Characterization of 19 Genes Encoding Membrane-Bound Fatty Acid Desaturases and their Expression Profiles in *Gossypium raimondii* Under Low Temperature

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Abstract

To produce unsaturated fatty acids, membrane-bound fatty acid desaturases (FADs) can be exploited to introduce double bonds into the acyl chains of fatty acids. In this study, 19 membrane-bound FAD genes were identified in *Gossypium raimondii* through database searches and were classified into four different subfamilies based on phylogenetic analysis. All 19 membrane-bound FAD proteins shared three highly conserved histidine boxes, except for GrFAD2.1, which lost the third histidine box in the C-terminal region. In the *G. raimondii* genome, tandem duplication might have led to the increasing size of the FAD2 cluster in the Omega Desaturase subfamily, whereas segmental duplication appeared to be the dominant mechanism for the expansion of the Sphingolipid and Front-end Desaturase subfamilies. Gene expression analysis showed that seven membrane-bound FAD genes were significantly up-regulated and that five genes were greatly suppressed in *G. raimondii* leaves exposed to low temperature conditions.

Introduction

Both saturated and unsaturated fatty acids are major components of membrane phospholipids in plants as well as triacylglycerols in seeds. Unsaturated fatty acids usually contain one or more double bonds in their acyl chains. The number and position of double bonds in fatty acids profoundly influence their physical and physiological properties [1,2]. The desaturation of fatty acids is catalyzed by a class of enzymes called fatty acid desaturases (FADs) [1,3]. The two major groups of fatty acid desaturases, soluble and membrane-bound, have been identified and have no evolutionary relationship with each other [4,5]. The soluble desaturases have two conserved histidine boxes and are represented by the plant stearoyl-ACP desaturase, which specifically desaturates stearoyl-ACP (18:0) to produce ACP-bound oleic acid (18:1) [6]. The membrane-bound
desaturases contain three histidine boxes and are ubiquitous in prokaryotes and eukaryotes [4,5]. They comprise a highly diversified family that includes many different types of regioselectivities, such as Δ4, Δ5, Δ6, Δ7, Δ8, Δ9, Δ12 and Δ15 [5,7].

Most of the fatty acids residing in plant membranes are unsaturated. Their level of unsaturation is highly dependent upon the tolerance of a given plant for various environmental stresses, especially temperature stress [8,9,10]. Previous studies have revealed that genes encoding membrane-bound FAD proteins are crucial for the sustenance of plants faced with different environmental stresses. In rice, OsFAD8 has been reported to have a functional role in stress tolerance at low temperatures [11]. FAD2 and FAD6 were found to be active in seedlings of Arabidopsis under salinity stress [12,13]. The ads2 mutant Arabidopsis plants showed increased sensitivity to chilling and freezing temperatures [14], and the mutants of SLD genes also showed enhanced sensitivity to prolonged low-temperature exposure [15]. In tomato, LeFAD3 over-expression enhanced the tolerance of tomato seedlings for salinity stress [16], whereas silencing the LeFAD7 gene alleviated high-temperature stress [17]. In transgenic tobacco plants, over-expressing FAD7 also showed enhanced cold tolerance [18], whereas antisense expression of the Arabidopsis FAD7 reduced salt and drought tolerance [19]. In soybean, the expression of FAD3 and FAD7 was tightly regulated in response to cold temperature [20].

Cotton is the major source of natural fibers used in the textile industry. It is also a promising oilseed crop. Cotton is mostly grown in tropical and subtropical regions of the world, and its cultivation has been achieved even in relatively cold regions. Low temperature (under 15°C) can adversely affect plant development, resulting in poor germination and higher seedling mortality due to disease infection, which ultimately cause significant losses in yield [21]. Although the plant has been grown in cold areas, little is known about the molecular responses of cotton to low temperature. The Δ12 desaturases (FAD2) were extensively characterized in Gossypium hirsutum [21,22,23,24], and expression analysis suggested that FAD2 genes play a direct role in cotton adaptation to cold stress [21]. More recently, Δ15 fatty acid desaturases (FAD3 and FAD7/8) were identified in Gossypium, and one of the genes, termed FAD7/8-1, was dramatically induced during cold temperature treatment of G. hirsutum seedlings [25].

Gossypium raimondii is a diploid cotton species, whose progenitor is the putative contributor of the D subgenome to the economically important fiber-producing cotton species G. hirsutum and G. barbadense [26]. Sequencing of the G. raimondii genome has provided an opportunity for genome-wide analysis of all the genes belonging to specific gene families in cotton. In this paper, our main objectives were to identify membrane-bound FAD genes in G. raimondii through homology searches, to classify them into different subfamilies according to phylogenetic analysis, as well as to investigate their expression profiles in different tissues and under a cold stress regime. The results may provide information valuable for understanding the biological roles of membrane-bound FAD genes in the response of cotton to cold stress, and may also help cotton breeders improve the quality of cotton oil via molecular design breeding.

Materials and Methods

Database search and gene retrieval

The genome database (release v2.1) [27] of G. raimondii was downloaded from Phytozome (http://www.phytozome.net/). Seventeen membrane-bound fatty acid desaturases of Arabidopsis (S1 Table) [28,29] were obtained from the Arabidopsis Information Resource (TAIR release 10, http://www.arabidopsis.org/). To identify all candidate membrane-bound FAD genes of G. raimondii, these FAD protein sequences of Arabidopsis were employed as queries to search the G. raimondii genome database using BlastP and tBlastN programs with default parameters. Subsequently, the Pfam (http://pfam.sanger.ac.uk/search) [30] and SMART databases
were used to confirm each putative member of the FAD family. The theoretical Mw (molecular weight) and pI (isoelectric point) of the full-length protein were predicted using the ProtParam tool (http://web.expasy.org/protparam/).

**Multiple sequence alignment and phylogenetic analysis**

Multiple sequence alignments of full-length protein sequences were performed using Clustal X version 2.0 [32] with default parameters. The Neighbor-Joining phylogenetic trees were constructed using MEGA 5.2 [33] with pairwise deletion option and poisson correction model. Bootstrap tests were carried out with 1000 replicates for statistical reliability.

**Gene structures, chromosomal locations and gene duplications**

To illustrate exon-intron organization for an individual gene, the Gene Structure Display Server (GSDS, http://gsds1.cbi.pku.edu.cn/) [34] was employed to compare the predicted coding sequences (CDSs) with their corresponding genomic sequences.

The location data of all membrane-bound FAD genes were acquired from the genome annotation document. The chromosome location image of membrane-bound FAD genes was generated using MapInspect software according to their starting positions on the *G. raimondii* chromosomes [35,36].

Gene duplication of membrane-bound FAD genes in *G. raimondii* was defined according to (1) the length of aligned sequence cover was > 80% of the longer gene, (2) the identity of the aligned regions was > 80%, and (3) only one duplication event was counted for tightly linked genes [36,37,38]. With the chromosomal locations of membrane-bound FAD genes, two types of gene duplications were recognized (i.e., tandem and segmental duplications).

**Plant materials and low temperature stress treatment**

All the plants of *G. raimondii* were grown in a temperature-controlled chamber at 28°C with a photoperiod of 16 hours light and 8 hours dark. After ten days, the leaves, stems, roots, and cotyledons of some seedlings were sampled to analyze tissue-specific expression. To examine the expression patterns of membrane-bound FAD genes under low temperature stress, the plant leaves of the remaining seedlings treated at 10°C in the temperature-controlled chamber were harvested at 0, 3, 6, and 12 hours, which represented normal plants, slight stress, moderate stress, and severe stress, respectively. All collected samples were immediately frozen in liquid nitrogen and stored at -80°C. Three biological replicates were conducted per sample.

**RNA isolation and quantitative real-time RT-PCR (qRT-PCR)**

Total RNA was extracted from all samples using the EASYspin Plus Total RNA Extraction Kit (Aidlab, Beijing, China), and first-strand cDNAs were synthesized with the PrimeScript 1st Strand cDNA Synthesis Kit (TakaRa, Dalian, China) according to the manufacturer’s protocols. For quantitative real-time RT-PCR (qRT-PCR) assay, gene-specific primers were designed for the membrane-bound FAD genes according to their CDSs (S2 Table). The qRT-PCR was performed with the SYBR Premix Ex Taq (TakaRa, Dalian, China) in the BioRad CFX96 Real-time PCR System following the manufacturer’s instructions. The cotton *UBQ7* gene was used as an internal reference for all the qRT-PCR analyses. Each sample was performed in three biological replicates. The relative expression levels were calculated according to the 2\(^{-\Delta\Delta C}T\) method [39]. The expression profiles were clustered using the Cluster 3.0 software [40].
Results

Identification of membrane-bound FAD genes in *G. raimondii*

The candidate membrane-bound FAD genes were identified from the *G. raimondii* genome using the BlastP and tBlastN programs with the query sequences of *Arabidopsis* membrane-bound FAD genes. The retrieved sequences were submitted to the Pfam and SMART databases to confirm the presence of conserved domains (Pfam: PF00487). As a result, 19 non-redundant membrane-bound FAD genes were confirmed in *G. raimondii*. For comparative analysis, the membrane-bound FAD genes in rice (S1 Table) were also identified from the Rice Genome Annotation Project Database (RGAP release 7, http://rice.plantbiology.msu.edu/index.shtml) following the same strategy. All identified FAD genes were named according to their orthology with reported counterparts in *Arabidopsis*. Detailed information about the 19 membrane-bound FAD genes in *G. raimondii* is provided in Table 1. The protein sequences encoded by these 19 FAD genes varied in length from 292 amino acids for GrFAD2.1 to 477 amino acids for GrFAD8.2, with an average of approximately 397 amino acids. The predicted molecular weight (Mw) of these proteins ranged from 33.44 kDa to 55.30 kDa, and the theoretical isoelectric point (pI) ranged from 6.95 to 9.61.

Phylogenetic analysis of membrane-bound FAD genes

To evaluate the phylogenetic relationships of membrane-bound FAD genes in different species, an unrooted phylogenetic tree was constructed according to the alignments of full-length protein sequences of membrane-bound FADs in *G. raimondii*, *Arabidopsis*, and rice. In previous reports, membrane-bound desaturases from eukaryotic genomes were divided into four functional subfamilies: First Desaturase, Omega Desaturase, Front-end Desaturase, and Sphingolipid Desaturase [7]. As shown in the phylogenetic tree (Fig 1), all of the membrane-bound desaturases used in this study fell into these four subfamilies.
Fig 1. Phylogenetic relationships and motif compositions of membrane-bound FAD genes from *G. raimondii*, *Arabidopsis* and rice. Unrooted phylogenetic tree (left panel): Four subfamilies marked with different color backgrounds are labeled as Omega, Sphingolipid, Front-end and First. Motif compositions (right panel): Protein sequences are indicated by thick gray lines, and the conserved motifs are represented by different colored boxes. The length (amino acids) of the protein and motif can be estimated using the scale bar at the top.

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The First Desaturase subfamily included Δ7 desaturases and Δ9 desaturases, encoded by ADS genes, which generally introduced the first double bond into the saturated acyl chain \[7,41\]. There were nine members of the subfamily in Arabidopsis, but only one gene was found in G. raimondii, which was a homolog of AtADS3 (also termed AtFAD5).

The Omega Desaturase subfamily contained Δ12 desaturases and Δ15 desaturases, which introduce a double bond between an existing double bond and the acyl end \[3\]. The Δ12 desaturases encoded by FAD2 and FAD6 were frequently called ω6 desaturases \[42,43\], and the Δ15 desaturases encoded by FAD3, FAD7 and FAD8 were also called ω3 desaturases \[44,45,46\]. In the phylogenetic tree, FAD2 and FAD6 genes were grouped in separate branches. G. raimondii, Arabidopsis and rice had one FAD6 gene each, but the number of FAD2 genes was diverse, with an expanded number (up to five) in G. raimondii, which was greater than that in Arabidopsis (one) and rice (three). FAD3, FAD7, and FAD8 formed the other cluster, which contained three FAD genes from Arabidopsis, four from rice, and five from G. raimondii.

The Front-end Desaturase subfamily was comprised of sphingolipid Δ8 desaturases, which were encoded by SLD genes \[7,15\]. Five SLD genes were found in G. raimondii, two SLD genes in Arabidopsis, and one SLD gene in rice.

The last group was the Sphingolipid Desaturase subfamily, which was represented by sphingolipid Δ4 desaturases \[7,47\]. The group contained one gene from Arabidopsis, one gene from rice, and two genes from G. raimondii.

Interestingly, the number of members identified in G. raimondii was greater than that in Arabidopsis and rice in three of four subfamilies (Table 2). This result suggested that the membrane-bound FAD genes in the G. raimondii genome might have undergone species-specific expansion over the course of evolution.

### Conserved motifs in membrane-bound FAD genes

The membrane-bound desaturases shared three highly conserved histidine boxes, which were thought to be involved in the formation of the active site of each desaturase \[1,48\]. All of the membrane-bound FAD proteins analyzed in this study contained these three histidine boxes, except for GrFAD2.1, which lost the third histidine box in the C-terminal region (Fig 1, S3 Table). Additionally, the relative positions of the three histidine boxes in the protein sequences were similar among desaturases. The first and second histidine boxes were located near each other, with only 31 or 32 amino acid residues between them. The intervening length in the First and Omega Desaturase subfamilies was 31 amino acid residues, and there were 32 amino acid residues in the Front-end and Sphingolipid Desaturase subfamilies. The third histidine box was positioned in the C-terminal region of the proteins. The number of amino acid residues between the second and third histidine boxes was different among subfamilies, but in each subfamily or cluster, the number was almost identical. For example, there were 127 amino

| Subfamily                  | Arabidopsis | Rice | G. raimondii |
|----------------------------|-------------|------|--------------|
| First Desaturase           | 9           | 0    | 1            |
| Omega Desaturase           | 5           | 8    | 11           |
| Front-end Desaturase       | 2           | 1    | 5            |
| Sphingolipid Desaturase    | 1           | 1    | 2            |
| Total                      | 17          | 10   | 19           |

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acid residues in the First Desaturase subfamily and 161 or 162 amino acid residues in the FAD3/FAD7/FAD8 cluster of the Omega Desaturase subfamily.

To further confirm the conservation of amino acid residues in the histidine boxes, the sequence logos of the three histidine boxes in each subfamily were generated using the WebLogo program (S1 Fig). As previously reported [49,50,51], it was observed that the first residue in the third histidine box was glutamine rather than histidine in the Front-end Desaturase subfamily. Apart from this divergence, the remaining histidines were strongly conserved among subfamilies. However, the other amino acids in the histidine boxes differed greatly. Remarkably, there were four amino acid residues between the histidines in the first histidine-box of the First Desaturase subfamily, but only three in the other three subfamilies.

FAD2, known as the ER-localized membrane-bound FAD, contained an ER (endoplasmic reticulum) retrieval motif consisting of F-X-X-K/R/D/E-F (F are large hydrophobic amino acid residues such as F/Y/W/I/L/V) at the C-terminus [52]. Expectedly, all five FAD2 proteins identified in G. raimondii had the ER retrieval motif (Fig 1). The motif was YHNKF in GrFAD2.1, YRNKF in GrFAD2.2, FRNKL in GrFAD2.3, FRNKL in GrFAD2.4, and FRNKI in GrFAD2.5, respectively. SLD, which functions as a sphingolipid Δ8 desaturase, was characterized by the presence of an N-terminal cytochrome b5 domain [53]. Through searches in Pfam database, it was found that all five SLD genes in G. raimondii contained the cytochrome b5 domain at the N-terminus (Fig 1).

**Chromosomal locations and structure of membrane-bound FAD genes**

The 19 membrane-bound FAD genes were mapped to the 11 chromosomes in G. raimondii (Fig 2). They were distributed unevenly among the chromosomes. Chromosomes 2 and 13 contained three membrane-bound FAD genes each and chromosomes 1, 7, 9, and 11 contained two genes each, while only a single FAD gene was localized on each of the chromosomes 4, 6, 8, 10, and 12. There were no FAD genes located on chromosomes 3 and 5. Four duplicated gene pairs, i.e., GrFAD2.3/GrFAD2.4, GrDSD1/GrDSD2, GrSLD1/GrSLD2, and GrSLD4/GrSLD5, were found in the G. raimondii genome. According to the chromosomal distribution of the membrane-bound FAD genes, three duplication events were assigned to the segmental duplication. GrFAD2.3 and GrFAD2.4, which were positioned adjacently on chromosome 2 with no intervening genes, were involved in a tandem duplication event.
A separate phylogenetic tree was generated using the protein sequences of all membrane-bound FAD genes identified in *G. raimondii* and the exon-intron structures of these genes were compared (Fig 3). All members of the FAD3/FAD7/FAD8 cluster contained eight exons. Their conserved gene structure supported their close evolutionary relationship. Most of the FAD2 genes, including the duplicated genes *GrFAD2.3* and *GrFAD2.4*, had only one exon, with the exception of *GrFAD2.1*, which contained three exons. For SLD, the genes of two duplicated pairs, *GrSLD1/GrSLD2* and *GrSLD4/GrSLD5*, had the same gene structures and contained one exon. *GrDSD1* and *GrDSD2*, another duplicated gene pair, contained two exons. However, *GrSLD3* had three exons, *GrFAD5* had five exons, and *GrFAD6* contained up to ten exons.

Expression of the membrane-bound FAD genes in different tissues

To investigate the expression profiles of membrane-bound FAD genes in different tissues of *G. raimondii*, qRT-PCR analysis was performed to examine the gene expression levels in the roots, stems, cotyledons, and leaves of 10-day-old seedlings. Because no transcripts could be detected for *GrFAD2.1* in the four representative tissues using up to five gene-specific primers in reverse transcription (RT)-PCR analysis (data not shown), this gene was not included in the qRT-PCR analysis. As shown in Fig 4, the expression patterns of the 18 membrane-bound FAD genes varied significantly in the tissues analyzed in this study. *GrFAD3.2*, *GrFAD8.1*, and *GrSLD5* were expressed at high levels in roots. *GrFAD2.3*, *GrSLD3*, *GrSLD2*, *GrSLD1*, *GrFAD3.1*, and *GrDSD1* shared high expression levels in young stems. *GrFAD5* and *GrFAD6* displayed the highest transcript abundance in cotyledons. And *GrFAD7* and *GrFAD2.4* were predominantly expressed in leaves. These results demonstrated that the majority of these membrane-bound FAD genes exhibited tissue-specific expression patterns, which was consistent with membrane-bound FAD genes in *Arabidopsis* and soybean, which also showed specific spatial expression patterns [28].

Furthermore, the tissue-specific expression patterns of the genes involved in duplication events were compared. Although the genes in all four duplicated gene pairs shared high
sequence similarity and the same gene structure, the gene expression patterns were highly diverse. For instance, \textit{GrFAD2.3} showed preferential expression in the stem and cotyledon, but that in the root and leaf was limited. And \textit{GrFAD2.4}, which was involved in a duplication event with \textit{GrFAD2.3}, showed preferential expression in the stem and leaf.

Expression patterns of membrane-bound FAD genes under low temperature

The expression patterns of the \textit{G. raimondii} membrane-bound FAD genes in the leaves of 10-day-old seedlings under low temperature stress (10°C) were investigated in this study, and all the gene expression levels responsive to slight (3 hours), moderate (6 hours), and severe (12 hours) cold stresses were compared with those of normal plants as shown in Fig 5, with the exception of \textit{GrFAD2.1}. Out of the 18 membrane-bound FAD genes, seven genes, i.e., \textit{GrFAD8.1}, \textit{GrFAD2.2}, \textit{GrFAD8.2}, \textit{GrSLD2}, \textit{GrSLD4}, \textit{GrDSD1}, and \textit{GrSLD5}, showed a marked increase in transcript level when treated with cold stress. Among them, the expression levels of \textit{GrFAD8.1} and \textit{GrSLD5} were higher at 12 hours after cold treatment, and \textit{GrSLD2} and \textit{GrSLD4} almost reached their highest levels at 6 hours after cold treatment, whereas \textit{GrFAD2.2}, \textit{GrFAD8.2}, and \textit{GrDSD1} were expressed highly at 3 hours of cold treatment. Additionally, the expression levels of 5 other genes, i.e., \textit{GrFAD2.4}, \textit{GrFAD3.1}, \textit{GrFAD3.2}, \textit{GrFAD2.5}, and \textit{GrDSD2}, were slightly up-regulated in response to cold stress. In contrast, the five genes \textit{GrFAD5}, \textit{GrFAD7}, \textit{GrFAD2.3},
GrSLD1 and GrSLD3 were significantly down-regulated after long periods of cold stress treatment, and one gene, GrFAD6, was suppressed slightly during treatment with cold stress. There were also some differences in expression patterns between duplicated genes under low temperature.

**Discussion**

FAD genes encode the enzymes that catalyze the desaturation of fatty acids, which affect the oxidative stability and nutritional value of seed storage oils [54]. Many studies have indicated that modifying the activity of FAD genes could create transgenic soybean lines with improved seed oil quality through altering relative amounts of fatty acids [54,55,56,57]. Cotton is also a significant oilseed crop, and cottonseeds are an important source of livestock feed, foodstuff and oil [58]. FAD2 genes have already been genetically manipulated for cottonseed oil improvement [59,60,61]. However, only several FAD genes encoding the Δ12 and Δ15 desaturases have been characterized in cotton [22,23,24,25]. In this study, a comprehensive set of 19 non-redundant membrane-bound fatty acid desaturases was identified from the available genome sequences of G. raimondii. Undoubtedly, the 19 membrane-bound FAD genes identified in the diploid cotton will provide candidate genes for the gene engineering of fatty acid biosynthesis in cotton.

The FAD genes were named based on their orthologous genes in *Arabidopsis*. However, following this nomenclature, it was difficult to distinguish *FAD7* and *FAD8*, due to their high degree of homology. It has been demonstrated that *FAD7* was highly expressed at high temperatures...
and that the transcript level of FAD8 increased at low temperatures [11,62,63,64]. Therefore, according to the expression patterns of FAD genes under low temperature stress analyzed in this study, GrFAD7 and two isoforms of GrFAD8 (GrFAD8.1 and GrFAD8.2) were designated. GrFAD7 was suppressed at low temperature, whereas GrFAD8.1 was consistently induced under long duration cold exposure and GrFAD8.2 was rapidly induced at 3 hours under low temperatures.

Analysis of protein structure showed that all of the 19 membrane-bound desaturases in G. raimondii, except for GrFAD2.1, had the three highly conserved histidine boxes, which contained strongly conserved histidine residues. GrFAD2.1 contained the first and second histidine boxes in the N-terminal region, but lost the third histidine box. Moreover, the gene structure of GrFAD2.1 was also different from other FAD2 genes. By using five gene-specific primers, GrFAD2.1 was not found to be expressed in this study. These observations suggested that GrFAD2.1 might not be a functional gene. Considering the adjacent location of GrFAD2.1 to GrFAD2.2 on chromosome 13, it could be deduced that GrFAD2.1 might have undergone significant functional divergence or even have become a pseudogene after originating from an ancient tandem duplication event of the FAD2 gene in the G. raimondii genome.

Gene duplications play a significant role in the expansion of gene families in the genome [65,66]. The total number of membrane-bound FAD genes identified in G. raimondii was much greater than that in Arabidopsis and rice. Phylogenetic analysis of membrane-bound desaturases in G. raimondii, Arabidopsis and rice indicated that all subfamilies except for the First Desaturase subfamily contained more gene members in G. raimondii than in Arabidopsis and rice. The increased number of members of the G. raimondii membrane-bound FAD gene family belonging to the three subfamilies suggested that they might have undergone species-specific expansion during the process of evolution. In this study, the gene duplication events, including tandem and segmental duplications, were investigated to elucidate the expansion mechanism of the membrane-bound FAD gene family in G. raimondii. Four duplicated gene pairs, including eight genes out of the 19 membrane-bound FAD genes, were identified. Among them, one tandem duplicated gene pair, GrFAD2.3/GrFAD2.4, belonged to FAD2 cluster of the Omega Desaturase subfamily. A segmental duplicated gene pair, GrDSD1/GrDSD2, belonged to the Sphingolipid Desaturase subfamily. And the remaining two segmental duplicated gene pairs, GrSLD1/GrSLD2 and GrSLD4/GrSLD5, belonged to the Front-end Desaturase subfamily. These results showed that the tandem duplication might contribute to the increasing size of the FAD2 cluster and that the expansion of the Sphingolipid and Front-end Desaturase subfamilies was due to the segmental duplication.

Duplicated genes may experience three outcomes, i.e., non-functionalization (loss of original functions), neo-functionalization (acquisition of novel functions), or sub-functionalization (partition of original functions), during the process of evolution [67]. Gene expression profiles can provide useful clues for understanding the function of these genes. According to the experiments characterizing tissue-specific expression or response to low temperature performed in this paper, the expression patterns of members in the four duplicated gene pairs were significantly diverse, which indicated that the functions of the duplicated genes were strongly differentiated after duplications. The fate of the duplicated genes could be described as neo-functionalization.

Low temperature is one of the serious environmental stresses affecting cotton development and production. Previous studies revealed that FAD2 and FAD7/8 genes participated in cotton adaptation to cold stress [21,25], and accumulating evidence has indicated that many members of the membrane-bound FAD gene family were involved in the response to cold stress in numerous plant species [9,10,11,14,15,18,20]. In this study, the expression patterns of the G. raimondii membrane-bound FAD genes in leaves under low temperature were investigated at the
whole family level. As a result, $GrFAD8.1$, $GrFAD2.2$, $GrFAD8.2$, $GrSLD2$, $GrSLD4$, $GrDSD1$ and $GrSLD5$ were found to be significantly up-regulated in response to cold stress, which suggested that these genes might be required to maintain appropriate levels of related unsaturated fatty acids in cotton plants under low temperature conditions. Conversely, $GrFAD5$, $GrFAD7$, $GrFAD2.3$, $GrSLD1$ and $GrSLD3$ were heavily down-regulated after long periods of cold stress treatment. In the previous study, two FAD2 genes, designated FAD2-3 and FAD2-4, were found to be induced in upland cotton ($G. hirsutum$) under cold stress [21]. In this study, four functional isoforms of FAD2 genes were identified in $G. raimondii$, of which $GrFAD2.2$ was induced under low temperature, $GrFAD2.4$ and $GrFAD2.5$ were slightly up-regulated in response to cold, and $GrFAD2.3$ was suppressed under low temperature. These results suggested that specific isoforms of FAD2 genes might play a vital role in cotton response to cold stress.

SLD genes encoding the sphingolipid $\Delta8$ desaturases have been well studied in Arabidopsis, and deficiency of the genes resulted in enhanced sensitivity to prolonged low-temperature exposure [15]. Here, $GrSLD2$, $GrSLD4$, and $GrSLD5$ were highly expressed after long periods of cold treatment, whereas $GrSLD1$ and $GrSLD3$ were suppressed under low temperatures, suggesting that SLD genes are critical for cotton response to cold stress.

Supporting Information

S1 Fig. Sequence logos of the three histidine boxes in four subfamilies. The height of the letter designating the amino acid residue at each position represents the degree of conservation. The numbers on the x-axis represent the residue positions within the boxes. The y-axis represents the information content measured in bits. Note that all protein sequences in each subfamily were included in the analysis, with the exception of $GrFAD2.1$, which was excluded from the Omega Desaturase subfamily.

S1 Table. The membrane-bound fatty acid desaturases in Arabidopsis and rice.

S2 Table. PCR primers used in this study.

S3 Table. The conserved histidine boxes of membrane-bound FAD proteins in $G. raimondii$, Arabidopsis and rice.

Author Contributions

Conceived and designed the experiments: SZ JC. Performed the experiments: W. Liu W. Li QH. Analyzed the data: W. Liu MKD. Contributed reagents/materials/analysis tools: SZ. Wrote the paper: W. Liu W. Li QH MKD JC SZ.

References

1. Shanklin J, Cahoon EB. Desaturation and related modifications of fatty acids. Annu Rev Plant Physiol Plant Mol Biol. 1998; 49: 611–641. PMID:15012248
2. Wu Q, Liu T, Liu H, Zheng G. Unsaturated fatty acid: metabolism, synthesis and gene regulation. African Journal of Biotechnology. 2009; 8: 1782–1785.
3. Sakamoto T, Murata N. Regulation of the desaturation of fatty acids and its role in tolerance to cold and salt stress. Curr Opin Microbiol. 2002; 5: 206–210.
4. López Alonso D, García-Maroto F, Rodríguez-Ruiz J, Garrido JA, Vilches MA. Evolution of the membrane-bound fatty acid desaturases. Biochemical systematics and ecology. 2003; 31: 1111–1124.
5. Sperling P, Ternes P, Zank TK, Heinz E. The evolution of desaturases. Prostaglandins Leukot Essent Fatty Acids. 2003; 68: 73–95. PMID: 12538072

6. Yukawa Y, Takaika F, Shoji K, Masuda K, Yamada K. Structure and expression of two seed-specific cDNA clones encoding stearoyl-acyl carrier protein desaturase from sesame, Sesamum indicum L. Plant Cell Physiol. 1996; 37: 201–205. PMID: 8665096

7. Hashimoto K, Yoshizawa AC, Okuda S, Kuma K, Goto S, Kanehisa M. The repertoire of desaturases and elongases reveals fatty acid variations in 56 eukaryotic genomes. J Lipid Res. 2008; 49: 183–191. PMID: 17921532

8. Somerville C, Browse J. Plant lipids: metabolism, mutants, and membranes. Science. 1991; 252: 80–87. PMID: 17739077

9. Nishida I, Murata N. Chilling sensitivity in plants and cyanobacteria: the crucial contribution of membrane lipids. Annu Rev Plant Physiol Plant Mol Biol. 1996; 47: 541–568. PMID: 15012300

10. Iba K. Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. Annu Rev Plant Biol. 2002; 53: 225–245. PMID: 12221974

11. Nair PMG, Kang I, Moon B, Lee C. Effects of low temperature stress on rice (Oryza sativa L.) plastid ω-3 desaturase gene, OsFAD8 and its functional analysis using T-DNA mutants. Plant Cell Tiss Organ Cult. 2009; 98: 87–96.

12. Zhang J, Liu H, Sun J, Li B, Zhu Q, Chen S, et al. Arabidopsis fatty acid desaturase FAD2 is required for salt tolerance during seed germination and early seedling growth. PLoS One. 2012; 7: e30355. doi: 10.1371/journal.pone.0030355 PMID: 2279586

13. Zhang JT, Zhu JQ, Zhu Q, Liu H, Gao XS, Zhang HX. Fatty acid desaturase-6 (FAD6) is required for salt tolerance in Arabidopsis thaliana. Biochem Biophys Res Commun. 2009; 390: 469–474. doi: 10.1016/j.bbrc.2009.09.095 PMID: 19799856

14. Chen M, Thelen JJ. ACYL-LIPID DESATURASE2 is required for chilling and freezing tolerance in Arabidopsis. Plant Cell. 2013; 25: 1430–1444. doi: 10.1105/tpc.111.111179 PMID: 23585650

15. Chen M, Markham JE, Cahoon EB. Sphingolipid Δ8 unsaturation is important for glucosylerceramide biosynthesis and low-temperature performance in Arabidopsis. Plant J. 2012; 69: 769–781. doi: 10.1111/j.1365-313X.2011.04829.x PMID: 22023480

16. Wang HS, Yu C, Tang XF, Zhu ZJ, Ma NN, Meng QW. A tomato endoplasmic reticulum (ER)-type omega-3 fatty acid desaturase (LeFAD3) functions in early seedling tolerance to salinity stress. Plant Cell Rep. 2014; 33: 131–142. doi: 10.1007/s00299-013-1517-z PMID: 24129846

17. Liu XY, Yang JH, Li B, Yang XM, Meng QW. Antisense-mediated depletion of tomato chloroplast omega-3 fatty acid desaturase enhances thermal tolerance. J Integr Plant Biol. 2006; 48: 1096–1107.

18. Khodakovskaya M, McAvoy R, Peters J, Wu H, Li Y. Enhanced cold tolerance in transgenic tobacco expressing a chloroplast ω-3 fatty acid desaturase gene under the control of a cold-inducible promoter. Planta. 2006; 223: 1090–1100. PMID: 16292565

19. Im YJ, Han O, Chung GC, Cho BH. Antisense expression of an Arabidopsis omega-3 fatty acid desaturase gene reduces salt/drought tolerance in transgenic tobacco plants. Mol Cells. 2002; 13: 264–271. PMID: 12018849

20. Román A, Andreu V, Hernández ML, Lagunas B, Picorell R, Martínez-Rivas JM, et al. Contribution of the different omega-3 fatty acid desaturase genes to the cold response in soybean. J Exp Bot. 2012; 63: 4973–4982. doi: 10.1093/jxbers/ers174 PMID: 22865909

21. Kargiotidou A, Deli D, Galanopoulou D, Tsatsaris 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; 59: 2043–2056. doi: 10.1093/jxb/ern065 PMID: 18453533

22. Liu Q, Singh SP, Brubaker CL, Sharp PJ, Green AG, Marshall DR. Molecular cloning and expression of a cDNA encoding a microsomal ω-6 fatty acid desaturase from cotton (Gossypium hirsutum). Aust J Plant Physiol. 1999; 26: 101–106.

23. Pirtle IL, Kongcharoensuntorn W, Nampaisansuk M, Knesek JE, Chapman KD, Pirtle RM. Molecular cloning and functional expression of the gene for a cotton Δ-12 fatty acid desaturase (FAD2). Biochim Biophys Acta. 2001; 1522: 122–129. PMID: 11750064

24. Zhang D, Pirtle IL, Park SJ, Nampaisansuk M, Neogi P, Wanjie SW, et al. Identification and expression of a new delta-12 fatty acid desaturase (FAD2-4) gene in upland cotton and its functional expression in yeast and Arabidopsis thaliana plants. Plant Physiol Biochem. 2009; 47: 462–471. doi: 10.1016/j.plaphy.2008.12.024 PMID: 19217793

25. Yurchenko OP, Park SJ, Iju DC, Inmon JJ, Millhollon JC, Liechty Z, et al. Genome-wide analysis of the omega-3 fatty acid desaturase gene family in Gossypium. BMC Plant Biol. 2014; 14: 312. doi: 10.1186/s12870-014-0312-5 PMID: 25403726
26. Wang K, Wang Z, Li F, Ye W, Wang J, Song G, et al. The draft genome of a diploid cotton *Gossypium raimondii*. Nat Genet. 2012; 44: 1098–1103. doi: 10.1038/ng.2371 PMID: 2292876

27. Paterson AH, Wendel JF, Gundlach H, Guo H, Jenkins J, Jin D, et al. Repeated polyploidization of *Gossypium* genomes and the evolution of spinnable cotton fibres. Nature. 2012; 492: 423–427. doi: 10.1038/nature11798 PMID: 23257886

28. Chi X, Yang Q, Lu Y, Wang J, Zhang Q, Pan L, et al. Genome-wide analysis of fatty acid desaturases in soybean (*Glycine max*). Plant Mol Biol Rep. 2011; 29: 769–783.

29. Li-Beisson Y, Shorrosh B, Beisson F, Andersson MX, Arondel V, Bates PD, et al. Acyl-lipid metabolism. Arabidopsis Book. 2013; 11: e161.

30. Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, et al. Pfam: the protein families database. Nucleic Acids Res. 2014; 42: D222–D232. doi: 10.1093/nar/gkt1223 PMID: 24283701

31. Letunic I, Doerks T, Bork P. SMART 7: recent updates to the protein domain annotation resource. Nucleic Acids Res. 2012; 40: D302–D305. doi: 10.1093/nar/gkr931 PMID: 22053084

32. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. Bioinformatics. 2007; 23: 2947–2948. PMID: 17846036

33. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011; 28: 2731–2736. doi: 10.1093/molbev/msr121 PMID: 21546353

34. Guo AY, Zhu QH, Chen X, Luo JC. GSDS: a gene structure display server. Yi Chuan. 2007; 29: 1023–1026. PMID: 17681935

35. Zhao Y, Zhou Y, Jiang H, Li X, Gan D, Peng X, et al. Systematic analysis of sequences and expression patterns of drought-responsive members of the HD-Zip gene family in maize. PLoS One. 2011; 6: e28488. doi: 10.1371/journal.pone.0028488 PMID: 22164299

36. Liu W, Li W, He Q, Daud MK, Chen J, Zhu S. Genome-wide survey and expression analysis of calcium-dependent protein kinase in *Gossypium raimondii*. PLoS One. 2014; 9: e98189. doi: 10.1371/journal.pone.0098189 PMID: 24867436

37. Wei H, Li W, Sun X, Zhu S, Zhu J. Systematic analysis and comparison of nucleotide-binding site disease resistance genes in a diploid cotton *Gossypium raimondii*. PLoS One. 2013; 8: e68435. doi: 10.1371/journal.pone.0068435 PMID: 23936305

38. Kong X, Lv W, Jiang S, Zhang D, Cai G, Pan J, et al. Genome-wide identification and expression analysis of calcium-dependent protein kinase in maize. BMC Genomics. 2013; 14: 433. doi: 10.1186/1471-2164-14-435 PMID: 23815483

39. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT Method. Methods. 2001; 25: 402–408. PMID: 11846609

40. de Hoon MJ, Imoto S, Nolan J, Miyano S. Open source clustering software. Bioinformatics. 2004; 20: 1453–1454. PMID: 14871861

41. Heilmann I, Pidkowich MS, Girke T, Shanklin J. Switching desaturase enzyme specificity by alternate subcellular targeting. Proc Natl Acad Sci U S A. 2004; 101: 10266–10271. PMID: 15240892

42. Okuley J, Lightner J, Feldmann K, Yadav N, Lark E, Browse J. Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. Plant Cell. 1994; 6: 147–158. PMID: 7907506

43. Falcone DL, Gibson S, Lemieux B, Somerville C. Identification of a gene that complements an Arabidopsis mutant deficient in chloroplast ω6 desaturase activity. Plant Physiol. 1994; 106: 1453–1459. PMID: 7846158

44. Browse J, McConn M, James DJ, Miquel M. Mutants of Arabidopsis deficient in the synthesis of ω-linolenic. Biochemical and genetic characterization of the endoplasmic reticulum linoleoyl desaturase. J Biol Chem. 1993; 268: 16345–16351. PMID: 8102138

45. Iba K, Gibson S, Nishiihi T, Fuse T, Nishimura M, Arondel V, et al. A gene encoding a chloroplast ω-3 fatty acid desaturase complements alterations in fatty acid desaturation and chloroplast copy number of the fad7 mutant of Arabidopsis thaliana. J Biol Chem. 1993; 268: 24099–24105. PMID: 8226956

46. McConn M, Hugly S, Browse J, Somerville C. A mutation at the fad8 locus of Arabidopsis identifies a second chloroplast ω-3 desaturase. Plant Physiol. 1994; 106: 1609–1614. PMID: 12232435

47. Michaelson LV, Zäuner S, Markham JE, Haslam RP, Desikan R, Mugford S, et al. Functional characterization of a higher plant sphingolipid Δ4-desaturase: defining the role of sphingosine and sphingosine-1-phosphate in Arabidopsis. Plant Physiol. 2009; 149: 487–498. doi: 10.1104/pp.108.129411 PMID: 18978071

48. Los DA, Murata N. Structure and expression of fatty acid desaturases. Biochim Biophys Acta. 1998; 1394: 3–15. PMID: 9767077
49. Sperling P, Libisch B, Zähringer U, Napier JA, Heinz E. Functional identification of a Δ8-sphingolipid desaturase from *Borago officinalis*. Arch Biochem Biophys. 2001; 388: 293–298. PMID:11368168

50. Moreno-Pérez AJ, Martínez-Force E, García R, Salas JJ. Sphingolipid base modifying enzymes in sunflower (*Helianthus annuus*): cloning and characterization of a C4-hydroxylase gene and a new paralogous Δ8-desaturase gene. J Plant Physiol. 2011; 168: 831–839. doi: 10.1016/j.jplph.2010.11.015 PMID:21256623

51. Li SF, Song LY, Yin WB, Chen YH, Chen L, Li JL, et al. Isolation and functional characterisation of the genes encoding Δ8-sphingolipid desaturase from *Brassica rapa*. J Genet Genomics. 2012; 39: 47–59. doi: 10.1016/j.jgg.2011.12.002 PMID: 22293117

52. McCartney AW, Dyer JM, Dhanoa PK, Kim PK, Andrews DW, McNew JA, et al. Membrane-bound fatty acid desaturases are inserted co-translationally into the ER and contain different ER retrieval motifs at their carboxy termini. Plant J. 2004; 37: 156–173. PMID:14690501

53. Sperling P, Heinz E. Plant sphingolipids: structural diversity, biosynthesis, first genes and functions. Biochim Biophys Acta. 2003; 1632: 1–15. PMID:12782146

54. Clemente TE, Cahoon EB. Soybean oil: genetic approaches for modification of functionality and total content. Plant Physiol. 2009; 151: 1030–1040. doi: 10.1104/pp.109.146282 PMID:19783644

55. Flores T, Karpova O, Su X, Zeng P, Bilyeu K, Sleper DA, et al. Silencing of *GmFAD3* gene by siRNA leads to low α-linolenic acids (18:3) of *fad3*-mutant phenotype in soybean (*Glycine max* (Merr.)). Transgenic Res. 2008; 17: 839–850. doi: 10.1007/s11248-008-9167-6 PMID: 18256901

56. Wagner N, Mroczka A, Roberts PD, Schreckengost W, Voelker T. RNAi trigger fragment truncation attenuates soybean *FAD2-1* transcript suppression and yields intermediate oil phenotypes. Plant Biotechnol J. 2011; 9: 723–728. doi: 10.1111/j.1467-7652.2010.00573.x PMID: 21083800

57. Haun W, Coffman A, Clasen BM, Demorest ZL, Lowy A, Ray E, et al. Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. Plant Biotechnol J. 2014; 12: 934–940. doi: 10.1111/pbi.12201 PMID: 24851712

58. Chen ZJ, Scheffler BE, Dennis E, Triplett BA, Zhang T, Guo W, et al. Toward sequencing cotton (*Gossypium*) genomes. Plant Physiol. 2007; 145: 1303–1310. PMID:18056866

59. Chapman KD, Austin-Brown S, Sparing SA, Kinney AJ, Ripp KG, Pirtle IL, et al. Transgenic cotton plants with increased seed oleic acid content. Journal of the American Oil Chemists' Society. 2001; 78: 941–947.

60. Liu Q, Singh SP, Green AG. High-stearic and High-oleic cottonseed oils produced by hairpin RNA-mediated post-transcriptional gene silencing. Plant Physiol. 2002; 129: 1732–1743. PMID:12177486

61. Sunil Kumar G, Campbell LM, Hossen M, Connell JP, Hernandez E, Reddy AS, et al. A comprehensive study of the use of a homologous promoter in antisense cotton lines exhibiting a high seed oleic acid phenotype. Plant Biotechnol J. 2005; 3: 319–330. PMID:17129314

62. Berberich T, Harada M, Sugawara K, Kodama H, Iba K, Kusano T. Two maize genes encoding ω-3 fatty acid desaturase and their differential expression to temperature. Plant Mol Biol. 1998; 36: 297–306. PMID: 9484441

63. Gibson S, Arondel V, Iba K, Somerville C. Cloning of a temperature-regulated gene encoding a chloroplast ω-3 desaturase from Arabidopsis thaliana. Plant Physiol. 1994; 104: 1615–1621. PMID: 7946164

64. Wang J, Meng F, Pittman J, Han Y, Hu J, Guo B, et al. Characterization of a rice (Oryza sativa L.) gene encoding a temperature-dependent chloroplast ω-3 fatty acid desaturase. Biochem Biophys Res Commun. 2006; 340: 1209–1216. PMID:16406238

65. Cannon SB, Miltra A, Baumgarten A, Young ND, May G. The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*. BMC Plant Biol. 2004; 4: 10. PMID: 15171794

66. Maere S, De Bodt S, Raes J, Casneuf T, Van Montagu M, Kuiper M, et al. Modeling gene and genome duplications in eukaryotes. Proc Natl Acad Sci U S A. 2005; 102: 5454–5459. PMID: 15800040

67. Lynch M, Conery JS. The evolutionary fate and consequences of duplicate genes. Science. 2000; 290: 1151–1155. PMID: 11073452