**(A)**

**>***GhFAD2-1A, Gh\_A13G1850*

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*>GhFAD2-1D, Gh\_D13G2238*

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*>GhFAD2-2A, Gh\_A01G2094*

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**>***GhFAD2-2D, Gh\_D01G1227*

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**>***GhFAD2-3A, Gh\_A11G2814*

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**>***GhFAD2-3D, Gh\_D11G3169*

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**>***GhFAD2-4A, Gh\_A01G2091*

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**>***GhFAD2-4D, Gh\_D01G1226*

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**(B)**

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Gh\_D13G2238 MGAGGRMPIDG-IKEENRGSVNRVPIEKPPFTLGQIKQAIPPHCFRRSLLRSFSYVVHDL 59

Gh\_A01G2091 MGAGGRTSVPPSPKKPEFYPLKRVPCSKPPFTLSQIKKAIPPHCFKRSVLRSFSYLIYDL 60

Gh\_D01G1226 MGAGGRTSVPPSPKKPEFNPLKRVPCSKPPFTLCQIKKAIPPHCFKRFVLRSFSYLIYDL 60

Gh\_A11G2814 MGAGGRMSVPPSQRKQESGSMKRVPISKPPFTLSEIKKAIPPHCFQRSLIRSFSYLVYDF 60

Gh\_D11G3169 MGAGGRMSVPPSQRKQESGSMKRVPISKPPFTLSEIKKAIPPHCFQRSLIRSFSYLVYDF 60

Gh\_A01G2094 ------MSVPPSPKKSEFNSLKRVPYSKPPFTLSEIKKAIPPHCFQRSVLRSFSYLLYDF 54

Gh\_D01G1227 ------MSVPTSPKKPEFNSLKRVPYSKPPFTLSEIKKAIPPHCFQRSVLRSFSYLLYDF 54

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Gh\_D13G2238 CLASLFYYIATSYFHFLPQPFSYIAWPVYWVLQGCILTGVWVIAHECGHHAFSDYQWVDD 119

Gh\_A01G2091 VLASLFYYVATNYFPSLPQPLPYVAWPLYWAMQGCIFTAFWALAHECGHQAFSDYQWLDD 120

Gh\_D01G1226 VLASLFYYVATNYFPSLPQALPYVAWPIYWAMQGCIFTAFWALAHECGHQAFSDYQWLDD 120

Gh\_A11G2814 ILVSIFYYVATTYFHNLPQPLSFVAWPIYWTLQGSVLTGVWVIAHECGHHAFSDYQWIDD 120

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Gh\_D01G1227 ILASLFYHVATNYFPNLPQALSNVAWPLYWAMQGCILTGVWVIAHECGHHAFSDYQWLDD 114

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Gh\_D01G1226 TIGFILHTFLLTPYFSLKYSHRRHHSNTGSLERDEVFVPKKKSALKWWAKHFNNPLGRFL 180

Gh\_A11G2814 TVGLILHSSLLVPYFSWKYSHRRHHSNTGSLERDEVFVPKKRSSIRWWAKYLNNPPGRFV 180

Gh\_D11G3169 TVGLILHSSLLVPYFSWKYSHRRHHSNTGSLERDEVFVPKKRSSIRWWAKYLNNPPGRFV 180

Gh\_A01G2094 TVGLILHSSLLVPYFSWKYSHRRHHSNTGSLERDEVFVPKKKSGLRWWAKHFNNPPGRFL 174

Gh\_D01G1227 TVGLILHSSLLVPYFSWKYSHRRHHSNTGSLERDEVFVPKKKSGLRWWAKHFNNPPGRFL 174

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Gh\_D13G2237 SLVVTLTLGWPMYLAFNVSGRYYDQLASHYN--GPIFSDCERLQVYISDASIFTVIYVLY 182

Gh\_A13G1850 SLVVTLTLGWPMYLAFNVSGRYYDRLASHYNPYGPIYSDRERLQVYISDTGIFAVIYVLY 239

Gh\_D13G2238 SLVVTLTLGWPMYLAFNVSGRYYDRLASHYNPYGPIYSERERLQVYISDAGIVAVIYVLY 239

Gh\_A01G2091 EISIQLTLGWPLYLLFNITGPPYDS----------------------------------- 205

Gh\_D01G1226 EISIQLTLGWPLYLLFNITGPPYDRLACHYDPYGPIFSDRQRLQIYVSDAGVLAVTYALY 240

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Gh\_A01G2091 ------------------------------------------------------------ 205

Gh\_D01G1226 LLVLAKGVGWVISVYGVPLLVVNAFLVMITYLQHTHPSLPHYESSEWDWLRGALSTVDRD 300

Gh\_A11G2814 RLVLAKGLAWVICVYGVPLLIVNAFLVMITYLQHTHPALPHYDSSEWDWLRGALATVDRD 300

Gh\_D11G3169 RLVLAKGLAWVICVYGVPLLIVNAFLVMITYLQHTHPALPHYDSSEWDWLRGALATVDRD 300

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Gh\_D01G1227 RLVLAKGVGWVISVYGVPLLVVNAFLVMITYLQHTHPSLPHYDSSEWDWMRGALSTVDRD 294

Gh\_D13G2237 YDSLSSVVRT---------------------SNLADRHGPGVSFPFDRTLIYKAMWRKAK 267

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Gh\_A01G2091 ------------------------------------------------------------ 205

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Gh\_A11G2814 YGILNKVFHNITDTHVAHHLFSTMPHYHAMEATKAIKPILGEYYSFDGTPVYKAIFREAK 360

Gh\_D11G3169 YGILNKVFHNITDTHIAHHLFSTMPHYHAMEATKAIKPILGEYYSFDGTPVYKAIFREAK 360

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Gh\_D01G1227 YGILNKVFHNITDTHVAHHLFSTMPHYHAMVATKAIKPILGEYYQFDGMPVYKAIWREAK 354

Gh\_D13G2237 ECLYVEPDVGGG--GSKCVFWYRNKF 291

Gh\_A13G1850 ECLYVEPDVGGGGGGSKGVFWYRNKF 385

Gh\_D13G2238 ECLYVEADVGG--GGSKGVFWYRNKF 383

Gh\_A01G2091 -------------------------- 205

Gh\_D01G1226 ECVYVEPDAGD--N-DKGVLWFRNKL 383

Gh\_A11G2814 ECIYVEPDEGE--QSSKGVFWFRNKI 384

Gh\_D11G3169 ECIYVEPDEGE--QSSKGVFWFRNKI 384

Gh\_A01G2094 ECLYVEPDEGD--K-DKGVFWFRNKL 377

Gh\_D01G1227 ECLYVEPDEGD--K-DKGVFWFRNKL 377

**(C)**



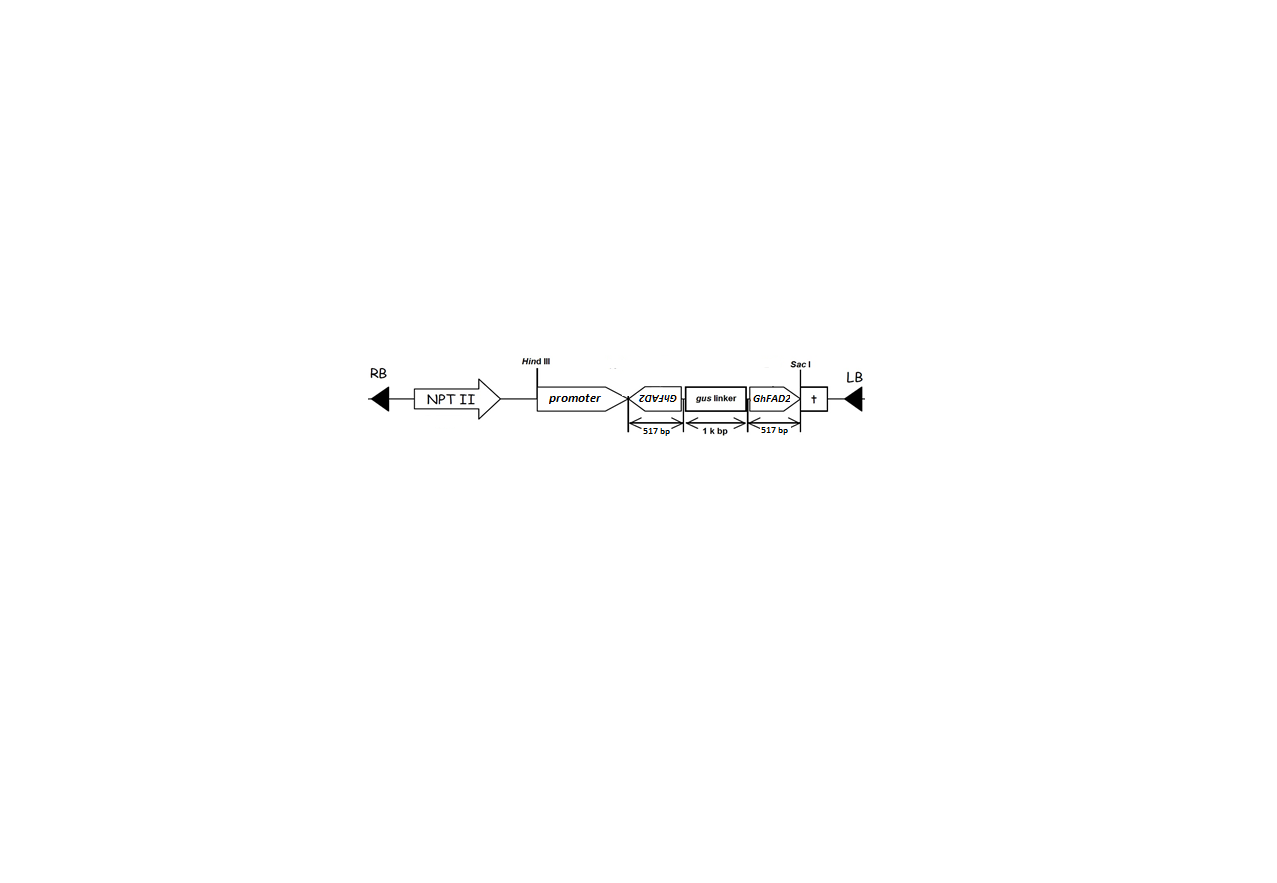
**Supplementary Fig. S1 The coding sequences and phylogenetic analysis of *GhFAD2***

**A. The coding sequences of *GhFAD2* genes.** The fragment in *GhFAD2-3D* and *GhFAD2-3A* targeted for RNAi is highlighted in red color. The underlined sequence in *GhFAD2-4A* was filled up in this study, which is a gap in the TM-1 genome (Zhang et al. 2015)

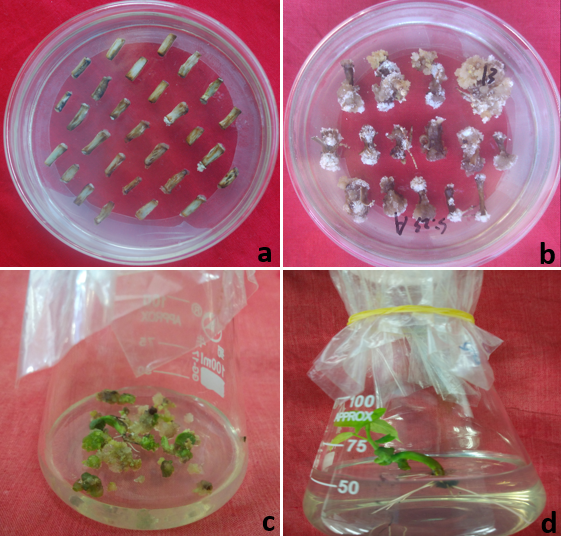
**B. Putative members of the *GhFAD2* family in the TM-1 genome identified based on blastp search using the protein sequence of the published GhFAD2-1 (X97016).** Gh\_D13G2237 contains three indels (50, 14 and 21 aa, respectively) compared to other proteins, and its 3rd deletion contains the 3rd conserved histidine-cluster observed in all FAD2 protein, this gene was thus considered as a non-functional FAD2 and not analysed further in this study. The three conserved histidine-clusters are highlighted in red. The annotated Gh\_A01G20191 was incomplete due to gap in the genomic sequence, and the missing sequence was filled up by sequence cloning in this study.

**C. Phylogenetic analysis of the cotton FAD2 family.** The tree was generated based on protein sequences of cotton FAD2 using the Maximum likelihood module of the MEGA6 software.

**(A)**



**(B)**



M 1 2 3 4 5 6 7 8 9 10

M 1 2 3 4 5 6 7 8 9 10

**(C)**



(bp)

4,500

3,000

2,000

1,200

800

500

200

*gus linker-GhFAD2*

*18s rDNA*

**Supplementary Fig. S2 Generation and molecular analysis of transgenic plants**

**A. Schematic representation of the pBIAP-ds*GhFAD2* expression cassettes used for cotton transformation**. promoter, Anther-specific promoter; NPT II, *neomycin phosphotransferase II* gene; *GhFAD2*, The partial fragment *GhFAD2-3D* coding sequence; RB, right border; LB, left border;

**B. Generation of transgenic plants.** a, induction of embryonic calli; b, calli produced from explants; c, embryoid produced from calli; d, regeneration of kanamycin resistant plantlets.

**C. Detection of the *GhFAD2-gus linker* fusion fragment in non-transformed control and transgenic plants by PCR**. A 1,113-bp fusion fragment was amplified and 18s rDNA was served as a control. The primers used in amplification were 5’-CTGTACAGCGAAGAGGCAGTC-3’ and 5’-CGTTGTAGATAGGACCGTAT-3’ for *GhFAD2-gus linker* fusion fragment, and 5’-GAGTCTGGTAATTGGAATGAG-3’ and 5’-TTCGCAGTTGTTCGTCTT-3’ for 18S rDNA. Lane M, DNA marker III; Lane 1-4; 18s rDNA sequence was amplified from transformants line; Lane 5, 18s rDNA sequence was amplified from untransformed control; Lane 6-9, The fusion fragment was amplified from transformants line; Lane 10, The fusion fragment was amplified from untransformed control.

**Supplementary Table S1 Representative differentially expressed genes involved in anther lipid metabolism**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Gene category | Gene ID | Genes description | Meiosis stage  (*fad2-3* *vs* WT) | | Tetrad stage  (*fad2-3* *vs* WT) | |
| log2(FC) | *P value* | log2(FC) | *P value* |
| Biosynthesis of unsaturated fatty acids | *Gh\_A11G2814* | Fatty acid desaturase 2 | -0.63 | 1.95E-10 | -1.89 | 6.02E-17 |
| *Gh\_D11G3169* | -0.31 | 0.0019 | -0.85 | 3.78E-10 |
| *Gh\_A13G1850* | -0.62 | 0.0060 | -2.01 | 1.88E-21 |
| *Gh\_D13G2238* | -2.52 | 6.23E-05 | -5.06 | 3.64E-27 |
| *Gh\_A01G2094* | -- | -- | -1.43 | 8.78E-05 |
| *Gh\_D01G1226* | -- | -- | -1.59 | 1.29E-08 |
| *Gh\_A09G0848* | Fatty acid desaturase 3 | -1.03 | 0.0003 | -3.60 | 2.39E-17 |
| *Gh\_A07G0946* | 0.42 | 0.0070 | -2.61 | 1.87E-20 |
| *Gh\_D07G1026* | -- | -- | -1.05 | 0.0027 |
| *Gh\_D09G0870* | -- | -- | -3.65 | 2.62E-13 |
| *Gh\_D05G0430* | Delta(8)-fatty-acid desaturase | -0.49 | 0.0030 | -- | -- |
| *Gh\_D11G0983* | 0.43 | 0.0010 | -- | -- |
| *Gh\_A11G0840* | -0.64 | 0.0030 | -- | -- |
| Linoleic acid metabolism and alpha-Linolenic  acid metabolism | *Gh\_D06G2175* | Lipoxygenase 3 | -- | -- | -4.28 | 0.0003 |
| *Gh\_D02G1542* | Lipoxygenase 6 | -0.48 | 0.0030 | -- | -- |
| *Gh\_D08G2225* | Linoleate 9S-lipoxygenase | 3.91 | 0.0013 | -- | -- |
| *Gh\_A09G2247* | Linoleate 9S-lipoxygenase 5 | 0.56 | 0.0062 | 3.07 | 4.31E-19 |
| *Gh\_D09G2080* | 0.51 | 0.0007 | 1.31 | 1.25E-22 |
| *Gh\_D07G1987* | Probable linoleate 9S-lipoxygenase 4 | 4.36 | 1.18E-23 | -- | -- |
| *Gh\_A08G1864* | Probable linoleate 9S-lipoxygenase 5 | 4.60 | 0.0042 | -- | -- |
| *Gh\_A13G0888* | -- | -- | 0.97 | 2.70E-07 |
| *Gh\_D05G0683* | Linoleate 13S-lipoxygenase 2-1 | -0.54 | 0.0021 | 1.00 | 6.11E-08 |
| *Gh\_A10G0504* | Linoleate 13S-lipoxygenase 3-1 | 1.34 | 0.0005 | 1.10 | 2.41E-12 |
| *Gh\_D10G2595* | 1.52 | 0.0002 | 1.24 | 9.65E-12 |
| Cutin, suberine and wax biosynthesis | *Gh\_D04G1447* | Cytochrome P450 86B1 | 1.35 | 1.05E-07 | -- | -- |
| *Gh\_A04G0930* | 1.16 | 1.34E-07 | -- | -- |
| *Gh\_A13G0518* | -- | -- | -1.30 | 1.89E-12 |
| *Gh\_A03G2129* | 1.43 | 8.66E-05 | 4.06 | 3.04E-08 |
| *Gh\_D02G1587* | 0.56 | 5.66E-05 | 1.41 | 0.0001 |
| *Gh\_A12G1506* | Cytochrome P450 704B1 | 4.33 | 1.20E-99 | -- | -- |
| *Gh\_D12G2768* | 4.70 | 1.10E-70 | -- | -- |
| *Gh\_D08G1463* | Cytochrome P450 86A8 | -- | -- | 1.37 | 0.0030 |
| *Gh\_A08G1178* | -- | -- | 3.11 | 1.69E-10 |
| *Gh\_A11G0806* | -0.54 | 5.77E-05 | -- | -- |
| *Gh\_A12G2100* | -1.46 | 0.0062 | -1.39 | 2.30E-07 |
| *Gh\_D08G1972* | -- | -- | -1.85 | 0.0008 |
| *Gh\_D12G2271* | -- | -- | -0.77 | 9.41E-06 |
| *Gh\_D10G0552* | ω-hydroxyacid dehydrogenase | -0.34 | 0.0014 | 0.92 | 4.54E-10 |
| *Gh\_D05G1294* | -0.89 | 1.66E-09 | 1.07 | 2.18E-06 |
| *Gh\_A05G1124* | -1.08 | 2.38E-10 | -- | -- |
| *Gh\_A10G2254* | -- | -- | 0.85 | 5.83E-08 |
| *Gh\_A08G1604* | -0.62 | 2.47E-06 | -- | -- |
| *Gh\_A11G0078* | 3.06 | 5.78E-15 | -2.25 | 2.11E-29 |
| *Gh\_D08G1915* | -0.73 | 3.12E-08 | -- | -- |
| *Gh\_D11G0083* | 1.52 | 3.35E-06 | -1.25 | 1.15E-22 |
| *Gh\_D12G2232* | 7.69 | 4.80E-67 | -- | -- |
| *Gh\_A12G2054* |  | 6.15 | 1.26E-57 | -- | -- |
| *Gh\_A06G1063* | Fatty acid 2-hydroxylase 1 | -1.92 | 0.0080 | -3.45 | 4.19E-27 |
| *Gh\_A06G1603* | -0.44 | 7.74E-05 | -- | -- |
| *Gh\_A02G0903* | -- | -- | 1.24 | 2.12E-05 |
| *Gh\_D06G1958* | -- | -- | 0.91 | 1.58E-13 |
| *Gh\_A09G0895* | Alcohol-forming fatty acyl-CoA reductase | -1.66 | 7.24E-05 | -2.84 | 1.85E-09 |
| *Gh\_D09G0571* | Peroxygenase 1 | -2.40 | 5.34E-08 | -4.82 | 4.39E-46 |
| *Gh\_A09G0571* | -2.09 | 1.11E-05 | -3.35 | 3.76E-29 |
| *Gh\_A10G0978* | Probable peroxygenase 3 | 0.71 | 0.0025 | -- | -- |
| *Gh\_A10G0977* | 0.61 | 3.56E-09 | -- | -- |
| *Gh\_D09G0572* | -1.46 | 0.0002 | -- | -- |
| *Gh\_D10G1585* | -- | -- | -0.88 | 3.66E-09 |
| *Gh\_D10G1584* | -- | -- | -0.77 | 3.59E-07 |
| *Gh\_A09G0574* | -- | -- | 0.67 | 0.0020 |
| *Gh\_A02G0527* | Aldehyde dehydrogenase family | -0.48 | 0.0020 | -0.53 | 7.16E-05 |
| *Gh\_A02G1616* | 1.83 | 1.07E-17 | -- | -- |
| *Gh\_A05G0568* | 0.82 | 6.94E-05 | 1.27 | 1.61E-24 |
| *Gh\_A06G0526* | 1.68 | 0.0016 | -- | -- |
| *Gh\_A06G1256* | 0.35 | 0.004497 | -- | -- |
| *Gh\_D03G0106* | 0.97 | 6.57E-10 | -0.66 | 3.90E-09 |
| *Gh\_D05G0697* | 0.81 | 2.44E-05 | -- | -- |
| *Gh\_D05G2245* | -1.48 | 2.92E-08 | -1.91 | 7.57E-06 |
| *Gh\_D06G0414* | -0.79 | 1.46E-05 | -0.96 | 0.000571 |
| *Gh\_D06G0580* | 0.85 | 0.0008 | -- | -- |
| *Gh\_D06G1578* | 0.42 | 0.0003 | -- | -- |
| *Gh\_A05G0157* | -- | -- | 1.43 | 1.13E-45 |
| *Gh\_D02G0793* | -- | -- | -0.36 | 0.0010 |
| *Gh\_D05G0221* | -- | -- | 0.82 | 5.27E-10 |
| *Gh\_A06G1389* | Protein ECERIFERUM 1 | -- | -- | 0.96 | 8.16E-09 |
| *Gh\_D04G0694* | -1.80 | 5.13E-28 | -- | -- |
| *Gh\_A09G1215* | Fatty acyl-CoA reductase 2 | -0.72 | 0.0006 | -1.17 | 5.89E-08 |
| *Gh\_D09G1221* | -1.36 | 5.47E-08 | -- | -- |
| *Gh\_A01G0664* | Long-chain-alcohol O-fatty-acyltransferase | -3.74 | 0.0014 | -- | -- |
| *Gh\_A13G1146* | Probable long-chain-alcohol O-fatty-acyltransferase 1 | -0.50 | 0.0016 | -- | -- |
| *Gh\_D06G1688* | Probable long-chain-alcohol O-fatty-acyltransferase 4 | 0.57 | 6.42E-05 | -- | -- |
| *Gh\_D13G1428* | Probable long-chain-alcohol O-fatty-acyltransferase 5 | -3.58 | 1.98E-10 | -- | -- |

Gene IDs and descriptions were based on Zhang et al. (2015). “--” represents no significant difference. FC, fold change.

**Supplementary Table S2 Primers of selected genes used for qRT-PCR analysis**

|  |  |  |  |
| --- | --- | --- | --- |
| Gene ID | Predict coding protein | Primer sequence (5’-3’) | Product length (bp) |
| Gh\_D10G1199 | Acyl-[acyl-carrier-protein] desaturase | F: CGGCAATGGCTACCTTC  R: CTTATCTGGACGAGGCATAT | 110 |
| Gh\_D11G3169 | Fatty acid desaturase 2 | F: CGTCACAATCACCATTCAG  R: CGTTGTAGATAGGACCGTAT | 119 |
| Gh\_A09G0848 | Omega-3 fatty acid desaturase | F: CTGTAATCGGTCCATCCAT  R: GCTTCTGCTCGTATCCAT | 111 |
| Gh\_D04G1447 | Cytochrome P450 86B1 | F: GGAACCTTGAGCATCTTCT  R: GTCGTCGGCGTTGAATA | 110 |
| Gh\_D12G2768 | Cytochrome P450 704B1 | F: AGATGGAACCAGAGGAACA  R: GATGCGGACAGGTATTCG | 119 |
| Gh\_A10G2254 | ω-hydroxyacid dehydrogenase | F: CAGTGTATTGATGTTGGAGAG  R: CAAGGCGATGTGGAAGTT | 82 |
| Gh\_D06G1578 | ω-oxo-acid dehydrogenase | F: GCACACTTCTTCTTCAAGG  R: CTATTATACCGCCACCAGTA | 92 |
| Gh\_A10G0978 | Peroxygenase | F: GGAGTTGTGGCAGATGAC  R: CTTGTCCTTGGCTACATAATAC | 106 |
| Gh\_D09G1325 | Cytochrome P450 94C1 | F: TGACGACGACATATTACCA  R: TTGAACTCCAAGCAATCTG | 117 |

Gene IDs and coding protein were based on Zhang et al. (2015).