Research Square

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Identification and Analysis of the FAD Gene Family in Walnuts (Juglans regia L.) Based on Transcriptome Data

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Research article

Keywords: Walnut, fatty acid, RNA-Seq, FAD gene family, FAD3

Posted Date: January 7th, 2020

DOI: https://doi.org/10.21203/rs.2.17547/v2

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Version of Record: A version of this preprint was published on April 15th, 2020. See the published version at https://doi.org/10.1186/s12864-020-6692-z.

Abstract

Background: Walnut fatty acids, the main component of walnut kernels, contain a large amount of unsaturated fatty acids, such as linoleic acid and linolenic acid, which are essential fatty acids in humans and have important effects on human growth and health. Fatty acid desaturase (FAD) is widely distributed throughout the biological world. Its main function is to remove hydrogen from carbon chains in the biosynthesis of unsaturated fatty acids to synthesize C=C double bonds. Results: In the current research, 24 members of the JrFAD gene family were identified by bioinformatics analysis; the expression of fatty acid synthesis genes in walnut kernels at different developmental stages was analysed by transcriptome sequencing, and the expression of JrFAD3-1 , an enzyme gene for linolenic acid synthesis, was particularly prominent. The results showed that the relative expression level of FAD3-1 changed dramatically with the kernel development stage, and the expression changes showed a "bell shape". There was a significant positive correlation between the expression of JrFAD3-1 from 90-100 DAF (days after flower) and the content of alpha-linolenic acid from 100-130 DAF, with a correlation coefficient of 0.991. JrFAD3-1 can be considered closely related to Betula pendula and Corylus heterophylla . Conclusion: 24 members of the JrFAD gene family consist of four subfamilies: the SAD desaturase subfamily, Δ7/Δ9 desaturase subfamily, Δ12/ω-3 desaturase subfamily and "front-end" desaturase subfamily. A key gene Jr FAD3-1 was obtained based on transcriptome data and there was a significant positive correlation between the expression of JrFAD3-1 from 90-100 DAF and the content of alpha-linolenic acid from 100-130 DAF. JrFAD3-1 can be considered closely related to Betula pendula and Corylus heterophylla .

1 Background

Walnut (*Juglans regia* L.) is an important economic tree species throughout the world and is widely cultivated worldwide as a traditional wood and oil crop. The walnut kernel oil content can be as high as 63%, higher than that of other leading oil crops, such as peanut, soybean, palm, olive, corn germ, and sunflower [1]. Fatty acids, which are the main nutritional component accumulated in walnut kernel, contain a large amount of unsaturated fatty acids, such as linoleic acid and linolenic acid [2], which are essential fatty acids for human beings and have an important role in human growth and health [3]. In addition, polyunsaturated fatty acids are also prodrugs of prostaglandins, which have blood pressure lowering, platelet adhesion reducing, and anticoagulant effects [4]. However, the body cannot synthesize linoleic acid and α-linolenic acid, which can only be obtained by ingesting food [5]. The unsaturated fatty acid is as high as 90% [3] of walnut kernel oil, which can not only inhibit the absorption of cholesterol in the small intestine but also inhibit the re-absorption of bile acid, promote the degradation and elimination of cholesterol in the liver, change the distribution of cholesterol in the body, and accelerate the transfer of plasma cholesterol to vascular tissues [4].

Fatty acid desaturase (FAD) is widely present in the biological world [6, 7, 8, 9]. Its main function is to remove hydrogen from carbon chains in the biosynthesis of unsaturated fatty acids to synthesize C=C double bonds. In plants, FADs can be classified into two categories according to their solubility, soluble desaturases and membrane-integrin desaturases [10]. The stearoyl-ACP-desaturase (SAD) unique to

higher plants is the only known FAD dissolved in a matrix in the biological world. All other types of desaturases belong to the membrane-integrins and are localized to the endoplasmic reticulum (FAD2 and FAD3) and plastids (FAD6, FAD7 and FAD8, etc.). The first step in the synthesis of unsaturated fatty acids is the introduction of a double bond at the Δ9 position [11], and the enzymes that catalyse the reaction include stearoyl-CoA-desaturase (SCD) [12] and SAD [13]. SAD, catalysed by ferredoxin, catalyses the stearoyl carrier protein (stearoyl-ACP) to remove two hydrogens to form oleoyl ACP [14]. The second unsaturated bond is introduced by the Δ12 desaturases based on the introduction of the first unsaturated bond, including both FAD2 and FAD6 [15]. Δ15 desaturases, including FAD3, FAD7 and FAD8, play a key role in further decreasing the degree of saturation [16, 17, 18]. In particular, the synthesis of linolenic acid is catalyzed by ω-3 fatty acid desaturases, in which the microsomal ω-3 fatty acid desaturase (FAD3) is located in the ER and uses phospholipids as acyl substrates, and NADH, NADH-cytochrome b_5 reductase and Cyt b_5 as electron donors. [19]

The enzyme encoded by the *FAD3* gene is a member of the ω-3 fatty acid dehydrogenase family, a membrane integrin distributed in the endoplasmic reticulum that catalyses the introduction of a third unsaturated bond. The FAD3 amino acid sequence is highly conserved with three histidine-rich conserved domains, which together with the divalent iron ions constitute the reaction centre of fatty acid dehydrogenase [20]. The Lys-Lys-X-X (KKXX) recovery signal contained at the C-terminus of the amino acid sequence is consistent with the properties of the endoplasmic reticulum integrin [20]. Since the first *FAD3* was isolated from *Arabidopsis* [21], researchers have successively cloned genes from plants such as rapeseed, perilla, peony and *Eucommia ulmoides*. In recent years, the functional regulation of *FAD3* in the synthesis and metabolism of unsaturated fatty acids has become a research hotspot.

With the release of walnut genome data, genes involved in the metabolism of walnut nutrients have been gradually discovered. A number of key enzyme genes involved in walnut fatty acid metabolism, such as ACCase [22, 23, 24], have been gradually cloned and characterized. In this research, walnut kernels at different stages of maturity were used as test materials and to analyse the key gene family involved in unsaturated fatty acid synthesis based on bioinformatics analysis and transcriptome sequencing. This study explored the formation mechanism of walnut fatty acids, especially the anabolic mechanism of unsaturated fatty acids, and provides a theoretical basis for the regulation of walnut fatty acid synthesis. Finally, this study can also provide a theoretical reference for the rational development of walnut oil and oil-producing walnut varieties.

2 Results

2.1 **Identification and analysis of FAD genes in the walnut genome**

We identified the FAD family using *Arabidopsis* FAD family protein sequence information to construct the hidden Markov model. The walnut protein data were searched, and 33 FAD family genes were screened in walnut. Using Pfam domain analysis, 24 FAD family genes were finally obtained, encoding 30 protein sequences, which were numbered according to their annotation information in walnut (Table 1).

Because of the limited depth of analysis of walnut genome data, we do not know the specific distribution of these genes in the 16 pairs of chromosomes in walnut. The shortest length among these *FAD* genes is 1262 bp, and the longest length is 14280 bp. The CDS lengths of these genes ranges from 453 bp-1368 bp, the lengths of FAD protein translation range from 150 to 455 amino acids, and their MW (molecular weight) range from 32.48 kDa (JrSLD-2) to 52.06 kDa (JrFAD7).

The length of *JrFAD3-1* was 2402 bp, its coding region was 1143 bp, and it encoded 380 amino acids, including 8 exons and 7 introns.

Table 1 Basic information of the FAD family in walnut

Gene	Gene ID	Gene				Amino acid		
		Gene length (bp)	Exon number	Intron number	CDS length (bp)	Protein length (AA)	PI	MW (KDA)
JrADS3	108998330	2497	5	4	1164	387	9.95	44.56
JrALD	109021249	2055	1	0	1344	447	8.56	51.34
JrDAL	109001694	4240	2	1	1149	382	8.78	44.11
JrDES1-1	109011517	2103	2	1	981	326	7.27	37.91
JrDES1-2	108990634	2623	2	1	978	325	7.83	37.74
JrFAD2-1	109021930	3411	3	2	1167	388	8.72	44.38
JrFAD2-2	109021930	3411	3	2	1167	388	8.72	44.38
JrFAD2-3	109011954	6737	3	2	1152	383	8.83	43.90
JrFAD2-4	109011954	6737	3	2	1152	383	8.83	43.90
JrFAD3-1	109002248	2402	8	7	1143	380	8.26	43.88
JrFAD3-2	108989905	2166	8	7	1053	350	7.79	40.51
JrFAD3-3	108989905	2166	8	7	1143	380	7.37	43.61
JrFAD3-4	108989903	2129	8	7	1077	358	7.8	41.43
JrFAD3-5	108989903	2129	8	7	1167	388	7.39	44.5
JrFAD4	108983937	1262	1	0	924	307	9.11	34.68
JrFAD6	108993197	4917	10	9	1329	442	9.18	51.38
JrFAD7	109007160	3038	8	7	1368	455	8.94	52.06
JrFAD8-1	108994930	2963	8	7	1368	455	8.98	51.77
JrFAD8-2	109015144	2965	8	7	1368	455	8.98	51.77
JrSAD-1	109005061	5401	3	2	1191	396	6.44	45.26
JrSAD-2	108984606	5286	4	3	1191	396	6.39	45.16
JrSAD-3	108984606	5286	4	3	1074	357	5.72	40.99
JrSAD-4	108984606	5286	4	3	999	332	5.4	38.05
JrSAD-5	108988959	2413	4	3	1176	391	7.72	44.43
JrSAD6	109012153	1549	2	1	1185	394	5.98	44.63
JrSAD-6	108989429	2075	3	2	1206	401	8.71	45.49
JrSAD-7	108988967	1981	3	2	1170	389	8.26	44.32
JrSAD-8	109018931	14280	4	3	1002	333	6.16	38.08
JrSLD-1	109009165	1905	1	0	1344	447	6.64	32.64
JrSLD-2	108002707	1846	1	0	1344	447	6.42	32.48

2.2 **Analyses of the evolution, exon-intron structure and motif distribution of JrFAD family members**

Using MEGA 5.0 software with the maximum likelihood method, the walnut FAD protein sequence was constructed together with the *Arabidopsis* FAD protein sequence to construct a phylogenetic tree (Fig. 1), indicating that the FAD gene families of *Juglans regia* and *Arabidopsis thaliana* are similar. There are

four main subfamilies: the SAD desaturase subfamily, Δ7/Δ9 desaturase subfamily, Δ12/ω-3 desaturase subfamily and "front-end" desaturase subfamily.

So far, SAD is the only subfamily of soluble enzymes found in the FAD family, and the remaining types of fatty acid desaturases are membrane integrins [25, 26]. There are 7 copies of the *SAD* gene in *Arabidopsis*, and 9 in walnut, and these genes are well clustered in the unified phylogenetic tree. In the Δ12/ω-3 desaturase subfamily, six branches of the walnut ω-6 desaturase and two from *Arabidopsis* were grouped together as Δ12 fatty acid desaturases; three *Arabidopsis FAD3* genes and eight annotated ω-3 desaturase genes in walnuts together formed the Δ12/ω-3 desaturase subfamily; in the five *FAD3* genes of walnut, *FAD3-1* was far from the other four evolutionary distances. By comparing the gene expression levels during the development of walnut kernels (Fig. 2), it was found that the expression of *FAD3-1* only was detected in the developing kernels, and the other four genes were not expressed or were expressed at very low levels, indicating that *FAD3-1* is a key enzyme gene that catalyses the synthesis of linolenic acid in walnut kernels. A similar expression profile was found in *FAD* genes of olive oil, and siRNA may act to suppress the expression of other *FADs* except *FAD3-1* [27].

To further investigate the structural evolution of walnut FADs, we first analysed the exon intron structure (Fig. 3 C). Analysis showed that the FAD genes contain introns, except for *JrALD, JrSLD-1, JrSLD-2* and *JrFAD4,* which had from 1-9 introns. Genes in the same subfamily have similar intron and exon structures. *FAD6* differs from other Δ12 desaturase genes in that it has the largest number of introns and exons. Both *DESI* genes had two exons, while the same group of *ALD* and *SLD* genes had only one exon. The number of exons in the SAD subfamily ranged between 2 and 4. *JrSAD-8* contained 4 exons, the highest amount, while *SAD6*, *SAD-3* and *SAD-4* contained two exons, and the remaining genes contained three exons.

Subsequently, we used MEME software to analyse the conservation of these protein sequences and structures (Fig. 3B). Most of the 20 conserved motifs (Fig. 3D) found belonged to the typical transmembrane helix region and unknown functional complex region in fatty acid desaturases. No common conserved motifs were found among the 30 members of the walnut FAD family, but the distribution of conserved motifs was very similar within different subfamilies. In the SAD subfamily, all genes except *JrSAD-8* contain conserved motifs 1, 3, 4, 7, and 10; in the Δ12/ω-3 subfamily, except for *JrFAD6*, the genes contain conserved motifs 2, 4, 5, 6, 8, and 9.

2.3 **Expression of genes related to unsaturated fatty acid synthesis**

A total of more than 1.7 billion reads (Supplementary Table 1) were sequenced across 9 libraries (70th, 90th and 110th DAF) by Illumina HiSeq X Ten, with Q20 values above 95% and GC content between 46-51%. More than 1.2 billion reads were aligned with the reference genome of walnut using the transcriptome data comparison by software STAR. The total alignment rate was 71.1%, indicating that the sequencing data obtained were of high accuracy and quality. Functional annotation was conducted on EggNOG database, and all data were located to 22708 transcripts in 22 subclasses of three

categories: cell process, signal transduction, information storage and processing, and metabolism (Supplementary Fig. 1).

The expression of genes (Supplementary Fig. 2) related to unsaturated fatty acid biosynthesis and arachidonic acid metabolism peaked at 110 DAF based on numeber of reads by transcriptome sequencing. The genes related to alpha-linolenic acid metabolism were first decreased and then increased and reached their maximum value at 110 DAF based on numeber of reads by transcriptome sequencing (Supplementary Fig. 2 , Supplementary Fig. 3A and 3B).

Seventeen genes enriched in the metabolic pathway of unsaturated fatty acid biosynthesis were selected (Fig. 3). *KCS2* (encoding 3-ketoacyl-CoA thiolase), *fabG* (encoding 3-oxoacyl-ACP reductase) are involved in carbon chain elongation; *fadE* (encoding acyl-ACP desaturase), *SSI2* (encoding acyl-ACP desaturase), *FAD2* (encoding fatty acid desaturase 2), *FAD3-1*(encoding fatty acid desaturase 3), *Acox1*(encoding peroxisomal acyl-CoA oxidase) and *SAD* (encoding stearoyl-ACP-desaturase)are involved in the desaturation process. The expression of 17 transcripts of these 8 genes can be found to be roughly divided into two categories. The expression level was lower at 70 DAF and higher at 110 DAF. There were 7 transcripts of *fabG, KCS2, fadE, FAD2* and *FAD3-1*, which were mainly involved in the biosynthesis of linoleic acid, linolenic acid and isounsaturated fatty acids. The other general trend is that the expression level was relatively high at 70 DAF and then gradually decreased. 10 transcripts of *fabG*, *KCS2*, *SAD*, *SSI2* and *FAD2* were mainly involved in the biosynthesis of oleic acid and linoleic acid. The expression of *FAD3-1* increased rapidly during the period from 70 DAF to 110 DAF. The dehydrogenation of linoleic acid to α- linolenic acid began 70 d after flowering while the α-linolenic acid content was almost zero, but the genes encodeing enzyme that can catalyze the dehydrogenase of linoleic acid to linolenic acid were highly expressed at this time. With the rapid increase in the expression of *FAD3-1*, the content of α-linolenic acid in the kernel began to increase gradually.

2.4 Tissue-specific expression of FAD family genes

By semi-quantitative detection (Fig. 4), *SLD-1* was found to be expressed in all 8 tissues, among which the expression levels were higher in catkins, old branches, mature leaves and kernels; *DAL* was most highly expressed in young and mature leaves. *JrFAD3-1* was expressed in catkins, young leaves and kernels, and the highest expression level was observed in mature embryos; *ADS3*, *SAD-2* and *SAD6* were only expressed in mature embryos. It can be preliminarily concluded that the FAD family is characterized by the desaturase subfamily and that the Δ7/Δ9 desaturase subfamily is specifically expressed in the embryo, while the "front-end" desaturase subfamily is expressed in all tissues. The Δ12/ω-3 desaturase subfamily is highly expressed in mature embryos. However, the determination of more specific expression patterns still requires further research and verification.

2.5 Expression of JrFAD3-1 and accumulation of α-linolenic acid in kernels at different developmental stages

The relative expression of *JrFAD3-1* increased slowly at 70 DAF in the 'QingXiang' kernel, and the relative expression increased rapidly after 90 DAF, peaking at 100 DAF (Fig. 5). Then, the expression quickly decreased, and it gradually stabilized at a lower level after 120 DAF. We also detected the content of α-linolenic acid in identical samples and found that the content of α-linolenic acid was maintained at a low level from 70 DAF to 95 DAF and gradually accumulated from 95 DAF to 120 DAF. It increased rapidly from 120 DAF to 130 DAF and peaked at 40.92 mg/g (Fig. 5), after which it decreased slightly. Combining the two results showed a 30-day difference between peak *JrFAD3-1* gene expression and peak of α-linolenic acid levels. The expression of *JrFAD3-1* at 70-100 DAF and 100-130 DAF was analysed. There was a significant positive correlation between the expression of *JrFAD3-1* and the content of α-linolenic acid at 5 periods (from 90-100 DAF) and the content of α-linolenic acid at 5 periods (from 100-130 DAF), with a correlation coefficient of 0.991. Therefore, it is possible that the decrease in *JrFAD3-1* expression and the increase in water content in the later stage are factors leading to the decline in the α-linolenic acid content in the later stage.

2.6 **Multiple sequence alignment of JrFAD3-1 proteins**

Multiple sequence alignment of the FAD3 proteins in 33 different species was performed using MEGA 5.0. The results show that FAD3 protein sequence was conserved between monocotyledonous and dicotyledonous plants and yeast. Conservative domain prediction was performed by a database search of the Pfam protein domain family, and all FAD3 proteins contained the fatty acid desaturase motif among 33 different species. Through comparative analysis, 62 completely conserved regions in the 33 protein sequences were found (Supplementary Fig. 4). It can be further confirmed that the primary structure of the JrFAD3-1 protein is closely related to the content of α-linolenic acid.

2.7 **Phylogenetic evolution of FAD3 proteins**

The retrieved homologous proteins were multiplexed with the JrFAD3-1 protein with MEGA 5.0 (Supplementary Fig. 4), and the reported *Olea europaea* (Genbank: AFX68461.1), *Glycine max* (Genbank: ABP01320.1; ABV00679.1; ACD56667.1), *Phaseolus lunatus* (Genbank: ADP07952.1), *Vigna unguiculata* (Genbank: ABY60736.1), *Betula pendula* (Genbank: AAN17504.1), *Corylus heterophylla* (Genbank: AEF80000.1), *Crepis alpina* (Genbank: ABA 55806.1), *Solanum lycopersicum* (Genbank: ABX24525. 1), *Eucommia ulmoides* (Genbank: ARQ20744.1), and *Arabidopsis thaliana* (Genbank: NP_180559.1) amount to 33 species that they all have a homology FAD proteins. Subsequently, we used MEME software to analyse the conservation of these protein sequences. Most of the 10 conserved motifs (Fig. 6) found belonged to the typical transmembrane helix region and unknown functional complex region in fatty acid desaturases. 3 common(motif2, motif3,motif8) conserved motifs were found among the 33 FAD3 of the 33 species, but the distribution of conserved motifs was very similar within different branches. In the 1 branches, 33 different species are divided into 2 branches(Fig. 7), *Candida tropicalis* is in one group only with 3 motif(motif2, motif3,motif8), and the other 32 plants are in one group; in the 2 branches, 32 different species are divided into 2 branches, *Paeonia suffruticosa* is a kind of flower or oil crop, which is divided into one group alone and the rest of the plants in one group; in the 2 branches,

Picea abies and Olea europaea are one group of higher plants with far-reaching evolution, while the rest are one group; in the 4 branches, the remaining 29 species were divided into 2 groups, 2 monocots (*Oryza sativa* and *Triticum aestivum*) and 27 dicots (*Glycine max*, *Phaseolus lunatus, Juglans regia* and so on). All FADs of 32 plants except *Crepis alpina*、*Physaria fendleri*、*Oryza sativa*, contain conserved motifs 1-10; the FAD3 of *Oryza sativa* contain conserved 8 motifs except motif6 and motif 9. JrFAD3-1 is closely related to *Betula pendula* and *Corylus heterophylla*. *Betula pendula*, *Corylus heterophylla* and *J. regia* all blong to the order Fagales, which further indicates that the conserved structure of FADs is consistent with classical plant architecture.

3 Discussion

To date, the FAD gene family has been identified and characterized in many plants, such including 17 FADs in *Arabidopsis* [21], 29 FADs in soybean[28], 31 FADs in peanuts[29], and 41 FADs in Raymond cotton [30]. The *FAD* gene was also isolated from land cotton[31], sesame[32], sunflower [33] and other plants. In this study, 24 FAD family members were identified and analysed based on their protein properties. Then, analysis of the genetic structure showed that each small branch has a similar genetic structure, and the subfamily is highly conservative. For example, the Δ12/ω-3 desaturase subfamily contains 7-8 exons, consistent with *Arabidopsis* [21] and *Carya cathayensis* [34]. There are 2-3 exons in the SAD subfamily, consistent with peanut [29]. Studies have shown that the origin of genetic introns is more ancient, and the insertion of introns is the result of exon rearrangement and plays an important role in gene evolution [35, 36]. By analysing the gene sequences, it can be found that the *JrFAD* family is highly conserved, but there are large differences between the subfamilies. The FAD family of walnut and *Arabidopsis thaliana* is composed of four major members of the SAD desaturase subfamily, the Δ7/Δ9 desaturase subfamily, the Δ12/ω-3 desaturase subfamily and the “front-end” desaturase subfamily [21]. However, there are large differences in the copy number of genes in each subfamily. The most common gene copy number of 9 was found in the Δ9 desaturase subfamily in *Arabidopsis* [21], but the gene copy number is only one in walnuts. Similar to the results in walnuts, Δ9 desaturase was not identified in the whole genome of soybean; instead, *SAD* [28], which also introduced the first unsaturated bond at Δ9 desaturase, was detected. This suggests that in more evolved higher plants, the Δ9 desaturase, which is widely distributed in the biological world, is replaced by the SAD subfamily with similar functions and more copy numbers, which may be related to the evolution of the *SAD* gene family in plants [21]. The number of members in the Δ12/ω-3 desaturase subfamily is also higher in walnut than in *Arabidopsis*, probably because these genes have not undergone related genome-wide replication during evolution.

Thus far, there is increasing evidence that the linolenic acid content is closely related to the *FAD3* gene [37, 38, 39, 40]. In *Arabidopsis* seeds, the specific overexpression of endogenous *FAD3* increased the α-linolenic acid content in the grain from 19% to 40% [41], while overexpression of *FAD3* genes in tomato resulted in α-linolenic acid levels being significantly increased in leaves and fruits of transgenic plants [42]. Ni *et al*.[43] proved that when *BnFAD3* was overexpressed, the content of α-linolenic acid in rapeseed increased. Studies have shown that RNAi technology inhibits the expression of the tobacco *FAD3* gene and can significantly reduce the accumulation of α-linolenic acid [44]. Liao *et al*.[45] inhibited the

expression of *FAD2*, *FAD3* and *FATB* in rapeseed by constructing a multi-gene interference vector containing the seed-specific promoter NapinA, which could increase the oleic acid content by more than 16%. The content of linolenic acid in walnut kernels was high, and 5 copies were identified in the genome-wide range. However, only *JrFAD3-1* expression was found in the seed kernel during development with the peak expression occurring approximately 100 DAF. Although *JrFAD3-1* is not a seed-specific gene, expression of this gene was significantly upregulated during seed development, showing a trend of first increasing and then decreasing, and tending to decline as the seed matures, consistent with the findings of previous studies [46, 47]. This change may be related to the important role of *FAD3* in the synthesis of unsaturated fatty acids in walnut [48]. In this study, after the expression of *JrFAD3* in walnuts, there may have been two reasons for the change in α-linolenic acid content after 30 days. First, a process in the cytoplasm may be involved. Protein must play a role in mRNA production and must be transported from the cytoplasm to the nucleus, and protein expression requires a process such as post-modification, that is, there is a time lag in the process of gene expression [49, 50]. Second, α-linolenic acid content is directly related to the amount of jasmonic acid produced, while ω-3 fatty acid dehydrogenase (FAD) can catalyse the synthesis of α-linolenic acid from linoleic acid [51]. There was a *JrFAD3-1* gene encoding Δ15 fatty acid desaturase in the seed, which catalysed the conversion of linoleoyl CoA to α-linoleyl CoA. Then, α-linolenic acid was transformed into jasmonic acid [52]. At this point, the protein content reached its highest level at 110 DAF and showed a positive correlation with the oil content [53]. It is also possible that the α-linolenic acid content caused by the lack of substrate in the early stage was not obvious in the early embryo development of walnut. As the embryo matured, the expression of *JrFAD3-1* was downregulated, and the α-linolenic acid content increased. In this process, the desaturation of the gene gradually decreased after the expression peaked, which may have been caused by the decrease in FAD3 activity and the consumption of α-linoleic acid. Through the verification of both positive and negative aspects, the gene function can be explained more comprehensively, which provides a theoretical basis for establishing a complete metabolic network.

4 Materials And Methods

4.1 Plant Materials

The test material was collected from Experimental Field of Hebei Agricultural University in 2016. The ‘Qingxiang’ variety, which has a full fruit bearing period, was selected as the test material.

The samples used for transcriptome sequencing were ‘Qingxiang’ kernels collected 70, 90 and 110 DAF. Nine samples of walnut kernel at each stages were gathered from ‘Qingxiang’ trees in the AUH Experimental Field. Several grams of different tissues from each plant were frozen in liquid nitrogen and then transferred to a freezer at -80°C.

FAD3-1 gene expression analysis was conducted with ‘Qingxiang’ samples from 10 different developmental stages, and 8 different tissue: pistils, catkins, young branches (bark), old branches (bark), young leaves, mature leaves, young embryos and mature embryos. After the fruits were collected, the

embryos were quickly removed by peeling and immersed in liquid nitrogen for quick freezing, and stored at -80 ℃ for RNA extraction, expression analysis and determination of fatty acid content.

The kits used for RNA extraction and DNA extraction were from Tiangen (product numbers: DP441 and DP350 respectively). The drugs used for reverse transcription, PCR and real-time PCR were from Takara (product numbers: RR047A, RR901A and RR820A, respectively).

4.2 Cloning of the JrFAD3-1 gene

The *JrFAD3-1* gene was amplified in kernels collected 100 DAF. *JrFAD3-1* partial gene sequence was identified from walnut genome data. So, homology-based cloning method was used to isolate *JrFAD3-1* coding sequence (CDS) from 'Qingxiang' walnut. According to the gene sequences of Soybeans and Castor beans. Premier 5.0 software was used to design specific primers. The *JrFAD3-1* gene specific primers as follow:

JrFAD3-1-F: 5'- GAGAAAGGAGGAGAGAATGC-3',

JrFAD3-1-R: 5'- GATAGCCTTGCTCTTCAAA-3'.

4.3 Identification of *FAD* Gene Family and Bioinformatics Analysis

The hidden Markov model was constructed using HMMER 3.1b software from the *Arabidopsis* FAD family protein sequence in the UniProt database and the protein sequence of walnut was downloaded from NCBI (www.ncbi.nlm.nih.gov/genome/?term=txid51240[orgn]). The conserved domains of the proteins were analysed using the PFAM protein family database [54] of the European Institute of Bioinformatics, using the online analysis tool Gene Structure Display Server [55] and MEME pair (http://meme-suite.org/) for the walnut FAD family. The *JrFAD* and *AtFAD* family phylogenetic trees were constructed using the maximum likelihood (ML) method in MEGA 5.0 software with a bootstrap value of 1000. The JrFAD and 31 other branches of phylogenetic trees were constructed using the maximum likelihood method in MEGA 6.0 software with a bootstrap value of 1000.

4.4 Data analysis of transcriptome sequencing

An Illumina high-throughput sequencing platform (HiSeq X Ten) was used for sequencing.

The RNA coding potential was predicted using CPAT v.1.2.2 software. The predicted coding transcripts were aligned with the GenBank NR, Swiss-prot, eggNOG, InterPro, GO, and KEGG databases for functional annotation. Differentially expressed genes were identified using R with the edgeR package, and q-values < 0.05 indicated differentially expressed genes.

4.5 Tissue-specific expression in the FAD family

For RNA isolation experiments, all samples were immediately frozen in liquid nitrogen and extracted using a Tiangen Plant RNA Extraction Kit according to the protocol. First-strand cDNA was synthesized

with a Tiangen Inverse Transcription Kit. The gene-specific primers used for RT-PCR and qRT-PCR were designed using NCBI (Table 2). Five genes belonging to four subfamilies were selected to analyse the expression of the FAD family in different tissues.

The PCR conditions were as follows: 94°C for 5 min; 33 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min; and 72°C for 10 min.

Table 2 Primers used in this study

Gene name	Sequence （5′–3′）
ACT2	F: TCCACCATGTTCCCTGGTAT; R: ACCTCCCAATCCAGACACTG
SLD-1	F: ACTTGGTTTGGGAGGCTGTC; R: TCGTCCTAACAACCCCAAGC
ADS3	F: TTCCTTGTGTGGGGAATGGG; R: AAATGCAAGCAATGCCACCC
SAD-2	F: TTTTTCCCCTTCTCAACCTCCAA; R: TGGTGTAAAAGGCTTCTTTGGATTG
SAD6	F: AAGCTGGAGGATTTGACGGG; R: CCAGCTAAACTTAACGCCGC
FAD3-1	F: ATCCACCATGATATCGGCACC; R: GAAGGGGAATTGGCCCAGA

4.6 Gene expression analysis by quantitative RT-PCR

The qRT-PCR experiments were performed on an FX96 Touch™ Real-Time PCR Detection System (Bio-Rad) using a SYBR Green I Master Kit (Tiangen). *JrActin* was used as the reference gene to calculate the relative fold differences based on the comparative cycle threshold ($2^{-\Delta\Delta ct}$). The PCR solution (20 μl) contained 10 μl of SYBR-Green PCR Master Mix (Tiangen), 0.2 μM of each primer, 100 ng of cDNA template, and nuclease-free water. The PCR conditions were as follows: 95°C for 5 min; 45 cycles of 94°C for 10 s, 60°C for 30 s, and 72°C for 30 s; followed by a final extension at 72°C for 3 min.

4.7 Determination of fatty acid content

The determination of unsaturated fatty acids referred to Bai [56]

4.8 Statistical Analysis

The data were analysed using SPSS 17.0 software.

5 Conclusions

24 members of the *JrFAD* gene family were identified by bioinformatics analysisfor the first time, it consists of four subfamilies: the SAD desaturase subfamily, Δ7/Δ9 desaturase subfamily, Δ12/ω-3 desaturase subfamily and "front-end" desaturase subfamily; A key gene *FAD3-1*, was obtained based on transcriptome data and its expression was analyzed, there was a significant positive correlation between the expression of *JrFAD3-1* from 90-100 DAF and the content of alpha-linolenic acid from 100-130 DAF. The function of a walnut kernels *FAD3-1* gene was also characterized from its location in the phylogeny. This study provides significant information for further in-depth molecular investigations into the biological of these genes.

Abbreviations

ADS:Arabidopsis desaturase; FAD:Fatty acids desaturase; SAD: Stearyl acyl-carrier-protein desaturase; DES: delta(7)-sterol-C5(6)-desaturase; ALD: acyl-lipid (9-3)-desaturase; SCD: stearoyl-CoA-desaturase; ACCase: Acetyl coenzyme A carboxylase; KCS2: 3-ketoacyl-CoA thiolase; fabG: 3-oxoacyl-ACP reductase; fadE: acyl-CoA dehydrogenase; ALD: acyl-lipid (9-3)-desaturase; ACP: acyl-carrier protein; DAL: delta(12)-acyl-lipid-desaturase; DES:sphingolipid delta(4)-desaturase; SLD: delta(7)-sterol-C5(6)-desaturase; ML: maximum likelihood; DAF: days after flowering

Declarations

Ethics approval and consent to participate

Walnut is one of widespread fruit trees in China, and it is not an endangered species. The walnut trees were from the Experimental Field of Hebei Agricultural University. No specific permits are required for sample collection on walnut.

Consent for publication

Not applicable.

Availability of data and materials

All data and materials are presented in the main paper and additional supporting file.

Competing interests

The authors declare that they have no competing interests.

Funding

Supported by the Natural Science Foundation of Hebei Province (C2019204270), the Science and Technology Research Project of the Universities of Hebei Province (QN2019159), and Research Foundations for Returned Scholars from Overseas of the Human Resources Dept. of Hebei Province (C20190345). These funding bodies had no role in the design of the study, sample collection, analysis or interpretation of data and in writing the manuscript.

Authors' contributions

HW and ZZ designed the research and wrote the paper. KL and SZ performed the experiments, analyzed the data and wrote the paper. SW participated in the data analysis.

KL and SZ are equal contributors. All authors read and approved the final the manuscript.

Acknowledgements

Not applicable.

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Figures

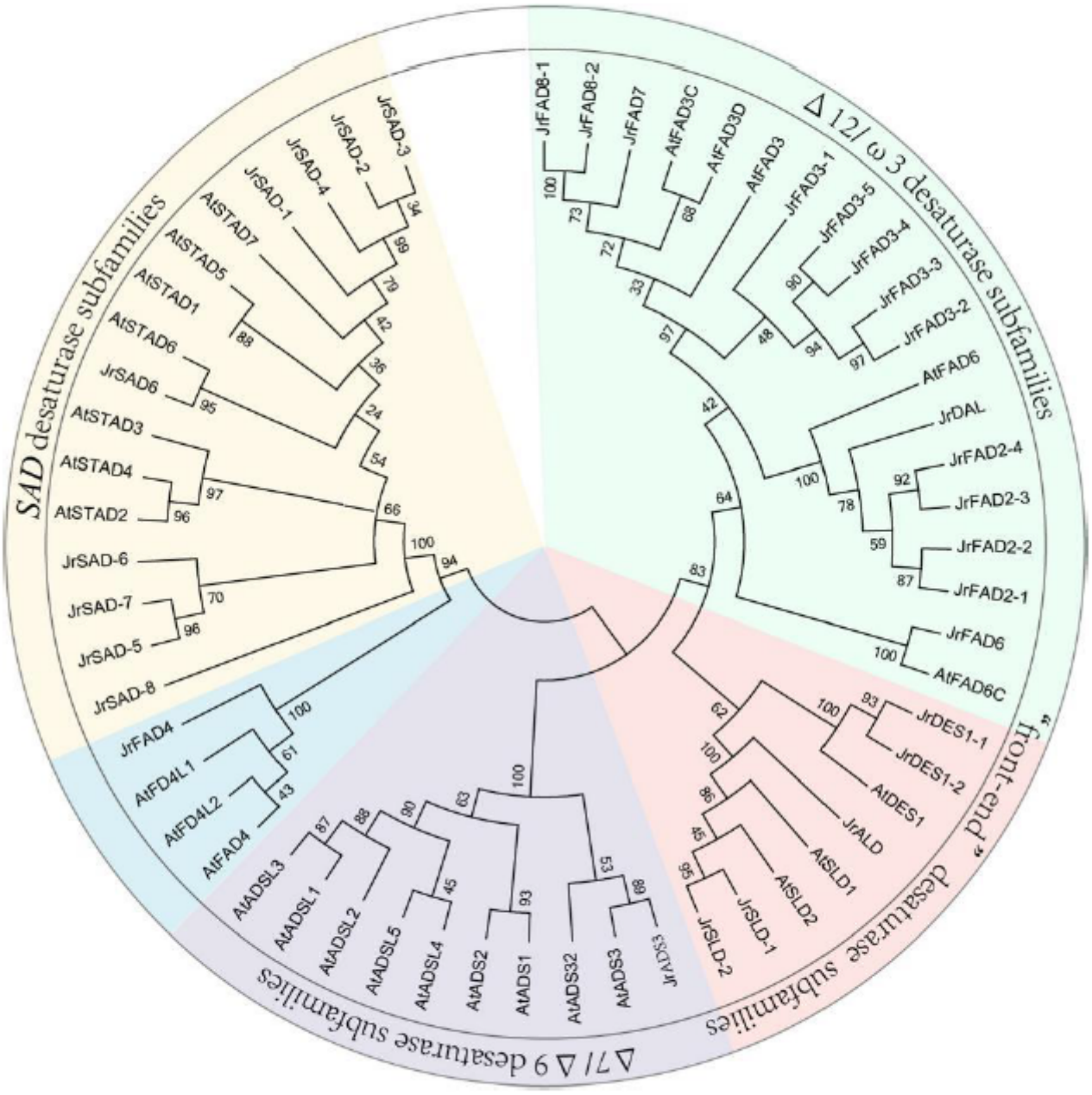


Figure 1

Phylogenetic analysis of Arabidopsis thaliana and Juglans regia FADs

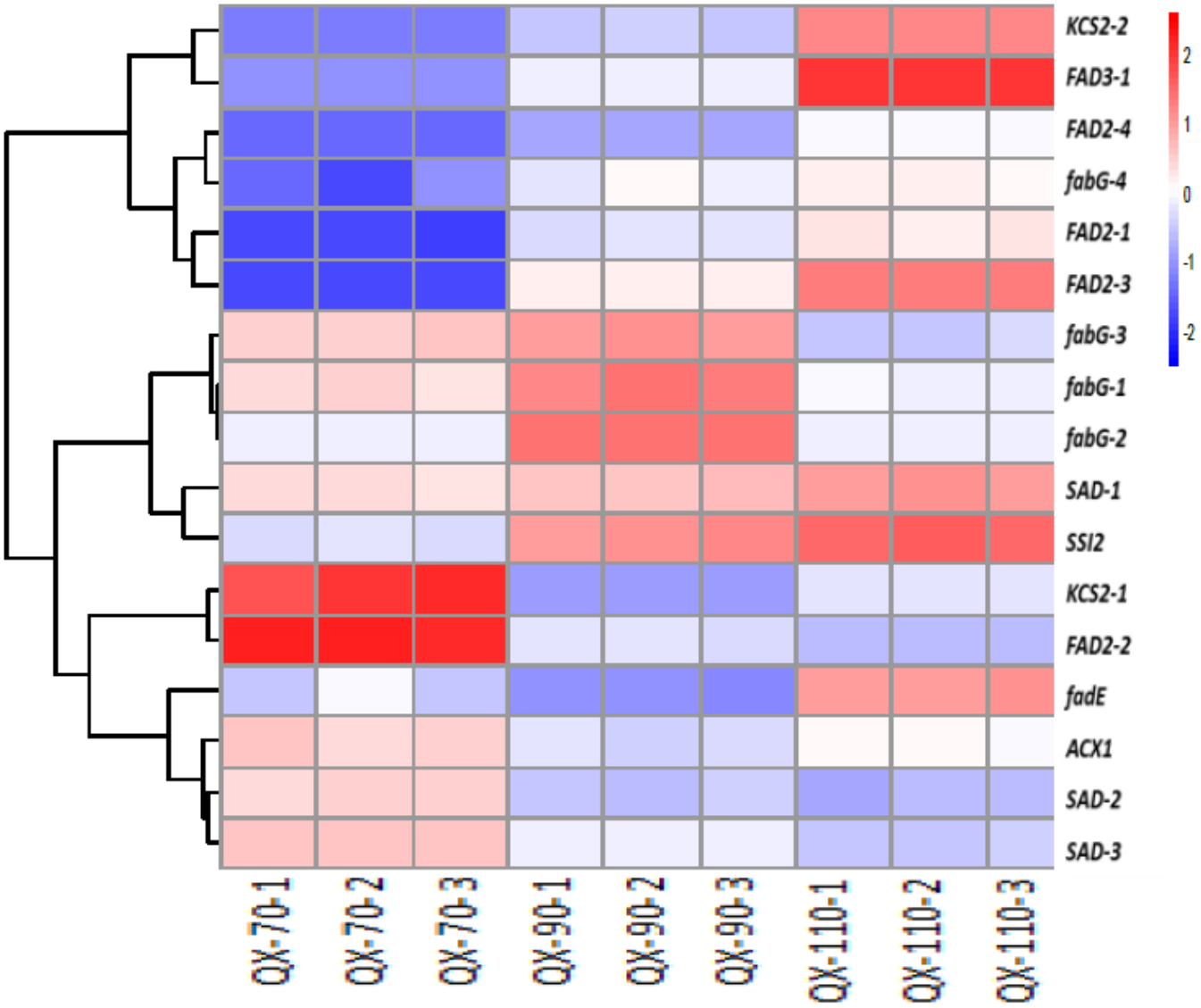


Figure 2

Expression profiles (heatmaps) of genes associated with unsaturated fatty acid biosynthetic

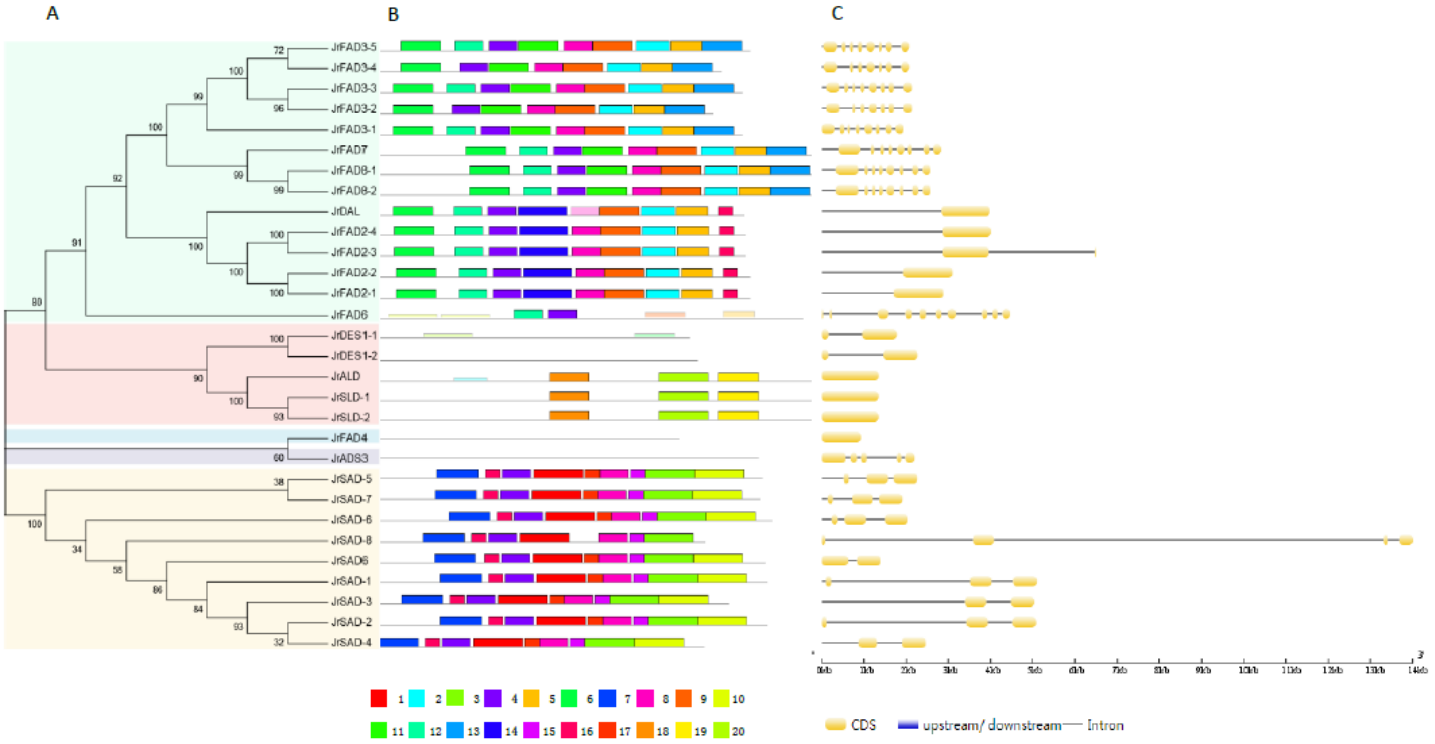


Figure 3

Gene structure and conserved motif analysis of the FAD family from Juglans regia Note: A: Phylogenetic analysis of JrFAD family; B: Conserved motif of JrFAD family; C Gene structure of JrFAD family

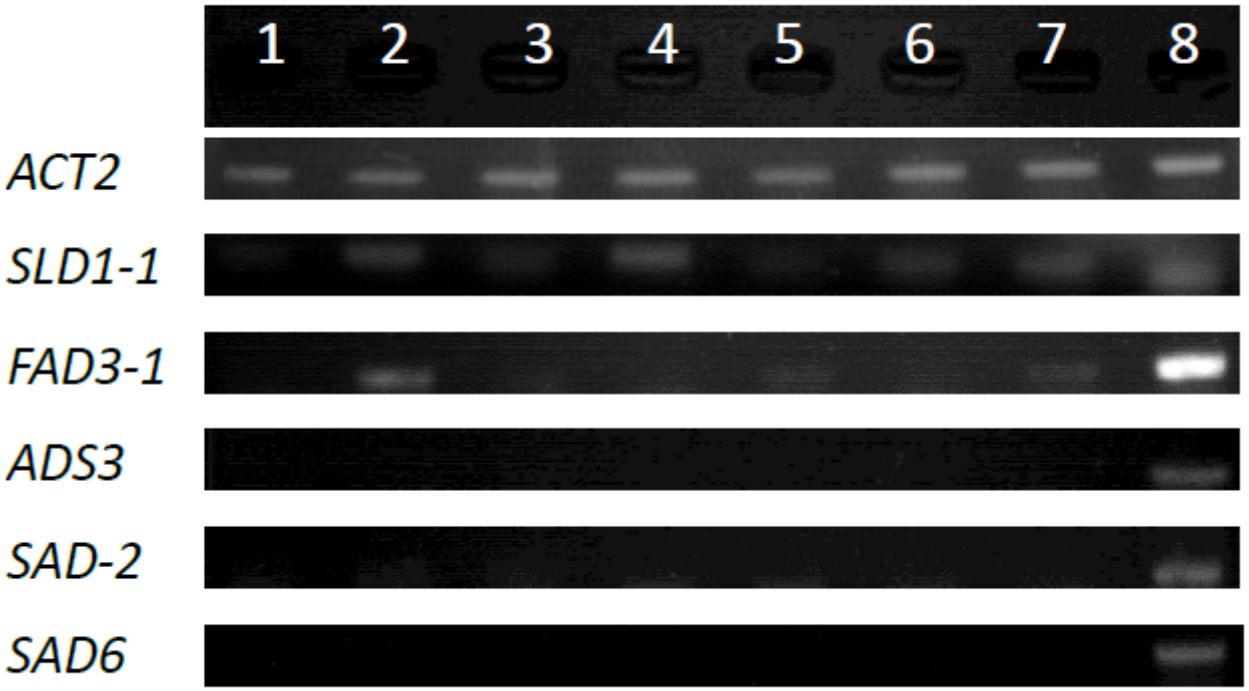


Figure 4

Tissue-specific expression of walnut FAD family Note：1: pistils; 2: catkin; 3: young stem; 4: older stem; 5: young leaves: 6: mature leaves; 7: young embryo; 8: mature embryo

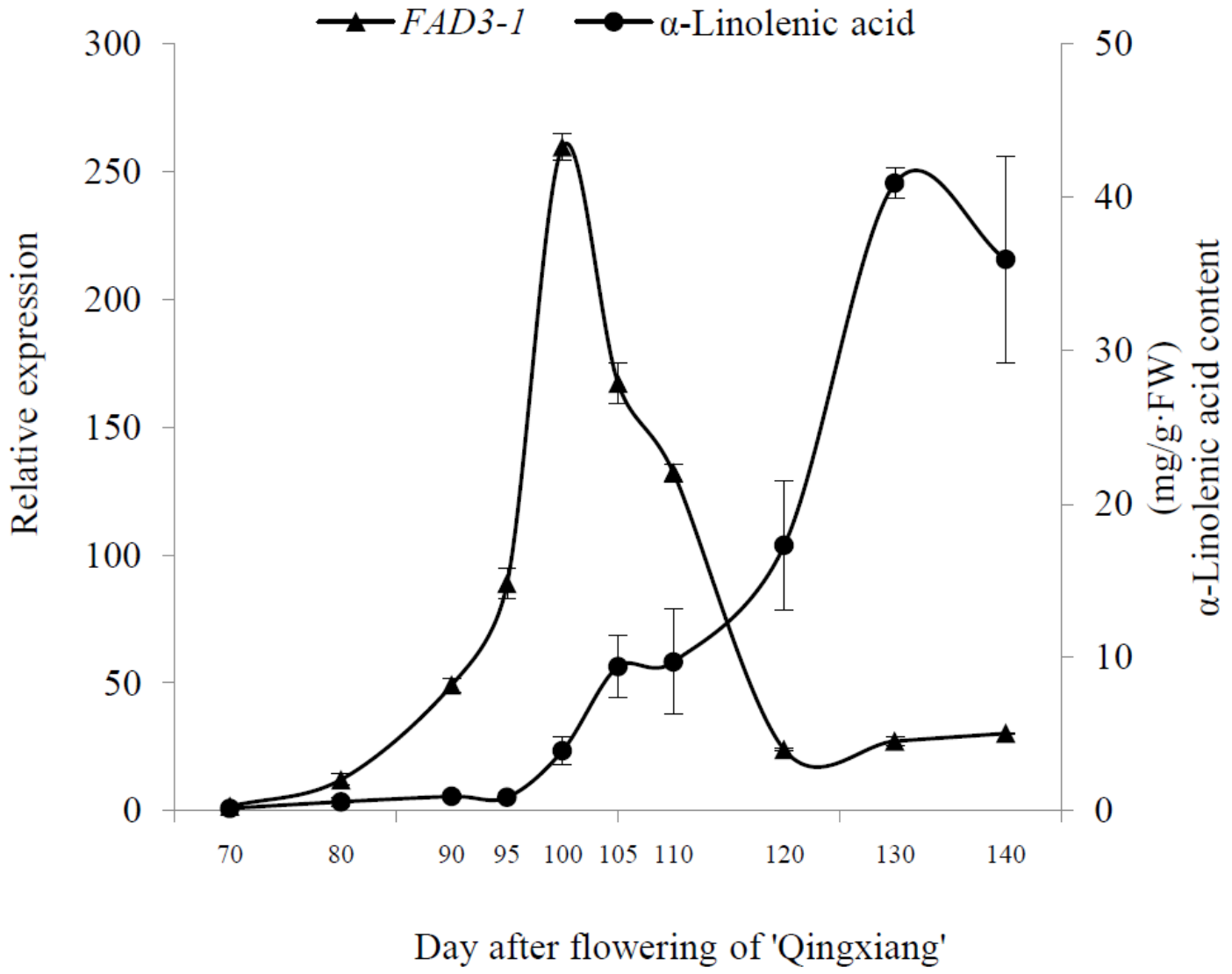


Figure 5

Expression of FAD3-1 and content of α-Linolenic acid in ‘Qingxiang’

Figure 6

Motif of FAD3 proteins in 33 different species

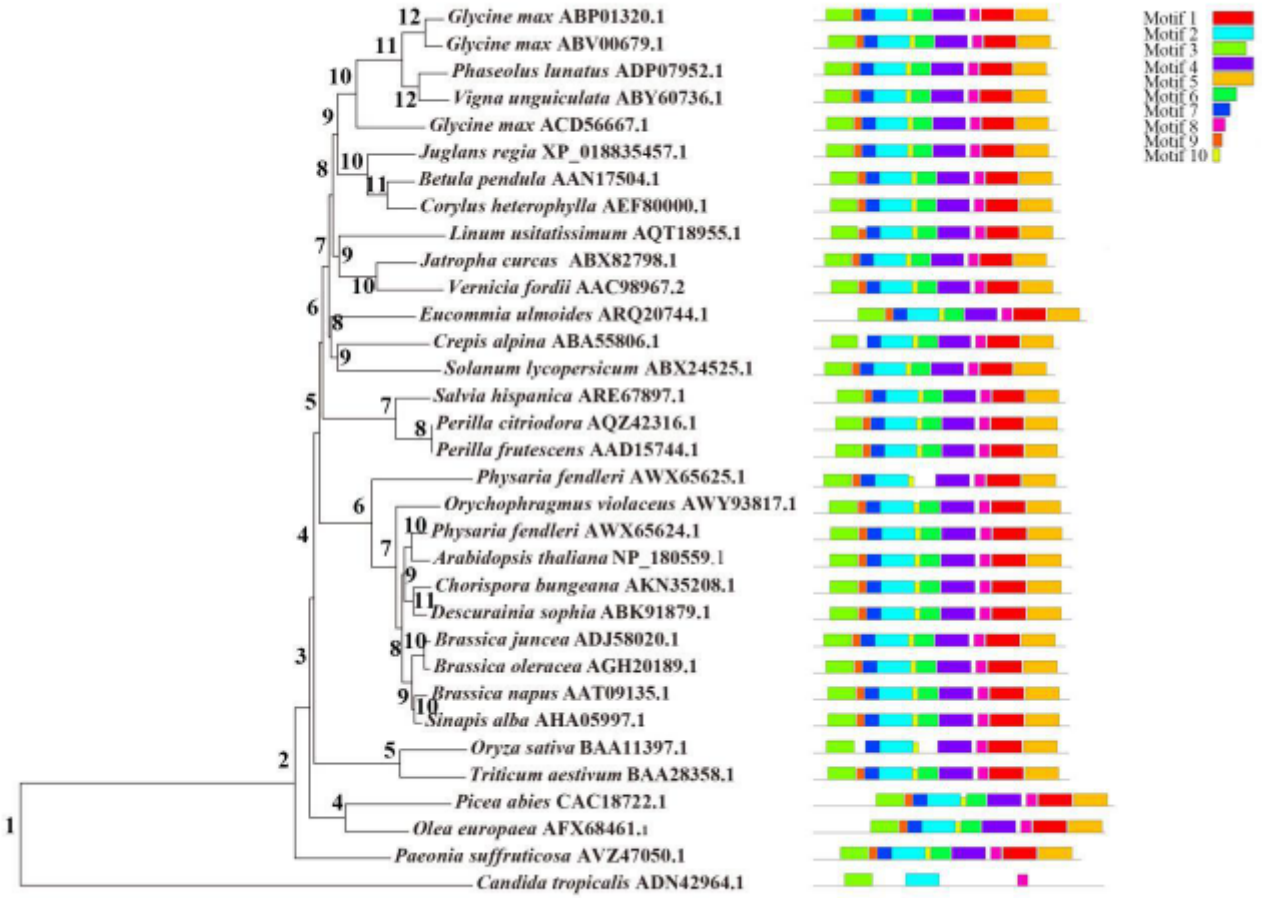


Figure 7

P hylogeny conserved motif of FAD3 proteins in different species Note:Number represents branch series

Supplementary Files

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