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Running title:

A Fatty Acid Hydroxylase from *Lesquerella lindheimeri.*

Title:

A FAD2 homologue from *Lesquerella lindheimeri* has predominantly fatty acid hydroxylase activity.

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Abstract

A genomic sequence encoding a polypeptide with 91% sequence identity to the *Lesquerella fendleri* bifunctional oleate 12-hydroxylase: desaturase was amplified by PCR from *Lesquerella lindheimeri*. Expression of the gene in the yeast *Saccharomyces cerevisae* resulted in the synthesis of ricinoleic acid and very low levels of diunsaturated fatty acids. Comparison of the amino acid sequences of the *L. lindheimeri* and castor bean oleate 12-hydroxylase enzymes to those of the *L. fendleri* bifunctional oleate 12-hydroxylase: desaturase and oleate 12-desaturase enzymes from 32 diverse species identified a single amino acid (M295) that was conserved in the hydroxylases and different but also conserved in the desaturases and the bifunctional enzyme. Site directed mutagenesis indicated that this residue was most likely not involved in determining the catalytic outcome of the hydroxylation/desaturation reaction.

Transformation of an *Arabidopsis fad2/fae1* mutant line with the *L. lindheimeri* hydroxylase gave further evidence that this enzyme, like the castor oleate 12-hydroxylase, has predominantly hydroxylase activity and should not be considered bifunctional. Total hydroxy fatty acid content of up to 18% of seed fatty acids was measured in homozygous transformants. Lines with the highest hydroxy fatty acid content showed significant reduction in total oil content.

Key words

Lesquerella lindheimeri, fatty acid hydroxylase, ricinoleic acid, hydroxy fatty acid, seed oil content, single seed TLC.

Abbreviations

FAMES; Fatty acid methyl esters. TLC; thin layer chromatography.

1. Introduction

Castor bean (*Ricinus communis*) and members of the genus *Lesquerella* both accumulate unusual hydroxy fatty acids in their seed oil. In castor the predominant fatty acid is ricinoleic acid (12-hydroxyoctadec-*cis*-9-enoic acid, 18:1-OH) which accounts for nearly 90% of the fatty acids in the storage triacylglycerol. *Lesquerella* species generally accumulate lesquerolic acid (14-hydroxyeicos-*cis*-11-enoic acid, 20:1-OH), although some also accumulate other hydroxy fatty acids including ricinoleic , densipolic acid (12-hydroxyoctadec-*cis*-9,15-dienoic acid, 18:2-OH) and auricolic acid (14-hydroxyeicos-*cis*-11,17-dienoic acid, 20:2-OH) [1].

In developing castor and *Lesquerella* seeds biochemical studies indicated that hydroxy fatty acids are formed by the hydroxylation of oleic acid to form ricinoleic acid [2-4]. In castor there is no further modification of ricinoleic acid whereas in *Lesquerella fendleri* ricinoleic acid is efficiently elongated to lesquerolic acid in a reaction catalyzed by a condensing enzyme specific to hydroxy fatty acids [5]. Genes encoding the oleate 12-hydroxylase have been isolated from castor [6] and *L. fendleri* [7]. The enzymes have 71% amino acid identity and belong to the FAD2 fold of enzymes [8, 9] which includes the plant endoplasmic reticulum (ER) oleate 12-desaturases, and a number of other fatty acid modifying enzymes.

Ectopic expression of the *L. fendleri* oleate 12-hydroxylase (LFAH12) in *Arabidopsis thaliana* demonstrated that this enzyme is bifunctional catalyzing both the Δ12 hydroxylation and Δ12 desaturation of oleate to produce either ricinoleic acid or linoleic acid (octadeca-*cis*-9,12-dienoic acid, 18:2) [7]. In contrast, the castor hydroxylase (CFAH12) can be considered a strict hydroxylase having only very low levels of desaturase activity [6, 10]. The bifunctional nature of the lesquerella hydroxylase and the high level of amino acid conservation between this enzyme, the castor hydroxylase and plant FAD2 desaturases (ER oleate 12-desaturases) has been used to identify key amino acids which are important in determining the catalytic outcome of the reaction [11]. For example, changing as few as 4 amino acids in *Arabidopsis* FAD2 to their equivalent in the *Lesquerella* hydroxylase using site directed mutagenesis can convert a strict desaturase to a bifunctional desaturase: hydroxylase enzyme. No single amino acid has yet been identified that has a major effect on the hydroxylation/desaturation ratio and it has not been possible to convert a strict desaturase to a strict hydroxylase.

To collect additional sequence information for oleate 12-hydroxylase enzymes and to examine whether hydroxylases from other *Lesquerella* species were also bifunctional we amplified a gene encoding a hydroxylase from *Lesquerella lindheimeri*, a species that accumulates over 80% lesquerolic acid in its seed oil. Expression in yeast and *Arabidopsis* was used to demonstrate that the encoded enzyme has predominantly fatty acid hydroxylase activity.

2. Materials and methods

2.1. Amplification of the L. lindheimeri hydroxylase gene.

Seeds of *L. lindheimeri*, generously donated by Dr D. Deirig (USDA-ARS, Maricopa AZ), were planted in moist potting soil and grown for 6 weeks at 20°C with continuous light. Genomic DNA was prepared from freshly harvested leaf pieces using the method of [12]. The putative hydroxylase gene was amplified by PRC using forward primer LFFE (5'-GCGAATTCAAGATGGGTGCTGGTGGAAGA) and reverse primer LFRSE (5'-GCGGAATTCGTCGACTCATAACTTATTGTTGTAATA), with genomic DNA as a template. Primers were based on the sequence of the bifunctional oleate hydroxylase from *L. fendleri* [7] and included restriction sites to facilitate subcloning. PCR was conducted using Expand High Fidelity DNA polymerase mixture (Roche, Laval, QC, Canada) with an annealing temperature of 55°C and extension of 100 seconds for 10 cycles followed by 30 cycles with an annealing temperature of 60°C with the same extension time. PCR products were gel purified by separation in a 1% agarose gel and recovered using the QIAquick gel extraction kit (Qiagen, Mississauga, ON, Canada).

2.2. Expression of Lesquerella hydroxylase genes in yeast

The PCR product (designated *LlinFAH12*) encoding the putative hydroxylase from *L. lindheimeri* was digested with the restriction enzyme EcoRI and cloned into the yeast expression vector pHVX2 under the control of the constitutive promoter PGK1 to create vector pMS696.

The same PCR product was also cloned into pGem11 zf(-) (Promega) to create vector pMS700. The gene encoding the bifunctional oleate 12-hydroxylase from *Lesquerella fendleri* (*LFAH12*, accession no. AF016103, kindly provided by Prof. C. Somerville, Carnegie Institute of Washington, Stanford, CA) was ligated separately into vector pHVX2 to generate vector pMS369). Yeast strain YPH499 (Stratagene, La Jolla, CA) was transformed essentially as described in the manual of the pYES2.1 TOPO® TA Expression Kit (Invitrogen; Carlsbad, CA). Transformants were selected on minimal medium without Leucine.

2.3. Site directed mutagenesis

Vector pMS700, encoding LlinFAH12, was used as a template for PCR site-directed mutagenesis of amino acid 295. Primers were designed according to the specifications of the QuikChange® Site-Directed Mutagenesis Kit (Stratagene; La Jolla, CA) and were M295LV (5'-GACTGGATTAGAGGAGCCTTGGTTACAGTAGACAG) and LV-RC (5'-CTGTCTACTGTAACCAAGGCTCCTCTAATCCAGTC). PCR was performed using PfuTurbo DNA Polymerase (Stratagene; La Jolla, CA) and 100 ng of template DNA. After an initial denaturation at 95°C for 30 seconds, 15 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, and elongation at 68°C for 15 minutes were performed. Twenty units of the restriction endonuclease *Dpn* I (New England Biolabs; Pickering, ON), which targets methylated and hemimethylated DNA, was added to each PCR reaction to digest the parental DNA which had been propagated in the dam+ *E. coli* DH5α strain. Two μls of the digested PCR reaction was used to transform One Shot® TOP10 competent *E. coli* cells (Invitrogen; Carlsbad, CA). The mutated gene was released by *Eco*R I digestion and cloned into yeast expression vector pHVX2. Successful mutagenesis was confirmed by DNA sequencing.

2.*4. Plant transformation vector construction*

Expression cassettes comprising the *Lesquerella* hydroxylase promoter [10] the *LlinFAH12* or *LFAH12* genes and an Arabidopsis oleosin terminator (kindly provided by Prof. M. Moloney, SemBioSys Inc, Calgary, Canada) were assembled in the vector pBluescript KS(+) (Stratagene, La Jolla, CA). Cassettes containing the *LlinFAH12* gene were ligated into the binary

vector pBar1 [13] to create the identical vectors pPL17 and pPL78 and the LFAH12 cassette was ligated into pBar1 to create vector pPL11. Construction of the binary vector containing the castor hydroxylase was described previously [10].

2.5. Arabidopsis lines and plant transformation

Wild type (Columbia ecotype) and *fad2/fae1* mutant *Arabidopsis* plants (Smith et al 2003) were transformed using the floral dip method [14]. For screening, T_1 seeds were sown onto moist potting soil, vernalized at 4°C for 72 hours then transferred to a growth chamber and allowed to grow until the first true leaves were clearly visible. Plants were sprayed 3 times at 2 day intervals with a solution containing 0.1ml/L Silwet (from) and 80mg/L glufosinate ammonium (WipeOut herbicide, CIL Nu-Gro IP Inc, Brantford, ON). Surviving plants were grown to maturity at 20°C in continuous light.

2.6. Lipid analysis

For analysis of yeast total fatty acids, cells from 5ml liquid cultures grown for 18 hours in minimal medium without leucine at 28°C, were pelleted by centrifugation and resuspended in 2ml 1M HCl in methanol (Supelco) in Pyrex, screw top tubes. The tubes were tightly capped and heated at 80°C for a minimum of 2 hours. After cooling, 2ml of 0.9% NaCl and 300µl of hexane were added and fatty acid methyl esters (FAMES) were recovered by collecting the hexane phase.

For determination of total seed fatty acid composition, approximately 100 seeds from individual *Arabidopsis* plants were transmethylated as described for yeast except that 300µl of hexane was added prior to heating. For quantitative analysis seeds were counted under a dissecting microscope, a known amount of 17:0 methyl ester was added to each tube before transmethylation and samples were heated at 80°C for 16 hours. Seeds of the two *Lesquerella* species were crushed before transmethylation. For all analyses FAMES were separated by gas chromatography (GC) as described previously [15].

Single seed TLC was conducted by arranging individual seeds on a pencil line drawn down the long axis of a 20cm x 5 cm Silica Gel G60 TLC plate (Whatman, Kent, UK). Seeds were covered with a sheet of paper and crushed directly into the silica gel layer. Seed debris was

removed by tapping the back of the plate and the TLC plate was developed in Hexane:Ethyl-ether: acetic acid (140:60:2 v/v/v). Lipid spots were visualized using Iodine and data recorded using a scanner.

3. Results

3.1. Fatty acid composition of Lesquerella seeds.

The fatty acid profiles of seeds of *L. fendleri* and *L. lindheimeri* were determined by *in situ* transmethylation of total seed lipids followed by gas chromatography of the resulting FAMEs. In both species the predominant hydroxy fatty acid was lesquerolic acid (Table 1). In *L.lindheimeri* this fatty acid accounted for nearly 86% of seed fatty acids. This species also accumulates very low levels of polyunsaturated fatty acids.

3.2. Isolation and characterization of a fatty acid hydroxylase from Lesquerella lindheimeri.

Using genomic DNA from *L. lindheimeri* as template, a PCR product of 1175 nucleotides was amplified encoding a FAD2 like protein of 383 amino acids (Fig 1). The putative fatty acid hydroxylase sequence had 91% identity to the hydroxylase from *L.fendleri* and contained the tripartite histidine rich motif characteristic of FAD2 fold desaturase enzymes [8,9]. Six of the 7 key residues previously reported as being strictly conserved in FAD2 desaturases, but divergent in hydroxylases [11] matched those of the *Lesquerella* and castor hydroxylases and not the FAD2 desaturases.

Expression of the *LlinFAH12* gene in yeast resulted in the accumulation of ricinoleic acid, a fatty acid not normally found in this organism (Fig. 2 chromatogram B). Very low levels of fatty acids with retention times corresponding to 16:1-OH and the di-unsaturated fatty acids 16:2 and 18:2 were also observed. The fatty acid profile of yeast cells expressing LFAH12 was determined for comparative purposes to demonstrate the bifunctional activity of this enzyme (Fig. 2 chromatogram A).

3.3 Mutagenesis of residue 295

Comparison of the amino acid sequences of the FAD2 desaturase enzymes from 32 diverse species with the castor and *Lesquerella* hydroxylases revealed a single amino acid residue (position 295, Fig.1) that was conserved in the strict hydroxylase enzymes but replaced by a different conserved residue in the desaturases and the bifunctional *Lesquerella* enzyme. To determine whether this residue played a role in determining catalytic outcome, site directed mutagenesis was used to convert a Methionine to Leucine at position 295 (M295L) in LlinFAH12. Expression of the altered LlinFAH12 gene (m1LlinFAH12) in yeast (Fig. 2 chromatogram D) did not result in any obvious changes in the ratio of hydroxy and di-unsaturated fatty acids accumulating. Cells expressing either the native or mutagenized enzymes accumulated hydroxy and di-unsaturated fatty acids in a ratio of approximately 9:1 with around 12% of these products having a chain length of 16 carbons.

3.4 Expression of LlinFAH12 in Arabidopsis seeds results in hydroxy fatty acid accumulation

To verify the predominantly hydroxylase activity of LlinFAH12 observed in yeast expression studies the gene was used to transform wild type (WT) and *fad2/fae1* mutant *Arabidopsis* plants. For comparative purposes we also transformed plants with LFAH12. Analysis of the fatty acid composition of T_2 seeds from transformed plants showed hydroxy fatty acid accumulation up to levels of 12-14% of total seed fatty acids. The fatty acid profiles of representative T_2 plants (WT background) and homozygous T_3 plants (*fad2/fae1* background) are given in Table 2. WT background plants showed reduced 18:2, 18:3 and 20:1 levels and increased 16:0, 18:0 and 18:1 content corresponding to hydroxy fatty acid production. In *fad2/fae1* lines a similar increase in 16:0 and 18:0 was observed and there was a substantial decrease in 18:1 content. Lines expressing LlinFAH12 showed a slight increase in the C18 polyunsaturated fatty acids 18:2 and 18:3. This increase was more pronounced in lines expressing the bifunctional LFAH12, where a significant accumulation of C18 PUFA was observed corresponding to hydroxy fatty acid production. Comparison of C18 PUFA and hydroxy fatty acid content for *fad2/fae1* lines expressing the two *Lesquerella* hydroxylases and

the castor hydroxylase suggested that the *L.lindheimeri* enzyme had relative desaturase activity comparable to that of the castor hydroxylase (Fig.3).

3.5 Accumulation of hydroxy fatty acids correlates with a decrease in seed oil content

Putative single insertion lines of *fad2fae1* plants transformed with the *Lesquerella* hydroxylases were identified by single seed TLC as illustrated in figure 4. This technique was developed because screening using the selection marker was found to be unreliable for T_2 and T_3 plants due to poor seedling establishment. For this procedure samples of around 100 T_2 seeds were collected from primary transformants expressing the hydroxylase genes. The average hydroxy fatty acid content of these seeds was determined by GC analysis of FAMES prepared from total seed lipids. Due to the nature of Agrobacterium mediated Arabidopsis transformation T_2 seeds will be segregating with respect to the transgene. The 100 seed sample is therefore a mixture of seeds with and without hydroxy fatty acids. Primary transformants with the highest average hydroxy fatty acid content were chosen for single seed TLC and the ratio of seeds containing hydroxy fatty acid to those without was determined. Lines giving a ratio of approximately 3:1 were selected as putative single insertion lines. Seeds from these lines were planted and allowed to grow to maturity. T_3 seeds were collected from individual plants and quantitative fatty acid analysis was conducted on carefully counted seed samples. By comparing the relative hydroxy fatty acid content of the T_3 seed samples the transgene genotype of the T_2 plants was determined. This was confirmed by single seed TLC of the T_3 seeds. Lines not showing the segregation ratio expected for a single locus transgene insertion were not included in this study.

A total of six independent lines expressing *LlinFAH12*, from 2 separate populations grown at different times, and 3 lines expressing *LFAH12* were identified as putative single insertion lines. For each of these lines the fatty acid content of individual homozygous transformants and their corresponding null segregants was determined by GC. Comparison of these fatty acid contents (Fig. 5) indicated that for all 6 lines expressing *LlinFAH12* the transformants had reduced seed fatty acid content. Line A25 for example had an average fatty

acid content of 775 μg of fatty acid/100 seeds in the null segregants but only 489 μg of fatty acid/100 seeds in the homozygous lines. In all but one case there was no overlap in standard deviations between the homozygous lines and null segregants indicating that the results were statistically significant. In the 3 lines expressing *LFAH12* there was also an apparent reduction in average fatty acid content in the homozygous transformants, however there was overlap in standard deviation between the homozygous lines and null segregants. Considerable variation was observed in fatty acid content between null segregants from different lines and also between individual plants of the same line. When seeds from the LlinFAH12 transformants were examined under a dissecting microscope some lines were observed to have shriveled seeds (data not shown).

4. Discussion

The *Lesquerella fendleri* bifunctional oleate 12-hydroxylase: desaturase was originally isolated as a genomic clone with a coding region that appeared to lack introns [7]. For this reason we used genomic DNA as a template for PCR to amplify a homologous gene from *L.lindheimeri*. The gene amplified from *L lindheimeri* encodes a polypeptide with very high amino acid sequence identity (91%) to the *L. fendleri* enzyme. The LlinFAH12 enzyme has the hydroxylase variant of 6 of the 7 amino acids previously suggested to play a role in determining the outcome of the hydroxylation/desaturation reaction but the desaturase variant of the 7th (the alanine at position 63). The strict hydroxylase activity of the LlinFAH12 enzyme observed in the yeast and Arabidopsis expression studies suggests that this residue may not play an important role in the catalytic outcome of the reaction. The role of amino acid 295 in hydroxylation was examined because the leucine in this position is conserved in all FAD2 family proteins examined except for a small number of yeast desaturase proteins, the *Trypanosoma brucei* FAD2 (Accession number AAQ74969) and the castor bean and *Lesquerella lindheimeri* fatty hydroxylases. Both hydroxylases have a methionine at this position whereas the bifunctional hydroxylase: desaturase has a leucine. The methionine to leucine change achieved by site directed mutagenesis altered the LlinFAH12 enzyme to match the sequence of the bifunctional LFAH12 in this region of the protein. No change in the strict hydroxylase activity of the *L. lindhiemeri* enzyme was observed.

The data therefore further supports the conclusion of [11] that differences in active site geometry determining the catalytic activity of FAD2 family enzymes may not be due to specific single amino acids. The isolation and comparison of additional oleate desaturase and hydroxylase enzymes from closely related species, such as members of the genus *Lesquerella*, will be of value in studying the factors that determine the activity of these enzymes.

Attempt to produce high levels of hydroxy fatty acids in oilseeds by the transfer and expression of the castor bean or *Lesquerella* hydroxylases have resulted in the generation of lines containing up to 20% hydroxy fatty acids (Smith et al unpublished data). Levels above this have not been achieved by single gene transfer. The reason for this apparent limit in hydroxy fatty acid accumulation is not clear, although the inefficient removal of newly synthesized hydroxy fatty acids from PC has been suggested as a possible factor [16]. Similar apparent constraints on the accumulation of unusual fatty acids have been seen in seeds engineered to express fatty acid modifying enzymes that utilize acyl groups esterified to PC [17-19]. To determine the effect of hydroxy fatty acid biosynthesis on seed oil content we examined homozygous lines in our populations that had the highest hydroxy fatty acid content. As there is often a large degree of variability in the fatty acid content of *Arabidopsis* seeds from different plants, putative single locus transgenic lines were identified and seeds from homozygous plants were compared to the corresponding null segregants grown at the same time. A clear decrease in seed fatty acid content was observed in the lines expressing the LlinFAH12. This is the first report of decreased seed fatty acid content in seeds expressing a hydroxylase and suggests that the synthesis of hydroxy fatty acids may be significantly disrupting the pathways of triacylglycerol synthesis in the seed. With the exception of conjugated fatty acid production in soybean [19], where the production of α-eleostearic acid corresponded to a wrinkled seed phonotype and reduced germination, the effect of ectopic expression of fatty acid modifying enzymes on seed oil content has largely gone unreported.

Further investigation is required to determine the significance of the observations from the LFAH12 lines where the decrease in fatty acid content did not appear to be as severe, to determine whether plants expressing the castor hydroxylase show similar decreases, and to measure fatty acid content in wild type and other lines expressing the hydroxylase transgenes.

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Tables .

Table 1.

Fatty acid composition of total seed lipids from mature seeds of *Lesquerella fendleri* and *Lesquerella lindheimeri*.

Table 2.

Fatty acid composition of total seed lipids from untransformed *Arabidopsis* plants, and plants expressing the LlinFAH12 or LFAH12 fatty acid hydroxylase enzymes.

* Total 18:1 = $18:1^{\Delta 9} + 18:1^{\Delta 11}$

**Total 20:1 = $20:1^{\Delta 11} + 20:1^{\Delta 13}$

Figure legends.

Fig. 1.

Amino acid sequence comparison of oleate 12-hydroxylase and desaturase enzymes. Sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw/) using the default parameters and shading was applied using Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Identical amino acids are shaded black, conserved substitutions are shaded gray. The tripartite Histidine rich motif characteristic of FAD2 like enzymes [8] is underlined. Amino acid residues identified as being conserved between FAD2 desaturases but divergent in the hydroxylases [11] are marked with an asterisk (*). Amino acid 295, changed from M to L by point mutation, is labeled with a triangle. Sequences are numbered with respect to the *L. lindheimeri* hydroxylase. Genbank accession numbers are; LFAH12, *Lesquerella fendleri* bifunctional oleate 12-hydroxylase, AAC32755; LlinFAH12, *Lesquerella lindheimeri* oleate 12-hydroxylase, XXXXX; AtFAD2, *Arabidopsis thaliana* FAD2 oleate 12-desaturase, AAA32782; BnFAD2, *Brassica napus* FAD2 oleate 12-desaturase, AAS92240; CFAH12, *Ricinus communis* oleate 12-hydroxylase, AAC49010.

Fig. 2.

Gas chromatograms of FAMES prepared from total lipids of yeast transformed with (A) LFAH12, (B) LlinFAH12, (C) empty vector and (D) M1LlinFAH12. Fatty acids were identified by comparison of retention times to those of FAME standards.

Fig. 3.

Comparison of desaturase activities of LFAH12, LlinFAH12 and CFAH12. Arabidopsis *fad2/fae1* mutant plants were transformed with the fatty acid hydroxylase genes and primary transformants were grown to maturity. The fatty acid composition of T2 seeds was determined by gas chromatography of FAMES prepared from total seed lipids. For each population of transformants the content of C18 polyunsaturated fatty acids (C18-PUFA = 18:2 + 18:3) in individual T2 seed samples was plotted against the total hydroxy fatty acid content of that sample.

Fig. 4.

Thin layer chromatograph of lipids from single *Arabidopsis* seeds. T2 seeds collected from a single T1 plant, heterozygous for a single locus insertion of the LlinFAH12 transgene, were placed on a TLC plate and crushed *in situ*. The plate was developed and lipids were detected by exposure to iodine vapour. The intensity of the stained area is a factor of both lipid content and unsaturation and is therefore not a good indication of the amount of lipid present. Triacylglycerol species separate according to the number of hydroxy fatty acids on the glycerol backbone. Unidentified spots close to the origin include 2-OH TAG, 3-OH TAG and diacylglycerols.

Fig. 5.

Seed fatty acid content of homozygous transformants and their corresponding null segregants from *Arabidopsis* lines transformed with fatty acid hydroxylases from *L. fendleri* and *L lindheimeri*. Hydroxy fatty acid content of the homozygous transformants are shown. Lines with prefix A and B were grown at different times. Error bars (+/- 1 standard deviation) are included where 3 or more separate determinations were conducted on seeds sampled from individual plants. Statistical analysis was conducted using Microsoft Excel.

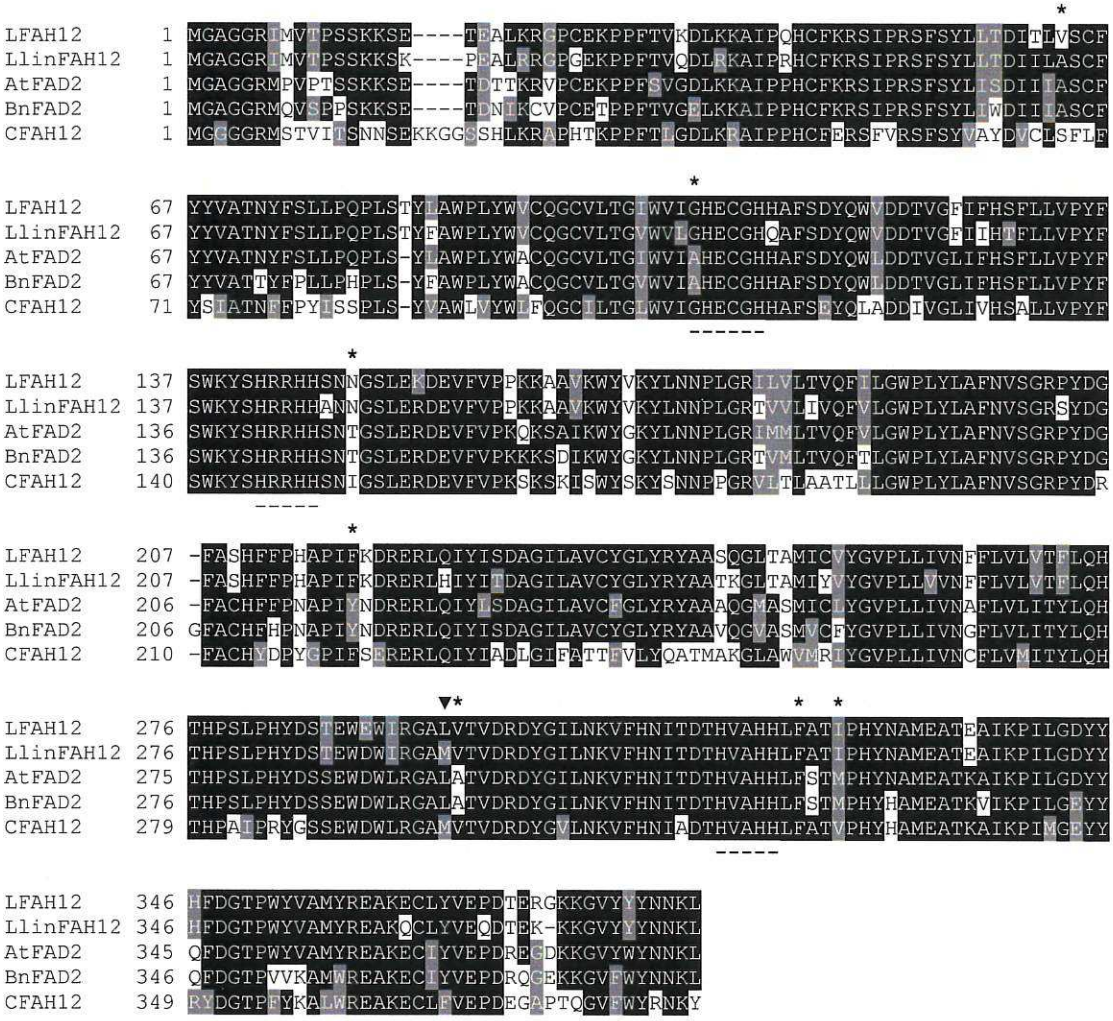
Table 1

Fatty acid	*L. fendleri*	*L. lindheimeri*
16:0	1.5	1.3
16:1	0.7	0.5
18:0	1.9	1.6
18:1	16.2	4.1
18:2	6.4	3.2
18:3	13.3	1.6
20:1	0.9	1.4
Ricinoleic (18:1-OH)	**0.7**	**1.5**
Lesquerolic (20:1-OH)	**54.5**	**85.9**
Auricolic (20:2-OH)	**3.8**	**0**

Table 2

Fatty acid	Wild type			*Fad2/fae1* mutant		
	Untransformed	LlinFAH12	LFAH12	Untransformed	LlinFAH12	LFAH12
16:0	7.2	9.8	10.0	5.7	8.7	8.2
16:1	0.3	0.3	0.3	0.3	0.4	0.4
18:0	2.9	4.3	4.4	2.6	4.3	4.2
Total 18:1 *	14.6	25.3	24.8	85.2	61.9	58.4
18:2	27.1	20.6	21.9	0.5	2.9	8.0
18:3	18.6	7.8	7.7	2.4	2.1	3.2
20:0	2.1	1.5	1.5	0.9	0.7	0.7
Total 20:1 **	22.5	16.7	16.3	0.6	0.4	0.4
20:2	2.1	0.6	0.6			
22:1	2.0	0.7	0.7			
18:1-OH	0	7.1	6.8	0	13.3	11
18:2-OH	0	3.6	3.5	0	4.1	4.8
20:1-OH	0	1.6	1.7	0		
Total Hydroxy	**0**	**12.3**	**12.0**	**0**	**17.4**	**15.8**

Figure 1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AtFAD2 1 MGAGGRMPVPTSSKKSE----TDTTKRVPCEKPPFSVGDLKKAIPPHCFKRSIPRSFSYLISDIIIASCF
BnFAD2 1 MGAGGRMQVSPPSKKSE----TLNIKCVPCETPPFTVGELKKAIPPHCFKRSIPRSFSYLIWDIILASCF
CFAH12 1 MGGGGRMSTVITSNNSEKKGGSSHLKRAPHTKPPFTIGDLKRAIPPHCFERSFVRSFSYVAYDVCLSFLF
LFAH12 67 YYVATNYFSLLPQPLSTYLAWPLYWVCQGCVLTGIWVIGHECGHHAFSDYQWVDDTVGFIFHSFLLVPYF
LlinFAH12 67 YYVATNYFSLLPQPLSTYFAWPLYWVCQGCVLTGVWVLGHECGHQAFSDYQWVDDTVGFIIHTFLLVPYF
AtFAD2 67 YYVATNYFSLLPQPLS-YLAWPLYWACQGCVLTGIWVIAHECGHHAFSDYQWLDDTVGLIFHSFLLVPYF
BnFAD2 67 YYVATTYFPLLPHPLS-YFAWPLYWACQGCVLTGVWVIAHECGHHAFSDYQWLDDTVGLIFHSFLLVPYF
CFAH12 71 YSIATNFFPYISSPLS-YVAWLVYWLFQGCILTGIWVIGHECGHHAFSEYQLADDIVGLIVHSALLVPYF
LFAH12 137 SWKYSHRRHHSNNGSLEKDEVFVPPKKAAVKWYVKYLNNPLGRILVLTVQFTLGWPLYLAFNVSGRPYDG
LlinFAH12 137 SWKYSHRRHHANNGSLERDEVFVPPKKAAVKWYVKYLNNPLGRTVVLIVQFVLGWPLYLAFNVSGRSYDG
AtFAD2 136 SWKYSHRRHHSNTGSLERDEVFVPKQKSAIKWYGKYLNNPLGRIMMLTVQFVLGWPLYLAFNVSGRPYDG
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CFAH12 140 SWKYSHRRHHSNIGSLERDEVFVPKSKSKISWYSKYSNNPPGRVLTLAATLLLGWPLYLAFNVSGRPYDR
LFAH12 207 -FASHFFPHAPIFKDRERLQIYISDAGILAVCYGLYRYAASQGLTAMICVYGVPLLIVNFFLVLVTFLQH
LlinFAH12 207 -FASHFFPHAPIFKDRERLHIYITDAGILAVCYGLYRYAATKGLTAMIYVYGVPLLVVNFFLVLVTFLQH
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BnFAD2 206 GFACHFHPNAPIYNDRERLQIYISDAGILAVCYGLYRYAAVQGVASMVCFYGVPLLIVNGFLVLITYLQH
CFAH12 210 -FACHYDPYGPIFSERERLQIYIADLGIFATTFVLYQATMAKGLAWVMRIYGVPLLIVNCFLVMITYLQH
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LlinFAH12 346 HFDGTPWYVAMYREAKQCLYVEQDTEK-KKGVYYYNNKL
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BnFAD2 346 QFDGTPVVKAMWREAKECIYVEPDRQGEKKGVFWYNNKL
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Figure 2

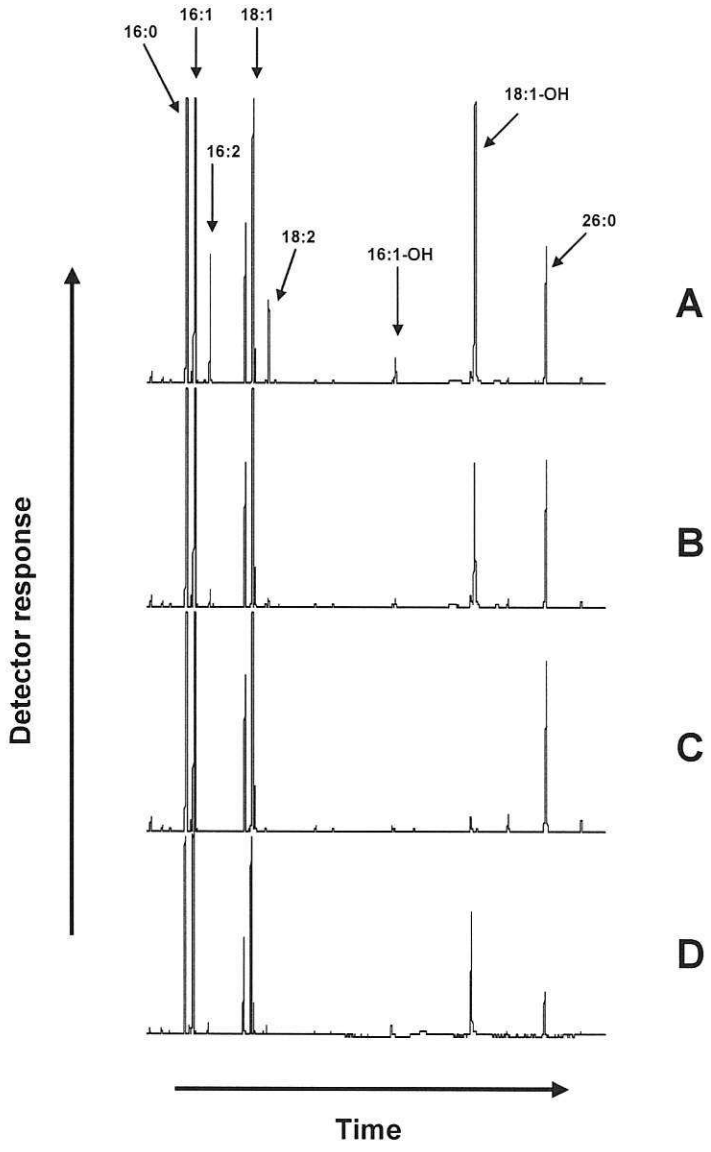

16:0
16:1
18:1
16:2
18:2
16:1-OH
18:1-OH
26:0
A
B
C
D
Detector response
Time

Fig 3

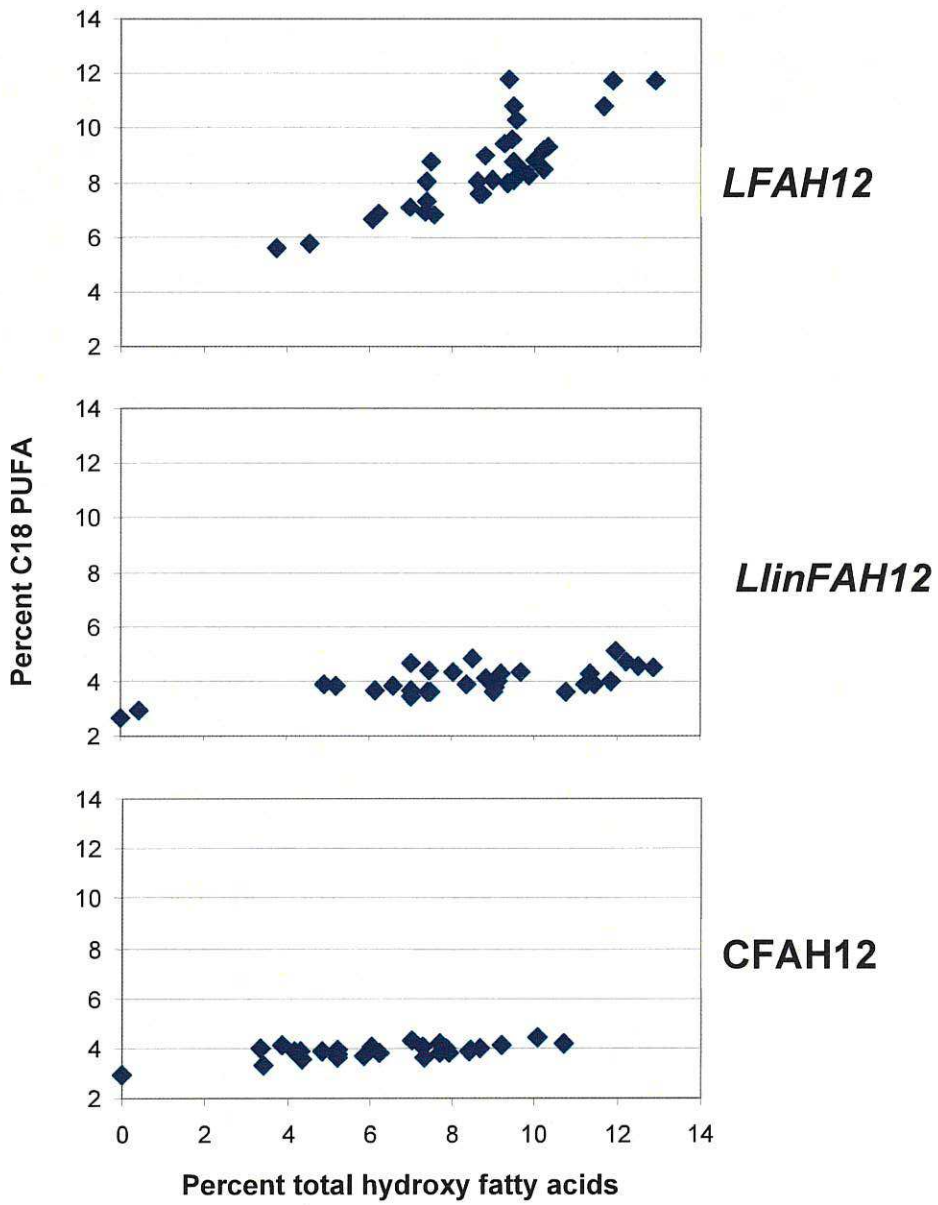

Percent C18 PUFA
Percent total hydroxy fatty acids
LFAH12
LlinFAH12
CFAH12
0
2
4
6
8
10
12
14

Figure 4

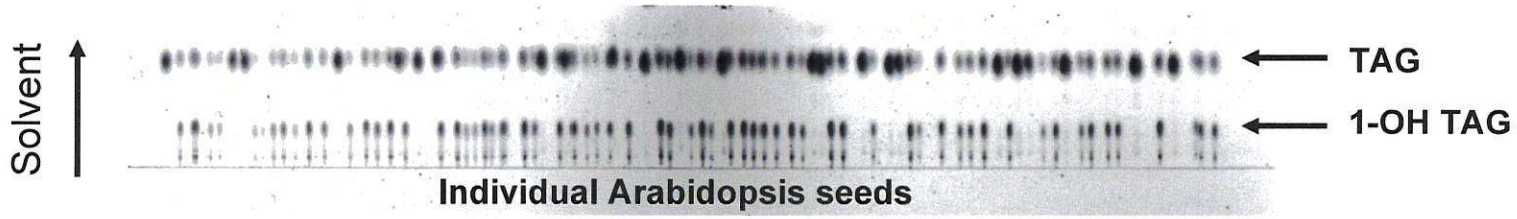
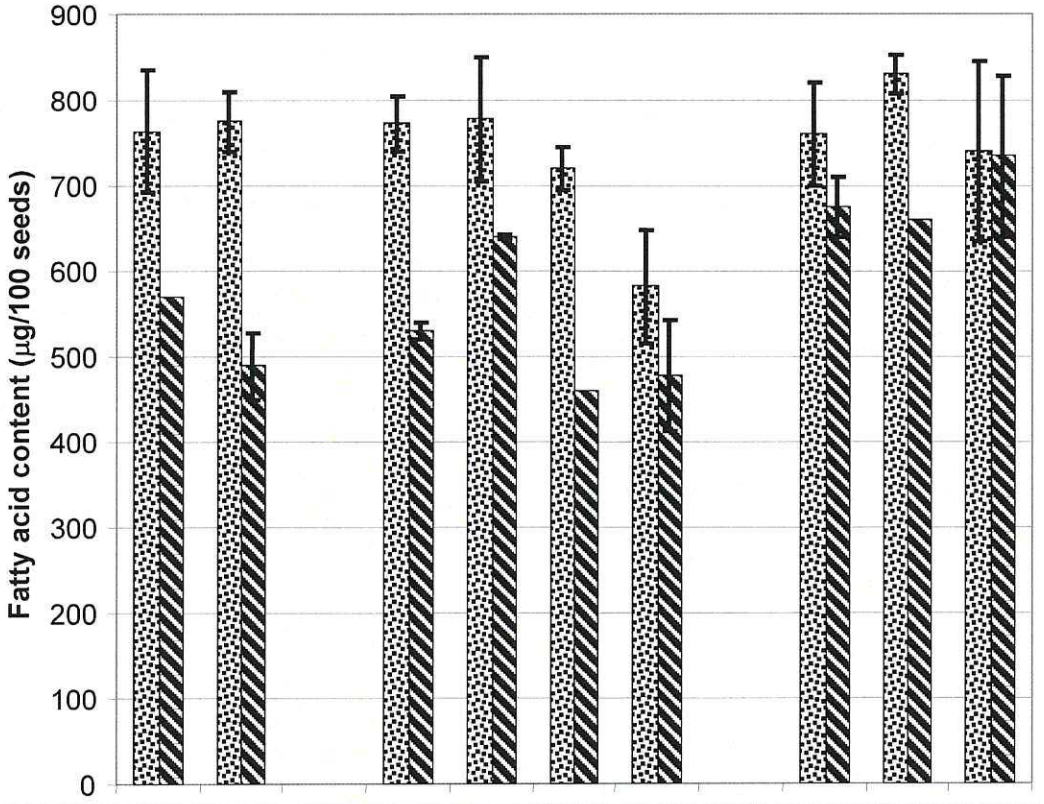


Fig 5



% Hydroxy fatty acids	16.7	16.3		16.7	17.8	16.5	12.2		15.9	16.6	15.3
Line	A10	A25		B3	B18	B19	B24		C14	C12	C9
Hydroxylase	*L. lindheimeri*								*L. fendleri*		