GhFAD2–3 is required for anther development in Gossypium hirsutum

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Abstract

Background: In higher plants, the FAD2 gene encodes the microsomal oleate Δ12-desaturase, one of the key enzymes essential for the biosynthesis of the polyunsaturated lipids that serve many important functions in plant development and stress responses. FAD2 catalyzes the first step, in the biosynthesis of the polyunsaturated fatty acids (PUFAs) found in the cell membrane and cell wall, and it is thus of great importance to investigate the regulatory role of FAD2 in anther development.

Results: We reported the molecular characterization of the cotton (Gossypium hirsutum) GhFAD2 gene family and the essential role of GhFAD2–3 in cotton anther development. G. hirsutum contains four pairs of homoeologous FAD2 genes (GhFAD2–1 to GhFAD2–4). GhFAD2–3 is ubiquitously and relatively highly expressed in all analyzed tissues, particularly in anthers. Specific inhibition of GhFAD2–3 using the RNA interference approach resulted in male sterility due to impaired anther development at the stages from meiosis to maturation. The cellular phenotypic abnormality observed at the meiosis stage of the GhFAD2–3 silenced plant (fad2–3) coincides with the significant reduction of C18:2 in anthers at the same stage. Compared with that of the wild type (WT), the content of C18:1 was 41.48%, which increased by 5 fold in the fad2–3 anther at the pollen maturation stage. Moreover, the ratio of monounsaturated to polyunsaturated fatty acid was 5.43 in fad2–3 anther, which was much higher than that of the WT (only 0.39). Through compositional analysis of anthers cuticle and transcriptome data, we demonstrated it was unfavorable to the development of anther by regulating GhFAD2–3 expression level to increase the oleic acid content.

Conclusions: Our work demonstrated the importance of C18:2 and/or C18:3 in the development of the pollen exine and anther cuticle in cotton and provided clue for further investigation of the physiological significance of the fatty acid composition for plant growth and development.

Keywords: Gossypium hirsutum, Fatty acid composition, GhFAD2, Male sterile, Linoleic acid, Oleic acid

Background

In higher plants, the microsomal oleate Δ12-desaturase (fatty acid desaturase 2, FAD2) is a hydrophobic endoplasmic reticulum protein, catalyzing the reaction from monounsaturated oleic acid (C18:1) to polyunsaturated linoleic acid (C18:2) by introducing a double bond between the 12th and 13th carbon atoms of C18:1 [1]. FAD2 was first identified in the model plant species Arabidopsis thaliana [2]. Although only a single copy of FAD2 was found in A. thaliana, multiple copies of FAD2 were identified in many other plants, such as canola (Brassica napus), cotton (Gossypium hirsutum), soybean (Glycine max), olive (Olea europaea), sesame (Sesamum indicum) and sunflower (Helianthus annuus) [3–8].

As the key gene controlling the conversion of C18:1 to C18:2, the FAD2 transcriptional level directly determines the relative content and proportion of polyunsaturated fatty acids (PUFAs) in plants [9]. FAD2 could be significantly induced by abiotic stresses [10, 11], suggesting that PUFAs could play a key role in plant stress responses and adaptation to environmental change. Due to the oxidative instability of PUFAs, studies on the regulation of FAD2 were mainly focused on reducing its expression level to decrease the C18:2 content in oil seeds, and thus to improve oil quality [4, 12–16].
In flowering plants, the anther cuticle and pollen wall are rich in lipids, mainly fatty acids and their derivatives [17–21]. Genetic analyses of Arabidopsis and rice have found that many genes with an important role in the development of the anther cuticle and pollen wall are involved in lipid metabolism, such as MALE STERILITY 2 (MS2), FACELESS POLLEN 1 (FPL1), CYP703, CYP704B1, CYP704B2, Acyl-CoA Synthetase 5 (ACOSS), NO EXINE FORMATION1 (NEFI), Wax-Deficient Anther1 (WDA1), β-ketoacyl-coenzyme A Synthase ECERIFERUM6 (CER6), Defective Pollen Wall (DPW), Fatty acyl-coenzyme A Reductase (FAR), 3-ketoacyl-CoA Synthase 9 (KCS9), and OsC6 [18, 22–34].

The upland cotton genome is large and complex allo-tetraploid (AADD; 2n = 52) and several rounds of genome duplication events have been identified in cotton genome [35], which makes it more difficult to analyze gene expression and regulation. In this study, based on the characterization of the cotton (G. hirsutum) FAD2 family genes, we investigated their expression patterns in various vegetative and reproductive tissues and found that GhFAD2–3 is the gene highly expressed in most tissues analyzed, particularly in anthers. We demonstrated that silencing of GhFAD2–3 resulted in male sterility, due to nonviable pollen grains and abnormal anther development resulting from significantly reduced levels of PUFAs at the meiosis and tetrad stages. This is also the first report on the dynamic changes of fatty acid constituents during cotton anther development, which were exactly opposite to those in developing and mature cottonseeds. Our work showed the effect of changes in fatty acid constituents on the physiological activity of anthers and revealed the essentialness of primary PUFAs in the development of the pollen wall and anther cuticle in cotton.

Results

Genome-wide identification of GhFAD2 in cotton

The protein sequence of a previously identified GhFAD2 gene (GenBank acc. no. X97016) was used to BLASTP the annotated proteins of G. hirsutum [35], G. raimondii [36] and G. arboreum [37]. Four, five and nine putative FAD2 genes were identified in G. arboreum (Ga), G. raimondii (Gr) and G. hirsutum (Gh), respectively. Based on phylogenetic analysis, each of the four Ga putative FAD2 and each of the five Gr putative FAD2 has a corresponding copy in the At and Dt subgenomes of Gh, respectively (Additional file 1: Figure S1), suggesting that the FAD2 gene family is highly conserved during the evolutionary history of cotton. Two Gr putative FAD2 on chromosome 13 (Gorai.013G248700 and Gorai.013G248800) are next to each other and Gorai.013G248700 lacks the 3rd conserved histidine-cluster observed in all plant FAD2 proteins [7]. We thus considered Gorai.013G248700 as a non-authentic (or pseudogenized) FAD2 gene. This observation suggests that Gorai.013G248700 could be a result of gene duplication followed by pseudogenization. Interestingly, a similar situation was observed in the two Gh orthologs (Gh_D13G2237 and Gh_D13G2238) of the two Gr genes. We therefore disregarded Gh_D13G2237 as an FAD2 gene and only used the remaining eight in the following analysis. The annotated GhFAD2–4A (Gh_A01G2091) is incomplete due to a sequence gap. We completed its cDNA sequence based on our RNA-seq data. Of these eight genes, five have previously been cloned. We renamed these eight GhFAD2 genes with the aim of keeping the previous nomenclature of the five cloned genes as intact as possible (Table 1).

Expression pattern of different members of the GhFAD2 gene family

We analyzed the expression levels of each GhFAD2 gene in different tissues and at different developmental stages of seeds and fibers using RNA-seq (Fig. 1). GhFAD2–1A and GhFAD2–1D were mainly expressed in developing seeds, particularly in 20–40 days post anthesis (DPA) seeds, expressed at very low levels in anther and ovary, and barely detectable in vegetative tissues and developing fibers, suggesting that the major role of GhFAD2–1 is responsible for C18:2 biosynthesis in seeds, consistent with previous results [7, 38]. The expression of GhFAD2–2, particularly GhFAD2–2A, was mainly observed in ovaries and leaves and was very low or undetectable in other tissues. GhFAD2–3 was constitutively highly expressed in all tissues, with a relatively low expression level in 40–60 DPA seeds. The two homoeologous GhFAD2–3 sequences were equally expressed in most tissues but were significantly biased in anther, stigma and leaves, in which the expression level of GhFAD2–3D was much higher than that of GhFAD2–3A. For GhFAD2–4, there was very little expression in other tissues apart from stem. These results indicate that different GhFAD2 genes are preferentially expressed in different tissues, and in some tissues the two homoeologs of the same GhFAD2 gene are differentially expressed, pointing to a potential different function of the GhFAD2 genes in cotton development. A very high expression level of GhFAD2–3 in anthers suggests that lipid desaturation catalyzed by GhFAD2–3 may have very important roles in anther development.

Molecular characterization of transgenic plants

A 517-bp fragment targeting both homoeologous GhFAD2–3 sequences was used in making the hairpin construct. In total, 12 independent transgenic cotton lines were obtained by Agrobacterium-mediated transformation
The transgenic cotton lines with the fusion fragment (1113 bp) from GhFAD2–3 and the gus linker were considered as positive transgenic events. To investigate the effect of downregulation of GhFAD2–3 on changes in the anther transcriptome, we compared transcriptomes of wild-type and fad2–3 using RNAs from anthers of the meiosis and tetrad stages, considering that the differences in cellular phenotypes between the wild-type and fad2–3 anthers began to be observed at the meiotic stage by transmission electron microscopy (see below). As expected, both homoeologous GhFAD2–3 (Gh_D11G3169 and Gh_A11G2814) were downregulated at both stages, although downregulation at the tetrad stage was more statistically significant (Additional file 1: Table S1). Among the other GhFAD2 genes, GhFAD2–1 (Gh_A13G1850 and Gh_D13G2238) was significantly downregulated at both stages in fad2–3 due to its closest sequence similarity with GhFAD2–3. However, compared to GhFAD2–3,

| Gene name | Also known as | Locus ID | Chromosome and coordinates | GenBank accession no. | CDS length (bp) | No. of amino acid |
|-----------|---------------|----------|-----------------------------|-----------------------|----------------|------------------|
| GhFAD2–1A | FAD2–1        | Gh_A13G1850 | A13:78167608..78168765      | X97016                | 1158           | 385              |
| GhFAD2–1D | FAD2–1        | Gh_D13G2238 | D13:58471954..58473105      | HQ259410              | 1152           | 383              |
| GhFAD2–2A | /             | Gh_A01G2094 | A01:23332066..2333199       | /                     | 1134           | 377              |
| GhFAD2–2D | FAD2–2        | Gh_D01G1227 | D01:30322983..30328022      | Y10112                | 1134           | 377              |
| GhFAD2–3A | FAD2–3        | Gh_A11G2814 | A11:91511504..91512658      | AF331163              | 1155           | 384              |
| GhFAD2–3D | FAD2–4        | Gh_D11G3169 | D11:64332280..64333434      | AY279314              | 1155           | 384              |
| GhFAD2–4A | /             | Gh_A01G2091 | scaffold111_A01:182694..183845 | /                     | 1152           | 383              |
| GhFAD2–4D | /             | Gh_D01G1226 | D01:30279978..30281129      | /                     | 1152           | 383              |

(Additional file 1: Figure S2). The transgenic cotton lines with the fusion fragment (1113 bp) from GhFAD2–3 and the gus linker were considered as positive transgenic events. To investigate the effect of downregulation of GhFAD2–3 on changes in the anther transcriptome, we compared transcriptomes of wild-type and fad2–3 using RNAs from anthers of the meiosis and tetrad stages, considering that the differences in cellular phenotypes between the wild-type and fad2–3 anthers began to be observed at the meiotic stage by transmission electron microscopy (see below). As expected, both homoeologous GhFAD2–3 (Gh_D11G3169 and Gh_A11G2814) were downregulated at both stages, although downregulation at the tetrad stage was more statistically significant (Additional file 1: Table S1). Among the other GhFAD2 genes, GhFAD2–1 (Gh_A13G1850 and Gh_D13G2238) was significantly downregulated at both stages in fad2–3 due to its closest sequence similarity with GhFAD2–3. However, compared to GhFAD2–3,
GhFAD2–2D and GhFAD2–4A had a negligent expression level in anthers, and could thus hardly have function in anther development.

Significantly changed genes encoding key enzymes of these pathways were shown in Additional file 1: Table S1. These results revealed that many significantly changed pathways are related to the metabolism of lipids and their deriviers; among them were those involved in biosynthesis of unsaturated fatty acids, alpha-linolenic acid metabolism, and the biosynthesis of cutin, suberin and wax. Apart from GhFAD2, DEGs related to biosynthesis of unsaturated fatty acids also included GhFAD3 (such as Gh_A09G0848 and Gh_A07G0946). The expression levels of genes involved in linoleic acid and alpha-linolenic acid metabolism was also statistically significant at the meiosis stage or tetrad stage in the fad2–3 anthers. In addition, fatty acyl-CoA reductase (FAR) catalyzes the reduction of fatty acyl-CoA to fatty alcohols, which are essential components of wax and cutin monomers. GhFAR2 genes (Gh_A09G1215), orthologous to Arabidopsis MS2 and rice DPW that are related to wax biosynthesis, were found to be downregulated at the tetrad stage in fad2–3. GhCYP86B1 (Gh_D04G1447, Gh_A04G0930, Gh_A03G2129 and Gh_D02G1587), a very long chain fatty acid hydroxylase specifically involved in cutin and suberin biosynthesis, was significantly upregulated in the fad2–3 anther. Furthermore, Cytochrome P450 CYP704B1, which participates in catalyzing omega-hydroxylation of long-chain fatty acids was also differentially expressed. Significant differences were also observed in the expression levels of some genes, such as those encoding peroxygenase and aldehyde dehydrogenase.

**Phenotypic analysis of the RNAi plants**

All fad2–3 produced lots of completely sterile flowers, although they were able to open as fully as the wild-type flowers. At anthesis, wild-type anthers dehisced to release pollen grains for pollination, whereas fad2–3 anthers did not dehisce, had a smooth and shiny epidermal surface and were plate-shaped (Fig. 2). In wild-type, anthers were clustered in fascicles, and stamens wrapped up stigma before pollen maturation. The fad2–3 had fewer anthers than wild-type, and its anthers did not wrap up stigma as did the wild-type anthers. As a result, stigma of fad2–3 stood out in the anther cluster. We also manually opened fad2–3 anthers and compared its pollen grains with those of wild-type. It was obvious that fad2–3 had fewer pollen grains. While the mature pollen grains of wild-type were spherical and became dark brown when treated with I2-KI (Fig. 2), the manually released fad2–3 pollen grains were smaller, shrunken, irregularly shaped, and were yellow brown when stained by I2-KI (Fig. 2). After acetolysis treatment, wild-type pollen grains remained intact, whereas fad2–3 pollens were severely damaged and became transparent (Fig. 2), suggesting that fad2–3 pollens were sensitive to
acetolysis probably due to lack of sporopollenin in the outer pollen wall; i.e., the exine. We compared the surface structure of wild-type and fad2–3 anthers harvested from 1 day before anthesis using scanning electron microscopy (Fig. 3). Compared with the well-formed, relatively smooth wild-type anthers, fad2–3 anthers had a severely shrunken, atrophied and disfigured outer surface.

To have a deeper understanding of the abnormalities of the fad2–3 anther development, we collected anthers at different developmental stages from fad2–3 and wild-type, and analyzed anther cross sections by transmission electron microscopy. Before the sporogenous cell stage, in both fad2–3 and wild-type, stamen primordia were initiated normally and large archesporial cells could be detected in developing anthers. Wild-type and fad2–3 anthers had similar cytological characteristics. At the sporogenous cell stage, wild-type and fad2–3 anthers had no obvious differences in cellular structures (Fig. 4a and b). At the microsporocyte stage, the four layers of the anther wall; i.e., epidermis, endothecium, middle layer and tapetum were well differentiated (Fig. 4c and d) in both wild-type and fad2–3. A clear defect in the fad2–3 anther was first observed at the meiosis stage. Compared with the wild-type anther, the fad2–3 anther showed cytoplasmic diffusion in microsporocytes and disintegration of the tapetum at this stage (Fig. 4e and f). At the early tetrad stage, the middle layer cells of the wild-type anther became narrow and deformed and began to show signs of degeneration (Fig. 4g). The tapetum cells were large, had thick cytoplasm rich in endoplasmic reticulum, mitochondria and plastids, and often contained double nuclei. Profuse vesicles with dense electron substances were continuously produced by the endoplasmic reticulum (Fig. 4i). Later, the endoplasmic reticulum of tapetum disappeared, resulting in accumulation of orbicules, polyvesiculate bodies and lipid bodies in the tapetum, and finally degradation of the tapetum. In contrast, the middle layer cells of the fad2–3 anther did not become thinner at the early tetrad stage (Fig. 4H). However, the tapetal cells of the fad2–3 anther showed significant abnormalities, including having many large vacuoles and defective plastids, without obvious accumulation of lipid droplets in elaioplasts and absence of dual nuclei (Fig. 4j). There were large numbers of vacuoles and dilated vesicles of endoplasmic reticulum in the tapetum cells (Fig. 4l). With the development of microspores, formation of primexine followed by bacula, tectum and nexine could be seen in the wild-type anther (Fig. 4m). Later, microspore exine and intine were fully thickened, and spinules protruding from the exine were formed (Fig. 4m and o). Further, vacuolization was observed in mononucleate-free microspores (Fig. 4q). In the fad2–3 anther, although bacula could form normally with its upper and lower ends extended laterally during the development of pollen exine, the development of microspore exine showed obvious abnormalities, including being unable to form tectum uniformly composed of small spinules (Fig. 4n and p), shrunken protoplasts showing breakage of the cell membrane, and external flow of cytoplasm. Other abnormalities were a concentration of partial cytoplasm in the center of the microspore, dissolution of nuclear membranes, and disintegration of the nucleus and cytoplasm (Fig. 4r).

Silencing of GhFAD2–3 decreased C18:2 content in anthers

The main fatty acids in cotton anthers are saturated myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) and arachidic acid (C20:0), monounsaturated oleic acid (C18:1), and polyunsaturated linoleic acid (C18:2) and α-linolenic acid (C18:3). In the wild-type

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*Fig. 3* Scanning electron microscopy of the mature anthers from the wild-type and the fad2–3 plants. a, c, and e mature wild-type anthers. The magnification was 500, 1000 and 2000 times, respectively. b, d, and f mature fad2–3 anther. The magnification was 500, 1000 and 2000 times, respectively
Fig. 4 (See legend on next page.)
C16:0 was the main fatty acid and reached the maximum in the mature pollen (Fig. 5a). C18:0 accumulated mainly at the early stage of anther development and its relative content significantly decreased from 25.56% at the sporogenous cell stage to 8.14% at the meiosis stage and slightly increased again at the pollen maturation stage. The relative content of C14:0, C20:0 and C18:3 was relatively low in all stages of anthers, although C14:0 was increased up to 8.97% at the mature pollen stage. C18:1 and C18:2 had a similar dynamic change pattern during anther development and reached their maximum at the microsporocyte and meiosis stage, respectively, suggesting that biogenesis of C18:2 largely depends on the amount of C18:1, which may be negatively correlated with that of C16:0.

In the fad2–3 anthers, due to the significantly reduced level of GhFAD2–3, conversion of C18:1 into C18:2 was compromised after the microsporocyte stage. Consequently, a significant reduction in C18:2 was observed at the meiosis and tetrad stages, while the relative content of C18:1 was significantly increased at these two stages (Fig. 5b). Significant reduction in C18:2 at around the meiosis stage coincides with the developmental abnormalities observed at this stage of anther development in fad2–3. A reduction (49.18%) in C18:2 was detected in the mature anthers of fad2–3 compared to that of wild-type, but C18:1 increased almost five-fold in fad2–3 compared to wild-type, suggesting that the biosynthesis steps from C16:0 to C18:1 were not greatly affected by silencing of GhFAD2–3, resulting in accumulation of C18:1.

The ratio of C18:2/C18:1 was analyzed during anther development. The results showed that there was no significant difference between fad2–3 and WT during early anther development (Fig. 5c). At the meiosis stage, the ratio of C18:2/C18:1 was 1.49 in WT anthers. However, the corresponding ratio was only 0.48 in fad2–3 anthers. Compared with the WT anther, there was a more significant decrease in the ratio of C18:2/C18:1 in the fad2–3 anther at the pollen maturation stage (Fig. 5c).

Cuticular wax constituents and cutin monomer of cotton anthers
To eliminate possible effects caused by the changed fad2–3 anther morphology, the experiments were performed using anthers harvested from 1 day before anthesis. The cuticular waxes and cutin of mature anthers was extracted and their components were analyzed accordingly by GC-MS (Fig. 6a).

Interestingly, in wild-type anthers, wax constituents contain wax esters at high content (over 50%), prominently monopalmitin and monostearate, which are products of the acyl reduction pathway. This is different from waxy components in anthers of rice and maize [31, 39], perhaps because cotton is a woody perennial plant, and it seems to be similar to that in wax constituents of Jojoba [40]. The content of alkanes was approximately 40%, mainly with chain lengths ranging from 23 to 35 carbons. Compared with the wild-type, there was no significant difference in the major components of wax in fad2–3. Although the content of fatty acid components was relatively low in wax constituents, fad2–3 had significantly lower levels of C18:2 and C18:3, but a relatively higher level of C18:1. The decrease in C16:0 and C18:0 was also observed in the anther wax of fad2–3. These results indicated that C18:2 and C18:3 were also important components of plant epidermis and downregulation of GhFAD2–3 affected the relative content of components in the cuticular layer.

Cortin monomers in the cuticular layer were methylated by methanolic HCl and reanalyzed by GC-MS. The major monomer was α, ω-octadecadienoic acid (C18:2 DCA), which is a usual constituent of cutin. 9,10,18-trihydroxy octadecenoic acid (9,10,18-triOH C18:1 FA), hexadecane-1,16-dioic acid (C16:2 DCA) and 9,10-epoxy-18-OH-C18:1 (9,10 Epoxy 18-OH Acid) at relatively high levels had also been detected by GC-MS in wild-type anthers (Fig. 6b). Cutin monomers also include fatty acid components such as C18:2 and C18:3. The strongest
Fig. 5 (See legend on next page.)
effects of the \textit{fad2–3} anther on cutin monomers were apparent in the unsaturated C18:2 DCA and 9,10,18-triOH C18:1FA. Especially, the C18:2 DCA content decreased by 82.16% in \textit{fad2–3}. Our results were also consistent with previous reports that the \textit{Arabidopsis} \textit{fad2} mutant showed a decrease in double unsaturated C18 α, ω-diacids in leaf polyester \cite{41}. Nevertheless, C18:2 and C18:3 are important substrates for biosynthesis of many other lipids that are essential structural components of anthers and the pollen wall.

![Fig. 5](See figure on previous page.)

\textbf{Fig. 5} The proportion of fatty acid compositions in anthers at different developmental stages. \textbf{a}, wild-type anthers; \textbf{b}, \textit{fad2–3} anthers; \textbf{c}, the ratio of C18:2 to C18:1. Anthers at different developmental stages was used for fatty acid assay. St1-St5: Anther at different developmental stages; St1, Sporogenous cell stage; St2, Microsporocyte stage; St3, Meiosis stage; St4, Tetrad stage; St5, Pollen maturation stage. The fatty acid methyl esters were prepared by alkaline transmethylation. The analyses were performed using GCMS-QP2020 at an electron ionization of 70 eV with an HP-88 column. The quantification was carried out according to the response value of quantitative ions and the established standard curve. Each test was repeated three times, and the content of each fatty acid composition was calculated as the percentage of total measured fatty acids. The ratio of C18:2/C18:1 is calculated by dividing the relative percentage content of C18:2 from that of C18:1 at the same developmental stage. Each bar represents the mean data of three biological replicates. Error bars are standard errors. Asterisks denote significant differences to wild-type (WT) as determined by Student’s t test: ***\( p < 0.001 \)

![Fig. 6](See figure on previous page.)

\textbf{Fig. 6} Analysis of anther wax and cutin in the wild type and \textit{fad2–3}. \textbf{(a)} Wax constituents in the wild-type and \textit{fad2–3}. C23 ALK, tricosane; C25 ALK, pentacosane; C27 ALK, heptacosane; C28 ALK, octacosane; C29 ALK, nonacosane; C31 ALK, hentriacontane; C35 ALK, pentatriacontane. C16:0 FA, hexadecanoic acid; C18:0 FA, octadecanoic acid; C18:1 FA, 9-octadecenoic acid; C18:2 FA, 9,12-octadecadienoic acid; C18:3 FA, 9,12,15-octadecatrienoic acid; C20 FA, eicosanoic acid; C22 FA, docosanoic acid; C24 FA, tetracosanoic. C16:0 DCA, hexadecane-1,16-dioic acid; C18:0 DCA, octadecane-1,18-dioic acid; C18:1 DCA, α,ω-octadecadienoic acid; C18:2 DCA, α,ω-octadecadienedioic acid; triOH C18:1 FA, 9,10,18-trihydroxy octadecenoic acid; 9,10 Epoxy 18-OH acid, 9,10-epoxy-18-OH-C18:1; DW, dry weight. The wax of anther at mature pollen stage was analyzed according to Jung et al. \cite{26}. The wax monomer was derivatized with 1 ml BFTSA in 1 ml pyridine (1:1) for 40 min at 70 °C before GC-MS analysis. The constituent analyses were performed using GCMS-QP2020 with a DB-1 column. Each compound was quantified against the internal standard by automatic integrating the peak areas. The protocol for lipid polyester analysis was performed according to Li-Beisson et al. \cite{50}. The cutin monomer fraction was derivatized with BFTSA/pyridine (1:1) for 60 min at 70 °C. The constituent were analyzed using GCMS-QP2020 with a DB-1 column. The GC-MS was conducted according to Li-Beisson et al. \cite{50} with helium carrier gas at 2 ml/min. Each compound was quantified on the basis of their total ion current as described by Li-Beisson et al. \cite{50}. Error bars are standard errors. Values represent the means ± SE, \( n = 3 \). Asterisks denote significant differences to wild-type (WT) as determined by Student’s t test: ***\( p < 0.001 \), **\( p < 0.01 \), *\( p < 0.05 \)
Discussion

Functional specificity and redundancy of GhFAD2 genes

Each individual GhFAD2 gene is expected to function as a desaturase to convert C18:1 into C18:2 in different cotton organs and/or tissues, a process that is presumably determined by the expression specificity and level of each GhFAD2. Among the four pairs of GhFAD2 genes, GhFAD2–2 and GhFAD2–4 were expressed at very low levels in the tissues analyzed in this study, suggesting a limited role or a specific role in the tissues not analyzed in this study of these genes in cotton development. GhFAD2–1 seems to be specifically expressed in developing seeds, with the highest expression level detected in the 40 DPA seeds (Fig. 1), consistent with its role in the accumulation of C18:2 in seeds [7]. In cotton seeds, the relative content of C18:2 could reach over 50% of the total fatty acid content [12]. In contrast, GhFAD2–3 seems to be ubiquitously expressed in all tissues analyzed, but its expression level was remarkably lower than that of GhFAD2–1 in the 40 DPA seeds although its expression levels in the 5 DPA and 20 DPA seeds were significantly higher than or similar to that of GhFAD2–1, respectively (Fig. 1). The highest expression level of GhFAD2–3, particularly GhFAD2–3D, was observed in anthers, whereas the other three pairs of GhFAD2 genes were not or expressed at very low levels in anther. This expression pattern suggests that GhFAD2–3 is the major, if not the sole, gene responsible for the synthesis of C18:2 in anthers and is important for anther development. The male sterile phenotype observed in fad2–3 supported this conclusion. GhFAD2–3, particularly GhFAD2–3D, was also relatively highly expressed in stigma and leaves, but we did not observe obvious phenotypic changes in these two organs in fad2–3, probably due to the presence of a functional GhFAD2–2 that was not a target of the 517-bp fragment used in generation of fad2–3. This observation suggests that different GhFAD2 genes may be functionally redundant.

One interesting observation was the significantly biased expression levels of the two homoeologous GhFAD2–3 in anthers, stigma and leaves (Fig. 1). The significantly higher expression level of GhFAD2–3D would suggest it is the major functional gene. This speculation could not be tested by using fad2–3 generated in this study because both gene homeologs are targets of the RNAi construct but can be tested by gene homolog-specific knock-out using the gene editing approach.

A role for GhFAD2–3 in anther and pollen development

Fatty acid metabolism is an essential physiological process throughout the plant life cycle. In higher plants, acetyl-CoA carboxylase carboxylates acetyl-CoA to form malonyl-CoA, which is further converted by fatty-acid synthase (FAS) to long-chain fatty acids via the six recurring reactions, until the C16:0 is produced. When the 16:0 carbon fatty acids (FAs) are formed, it then undergoes some modifications leading to desaturation and/or elongation. The elongation begins with stearate (C18:0) and is mainly performed by several membrane-bound enzymes in the endoplasmic reticulum (ER). C18:0 was also further dehydrogenated by Δ9-stearyl-ACP desaturase (SAD) to form monounsaturated C18:1. After that, most PUFAs are synthesized by desaturases located in the ER, namely, FAD2 (C18:1 to C18:2 desaturation) and FAD3 (C18:2 to C18:3 desaturation). Most cuticular wax and cutin are derived from fatty acid precursors and play important roles in developmental events and physiological functions. Our results also showed that the major wax constituents in the cotton anthers were wax esters and alkanes. Importantly, it had been reported that fatty acid desaturases, including FAD2, are responsible for the biosynthesis of 30–35% of the cutin monomers from unsaturated C18 aliphatics [42]. Similar to the observation of a decrease in the double unsaturated C18 diacids in leaf polyester of the Arabidopsis fad2 mutant [41], we showed that the contents of polyunsaturated C18 dioic acid were significantly reduced in the anther cutin of fad2–3, suggesting that maintaining a certain level of polyunsaturated C18 is important for proper development of the cuticular structure of cotton anthers.

In our study, silencing GhFAD2–3 induced transcriptional changes during anther development (Additional file 1: Table S1). Our qRT-PCR results were in accordance with transcriptional analysis (Fig. 7), as silencing of GhFAD2–3 in anthers resulted in changing expression levels of many genes, such as GhCYP86B1, GhCYP704B1 and GhCYP94C1. With our study, the probable scheme of FAD2 involved in the primary pathways for cutin monomers synthesis in Gossypium was proposed (Fig. 8). In this pathway, the ω-hydroxylation reaction is typically catalyzed by cytochrome P450 monooxygenases, particularly of the GhCYP86B1 and GhCYP704B1. The ω-hydroxy FAs could be further oxidized by ω-hydroxycacid dehydrogenase (HTH) to ω-oxo FAs. GhALDH4 encodes an aldehyde dehydrogenase that further catalyzes ω-oxo FAs to produce α, ω-dicarboxylic FAs. On the other hand, peroxygenase (PXG) catalyzes the hydroperoxide-dependent epoxidation of unsaturated fatty acids, and then GhCYP94C1 with high omega-hydroxylase activity to 9, 10-epoxyoic acid metabolized C18 unsaturated FAs to produce polyhydroxy-octadecenoic acid. Mutation in any gene encoding the enzymes involved in the pathway could cause a lack of synthesis of cutin/wax and sporopollenin precursors, resulting in failure to form normal pollen exine and anther cuticle that had also been reported [18, 23, 33].

In our model, GhFAD2 could play an important role in the biosynthesis of the cutin and suberin monomers.
The fad2–3 is deficient in its ability to catalyze C18:1 to C18:2, which finally results in anther polyesters with less \( \alpha, \omega \)-octadecadiendioic acid (C 18:2) and polyhydroxy-octadecenoic acid (9,10,18-triOH C18:1 FA). Our qRT-PCR results showed that the expression of \( \text{GhFAD2} \) was relatively low at the sporogenous cell stage and microsporocyte stage, significantly increased at the tetrad stage, and peaked at the pollen maturation stage in the control plants (Fig. 7). In \( \text{fad2–3} \), \( \text{GhFAD2} \) had a very similar dynamic expression change at different anther developmental stages; however, its expression level was significantly lower than that of the wild-type at the time points investigated, particularly from the tetrad stage to the pollen maturation stage (Fig. 7). \( \text{GhFAD3} \) also had a similar expression change in the wild-type and \( \text{fad2–3} \) during anther development. Correspondingly, from the sporogenous cell stage to the microsporocyte stage, there was no difference in the relative content of C18:2 and C18:3 between the wild-type and \( \text{fad2–3} \) anthers, but from the microsporocyte stage until the mature pollen stage, the relative content of C18:2 and C18:3 was significantly lower in \( \text{fad2–3} \) than in wild-type (Fig. 5).

Overall, in \( \text{fad2–3} \), the expression of \( \text{GhFAD2} \) and \( \text{GhFAD3} \) genes was significantly inhibited; however, the expression levels of the above determined related genes in the pathway, such as \( \text{GhSAD} \), \( \text{GhCYP86B1} \), \( \text{GhCYP94C1} \), \( \text{GhHTH} \) and \( \text{GhALDH} \), were apparently upregulated, especially at the meiosis stage and/or tetrad stage. Thus, it is interesting that the upregulation of the expression of these genes in \( \text{fad2–3} \) was accompanied by the increase in C18:1 content relative to the control anther. Similar phenomena have also been previously observed with transgene expression of \( \text{FAD2} \), which produces unusual FAs, including epoxidation, hydroxylation and double bonding conjugation [40, 41]. One hypothesis explaining this phenotype is that unusual FA products inhibit the activity of FAD2, thereby effectively preventing the conversion of C18:1 to C18:2 [43–46].
cottonseed, the relative content of C18:2 peaked in the mature seeds, accounting for over 50% of the total fatty acid content of seed [12]. However, the accumulated unusual FA products, such as epoxy fatty acid, hydroxyl acid, and dioic acid, may act as inhibitors of \textit{GhFAD2} expression in anthers. Thus, this regulatory mechanism may account for part of why the relative proportion of C18:2 is not very high in anthers. In \textit{fad2–3}, the expression of \textit{GhSAD} was significantly upregulated at the meiosis stage and the tetrad stage, which would further lead to a much higher proportion of C18:1 content. Under this metabolic scenario, \textit{GhFAD2} would be further inhibited by the increased expression of related genes in the pathway, such as \textit{GhCYP86B1}, \textit{GhALDH} and \textit{GhCYP94C1}, resulting in relatively low C18:2 content at the meiosis stage and the tetrad stage in \textit{fad2–3}. In contrast, the expression of related genes in the pathway to synthesize waxy and cutin monomers could be induced by a low level of C18:2 content. This may be a self-protective mechanism of plant cells. Compared to wild type, \textit{GhHTH} and \textit{GhCYP704B1} shows significantly higher expression level at the meiosis stage. However, this does not mean that it could result in an increase in the content of hydroxy and epoxy fatty acids. At the tetrad stage, the expression of \textit{GhHTH} and \textit{GhCYP704B1} was very low in both wild type and \textit{fad2–3}, and there was no significant difference between them. The anthers of \textit{fad2–3} were smaller than those of wild-type, and the \textit{fad2–3} pollen grains appeared to lack the exine layer. These results suggest that the lack of C18:2 and C18:3 have adverse effects on the establishment of functional anther cuticles and pollen exine in \textit{fad2–3}, probably a result of insufficient biosynthesis and/or deposits of sporopollenin in these protective walls, as suggested by the observation that the \textit{fad2–3} pollen grains were unresistant to acetolysis treatment. The high C18:1 content in cottonseeds by specific inhibition of the expression of \textit{GhFAD2–1} had disadvantageous effects on seed vigor [12]. As in cottonseed, it seemed to suggest that the too high accumulation of C18:1 was also unfavorable to the development of anthers.

In plants, the synthesis of various fatty acid components has a complex interrelationship, and the synthesis of other fatty acids will be affected when the synthesis of a particular fatty acid component is regulated. In general, with the increase in C18:1 content, the content of C16:1 decreased in this study. The decrease in C16:0 was also observed in the anther wax of \textit{fad2–3}. Accordingly, the content of C16:0 DCA was reduced in the anther cutin of \textit{fad2–3}. The relative proportion of fatty acid components accumulated in plant tissues is very important for ensuring that they could preserve the basic physiological activities. Changing the relative content and proportion of one or some fatty acid components in plant tissues may adversely affect vegetative or reproductive growth of plants. It had been reported that the
Arabidopsis fad2 mutants showed dwarf phenotypes and the levels of PUFAs in phospholipids were relatively low. The limited membrane fluidity of the fad2 mutant resulted in its inability to survive at a low temperature [47]. We had also previously found that the decreased C18:2 content in cottonseeds by specific inhibition of the expression of GhFAD2–1 had disadvantageous effects on seed vigor [12]. C18:2 and C18:3 could serve as the essential structural components of anthers and pollen walls and/or as the substrates for biosynthesis of signaling molecules and/or hormones, such as Jasmonic acid (JA), essential for anther and pollen development. Jasmonic acid is a carboxylic fatty acid and plays an essential role in pollen and anther development. Several Arabidopsis male sterile mutants have been shown to be caused by mutation in genes involved in JA biosynthesis and/or signaling pathways, such as coi1 [48] and opr3/ dde1 [49, 50]. It has been reported that the crucial requirement for C18:3 in A. thaliana pollen development and anther dehiscence seems to be as a substrate for JA biosynthesis through the octadecanoid pathway [51]; however, the threshold requirement for C18:3 is very low, and anthers with only 1–2% of C18:3 could remain fertile. The wild-type cotton anthers contain a relatively low level of C18:3 compared to C18:1 and C18:2 but could have a level higher than the low threshold reported in Arabidopsis. The C18:3 content was significantly reduced in the fad2–3 anthers and became as low as 0.3% (~10-fold reduction) at the mature pollen stage (Fig. 5). Taken together, C18:2 and C18:3 are essential structural components of anthers and are also important substrates for biosynthesis of many other lipids in anther development.

Conclusion
In this study, we show functional specificity and redundancy of GhFAD2 genes that would be helpful to understand gene expression and regulation in allopolyploid crops. We report for the first time on the dynamic changes in fatty acid constituents during anther development and showed that silencing of GhFAD2–3 reduced PUFAs (mainly C18:2 and C18:3) in anthers, severely hampered pollen development and significantly reduced the number and viability of pollen grains. Our results demonstrated that the relative proportions of fatty acid components accumulated in plant tissues is very important to ensure that they could preserve the basic physiological activities. In the future, a comprehensive and in-depth study of unsaturated fatty acids and their derivatives in plant cells would help us to better control plant growth and development on the basis of understanding the physiological significance of the formation of plant fatty acid components.

Methods
Characterization of the GhFAD2 gene family
The genome sequences and annotation files of G. hirsutum (AD1_NBI), G. raimondii (D5_JGI) and G. arboreum (A2_BGI) were downloaded from CottonGen (https://www.cottongen.org). To identify the members of the GhFAD2 family genes, the amino acid sequence of the previously reported GhFAD2 (GenBank accession no. X97016) was used to search for its homologs in the annotated proteins of the three genomes using BLASTP (E-value ≤10\(^{-50}\)). The hits with all three conserved histidine-clusters observed in all reported plant FAD2 sequences were considered as GhFAD2. The relationship of the identified GhFAD2 genes was investigated by sequence alignment and phylogenetic analysis.

Transcriptome analyses
We did two transcriptome analyses. One aimed to investigate the expression profiles of individual GhFAD2 in various tissues and the other compared the difference in anther transcriptomes between the GhFAD2–3 silenced transgenics and wild-type. For the first experiment, total RNA was isolated from root, leaf, stem, anther, stigma, ovary, developing and mature seed (at 5, 20, 40 and 60 days post anthesis) and fiber (at 12 and 24 days post anthesis). Each sample had three biological replicates. For each sample, a total of 3 μg RNA was used in preparing the RNA-seq library. Barcoded multiplexed RNA-seq libraries were created using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA) according to the manufacturer’s protocol. Clean paired-end reads were aligned to the TM-1 reference genome [35], and the number of reads aligned to each gene was measured using HTSeq v0.6.1. The expression levels of individual genes were quantified using FPKM (fragments per kilobase of transcript per million mapped reads).

For the anther transcriptome experiment, total RNA was isolated from anthers of wild-type (control) and transgenic plants with silenced GhFAD2–3 (fad2–3). Anthers from two developmental stages, meiotic and tetrad, were investigated. Three samples were collected from each stage for both wild-type and fad2–3. A total amount of 3 μg RNA from each sample was used in generating index-coded RNA-seq libraries using the Illumina TruSeq RNA Library Prep Kit. The clustering of the index-coded libraries was performed using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina) on a cBot Cluster Generation System following the manufacturer’s manual. The libraries were then sequenced using the Illumina HiSeq 2500 platform. Read mapping and FPKM calculations were performed as described previously. The model based on negative binomial distribution was used to determine differentially expressed genes (DEG) with an adjusted p-value <0.05 [52]. The KOBAS
software was used to test statistically the enrichment of DEGs in KEGG pathways.

Plant material and generation of RNAi plants
The cotton variety ‘Xinluzao 33’ provided by the Cotton Research Institute of Shihezi University was used in this study. A 517-bp fragment (Additional file 1: Figure S1) was amplified by PCR using PrimeSTAR™ HS DNA polymerase. The primers used were 5′-CACCCGCTCA CTATCCGTCA-3′ (CACC was added at the 5′ end for directional cloning of the amplified fragment into the RNAi construct) and 5′-CGTTGTAGATAGGACCGT AT-3′. The PCR cycles were performed as follows: 95°C for 5 min, followed by 29 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 10 min. The amplified DNA fragment was subcloned into pENTR/D-TOPO (Invitrogen) to generate the entry vector pENTR/D-GhFAD2 that was confirmed by sequencing. The LR recombination reaction between pENTR/D-GhFAD2 and the gateway vector pAN- DA35HK was then used to create pANDA35HK-dsGhFAD2 using the Gateway™ LR Clonase™ plus enzyme mix (Invitrogen). The native promoter of GhFAD2-3D isolated from cotton genomic DNA (Additional file 1: Figure S2a) was cloned into the Hin dIII-Bam HI site of pBI121 to generate pBIAP. Finally, the fragment with the 517-bp fragment inserted forwardly and reversely at two sides of the intron was excised from pANDA35HK-dsGhFAD2 and used to replace the Gus gene in pBIAP to create pBIAP-dsGhFAD2 (Additional file 1: Figure S2b), which was then electroporated into the Agrobacterium tumefaciens strain LBA4404. Cotton transformation was carried out using hypocotyl explants from G. hirsutum cultivar Xinluzao 33 as described by Jin et al. [53].

A fragment (1,113 bp) containing part of the 517-bp GhFAD2-3D segment and part of the gus linker was amplified by PCR to identify positive transgenic plants. Genomic DNA was isolated from cotton leaf tissues and amplified by PCR to identify positive transgenic plants.

For testing pollen viability, pollen grains were stained with 1% l-KI solution and photographed using a SteREO Discovery microscope (Carl Zeiss). For the control plants, sufficient mature pollens were obtained by placing 3–4 open flowers in a microfuge tube. For the fad2–3 plants, mature anthers were dissected from flowers and gently squashed in staining solution using dissecting needles. Acetolysis treatment was carried out according to Aarts et al. [19], and pollen grains were treated with a mixture of sulfuric acid and acetic anhydride at 100°C. The pollen pellet was then transferred to a microscope slide and viewed with a SteREO Discovery microscope.

Scanning and transmission electron microscopy
For scanning electron microscopy, anthers at different developmental stages were fixed overnight in 2.5% glutaraldehyde, and then washed and postfixed in 1% osmium tetroxide in 0.1 M sodium phosphate buffer (PBS, pH 7.2). Samples were then dehydrated in a graded ethanol concentration (30, 50, 70, 95 and 100%) and dried with liquid CO2. Before examination with the scanning electron microscope, dried anthers were sputtered with gold palladium for 300 s at 25 mA. Samples were finally visualized using a Hitachi S4500 microscope.

Transmission electron microscopy was performed using a Hitachi H7600 transmission electron microscope. Anthers were fixed in 2.5% glutaraldehyde (stored overnight at 4°C), washed three times (5 min for each) with 0.1 M PBS, postfixed in 1% OsO4 for 2 h, and washed with PBS (three times, 5 min for each). Samples were then dehydrated as described above, treated with propylene oxide, and embedded in Spurr’s resin. Thin sections (70 nm) were then taken using the Leica UC6 cryo ultramicrotome. Sliced sections were placed on 100-mesh copper grids and sequentially stained with uranyl acetate (30 min) and lead citrate (Sato’s Lead; 15 min).

Anther collection and fatty acid analysis
To analyze the composition of fatty acids in developing anthers, different sizes of flower buds (without bracts) with a diameter < 9 mm were collected from wild-type (fertile) and fad2–3 (sterile) and used in isolation of anthers with pollen grains at the following five developmental stages: sporogenous cells, microsporocyte, meiosis, tetrad and pollen maturation. Identification of these developmental stages was done using optical microscopy.

The whole anther (including both anther wall and pollen grains) of the five stages mentioned above was used in fatty acid analysis. The fatty acid methyl esters were prepared by alkaline transmethylation. Briefly, 0.5 g freeze-dried anthers were transferred into glass tubes,
and used in oil extraction by using a Soxtherm apparatus (Gerhadt). Then, 5 ml 0.4 M KOH-methanol and 5 ml hexane were added and mixed. The solution was transferred into vials and shaken for 30 min at 40 °C. After adding ~1 g of anhydrous sodium sulfate to remove water, the upper hexane layer was used in GC-MS analysis. The quantitative standard curve was established by mixing 37 fatty acid methyl esters (Sigma) determined by GC-MS, and the external standard method was used for quantitative determination. The analyses were performed using GCMS-QP2020 at an electron ionization of 70 eV with an HP-88 capillary column (100 m × 0.2 mm) and film thickness of 0.2 μm. The column program used was: the injection temperature 250 °C, oven temperature kept at 40 °C for 2 min, then increased to 240 °C at a rate of 4 °C/min, and kept constant at 240 °C for 15 min. Operating conditions: helium carrier gas 2 mL/min, split ratio 10:1.

Analysis of anther cuticular waxes and cutin-like polyester
The wax of anther at mature pollen stage was analyzed using a published protocol [29] with some modifications. Briefly, 100 mg of freeze-dried anthers was submersed in 10 ml of chloroform containing 100 μg of tetracosane (Fluka; serving as an internal standard) for 1 min. The solution was transferred to a new vial, and then the solvent was evaporated under a nitrogen gas stream. The remaining compounds were incubated with 200 μl bis-N, N-(trimethylsilyl)-trifluoroacetamide (Sigma-Aldrich) in 200 μl pyridine for 60 min at 70 °C before GC-MS analysis. The constituent analyses were performed using GCMS-QP2020 with a DB-1 column of 30 m × 0.32 mm and film thickness of 0.1 μm. GC-MS analyses were performed as described by Jung et al. [29]. Each compound was quantified against the internal standard by automatic integration of the peak areas.

The protocol for lipid polyester analysis was performed according to Li-Beisson et al. [54]. First, 100 mg of freeze-dried anthers was delipidated. After that, depolymerization was performed by acid catalysis as described by Li-Beisson et al. [50]. The resulting cutin monomer fraction was derivatized with BFTSA/pyridine (1:1) for 60 min at 70 °C, and then samples were analyzed using GCMS-QP2020 with a DB-1 column of 30 m × 0.32 mm and film thickness of 0.1 μm. The GC-MS was conducted according to Li-Beisson et al. [50] with helium carrier gas at 2 ml/min. Each compound was quantified on the basis of their total ion current as quantified on the basis of their total ion current as described by Li-Beisson et al. [54].

Quantitative real-time PCR (qRT-PCR) analysis
Total RNA was extracted from anthers at different developmental stages using RNAiso Plus (Takara), and then the RNA was reverse transcribed to obtain first-strand cDNA using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, China). The transcript levels of genes were analyzed by qRT-PCR using the LightCycler® 480 II (Roche, Germany). Each reaction was performed in 10 μl volumes using SYBR Green Master Mix (Takara, China) under the following PCR conditions: 94 °C for 3 min followed by 40 cycles of 94 °C for 15 s, 56 °C for 15 s, and 72 °C for 15 s. All gene specific primers for qRT-PCR were designed using the Primer 6.0 program (Additional file 1: Table S2). The cotton poly-ubiquitin gene (GhUBQ14, accession number in GenBank: DW/505546) was used as an internal control. The primers for GhUBQ14 were 5′-CAACGCTCCATCTGTCTC-3′ and 5′-TGATCCT GTTTCGCCGTAGC-3′. All qRT–PCR reactions were performed in triplicate. The relative expression levels of target genes were calculated with the 2−ΔΔCT method [55].

Additional files

Additional file 1: Figure 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_D132237 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 proteins, 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_A0120931 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.

Additional file 2: Raw data. This file contains raw data with individual data points or replicates for Figs. 1, 5, 6, and 7. (XLS 126 kb)

Abbreviations
C14:0. Myristic acid; C16:0 DCA: Hexadecane-1,16-dioic acid; C16:0. Palmitic acid; C18:0. Stearic acid; C18:1 DCA: α- octadecenoic acid; C18:1: 9-octadecenoic acid; C18:2 DCA: α, ω-octadecadienoic acid; C18:2: 9,12-octadecadienoic acid; C18:3: 9,12,15-octadecatrienoic acid; C20:0. Arachidic Acid
acid; DPA: Days post anthesis; DW: Dry weight; ER: Endoplasmic reticulum; FAD2: Fatty acid desaturase 2; FAD3: Fatty acid desaturase 3; FAE1: Fatty acid epoxide hydrolase; FAR: Fatty acyl-coenzyme A Reductase; FAS: Fatty acid synthase; FA: Fatty acids; HTH: ω-hydroxyacid dehydrogenase; NPT II: Neomycin phosphotransferase II gene; OADH: ω-oxo-acid dehydrogenase; P450: Cytochrome P450 monooxygenase; PLFAs: Polyunsaturated fatty acids; PXG: Peroxygenase; SAD: Stearoyl-ACP desaturase; triOH C18:1 FA: 9,10,18-trihydroxy octadecenoic acid

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Authors’ contributions

JS, FL and QZ planned and designed the research. FL, LM, YL, FX, XZ, XN and YW performed experiments, conducted fieldwork, analysed data etc. FL, QZ and JS wrote the manuscript. JS and QZ contributed equally. All authors read and approved the final version of the manuscript.

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Availability of data and materials

All Gene ID and annotation files could be obtained from CottonGen (https://www.cottongen.org). Raw data for Figs. 1, 5, 6, and 7 can be found in ‘Additional file 2: Raw data’. All other data generated or analyzed during this study are included in this manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Shanklin J, Cahoon EB. Desaturation and related modifications of fatty acids. Annu Rev Plant Physiol Plant Mol Biol. 1998; https://doi.org/10.1146/annurev.arplant.49.1.611.
2. Okuley J, Lightner J, Feldmann K, Yadav N, Lark E, Browse J. Arabidopsis FAD2 gene encodes the enzyme that is essential for polysaturated lipid synthesis. Plant Cell. 1994; https://doi.org/10.1105/tpc.6.1.147.
3. Jin UH, Lee JW, Chung YS, Lee JH, Yi YB, Kim YK, Hyung NI, Pyee JH, Chung SH. Characterization and temporal expression of a ω-6 fatty acid desaturase cDNAfrom sesame (Sesamum indicum L) seeds. Plant Sci. 2001; https://doi.org/10.1016/S0168-9452(01)00489-7.
4. Jung JH, Kim H, Go YS, Lee SB, Hur CG, Kim HU, Suh MC. Identification of functional BrFAD2-1 gene encoding microsomal delta-12 fatty acid desaturase from Brassica rapa and development of Brassica napus containing high oleic acid contents. Plant Cell Rep. 2001; https://doi.org/10.1007/s00299-011-0945-x.
5. Li LY, Wang XL, Gai JY, Yu DY. Molecular cloning and characterization of a novel microsomal oleate desaturase gene from soybean. J Plant Physiol. 2007; https://doi.org/10.1016/j.jplph.2006.08.007.
6. Martínez-Rivas JM, Sperring P, Luhs W, Heinz E. Spatial and temporal regulation of three different microsomal oleate desaturase genes (FAD2) from normal-type and high-oleic varieties of sunflower (Helianthus annuus L). Mol Breed. 2001; https://doi.org/10.1023/A:1013324329322.
7. Liu Q, Singh SP, Brubaker CL, Sharp PJ, Green AG, Marshall D. Molecular cloning and expression of a CDNA encoding a microsomal ω-6 fatty acid desaturase from cotton (Gossypium hirsutum). Aust J Plant Physiol. 1999; https://doi.org/10.1071/PP98118.
8. Pirte L, Kangcharoensuntorn W, Nampaisansuk M, Knesek JE, Chapman KD, Pirte RM. Molecular cloning and functional expression of the gene for a cotton ωII fatty acid desaturase (FAD2). Biochim Biophys Acta. 2001; https://doi.org/10.1016/S0167-4781(01)00312-8.
9. Dar AA, Choudhury AR, Kanchal PA, Anumugam N. The FAD2 gene in plants: occurrence, regulation, and role. Front Plant Sci. 2017; https://doi.org/10.3389/fpls.2017.01789.
10. Marieucci M, D’Angeli S, Errico S, Lamanna R, Perrotta G, Altamura MM. Cold affects the transcription of fatty acid desaturases and oil quality in the fruit of Olea europaea L. genotypes with different cold hardness. J Exp Bot. 2011; https://doi.org/10.1093/jxb/erq013.
11. Kargioittou A, Deli D, Galanopoulos D, Tsafiras A, Farmaki T. Low temperature and light regulate delta 12 fatty acid desaturases (FAD2) at a transcriptional level in cotton (Gossypium hirsutum). J Exp Bot. 2008; https://doi.org/10.1093/jxb/erm065.
12. Liu F, Zhao YP, Zhu HG, Zhu QH, Sun J. Simultaneous silencing of GrFAD2-1 and GrFATB enhances the quality of canola seed oil with high oleic acid. J Plant Physiol. 2017; https://doi.org/10.1016/j.jplph.2017.06.001.
13. Wells R, Trick M, Sounpoubour-eu C, Clissold L, Morgan C, Werner G, Gibbard C, Clarke M, Jennaway R, Bancroft I. The control of seed oil polysaturated content in the polyploid crop species Brassica napus. Mol Breed. 2014; https://doi.org/10.1007/s11032-013-9954-5.
14. Qu J, Mao HZ, Chen W, Gao SQ, Bai YN, Sun YW, Geng YF, Ye J. Development of marker-free transgenic Jatropha plants with increased levels of seed oleic acid. Biotech Biofuels. 2012; https://doi.org/10.1186/1754-6834-5-10.
15. Pham AT, Shannon JG, Bilyeu KD. Combinations of mutant FAD2 and FAD3 genes to produce high oleic acid and low linolenic acid soybean oil. Theor Appl Genet. 2012; https://doi.org/10.1007/s00122-012-1849-z.
16. Lu C, Napier JA, Clemente TE, Cahoon EB. New frontiers in oilseed biotechnology: meeting the global demand for vegetable oils for feed, biofuel, and industrial applications. Curr Opin Biotechnol. 2011; https://doi.org/10.1016/j.copbio.2011.09.008.
17. Piñafiel P, Ross HJ, Murphy DJ. Intra- and extracellular lipid composition and associated gene expression patterns during pollen development in Brassica napus. Plant J. 1997; https://doi.org/10.1046/j.1365-313X.1997.11030549.x.
18. Dobritsa AA, Shrestha J, Morant M, Pinot F, Matsuno M, Swanson R, Maller BL, Preuss D. CYP704B1 is along-chain fatty acid ω-hydroxylation essential for sponspollenin synthesis in pollen of Arabidopsis. Plant Physiol. 2009; https://doi.org/10.1104/pp.109.144459.
19. Jiang J, Zhang Z, Cao J. Pollen wall development: the associated enzymes and metabolic pathways. Plant Biol (Stuttg). 2013; https://doi.org/10.1111/j.1365-8389.2013.03369.x.
20. Shi J, Cui M, Yang L, Kim YJ, Zhang D. Genetic and biochemical mechanisms of pollen wall development. Trends Plant Sci. 2015; https://doi.org/10.1016/j.tplants.2015.07.010.
21. Zhang D, Shi J, Yang X. Role of lipid metabolism in plant pollen exine development. In: Nakamura Y, Li-Beisson Y, editors. Lipids in plant and algae development: Subcellular Biochemistry; 2016. https://doi.org/10.1007/978-3-319-25979-6_13.
22. Aarts MG, Hodge R, Kalantidis K, Florack D, Wilson ZA, Mulligan BJ, Stiekema MALE STERILITY 2 protein shares similarity with reductases in elongation/condensation complexes. Plant J. 1997; https://doi.org/10.1046/j.1365-313X.1997.00615.x.
23. Chen W, Yu XH, Zhang K, Shi J, De Oliveira S, Schreiber L, Shanklin J, Zhang D. Male Sterile2 encodes a plastid-localized fatty acyl carrier protein reductase required for pollen exine development in Arabidopsis. Plant Physiol. 2011; https://doi.org/10.1104/pp.111.181693.
24. Anzumi T, Hatakeyama K, Hinata K, Inatougi R, Nishida I, Sato S, Kato T, Tabata S, Torigawa K. Disruption of the novel plant protein NBR1 affects lipid accumulation in the plastids of the tapetum and exine formation of pollen, resulting in male sterility in Arabidopsis thaliana. Plant J. 2004; https://doi.org/10.1111/j.1365-313X.2004.02118.x.
25. Morant M, Jørgensen K, Schaller H, Pinot F, Maller BL, Weerk-Reichhart D, Bak S. CYP703 is an ancient cytochrome P450 in land plants catalyzing in-
chain hydroxylation of lauric acid to provide building blocks for sporopollenin synthesis in pollen. Plant Cell. 2007; https://doi.org/10.1105/tpc.106.049548.

26. Li H, Pirrot F, Sauveplane V, Werck-Reichhart D, Diehl P, Schreiber L, Franke R, Zhang P, Chen L, Gao YM, et al. Cytochrome P450 family member CYP70B2 catalyzes the omega-hydroxylation of fatty acids and is required for anther cutin biosynthesis and pollen exine formation in rice. Plant Cell. 2010; https://doi.org/10.1105/tpc.108.062352.

27. de Azevedo SC, Kim SS, Koch S, Kienow L, Schneider K, McKim SM, Haughn GW, Kombrink E, Douglas CJ. A novel fatty acyl-CoA synthetase is required for pollen development and sporopollenin biosynthesis in Arabidopsis. Plant Cell. 2009; https://doi.org/10.1105/tpc.108.062513.

28. Arizumi T, Harakaya K, Hinata K, Sato S, Kato T, Tabata S, Toriyama K. A novel male-sterile mutant of Arabidopsis thaliana, faceless pollen-1, produces pollen with a smooth surface and an acetylcosyl-sensitive exine. Plant Mol Biol. 2003; https://doi.org/10.1023/B:PLANT.0000026997.7737.70.

29. Jung KH, Han MJ, Lee DY, Lee YS, Schreiber L, Franke R, Faust A, Yephremov S, Alhattab R, Lowe C, Pascal S, Lessire R, Rowland O. Three acyl-lipid metabolism. Plant Physiol. 2013; https://doi.org/10.1104/pp.111.206656.

30. Smirnova A, Leide J, Riederer M. Deficiency in a very-long-chain fatty acid β-ketoacyl-coenzyme A synthase of tomato impairs microgametogenesis and causes floral organ fusion. Plant Physiol. 2013; https://doi.org/10.1104/pp.111.207528.

31. Shi J, Tan H, Yu XH, Liu Y, Liang W, Ranathunge K, Franke RB, Schreiber L, Wang Y, Kai G, et al. Defective pollen wall is required for anther and microspore development in rice and encodes a fatty acyl carrier protein reductase. Plant Cell. 2011; https://doi.org/10.1105/tpc.111.087528.

32. Domergue F, Vishwanath SJ, Joubès J, Ono J, Lee JB, Bourdon M, Alhattab R, Lowe C, Pascal S, Lessire R, Rowland O. Three Arabidopsis fatty acyl-coenzyme A reductases, FAR1, FAR4, and FAR5, generate primary fatty alcohols associated with suberin deposition. Plant Physiol. 2010; https://doi.org/10.1104/pp.109.152398.

33. Kim J, Jung JH, Lee SB, Go YS, Kim HJ, Cahoon R, Markham JE, Cahoon EB, Suh MC. Arabidopsis 3-ketoacyl-coenzyme A synthase is involved in the synthesis of tetracosanoic acids as precursors of cuticular waxes, suberins, sphingolipids, and phospholipids. Plant Physiol. 2013; https://doi.org/10.1104/pp.113.210456.

34. Zhang DS, Liang W, Yin C, Zong J, Gu F, Zhang D. OsCg5, encoding a lipid transfer protein, is required for postmeiotic anther development in rice. Plant Physiol. 2010; https://doi.org/10.1104/pp.110.158865.

35. Zhang T, Hu Y, Jiang W, Fang L, Guan X, Chen J, Zhang J, Sasaki CA, Scheffler BE, Stelly DM, et al. Sequencing of allotetraploid cotton (Gossypium hirsutum L. acc. TM-1) provides a resource for fiber improvement. Nat Biotechnol. 2015; https://doi.org/10.1038/nbt.3207.

36. Paterson AH, Wendel JF, Gundlach H, Guo H, Jenkins J, Jin D, Llewellyn D, Llewellyn J. The genome sequence of the cultivated cotton Gossypium arboretum. Nat Genet. 2014; https://doi.org/10.1038/ng.3084.

37. Sanders PM, Lee PY, Biesgen C, Boone JD, Beals TP, Weiler EW, Goldberg RB. The Arabidopsis DELAYED DEHISCENCE 1 gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell. 2000; https://doi.org/10.1105/tpc.12.7.1041.

38. Stintzi A, Jiang B. The Arabidopsis male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc Natl Acad Sci U S A. 2000; https://doi.org/10.1073/pnas.97.16.8309.

39. Laffly KJ, Schmitgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the ΔΔCt method. Methods. 2001; https://doi.org/10.1016/s1043-2760(01)00030-1.

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