degree of residue conservation relative to known FAD2 and FAH12 protein sequences in dicotyledonous plants. The predicted transmembrane helices (TM1, TM2, TM3 and TM4) are shown in green

Fig. 4 Proposed topological model of the *Ricinus communis* oleate 12-hydroxylase. The positions mutated in the OLE-1 high-oleic line are indicated by red circles and referred to as: a (F49S), b (V242A), and c (H319Q). TM, Transmembrane helix and PMS, peripheral membrane-associated segment

Fig. 5 Proposed structural effect of the V242A mutation in RcFAH12 from the OLE-1 high-oleic castor bean line. Helical wheel representations and schemes of the predicted TM3 and TM4 transmembrane helices from the wild type (**a**) and V242A mutant (**b**) RcFAH12 proteins. The arrows point to residue 242. Hydrophobic residues are shown in black, polar residues in blue, and positively and negatively charged residues in bold blue and red, respectively

Fig. 6 Scheme of the biochemical pathways involved in triacylglycerol assembly. CPT, CDP-choline:diacylglycerol cholinephosphotransferase; DAG, Diacylglycerol; DAGAT, Diacylglycerol acyltransferase; G3P, Glycerol-3-phosphate; GPAT, Glycerol-3-phosphate acyltransferase; LPA, Lysophosphatidic acid; LPAAT, Lysophosphatidic acid acyltransferase; LPC, Lysophosphatidylcholine; LPCAT, Lysophosphatidylcholine acyltransferase; PA, Phosphatidic acid; PC, Phosphatidylcholine; PDAT, Phospholipid:diacylglycerol acyltransferase; PDCT, Phosphatidylcholine:diacylglycerol cholinephosphotransferase; PL-C, Phospholipase C; PP, Phosphatidic acid phosphatase; and TAG, Triacylglycerol

Fig. 7 TAG species from the OLE-1 mutant and wild type castor bean oils analysed by gas chromatography. (**a**) Non-silanized and (**b**) silanized OLE-1 mutant TAGs, and (**c**) silanized wild type castor bean TAGs

Highlights:

The OLE-1 high oleic castor bean mutant has been characterized genetically, providing insight into the biochemistry of castor oil synthesis.

Point mutations in the FAH12 gene are responsible for the high oleic phenotype.

The contribution of each mutation has been evaluated by heterologous expression in yeast.

Table 1. Sequences of the PCR primers.

Primer name	Sequence	Use
RcFAH-F	5´-ATGGGAGGTGGTGGTCGC-3´	C
RcFAH-R	5´-TTAATACTTGTTCCGGTACCAGAAA-3´	
RcFAD2-F	5´-ATGGGTGCTGGTGGCAGA-3´	
RcFAD2-R	5´-TCAAAATTTGTTGTTATACCAGAAA-3´	
q*RcFAH*-F	5´-TAACCAGCAACAACAGTGAG-3´	QPCR
q*RcFAH*-R	5´-ATAGGCAACATAGGAGAATGAG-3´	
q*RcFAD2*-F	5´-GCCATCCCACCTCATTGTT-3´	
q*RcFAD2*-R	5´-TGTGCTATAACCCAAACACCAG-3´	
q*RcAct2*-F	5´- CCAGGGAGGAGTATGGAGGT -3´	
q*RcAct2*-R	5´- ACCACATCCACAGGAACCAT -3´	
q*RcPDAT1.1*-F	5'- AGATTTGGGAGGCGATCAAG -3'	
q*RcPDAT1.1*-R	5'- CAGTGGCTAGGGTTGAGAAAG -3'	
q*RcPDAT1.2*-F	5'- CCACTTCTCCCATTGCAGTT-3'	
q*RcPDAT1.2*-R	5'- CCAAAACAATGGAGGAGCAT-3'	
q*RcPDAT2*-F	5'- GAGGGGGACAGAGAGTGGAT-3'	
q*RcPDAT2*-R	5'- TCCAGCAGCAACTCTTAGCA-3'	
q*RcROD*-F	5'-AATGCAGAGATGGGAATTGG-3'	
q*RcROD*-R	5'-TATTTCCCCGCAAGTGAATC-3'	
Hind*RcFAH12*-F	5´-AA**AAGCTT**ACC**ATGG**GAGGTGGTGGTCGCATGTC-3´	YE
Xba*RcFAH12*-R	5´-GG**TCTAGA**TTAATACTTGTTCCGGTACCAGAAAACG-3´	
S49FMutHO*FAH12*-F	5´-CCATCCCACCCCATTGC***TT****T*GAACGCTCTTTTGT-3'	M
S49FMutHO*FAH12*-R	5´-ACAAAAGAGCGTTC***AA****A*GCAATGGGGTGGGATGG-3'	
Q319HMutHO*FAH12*-F	5´-GCAGACACTC***AT****T*GTAGCTCATC-3´	
Q319HMutHO*FAH12*-R	5´-GATGAGCTAC***A****TG*AGTGTCTGC-3´	

Restriction sites are shown in bold, Kosak sequence is underlined and mutagenesis nucleotides are in italics. C, Cloning; QPCR, Quantitative PCR; YE, Yeast expression and M, Site directed mutagenesis.

Table 2. Fatty acid composition of *S. cerevisiae* cells expressing control and recombinant plasmids. Data are the average of three independent samples. SD <5% of mean value.

		% hydroxylated	mol %					
	induction	**product**	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**	**18:1-OH**
pYES2 empty vector	+	0.0	15.4	38.1	4.0	28.9	0.0	0.0
RcFAH12	+	6.5	18.7	47.7	4.2	27.3	0.2	1.9
	-	0.0	16.1	47.0	4.4	32.4	0.1	0.0
OLE1 FAH12 (F49S, V242A, H319Q)	+	0.0	18.0	45.8	4.7	31.4	0.0	0.0
	-	0.0	16.2	39.0	6.0	38.8	0.0	0.0
m_1OLE1 FAH12 (F49S, V242A)	+	0.1	18.9	40.7	5.5	34.7	0.1	0.1
	-	0.0	17.3	39.6	4.5	38.5	0.0	0.0
m_2OLE1 FAH12 (V242A, H319Q)	+	0.2	19.7	48.0	4.2	28.0	0.1	0.1
	-	0.3	17.7	47.6	4.3	30.2	0.1	0.1
m_3OLE1 FAH12 (V242A)	+	3.0	19.2	50.0	3.4	26.5	0.1	0.8
	-	0.2	18.4	45.9	4.3	31.3	0.1	0.1

16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; and 18:1-OH, ricinoleic acid.

Table 3. Fatty acid composition of seed oil and oil content of seed from OLE-1 and control mature seeds. Each value is the mean of three independent samples. SD < 2.5% of mean value.

			Fatty acid composition (mol%)				
		%	16:0	18:0	18:1	18:2	18:1-OH
TL	OLE1	53.6	2.4	0.7	91.6	4.7	0.5
	WT	60.5	1.3	2.1	3.1	4.9	88.6
NL	OLE1	98.5	1.7	0.7	92.3	4.2	1.1
	WT	98.9	1.2	1.3	3.2	4.9	89.4
PL	OLE1	1.5	15.1	4.6	75.0	4.4	1.1
	WT	1.1	32.0	10.0	20.9	34.1	2.9
PC	OLE1	57.2	8.5	2.5	78.0	10.9	0.1
	WT	59.5	15.3	10.9	18.9	52.1	2.6
PE	OLE1	21.7	16.4	1.6	64.0	15.8	2.2
	WT	25.3	21.5	6.3	10.4	58.9	2.9
PI	OLE1	2.6	31.3	3.7	56.9	6.8	1.2
	WT	9.8	35.9	11.0	8.3	42.6	2.2

16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; and 18:1-OH, ricinoleic acid. TL, Total lipids; NL, Neutral lipids; PL, Polar lipids; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol.

Table 4. TAG molecular species composition of normal (WT) and high-oleic (OLE1) castor seed oils (mol percentage) determined by GLC, expected TAG profile for a *sn*-1,3 random *sn*-2 non-random fatty acid distribution (Martínez-Force et al. 2009) and linoleic percentage in each TAG species containing that fatty acids in OLE1 mutant versus the expected one.

WT			OLE1	Expected*	%L **OLE1** vs Expected
RRP	3.44	POO	3.32	3.21	
RRS	3.56	POL	0.38	0.34	**3.10**/3.82
RRO	7.57	POR	1.51	1.38	
RRAs	0.96	SOO	1.89	1.85	
RRL	9.10	OOO	44.31	45.39	
RRR	75.35	SOL	0.96	0.24	**7.84**/2.64
		SOR	0.08	1.21	
		OOL	5.59	7.60	**45.67**/84.23
		OOR	31.35	32.24	
		OLL	1.27	0.41	**20.75**/9.08
		ORR	8.30	6.03	
		LLL	0.97	0.01	**23.77**/0.23
		RRR	0.08	0.11	

As, asclepic acid; L, linoleic acid; O, oleic acid; P, palmitic acid; R, ricinoleic acid; S, stearic acid.

Table 5. Fatty acid composition of *sn*-2 and *sn*-(1 + 3) positions of high-oleic castor triacylglycerols.

	WT		OLE1	
	sn-2	*sn*-1,3	*sn*-2	*sn*-1,3
P	1.45	2.32	1.25	1.95
S	5.43	0.93	3.25	0.16
O	2.60	6.88	89.69	68.72
As	0.00	1.41	0.91	0.89
L	3.44	9.21	3.15	4.55
R	87.08	77.40	1.76	23.73

Figure 1

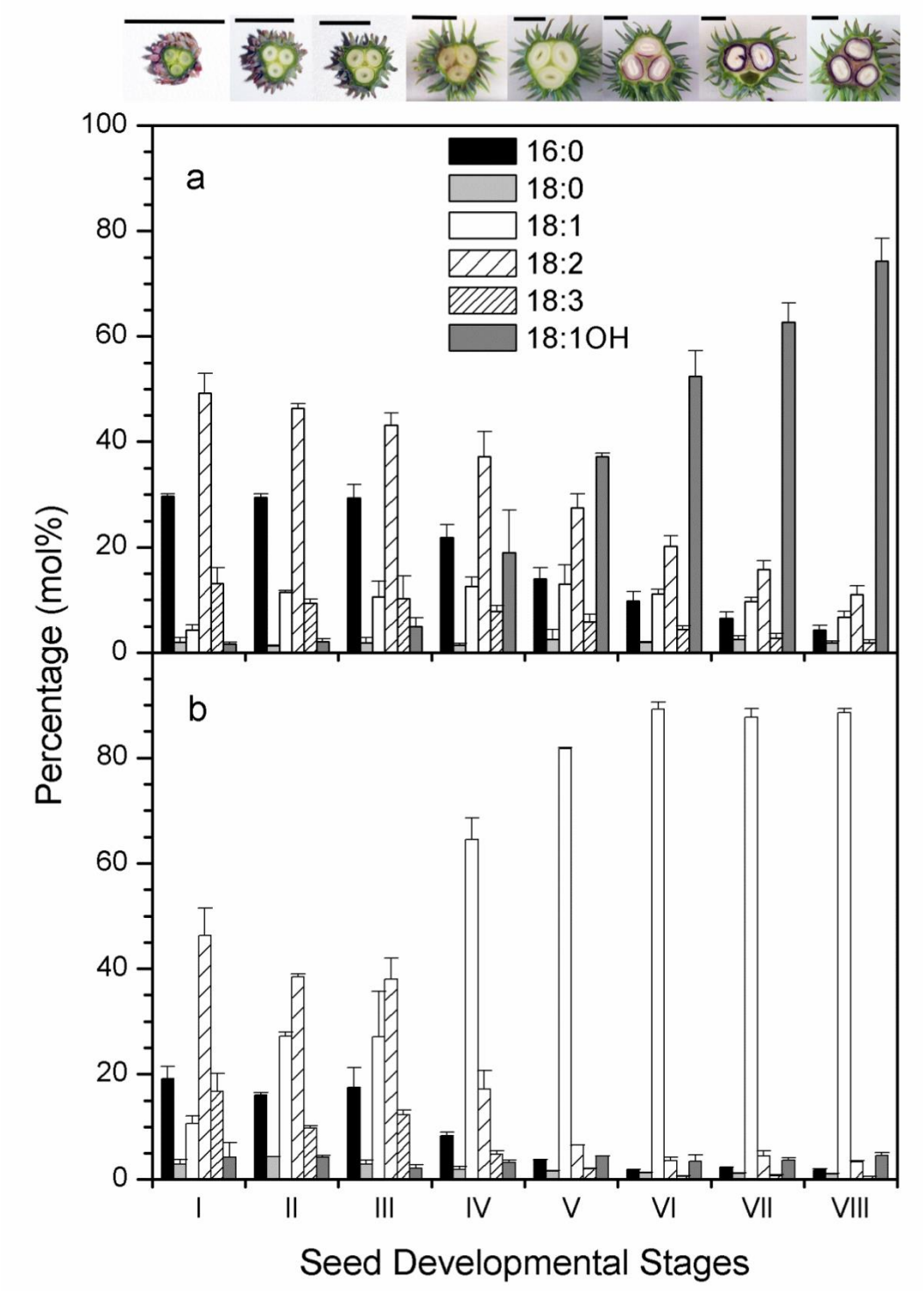

a
b
16:0
18:0
18:1
18:2
18:3
18:1OH
Percentage (mol%)
0
20
40
60
80
100
I
II
III
IV
V
VI
VII
VIII
Seed Developmental Stages

Figure 2

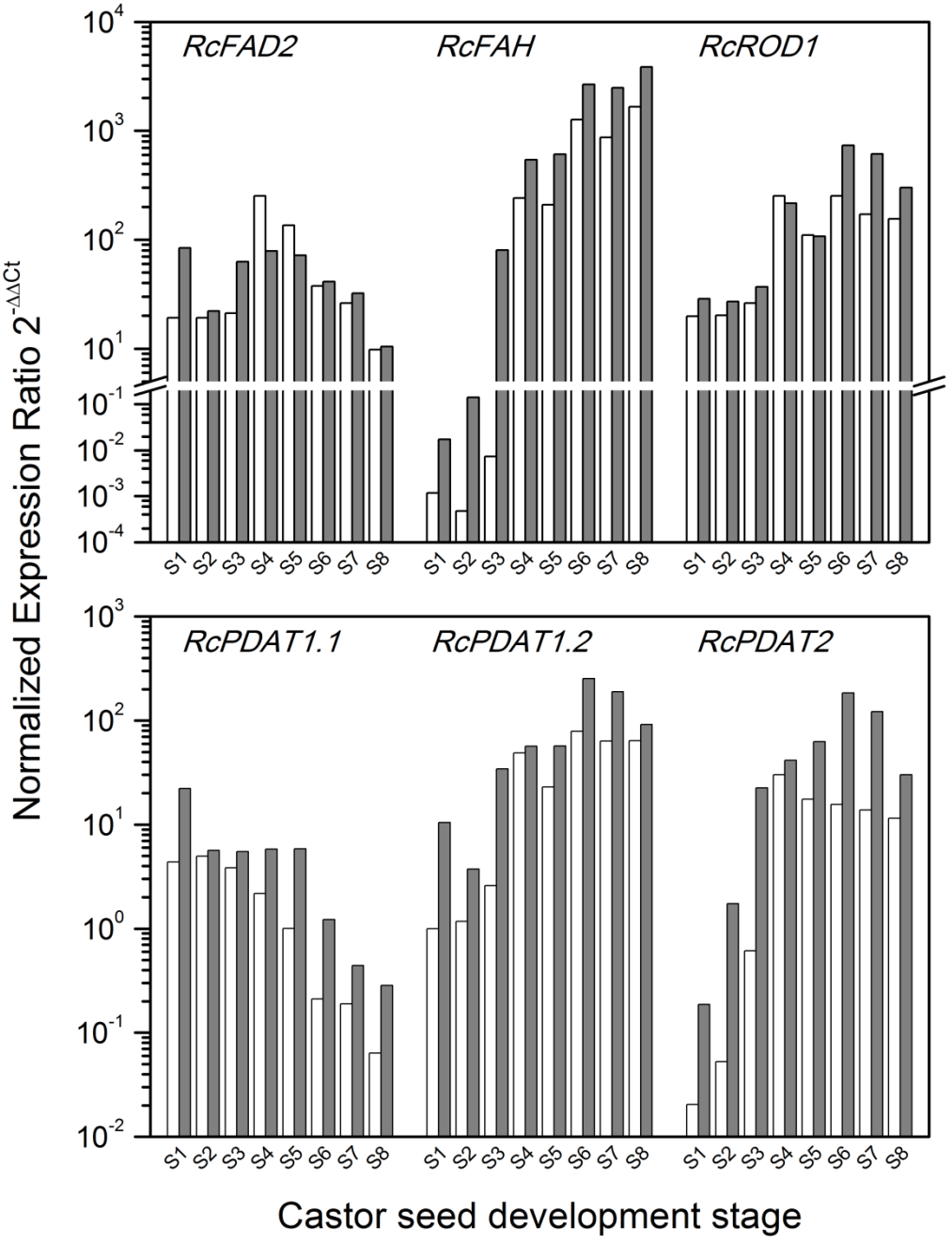

RcFAD2
RcFAH
RcROD1
RcPDAT1.1
RcPDAT1.2
RcPDAT2
Normalized Expression Ratio 2-ΔΔCt
Castor seed development stage
S1 S2 S3 S4 S5 S6 S7 S8

Figure 3

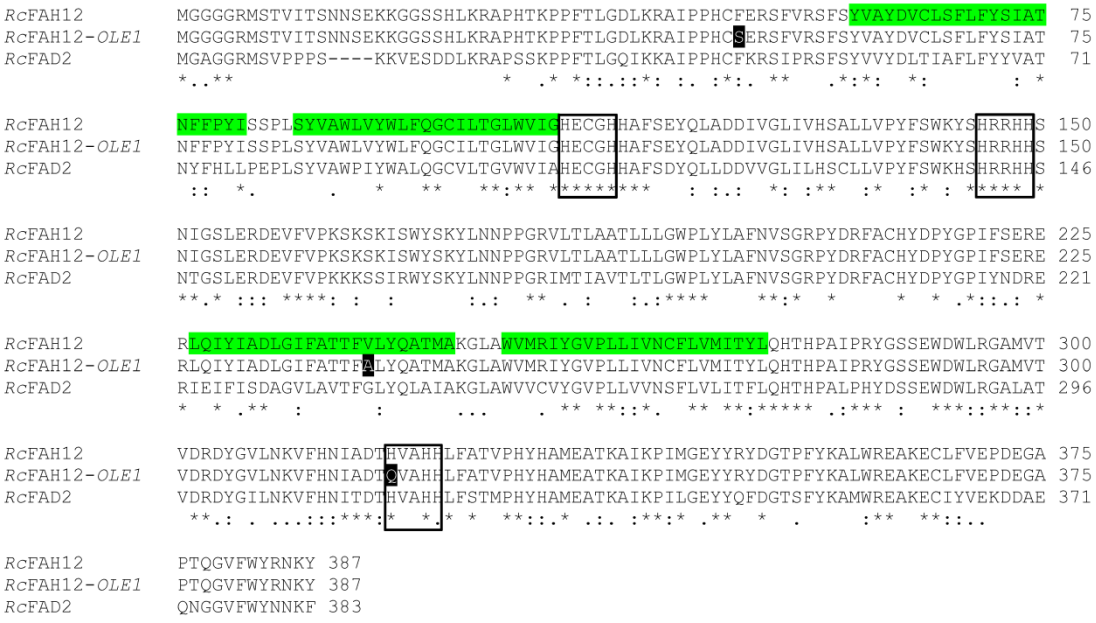
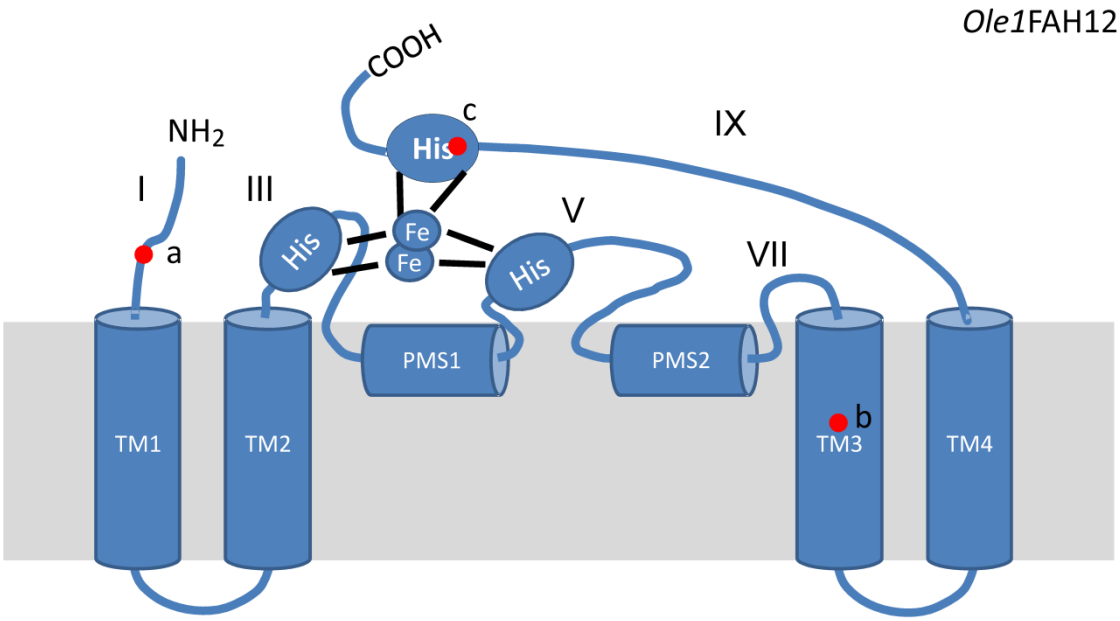

RcFAH12 MGGGGRMSTVITSNNSEKKGGSSHLKRAPHTKPPFTLGDLKRAIPPHCFERSFVRSFSYVAYDVCLSFLFYSIAT 75
RcFAH12-OLE1 MGGGGRMSTVITSNNSEKKGGSSHLKRAPHTKPPFTLGDLKRAIPPHCSERSFVRSFSYVAYDVCLSFLFYSIAT 75
RcFAD2 MGAGGRMSVPPPS----KKVESDDLKRAPSSKPPFTLGQIKKAIPPHCFKRSIPRSFSYVVYDLTIAFLFYYVAT 71
RcFAH12 NFFPYISSPLSYVAWLVYWLFQGCILTGLWVIGHECGHHAFSEYQLADDIVGLIVHSALLVPYFSWKYSHRRHHS 150
RcFAH12-OLE1 NFFPYISSPLSYVAWLVYWLFQGCILTGLWVIGHECGHHAFSEYQLADDIVGLIVHSALLVPYFSWKYSHRRHHS 150
RcFAD2 NYFHLLPEPLSYVAWPIYWALQGCVLTGVWVIAHECGHHAFSDYQLLDDVVGLILHSCLLVPYFSWKHSHRRHHS 146
RcFAH12 NIGSLERDEVFVPKSKSKISWYSKYLNNPPGRVLTLAATLLLGWPLYLAFNVSGRPYDRFACHYDPYGPIFSERE 225
RcFAH12-OLE1 NIGSLERDEVFVPKSKSKISWYSKYLNNPPGRVLTLAATLLLGWPLYLAFNVSGRPYDRFACHYDPYGPIFSERE 225
RcFAD2 NTGSLERDEVFVPKKKSSIRWYSKYLNNPPGRIMTIAVTLTLGWPLYLAFNVSGRPYDRFACHYDPYGPIYNDRE 221
RcFAH12 RLQIYIADLGIFATTFVLYQATMAKGLAWVMRIYGVPLLIVNCFLVMITYLQHTHPAIPRYGSSEWDWLRGAMVT 300
RcFAH12-OLE1 RLQIYIADLGIFATTHALYQATMAKGLAWVMRIYGVPLLIVNCFLVMITYLQHTHPAIPRYGSSEWDWLRGAMVT 300
RcFAD2 RIEIFISDAGVLAVTFGLYQLAIAKGLAWVVCVYGVPLLVVNSFLVLITFLQHTHPALPHYDSSEWDWLRGALAT 296
RcFAH12 VDRDYGVLNKVFHNIADTHVAHHLFATVPHYHAMEATKAIKPIMGEYYRYDGTPFYKALWREAKECLFVEPDEGA 375
RcFAH12-OLE1 VDRDYGVLNKVFHNIADTQVAHHLFATVPHYHAMEATKAIKPIMGEYYRYDGTPFYKALWREAKECLFVEPDEGA 375
RcFAD2 VDRDYGILNKVFHNITDTHVAHHLFSTMPHYHAMEATKAIKPILGEYYQFDGTSFYKAMWREAKECIYVEKDDAE 371
RcFAH12 PTQGVFWYRNKY 387
RcFAH12-OLE1 PTQGVFWYRNKY 387
RcFAD2 QNGGVFWYNNKF 383

Figure 4



Mutation	Change	Location
a	F49S	Domain I
b	V242A	TM3
c	H319Q	**His box III (domain IX)**

Figure 5

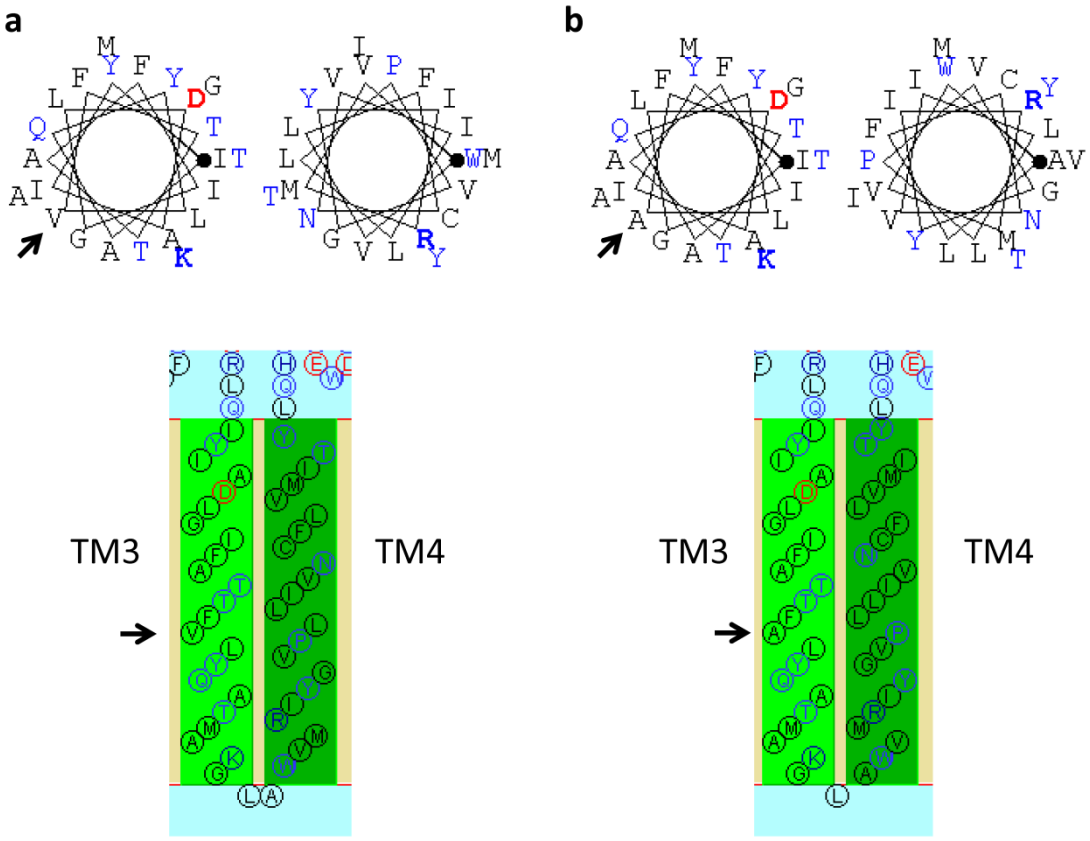


Figure 6

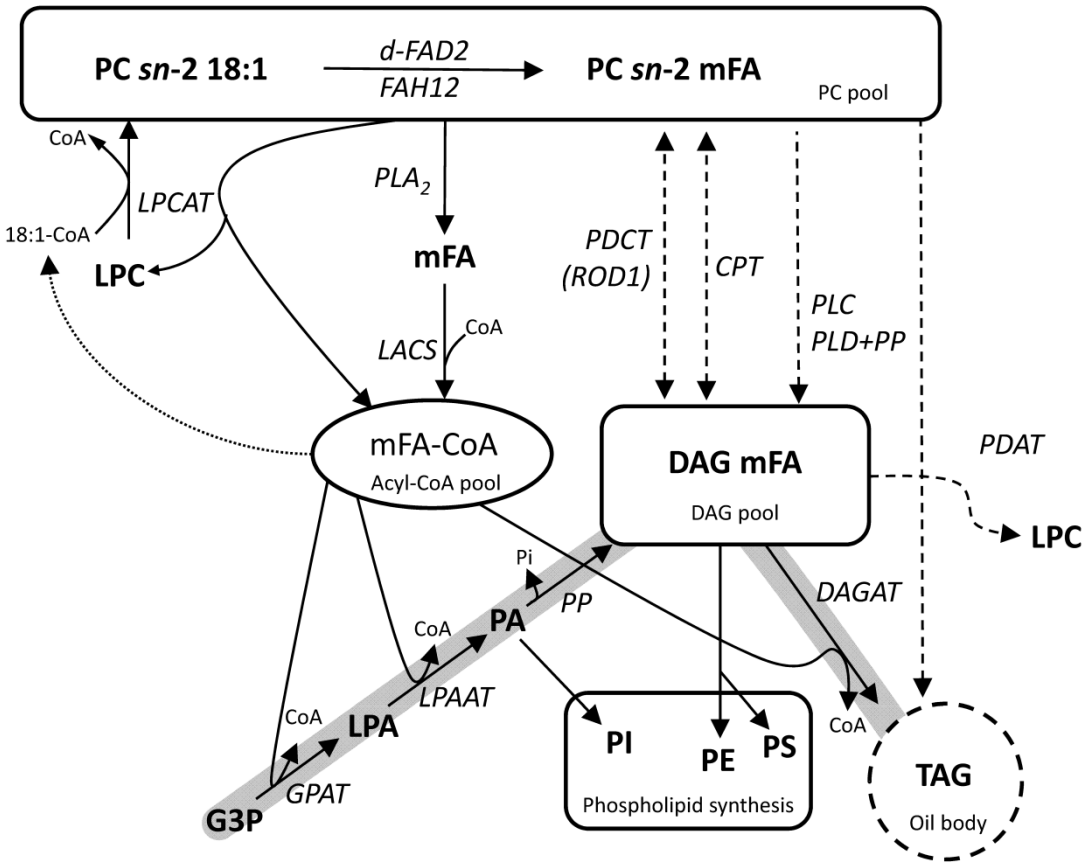
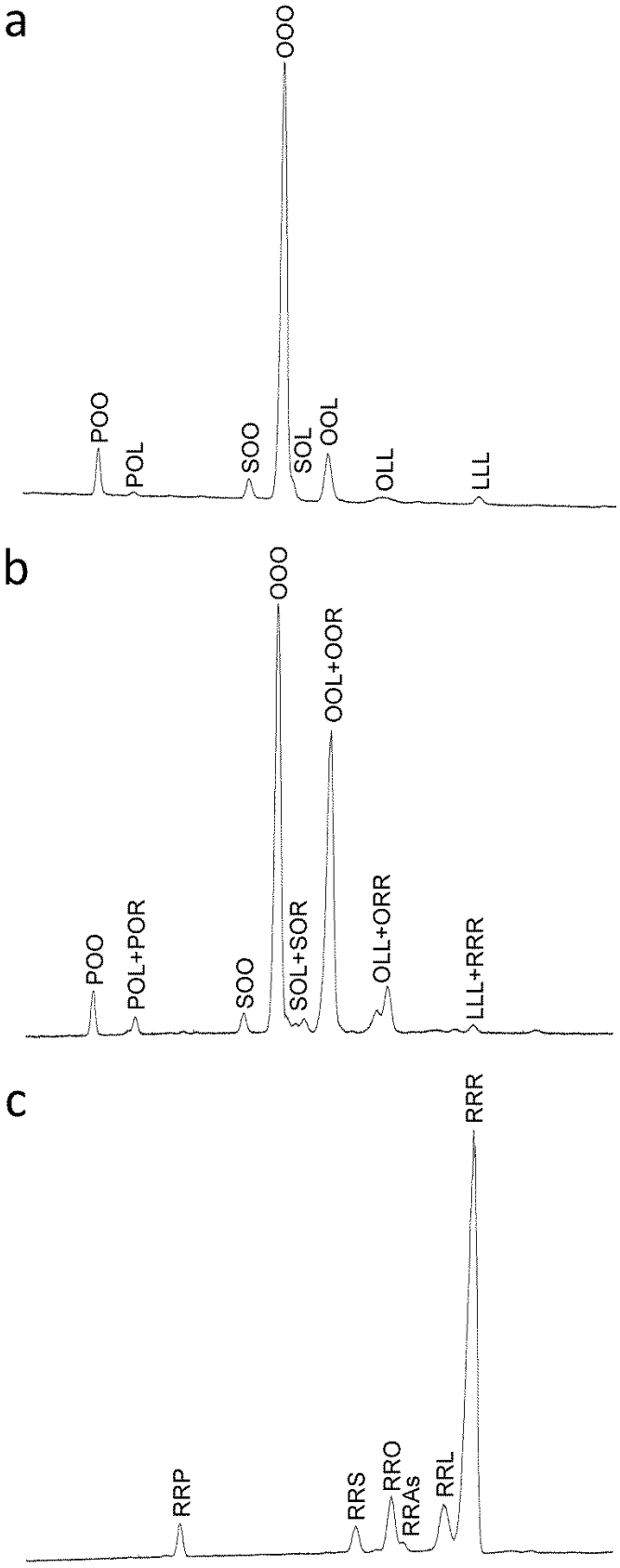

PC sn-2 18:1
d-FAD2
FAH12
PC sn-2 mFA
PC pool
CoA
LPCAT
18:1-CoA
LPC
PLA2
mFA
LACS
CoA
PDCT
(ROD1)
CPT
PLC
PLD+PP
mFA-CoA
Acyl-CoA pool
DAG mFA
DAG pool
PDAT
LPC
Pi
PP
DAGAT
CoA
PA
LPAAT
CoA
LPA
GPAT
G3P
PI
PE
PS
Phospholipid synthesis
CoA
TAG
Oil body

Figure 7


a
OOO
POO
POL
SOO
SOL
OOL
OLL
LLL
b
OOO
OOL+OOR
POO
POL+POR
SOO
SOL+SOR
OLL+ORR
LLL+RRR
c
RRR
RRP
RRS
RRO
RRAs
RRL