



RESEARCH ARTICLE

REVISED Homologous electron transport components fail to increase fatty acid hydroxylation in transgenic *Arabidopsis thaliana* [v2; ref status: indexed, <http://f1000r.es/2a3>]

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Abstract

Ricinoleic acid, a hydroxylated fatty acid (HFA) present in castor (*Ricinus communis*) seeds, is an important industrial commodity used in products ranging from inks and paints to polymers and fuels. However, due to the deadly toxin ricin and allergens also present in castor, it would be advantageous to produce ricinoleic acid in a different agricultural crop. Unfortunately, repeated efforts at heterologous expression of the castor fatty acid hydroxylase (RcFAH12) in the model plant *Arabidopsis thaliana* have produced only 17-19% HFA in the seed triacylglycerols (TAG), whereas castor seeds accumulate up to 90% ricinoleic acid in the endosperm TAG. RcFAH12 requires an electron supply from NADH:cytochrome b5 reductase (CBR1) and cytochrome b5 (Cb5) to synthesize ricinoleic acid. Previously, our laboratory found a mutation in the *Arabidopsis CBR1* gene, *cbr1-1*, that caused an 85% decrease in HFA levels in the RcFAH12 *Arabidopsis* line. These results raise the possibility that electron supply to the heterologous RcFAH12 may limit the production of HFA. Therefore, we hypothesized that by heterologously expressing RcCb5, the reductant supply to RcFAH12 would be improved and lead to increased HFA accumulation in *Arabidopsis* seeds. Contrary to this proposal, heterologous expression of the top three RcCb5 candidates did not increase HFA accumulation. Furthermore, coexpression of RcCBR1 and RcCb5 in RcFAH12 *Arabidopsis* also did not increase in HFA levels compared to the parental lines. These results demonstrate that the *Arabidopsis* electron transfer system is supplying sufficient reductant to RcFAH12 and that there must be other bottlenecks limiting the accumulation of HFA.

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REVISED Amendments from Version 1

The text in the Discussion (p10) has been edited to acknowledge the possibility that redox-competent RcCb5 and/or RcCBBR1 are not produced in our transgenic plants.

See referee reports

Introduction

Ricinoleic acid ((9Z,12R)-12-hydroxyoctadec-9-enoic acid), a hydroxylated fatty acid (HFA), is an important industrial feedstock for products such as lubricants, polyamide 11 (Nylon 11), coatings, inks, surfactants, emulsifiers, and biodiesel¹. Castor oil is composed of up to 90% ricinoleic acid that is stored in the developing endosperm of the castor plant (*Ricinus communis*) in the form of triacylglycerol (TAG). However, castor seeds also contain the deadly toxin ricin, as well as a 2S albumin that is a major allergen^{2,3}. These proteins, as well as the poor agronomic characteristics of the plant, make castor unsuitable as a major crop. While United States farmers are reluctant to grow castor, the demand for castor oil is increasing. Currently, industrial demand for castor oil is met from plants grown and manually harvested in India, China, and Brazil. Therefore, it would be advantageous to produce ricinoleic acid in a suitable crop species, such as canola, soybean, or camelina that lack ricin and the 2S albumin and can be widely grown in the United States. Furthermore, the knowledge gained from studying the biosynthesis of HFA in transgenic plants could be used to explore the synthesis of other novel fatty acids, such as epoxy fatty acid or conjugated fatty acids, which are made through similar biosynthetic mechanisms that rely on catalysis by modified desaturase enzymes⁴.

The castor fatty acid hydroxylase (RcFAH12) is a diiron, mixed-function oxidase responsible for the synthesis of ricinoleic acid from oleate (18:1) on phosphatidylcholine (PC) in the endoplasmic reticulum membrane^{4,5}. The reaction mechanism requires transfer of two electrons from NADH through cytochrome b5 reductase (CBR1) and cytochrome b5 (Cb5) and then to the catalytic enzyme RcFAH12, which oxidizes 18:1 to 18:1-OH with the reduction of oxygen to water^{6,7}.

Repeated attempts to express a *RcFAH12* cDNA in *Arabidopsis* under the control of seed-specific promoters have yielded a maximum of only 17% HFA in seed oil^{8,9}. Ricinoleic acid has also been produced in tobacco but at a very low yield⁷. In *Arabidopsis*, ricinoleic acid can be further desaturated to densipolic acid (18:2-OH) or elongated to lesquerolic acid (20:1-OH) and auricolic acid (20:2-OH) by the fatty acid elongase FAE1 and associated enzymes⁸. To reduce the range of HFAs produced, we transformed *RcFAH12* into the *fae1* background¹⁰ under the control of the seed-specific phaseolin promoter^{11,12}. One of the lines generated, CL37, has a total HFA accumulation (18:1-OH plus 18:2-OH) of 17–19%¹³ and has been chosen for experimental investigations aimed at increasing the accumulation of HFA in the seed oil.

In CL37 plants, we assume that RcFAH12 relies on the endogenous *Arabidopsis* electron transfer components, but these may not interact as efficiently with the RcFAH12 protein as do the components

of the castor electron transfer system. The *Arabidopsis* fatty acid desaturases FAD2 and FAD3, which convert 18:1 into linoleic acid (18:2) and 18:2 into α -linolenic acid (18:3), respectively, are also diiron proteins that require reductant from CBR1 and Cb5^{14–17}. Previously, we have shown that a hypomorphic mutation in the *CBR1* gene (*cbr1-1*) led to an 85% decrease in HFA levels in RcFAH12 *Arabidopsis* seeds, but much smaller decreases in 18:3 and 18:2¹⁵. We concluded that, in the *cbr1-1* mutant, the very substantial decrease in HFA was caused by an inadequate supply of electrons reaching the hydroxylase via Cb5, demonstrating that the *Arabidopsis* cytochrome b5 electron supply was critical to the activity of RcFAH12. It is possible that the decreased accumulation of HFA is a result of poor interaction between heterologous RcFAH12 enzyme and the endogenous *Arabidopsis* Cb5 proteins. Furthermore, in a separate study, we have shown that the activity of a *Tetrahymena thermophila* desaturase expressed in yeast is limited by weak interaction with the endogenous yeast Cb5. Activity of this desaturase was increased nearly tenfold by coexpression with a *T. thermophila* Cb5 protein¹⁸. Together, these results suggest that productive protein-protein interactions within the endoplasmic-reticulum electron transport chain are critical to supporting hydroxylase and desaturase activities.

To test the possibility that electron supply to the RcFAH12 enzyme may be a constraint on hydroxylase activity and accumulation of HFA in seeds of the CL37 line, we set out to identify castor genes encoding components of the endoplasmic reticulum electron transport chain and express them in the CL37 line, under control of seed-specific promoters. We tested three *RcCb5* genes, and we also expressed each of these alongside the gene encoding castor cytochrome b5 reductase (*RcCBBR1*). None of the gene combinations that we tested in a total of 270 independent transgenic lines provided any substantial increase in seed HFA content. Our results strongly indicate that electron supply is not limiting the activity of the RcFAH12 hydroxylase in these transgenic *Arabidopsis* lines.

Methods**Plant materials and growth conditions**

Seeds from *Arabidopsis* (ecotype Columbia-0) containing the *RcFAH12* transgene line CL37¹³ were stratified for 2–3 d at 4°C and germinated on 1× MS medium (Sigma-Aldrich) supplemented with 1% (w/v) sucrose and 0.75% agar. Seeds were germinated with a 16 h day/8 h night cycle at 22°C with 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light. Ten to 12 day old seedlings were then transferred to soil and grown under 24 h light at 22°C with 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light at rosette height in a growth chamber or under a 16 h day/8 h night cycle at 22°C with greater than 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light in the greenhouse.

Real-time PCR

RNA was previously extracted by Dr. Chaofu Lu of Montana State University from castor developing endosperm, when the RcFAH12 is most highly expressed¹⁹. This RNA was then used to synthesize cDNA using Superscript III (Life Technologies) reverse transcriptase. Quantitative reverse transcription PCR (RT-PCR) was then performed with a MX3005P QPCR System (Stratagene) using the DNA binding fluorescent dye SYBR Green I (Life Technologies) and five sets of primers, amplifying full-length cDNA from each gene, see [Supplementary Table 1](#) for list of primers. The collected

data was then normalized to the *RcACTIN* control gene and the relative fold change was calculated ($=1/(2^{(\text{experimental}-\text{control})})$).

Identification of castor genes and cloning procedures

Castor genes were subcloned from a castor seed cDNA library¹⁹ or cloned using castor endosperm RNA extracted by Dr. Lu. Reverse transcriptase (Superscript™ III First-Strand Synthesis System; Life Technologies) was used to make cDNA from the castor RNA. Full-length transcripts were amplified from cDNA with KOD polymerase (Takara). The 5' start primer contained the CACC sequence for directional topoisomerase cloning into pENTR-D-Topo (Life Technologies) see [Supplementary Table 1](#) for the list of primers. The castor genes were then sequenced with vector primers (M13 forward and M13 reverse, Life Technologies) and compared with the castor genome at The Institute of Genomic Research (TIGR) castor genome database (<http://castorbean.tigr.org/>)²⁰. These castor genes were renamed as follows: *RcCb5-1*, 28014.m000117; *RcCb5-2*, 30213.m000673; *RcCb5-3*, 29904.m002991; *RcCb5-4*, 30204.m001761; *RcCb5-5*, 29912.m005430; and *RcCb5-6*, 30174.m009087. From the entry vectors, these genes were subcloned into gateway compatible plant transformation vectors containing the seed specific β -phaseolin promoter, pGate-Phas-Basta (pGPB) or pGate-Phas-dsRed (pGPD), using LR Clonase I (Life Technologies). These constructs were then transformed into *Agrobacterium* GV3101 and used for plant transformations.

Dr. Edgar Cahoon (University of Nebraska-Lincoln) graciously provided a dual-gene plant transformation vector (renamed pEC-dsRed) along with a cassette vector pKMS2, which contains the seed-specific oleosin promoter. The pEC-dsRed vector contains two multiple cloning sites and the dsRed selection marker. This pEC-dsRed vector was made gateway compatible at the multiple cloning site containing the storage protein promoter glycinin. For cloning into pEC-dsRed, the *RcCBRI* gene was subcloned from pENTR into pCR-Script (Stratagene) with NotI restriction sites on both primers. The restriction enzyme NotI was then used to cut the *RcCBRI* gene out of pCR-Script and subclone it into the NotI site of pKMS2, which contains the oleosin promoter. The AscI sites were used to transfer the oleosin-*RcCBRI* cassette from pKMS2 and into pEC-dsRed. The pEC-dsRed-*RcCBRI* was then used for three individual gateway reactions with each of the three *RcCb5* entry vectors using LR Clonase I (Life Technologies), so that the oleosin promoter controlled the *RcCBRI* expression and the glycinin promoter controlled the expression of the *RcCb5* genes. This cloning resulted in the three binary plant transformation vectors pEC-dsRed-*RcCBRI*-*RcCb5-2*, pEC-dsRed-*RcCBRI*-*RcCb5-3*, and pEC-dsRed-*RcCBRI*-*RcCb5-4* that were transformed into the *Agrobacterium* strain GV3101.

Transformations

Line CL37¹³ was transformed with constructs mentioned above using the floral dip method²¹. In brief, a 72 h 500 mL culture of *Agrobacterium* containing the plasmid was resuspended in 5% sucrose and 0.05% Silwet L-77 (Lehle Seeds), flowers were dipped in the solution for 30 seconds, and plants were covered with plastic wrap over-night. For constructs containing the dsRed selection marker (pGPD and pEC-dsRed), T₁ and T₂ selection was conducted

using a green LED light with a red filter¹³. For the pGPB constructs, T₁ transformants were selected for Basta® (Bayer) resistance on 1× MS-agar supplemented with 1% sucrose, containing 20 µg/mL glufosinate-ammonium.

Fatty acid analysis

Fatty acid methyl esters were prepared from 20–50 seeds and analyzed by gas chromatography²². Statistical analyses were conducted by an unpaired two-tailed *t*-test in Excel, with a 95% confidence ($P < 0.05$).

Gene expression analysis

Ten to 12 developing siliques from select lines were frozen in liquid nitrogen and used for RNA extraction according to the protocol described by Onate-Sanchez and Vicente-Carbajosa²³, with minor modifications: tissue was ground in liquid nitrogen with a mortar and pestle and transferred into a pre-chilled microcentrifuge tube where 550 µL of extraction buffer (0.4 M LiCl, 0.2 M Tris pH 8, 25 mM EDTA, 1% SDS) and 550 µL of chloroform were added²³. The tubes were vortexed and then centrifuged for 3 min at 14,000 × g at 4°C. 500 µL of water-saturated acidic phenol, 200 µL of chloroform, and 8 µL of iso-amyl alcohol were added to the supernatant and the tubes were vortexed followed by centrifugation for 3 min. The supernatant was extracted twice with 500 µL of chloroform and 1/3 of a volume of 8 M LiCl was added to the resulting supernatant. The tubes were incubated at 20°C for 1.5 h and then centrifuged for 30 min at 14,000 × g at 4°C. The pellet was dissolved in 20 µL DEPC-water and subjected to DNaseI treatment according to the DNA-free RNA kit (Zymo). Complementary DNA was synthesized using Superscript III reverse transcriptase (Life Technologies) and the full-length castor genes were amplified using KOD polymerase (Takara), see [Supplementary Table 1](#) for the list of primers.

Results

Identification of castor cytochrome b5 genes

In *Arabidopsis*, five cytochrome b5 proteins (AtCb5-A to AtCb5-E) and one AtCb5-like protein receive electrons from NADH:cytochrome b5 reductase encoded by *AtCBRI*^{24,25}. In developing *Arabidopsis* seeds, the most highly expressed genes encode AtCb5-E (At5g53560) and AtCb5-D (At5g48810); *AtCb5-B* (At2g32720) is also strongly expressed, but there is only weak expression of the remaining three genes^{26,27}. The *AtCb5-E* and *AtCb5-D* genes are also highly expressed in other tissues of the plant. Proteins encoded by these three strongly expressed isoforms are predicted to localize to the endoplasmic reticulum, based on their homology to Cb5 proteins from tung tree (*Vernicia fordii*) VfCb5-A, VfCb5-B and VfCb5-C that have been shown to be targeted exclusively to the endoplasmic reticulum²⁸.

The *Arabidopsis* and *Vernicia* protein sequences were used to search the castor genome²⁰ and identify likely *RcCb5* orthologues. Sequences from all three species were used to derive an unrooted dendrogram ([Figure 1](#)) showing the phylogenetic relationships among the proteins. The castor isoforms designated *RcCb5-2*, *RcCb5-3* and *RcCb5-4* (see Materials and methods for accession numbers) fall into a distinct clade with the *Vernicia* and *Arabidopsis* endoplasmic reticulum Cb5 proteins. Quantitative PCR (qPCR) was performed to measure the expression of *RcCb5* genes in developing endosperm of castor seeds during the period of HFA synthesis in this tissue¹⁹. We found that *RcCb5-2*,

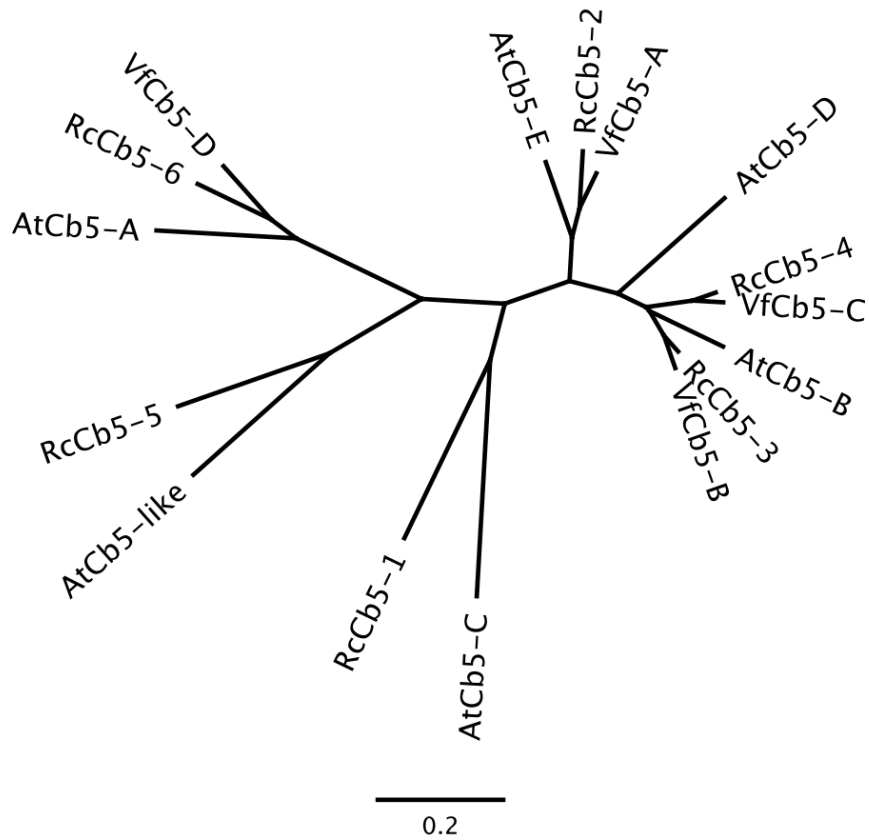


Figure 1. Phylogenetic relationships among 16 Cb5 proteins. Protein sequences from *Arabidopsis thaliana* (At), *Ricinus communis* (Rc), and *Vernicia fordii* (Vf) were used to build an unrooted dendrogram in Geneious v5.1 (www.geneious.com). Size bar indicates genetic distance between proteins using the Neighbor-Joining method.

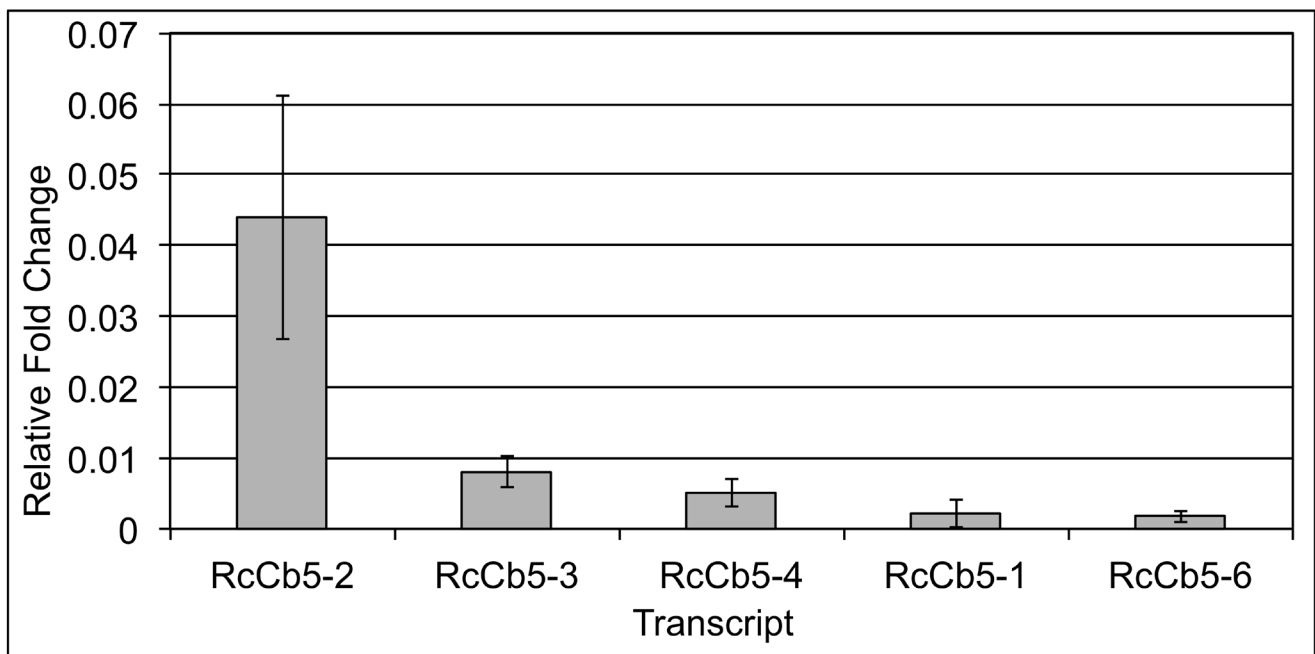


Figure 2. Quantitative PCR of RcCb5 transcripts from developing castor endosperm. Transcripts were normalized to *RcACTIN*. Error bars represent standard error of the mean of three independent experiments with three replicates for each experiment.

RcCb5-3 and *RcCb5-4* transcripts were all strongly expressed, with highest transcript levels found for *RcCb5-2* (Figure 2). By contrast, the *RcCb5-1* and *RcCb5-6* genes showed very low expression. Data from a transcript profiling database with $\sim 10^6$ sequences covering five stages of endosperm development²⁹ includes 21 reads for *RcCb5-2* and 26 reads for *RcCb5-4*, while *RcCb5-3*, *RcCb5-1* and *RcCb5-6* were not represented in this data set. Taken together, these results indicate that *RcCb5-2*, *RcCb5-3* and *RcCb5-4* likely have redundant roles in providing electrons to the FAH12 hydroxylase enzyme in the endoplasmic reticulum of endosperm cells of developing castor seeds.

Transformation of the CL37 line with three *RcCb5* proteins

The cDNAs encoding the three *RcCb5* isoforms that are strongly expressed in seeds (*RcCb5-2*, *RcCb5-3* and *RcCb5-4*) were each cloned under control of the phaseolin promoter in vector pGate-Phas-Basta and transformed into CL37 plants expressing RcFAH12¹³. Putative T₁ transformants were selected for Basta resistance. Analysis of the bulk T₂ seed from 35 independent *RcCb5-2* CL37 primary transformants showed no significant difference in total HFA (18:1-OH plus 18:2-OH) levels compared to the control CL37 plants grown at the same time, with a mean of 19.5% HFA (P value = 0.5979) for the 35

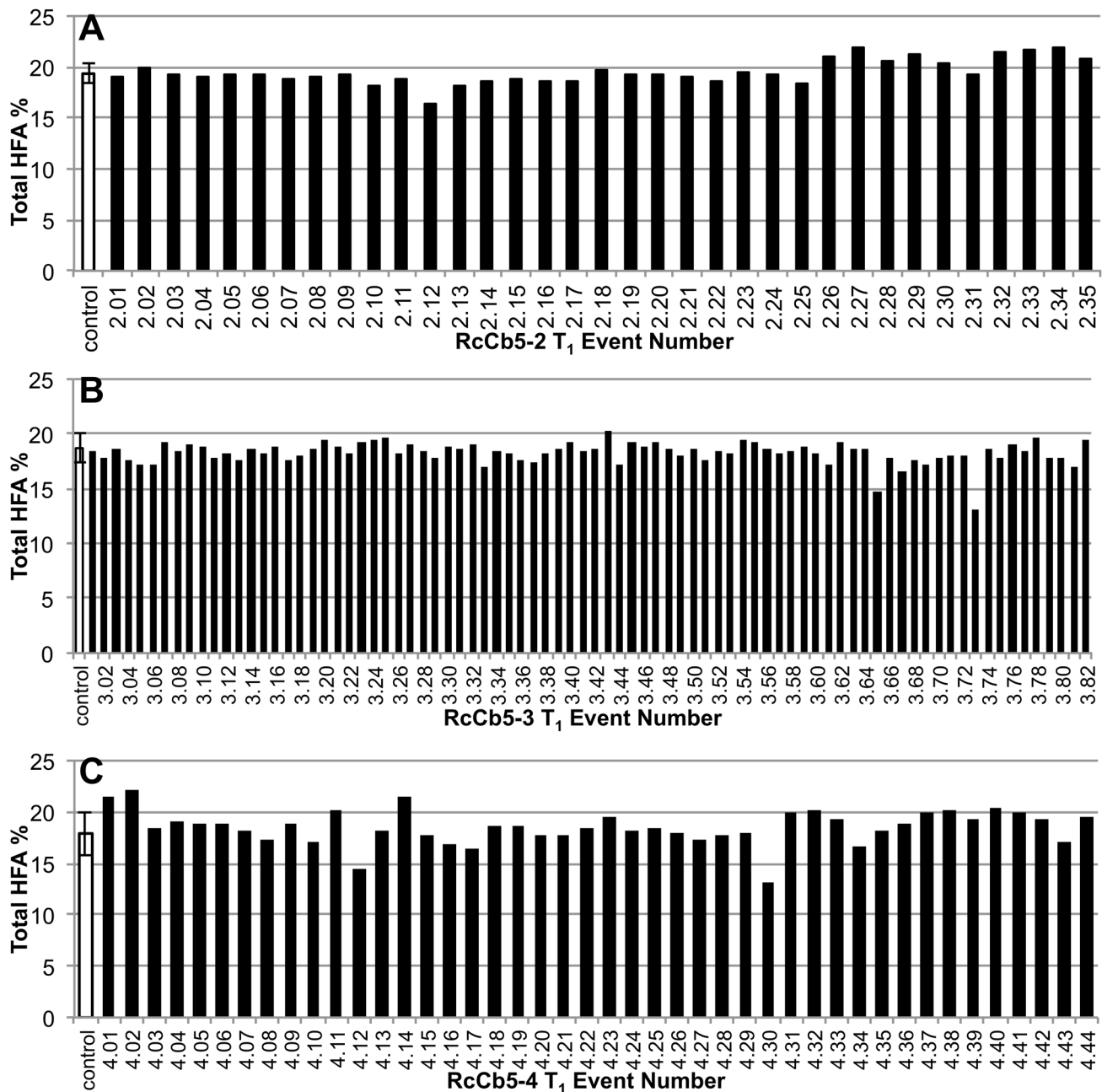


Figure 3. Total HFA accumulation in seed of individual *RcCb5* CL37 primary transformants. Total HFA (18:1-OH+18:2-OH) contents of mature T₂ seeds of individual transformants expressing (A) *RcCb5-2*, (B) *RcCb5-3*, or (C) *RcCb5-4* in the CL37 line. Control at left is mean HFA in untransformed CL37 plants grown alongside T₁ plants. For controls, error bars indicate standard deviation; n=28 in A, n=11 in B, and n=15 in C.

RcCb5-2 events (Figure 3A). There were also no significant differences in HFA levels for the 82 independent T_1 events for RcCb5-3 CL37 compared to untransformed CL37, with a mean of 18.3% HFA (P value = 0.2155) (Figure 3B), nor for the 44 independent T_1 events of RcCb5-4 CL37 (mean of 18.6% HFA) compared to untransformed CL37 (P value = 0.2470; Figure 3C).

This analysis of T_2 seed samples indicates that there is no dramatic increase in HFA accumulation through the co-expression of any of the three RcCb5 isoforms with the RcFAH12 in *Arabidopsis*. However, gene expression is typically variable across a population of individual T_1 transformants and T_2 seeds are segregating for the newly introduced transgene. We therefore identified five T_1 events that produced seed with relatively high HFA and that contained a single *RcCb5* transgene insert, based on segregation of the Basta-resistance marker in the T_2 generation. For each of the chosen lines, RcCb5-2 #34, RcCb5-2 #27, RcCb5-2 #33, RcCb5-3 #43, and RcCb5-4 #40, T_2 progeny were grown to maturity and nine to 10 plants homozygous for the *RcCb5* transgenes, together with four to nine segregants lacking the transgene from each event, were identified by pedigree analysis of T_3 progeny for Basta resistance. Comparisons of the total HFA content between these two sets of sibling segregants (Figure 4) failed to identify any statistically significant increase in HFA as a result of *RcCb5* expression. Thus, plants from the three lines of RcCb5-2 CL37, #34, #27, and #33, were not significantly different from their segregating untransformed CL37 counterparts, with $P = 0.2204$, 0.0942 , and 0.3965 respectively (Figure 4). The RcCb5-3 CL37 #43 was not significantly different from segregating untransformed CL37 ($P = 0.4211$), and the RcCb5-4 CL37 #40 was also not significantly different from segregating untransformed CL37 ($P = 0.2102$) (Figure 4). Although no changes were observed in the total HFA levels from these lines, we did observe some minor changes in the fatty acid profile; there were increases in the level of 18:1 and minor decreases in 18:2 and 18:3 (Supplementary Table 2).

Since it is assumed that the RcFAH12 in the CL37 line is receiving reductant via the endogenous *Arabidopsis* CBR1 (*AtCBR1*) and an *AtCb5*, we attempted to increase reductant supply to the *AtCb5* proteins via overexpressing *AtCBR1*. However, again we found no changes in total HFA in the T_2 seeds (Supplementary Figure 1). Taken together, these results indicate that none of the three RcCb5 proteins or any of the *AtCBR1* electron acceptors can provide increased RcFAH12 activity in CL37.

Coexpression of RcCBR1 and RcCb5s in CL37

We transformed CL37 plants with *RcCb5* genes on the basis that the RcFAH12 hydroxylase enzyme may not interact optimally with endogenous *AtCb5* proteins to receive electrons required for the hydroxylation reaction. However, it is also possible that interactions of the RcCb5 isoforms with the *Arabidopsis* cytochrome b5 reductase (*AtCBR1*) are weak, resulting in incomplete reduction of the RcCb5 proteins in the transgenic plants. To test this possibility, and to provide a fully compatible electron transport chain from NADH to the hydroxylase, we transformed CL37 plants with expression constructs containing both *RcCBR1*, encoding the castor cytochrome b5 reductase isozyme and a *RcCb5* coding sequence, under control of strong, seed-specific promoters (see Materials and

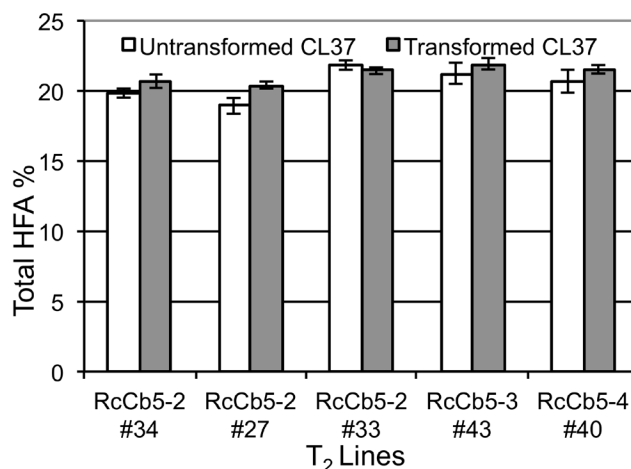


Figure 4. Total HFA accumulation in RcCb5 CL37 T_2 seed from selected T_2 lines. Total HFA (18:1-OH+18:2-OH) contents of T_2 seed of selected homozygous lines expressing RcCb5-2, RcCb5-3, or RcCb5-4 in CL37 were compared with segregating untransformed CL37 siblings. Error bars represent standard error of the mean for four to ten individual plants.

methods for details). The *RcCBR1* and each of the top three *RcCb5* genes were subcloned into pEC-dsRed plant transformation vector, resulting in three dual-gene vectors expressing *RcCBR1+RcCb5-2*, *RcCBR1+RcCb5-3*, and *RcCBR1+RcCb5-4*. The T_1 transformants were selected by screening for dsRed fluorescence in seeds and grown to maturity. Screening of individual transformation events for total HFA accumulation was performed by gas-chromatography of bulk T_2 seeds. The 45 *RcCBR1+RcCb5-2* CL37 events generated were not statistically different (mean of 18.4% HFA) from the untransformed CL37 control lines ($P = 0.6820$) (Figure 5). For the 16 *RcCBR1+RcCb5-3* CL37 events the mean of 18.5% HFA was not significantly changed in HFA accumulation compared to the CL37 control plants ($P = 0.6834$). Similarly, the 30 *RcCBR1+RcCb5-4* CL37 events (mean of 17.4% HFA) also were not significantly different from the CL37 controls ($P = 0.0898$).

Three single-insert lines with relatively high HFA were selected from the T_1 events shown in Figure 5. Progeny from each of the three lines were grown to maturity and four to seven plants homozygous for the *RcCBR1* and *RcCb5* transgenes were identified by the presence of the DsRed marker in their seeds and were grown together with CL37 control plants. Analyses of total HFA content of the homozygous seed showed no statistically significant difference between *RcCBR1+RcCb5-2* #24 double transgenic and seed of CL37 lacking these transgenes ($P = 0.0571$; Figure 6). Similarly, HFA in seed of homozygous plants of lines *RcCBR1+RcCb5-3* #12 and *RcCBR1+RcCb5-4* #20 were not statistically different from HFA in CL37 controls ($P = 0.1388$ and $P = 0.5774$ respectively; Figure 6).

To confirm that the *RcCBR1* and *RcCb5* transgenes were being expressed, we isolated RNA from developing seeds of representative plants from these three homozygous transgenic lines, and from the untransformed CL37 siblings. The RNA samples were used as templates for RT-PCR using primers specific for *RcCBR1* and each of

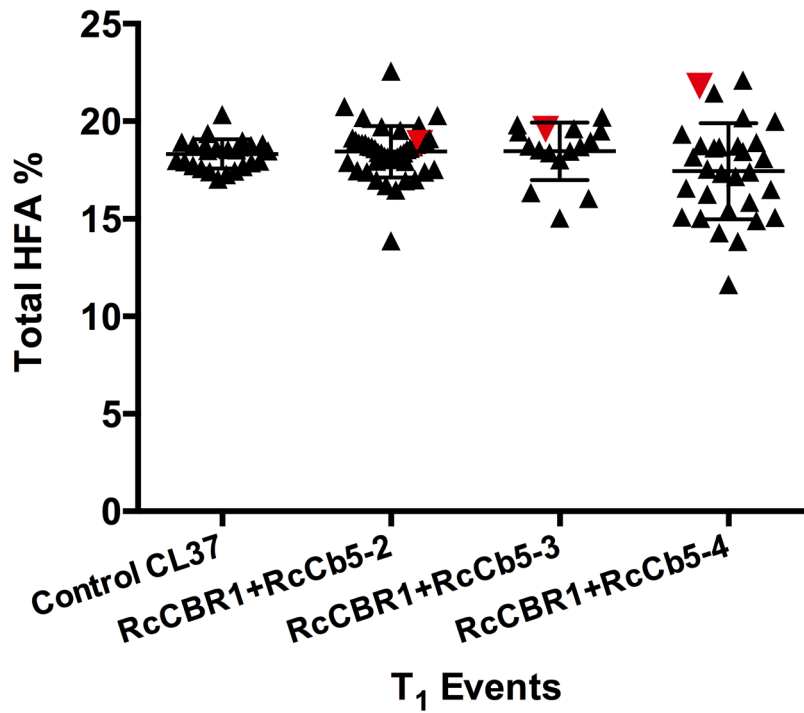


Figure 5. Total HFA accumulation in seed of individual RcCBR1+RcCb5 CL37 primary transformants. Total HFA (18:1-OH+18:2-OH) contents of mature T₂ seeds from individual transformants expressing RcCBR1+RcCb5-2, RcCBR1+RcCb5-3 or RcCBR1+RcCb5-4 in the CL37 line. Results are compared with untransformed CL37 controls grown alongside T₁ plants. Red inverse triangles indicate events that were further analyzed. Error bars represent standard deviation; n=25 for control CL37, n=45 for RcCBR1+RcCb5-2, n=16 for RcCBR1+RcCb5-3, and n=30 for RcCBR1+RcCb5-4.

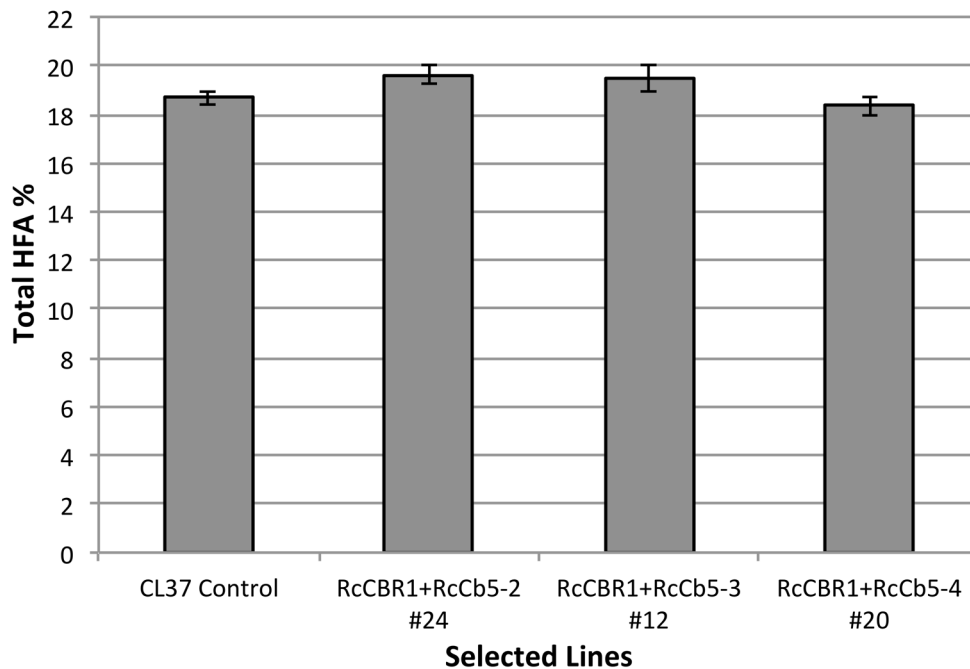


Figure 6. Total HFA accumulation in RcCBR1+RcCb5 CL37 from selected lines. Total HFA (18:1-OH+18:2-OH) contents of seed from selected lines expressing RcCBR1+RcCb5-2, RcCBR1+RcCb5-3 or RcCBR1+RcCb5-4 in CL37. Plants homozygous for each dual-gene construct were compared with untransformed CL37 controls. Error bars represent standard error of the mean; n=14 for CL37 controls, n=6 for #24, n=7 for #12, and n=4 for #20.

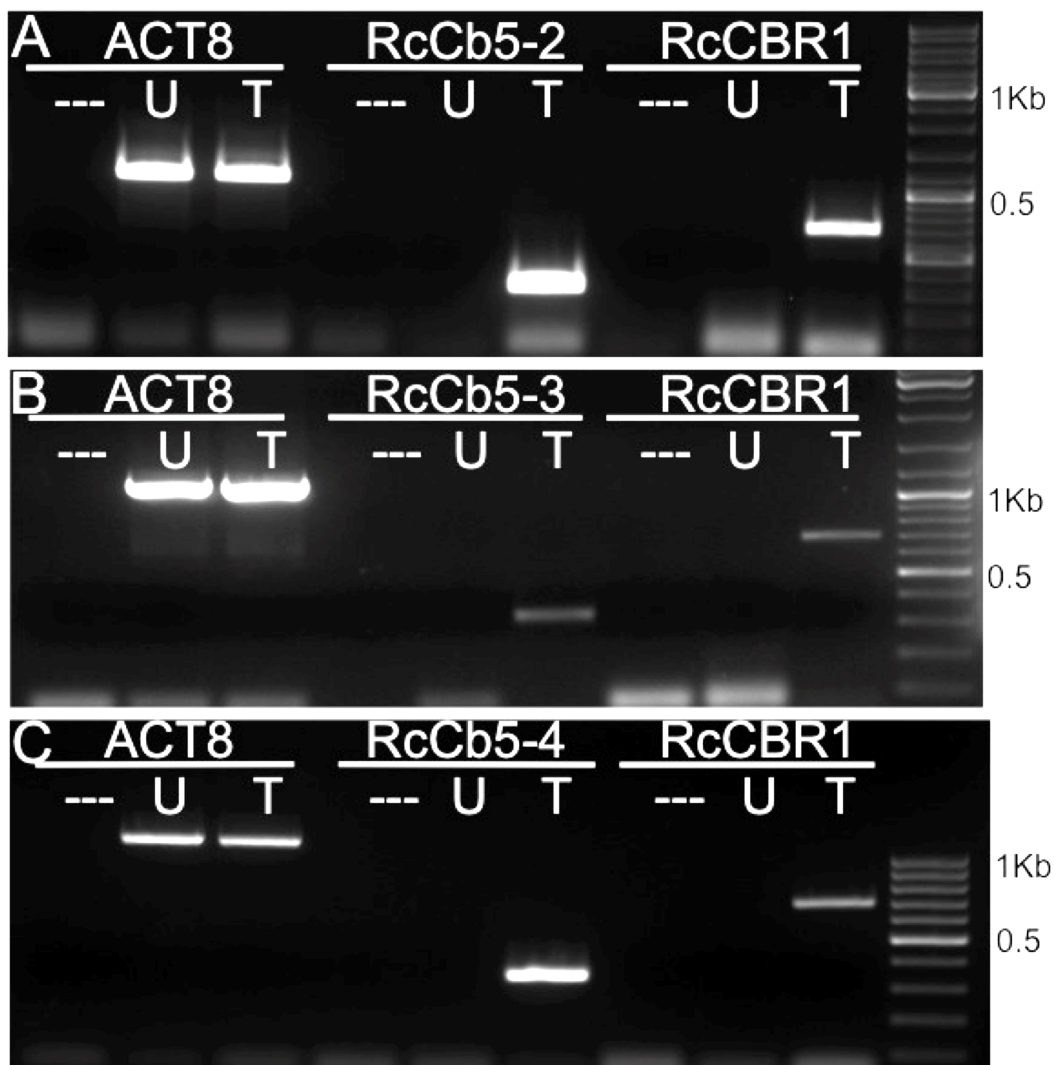


Figure 7. Reverse transcriptase-PCR from representative RcCbr1+RcCb5 CL37 T₃ plants. (A) *RcCbr1*+*RcCb5-2* #24, (B) *RcCbr1*+*RcCb5-3* #12 and (C) *RcCbr1*+*RcCb5-4* #20. RNA prepared from developing siliques was subject to RT-PCR using gene-specific primers. In each panel, --- indicates no-template control. U indicates untransformed CL37, T indicates homozygous transformant. The *ACTIN8* gene (*ACT8*, At1g49240) was used as a control to test RNA quality.

the *RcCb5* isoforms. Bands of the expected full-length sizes were observed for the transgenic plants but were not detected in the untransformed CL37 siblings (Figure 7). Successful amplification of a band using primers to the *ACT8* gene indicated that none of the RNA samples was degraded.

Discussion

The endoplasmic reticulum diiron enzymes that catalyze desaturation and hydroxylation of fatty acids and other substrates require electrons from cytochrome b5 (Cb5) proteins^{6,30}. In seeds of transgenic *Arabidopsis* expressing the RcFAH12 hydroxylase from castor, a hypomorphic mutation in the *CBR1* gene encoding cytochrome b5 reductase led to an 85% reduction in total HFA, but had smaller effects on 18:1 and 18:2 desaturation, <25% and <60% respectively¹⁵. These results indicated that there is competition among the

reactions that utilize electrons from reduced Cb5, and raised the possibility that the RcFAH12 enzyme is not able to efficiently accept electrons from the endogenous AtCb5 proteins. In another study, we have shown that the activity of a *T. thermophila* desaturase expressed in yeast is strongly limited by the failure of the desaturase to properly interact with, and receive electrons from, the yeast Cb5. A nearly ten-fold increase in desaturase activity was achieved by coexpressing *T. thermophila* Cb5 proteins¹⁸.

When RcFAH12 is heterologously expressed in *Arabidopsis*, the reductant supply for this enzyme comes from the endogenous *Arabidopsis* proteins. Here, we have tested the proposal that, since the castor orthologs of CBR1 and Cb5 have coevolved with RcFAH12, these proteins may be more efficient at supplying reductant to RcFAH12 and lead to a greater accumulation of HFA. The

coevolution hypothesis has been shown to be valid for two castor acyltransferases, RcDGAT2 and RcPDAT1A; when these castor enzymes were heterologously expressed with the RcFAH12 in *Arabidopsis*, the HFA content of seeds increased from 17% to as much as 30% of total fatty acids, due to the increased efficiency of HFA incorporation into TAG^{22,31}. However, contrary to our hypothesis, we found that there was no substantial increase in HFA accumulation when any of three RcCb5 proteins were expressed in CL37 (Figure 3 and Figure 4). These results argue against there being a substantial difference in the effectiveness of interaction of RcFAH12 with RcCb5 versus AtCb5 protein isoforms. Overexpressing the AtCBR1 in CL37 also did not increase HFA levels (Supplementary Figure 1), indicating that reductant supply via AtCBR1 is not limiting to the hydroxylase activity. However, these experiments cannot exclude the possibility that AtCBR1 does not efficiently reduce RcCb5 proteins.

To reconstitute the complete electron transport chain from NADH to the hydroxylase present in castor seeds, we transformed *RcCBR1* and *RcCb5* genes into the CL37 line, using a dual-gene plant expression system. The proportion of HFA in seeds of plants expressing *RcCBR1*+*RcCb5*-2 CL37, *RcCBR1*+*RcCb5*-3 CL37, or *RcCBR1*+*RcCb5*-4 CL37 was not significantly increased in comparison to the parental CL37 line. Although we confirmed expression of *RcCBR1* and *RcCb5* transcripts (Figure 7), production of functional reductase and cytochrome b5 requires translation, correct protein folding in the endoplasmic reticulum, and insertion of the required cofactor. It is possible that either *RcCBR1* or (all of) the Cb5 proteins fail to assemble to the mature form. Assuming the proteins are correctly expressed, our results suggest that electron transfer to RcFAH12 is not limiting in the CL37 line, and that the endogenous *Arabidopsis* electron transfer system appears to be as efficient in transferring electrons to RcFAH12 as the transgenic castor electron transfer system in *Arabidopsis*.

Taken as a whole, our results fail to show any positive effect of expressing genes of the castor electron-transport components on the synthesis and accumulation of HFA in transgenic *Arabidopsis* expressing the castor RcFAH12 hydroxylase. However, our CL37 transgenics only accumulate 17–19% HFA in the seed oil, compared to almost 90% HFA found in oil from castor^{22,31}. Some of

the constraints on HFA synthesis and accumulation have been identified^{22,32,33}. If these constraints can be alleviated, by more efficient shuttling of HFA into triacylglycerol or reducing feedback inhibition of metabolism for example, it is possible that electron supply to the hydroxylase will then become limiting. If so, the constructs and lines generated in this study can be used for transforming and crossing with the elite lines containing higher levels of HFA.

Fatty acid hydroxylation in transgenic *Arabidopsis thaliana*

8 Data Files

<http://dx.doi.org/10.6084/m9.figshare.808590>

Author contributions

LLW and JB conceived the study; LLW collected and analyzed the data; LLW and JB wrote the manuscript.

Competing interests

No competing interests were disclosed.

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Supplementary figure

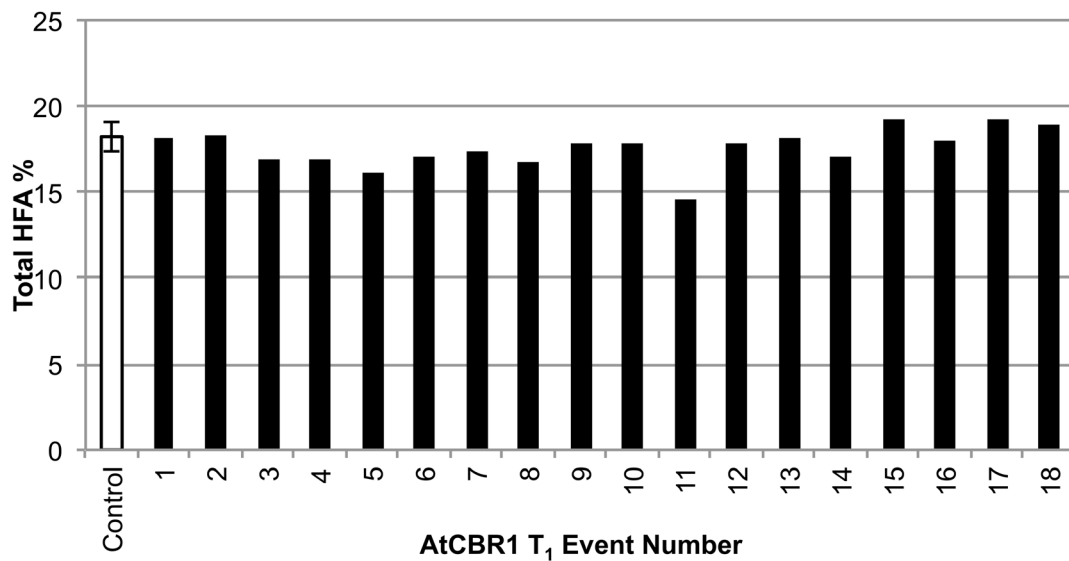


Figure S1. Total HFA accumulation in AtCBR1 CL37 primary transformants. Total HFA (18:1-OH+18:2-OH) contents of mature T_2 seeds of individual transformants expressing AtCBR1 in CL37. Control at left is mean HFA in untransformed CL37 plants grown alongside the T_1 plants. For the control, error bar indicates standard deviation, $n=12$.

Supplementary tables

Table S1. List of primers used in experiments.

Experiment	Primers	Sequence	
Q-PCR	RcCb5-2 QFW	AAACTTCGAAGAGGTGGCTAA	
	RcCb5-2 QRV	TGTAGTTGGCTTGTGGTTTAGG	
	RcCb5-4 QFW	GGGTGGTCAAGGAAAGGTTTAT	
	RcCb5-4 QRV	GAGGCTGCTTAGGAGGAGTATATG	
	RcCb5-3 QFW	GTTTTCACTTTGGCTCAGGTGT	
	RcCb5-3 QRV	GGCTTATATGTCGCCTTCTTAGG	
	RcCb5-1 QFW	GGCTTCAGAACCCTAAAATCT	
	RcCb5-1 QRV	GGTTCTTAGGATGTTCTGAAGGTG	
	RcCb5-6 QFW	GCTCAGCACAAACCAAAGAG	
	RcCb5-6 QRV	CTTCGTCAAGTCCTTGAGCTTC	
	RcACTIN QFW	AAGGTTATGCTCTCCACATGCCA	
	RcACTIN QRV	ACTTCTGGGCAACGGAATCTCTCA	
	Cloning & RT-PCR	RcCb5-2 5topo	CACCATGGCGAGCGATCCGAAAAT
		RcCb5-2 3end	CTACTCTTTCTTGGTGTAGTGCCTGAC
RcCb5-4 5topo		CACCATGGGTGGTCAAGGAAAGGTTTATA	
RcCb5-4 3end		CTATTATGCTTCAGCTGATTTGGTGT	
RcCb5-3 5topo		CACCATGAGTGGTGAAGGCAAAGTTT	
RcCb5-3 3end		TTAAGTTGATTTGTATAGATGCGGAT	
Sub-cloning	RcCBR1 5'Not1	CACCGAATGCGGCCGCATGGATTAGATT TCTTGCAAACAC	
	RcCBR1 3'Not1	ATAGTTTAGCGGCCGCTCAAATGGAAC GCATCTCA	

Table S2. Fatty acid profile from RcCb5 CL37 T₃ seed from selected T₂ lines.

	16:0	16:1	18:0	18:1	18:2	18:3	20:1	18:1-OH	18:2-OH	Total HFA ^a
RcCb5-2 #34	10.8%	0.5%	4.3%	44.0%	13.8%	5.3%	0.6%	14.9%	5.8%	20.7%
n=10	0.3%	0.0%	0.2%	0.6%	0.4%	0.1%	0.0%	0.2%	0.4%	0.5%
Untransformed #34	12.5%	0.6%	5.2%	34.7%	19.5%	6.8%	0.8%	15.4%	4.5%	19.9%
n=9	0.4%	0.0%	0.2%	0.8%	0.5%	0.1%	0.0%	0.2%	0.3%	0.3%
RcCb5-2 #27	11.1%	0.5%	4.8%	44.6%	13.2%	4.5%	0.8%	15.5%	4.9%	20.4%
n=9	0.3%	0.0%	0.2%	0.9%	0.5%	0.6%	0.0%	0.4%	0.2%	0.3%
Untransformed #27	13.3%	0.6%	5.5%	33.2%	20.9%	6.7%	0.9%	14.8%	4.1%	18.9%
n=6	0.7%	0.0%	0.3%	1.3%	0.7%	0.1%	0.1%	0.3%	0.4%	0.5%
RcCb5-2 #33	10.3%	0.6%	4.8%	45.3%	12.0%	4.8%	0.7%	16.2%	5.3%	21.4%
n=10	0.3%	0.0%	0.2%	1.2%	0.8%	0.2%	0.0%	0.3%	0.5%	0.2%
Untransformed #33	11.0%	0.5%	4.8%	39.9%	15.7%	5.7%	0.7%	16.5%	5.3%	21.8%
n=8	0.1%	0.1%	0.2%	1.3%	1.4%	0.3%	0.0%	0.4%	0.3%	0.4%
RcCb5-3 #43	12.4%	0.6%	5.5%	38.5%	15.3%	5.0%	0.8%	17.6%	4.3%	21.9%
n=10	0.3%	0.0%	0.2%	1.1%	0.6%	0.6%	0.0%	0.2%	0.2%	0.4%
Untransformed #43	13.0%	0.6%	5.5%	36.9%	17.5%	4.5%	0.8%	16.5%	4.8%	21.2%
n=9	1.1%	0.0%	0.5%	1.7%	1.2%	0.8%	0.1%	0.3%	0.5%	0.8%
RcCb5-4 #40	11.6%	0.6%	4.7%	39.4%	16.6%	5.0%	0.6%	16.4%	5.1%	21.5%
n=10	0.3%	0.0%	0.2%	0.4%	0.4%	0.5%	0.0%	0.2%	0.3%	0.3%
Untransformed #40	13.7%	0.7%	6.1%	35.2%	18.2%	4.6%	0.9%	16.8%	3.8%	20.7%
n=4	0.6%	0.0%	0.3%	1.2%	0.9%	1.5%	0.1%	0.6%	0.3%	0.8%

^aTotal HFA is the sum of 18:1-OH and 18:2-OH.
Standard error of the mean is below each value.

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Open Peer Review

Current Referee Status:



Version 2

Referee Report 17 January 2014

doi:10.5256/f1000research.2955.r3175



Mark Smith

National Research Council Canada, Saskatoon, SK, Canada

I agree with the authors response to my comments, the evidence supports the conclusion that expression of genes encoding homologous electron transport components does not appear to be of value in this case. Further work would provide an improved characterization of the transgenic plants, but the conclusion would most likely remain the same. This is an interesting study and adds some valuable information to plant lipid research.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 18 November 2013

doi:10.5256/f1000research.2955.r2440



Daniel Gallie

Department of Biochemistry, College of Natural and Agricultural Sciences, University of California, Riverside, CA, USA

I have no comments other than those I made for the first version.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 06 November 2013

doi:10.5256/f1000research.2397.r2243

**Mark Smith**

National Research Council Canada, Saskatoon, SK, Canada

FAD2 family proteins in plants require a reductant that is channelled via an electron transport chain comprising of cytochrome b5 (Cb5) and cytochrome b5 reductase (CBR). This study explores the hypothesis that lower than expected levels of hydroxy fatty acid (HFA) accumulation in engineered Arabidopsis could in part be due to inefficient interaction between the expressed castor hydroxylase and the endogenous Cb5 electron transport chain. The authors used a well characterized line of Arabidopsis (CL37) previously engineered to express the castor hydroxylase. Retransformation with a various cDNAs encoding Cb5 or Cb5+CBR1 failed to generate lines with increased HFA content.

The study addresses an important and somewhat overlooked aspect of the ER fatty acid modification processes. The experiments are well planned, and the comparison of null and transgenic lines in single insertion lines (Fig.4) adds confidence to the data. My only concern is that although there is evidence of transgene expression by RT-PCR, there is no evidence to show the assembly of a functional electron transport chain containing the expressed proteins, or an increase in electron transport activity resulting from Cb5 and CBR overexpression. A biologically active Cb5 requires the assembly of cytochrome b5 apoprotein and haem to generate the Cb5-holoenzyme. Demonstrating over-expression of active Cb5 would require a spectrophotometric assay of microsomal membranes to quantify the amount of Cb5 present and to determine electron transport activity (not easy in Arabidopsis). Alternatively, a Western blot showing increased Cb5 protein and a good argument that haem-protein assembly in developing Arabidopsis seeds is not limiting would help.

An added complication from measuring total HFA in mature seed is that changes in HFA production could be masked by bottlenecks in HFA assembly into TAG.

Further development of this work will be very interesting; the conclusions would be better supported by characterization of the electron transport chain.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Author Response 07 Nov 2013

John Browne, Institute of Biological Chemistry, Washington State University, USA

Mark Smith is correct in pointing out that in biochemistry (and in life) it is protein function that matters. We have added a caveat on p.10 to acknowledge the possibilities he raises.

Unfortunately, microsomal membranes from Arabidopsis seeds are contaminated with chlorophyll-containing thylakoids and the pigment confounds the spectrophotometric measurement of cytochrome b5. Even with the possibility that redox-competent RcCb5 and RcCBR1 are not produced, our conclusion remains the same: Expression of genes encoding homologous electron-transport components does not present itself as a useful route to increasing HFA accumulation in transgenic plants.

Competing Interests: No competing interests were disclosed.

Referee Report 07 October 2013

doi:10.5256/f1000research.2397.r1998



Daniel Gallie

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The castor fatty acid hydroxylase (RcFAH12) is responsible for the synthesis of ricinoleic acid which has important industrial applications. Heterologous expression of RcFAH12 in *Arabidopsis thaliana* resulted in only 17-19% hydroxylated fatty acid (HFA) in seed triacylglycerols (TAG). In this work, the authors suggest that electron supply to RcFAH12 in *Arabidopsis* is limiting due to its heterologous expression and they test this hypothesis by co-expressing castor NADH:cytochrome b5 reductase (RcCBR1) and cytochrome b5 (RcCb5) that supply electrons to RcFAH12.

The work is well presented and performed and indicates that the endogenous electron transfer system in *Arabidopsis* is not rate limiting in HFA production.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.
