



Article

Transcriptional Analysis of Different Cultivars and Genome-Wide Identification of FAD Gene Family in Tree Peony

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Abstract: The tree peony (*Paeonia ostii*), a newly recognized woody oil plant endemic to China, is noteworthy for its high content of unsaturated fatty acids (UFA), particularly alpha-linolenic acid (ALA). Fatty acid desaturases (FADs) are integral to plant development and defense mechanisms. Nonetheless, there is limited understanding of (i) the molecular mechanism underlying FA biosynthesis in various varieties during seed maturation and (ii) a genome-wide analysis of FAD family genes within the tree peony. We selected three distinct cultivars of tree peony for transcriptome sequencing and performed an extensive analysis of *PoFAD* genes. In total, 67,542 unigenes were acquired and annotated with six protein databases available to the public. Forty-one differentially expressed genes (DEGs) pertinent to FA biosynthesis and lipid metabolism were identified in this study. Notably, genes such as *PoFAD2*, *PoFAD6*, and *PoSAD* were found to be significantly upregulated, contributing to a differential linolenic acid and linoleic acid content across the three cultivars. Herein, 24 *PoFADs* from the *P. ostii* genome were recognized and categorized into four distinct clusters according to their conserved structural features. The distribution of *PoFADs* was found to be random and uneven across five chromosomes, indicating a complex genomic architecture. Six colinear gene pairs were found between *P. ostii* and *V. vinifera*, indicating a potential link due to their close relationship. Together, these findings significantly enhance our knowledge of the molecular processes governing fatty acid synthesis, elucidate the functional roles of the FAD gene family, and lay the groundwork for using genetic manipulation to boost lipid levels.

Keywords: tree peony; transcriptome; α -linolenic acid; FAD gene family



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1. Introduction

Edible vegetable oils are essential for human health, providing energy and critical nutrients that support bodily functions [1]. Among these nutrients, alpha-linolenic acid (ALA) stands out as a vital omega-3 fatty acid that the human body cannot synthesize independently, thereby necessitating its acquisition through plant-based food sources [2]. It promotes brain development [3] and protects against inflammation [4], cardiovascular diseases [5], and neurological diseases [6]. Consequently, the quest for plants with a high content of edible oil and elevated levels of ALA is vital for ensuring a sufficient supply of edible oils.

Fatty acid desaturases (FADs) are key enzymes that facilitate the introduction of double bonds into fatty acid chains, leading to the formation of unsaturated fatty acids

(UFAs). These enzymes are vital not only for the overall growth and development of plants, but also for their ability to respond to environmental stressors [7]. FADs can be categorized based on their solubility, with soluble desaturases, such as FAB2/SAD, and membrane-binding desaturases, like FAD [8]. The latter, which are localized on the membranes of the endoplasmic reticulum and plastids, can be divided into four distinct subfamilies depending on their functional characteristics, including FAD2/FAD6, FAD4, FAD3/FAD7/FAD8, and ADS/SLD/DES. The sequences of amino acids in FADs exhibit a high degree of conservation within the same subfamily.

In crop seeds, the conversion of oleic acid to linoleic acid, and subsequently to α -linolenic acid, is facilitated by the enzymes FAD2 and FAD3, respectively [9,10]. Research on FAD genes has included a variety of plant species, such as arabidopsis [11], flax [12], and peanut [13]. The FAD genes are categorized into either small or large gene subfamilies, with expression patterns that can differ between specific tissues and exhibit a general constitutive expression. For instance, in flax, the genes *FAD2b-1*, *FAD2b-2*, *SAD2-1*, and *SAD2-2* demonstrate high expression levels across all tissues [14]. In contrast, the expression of the two *OeFAD2* genes during the ripening of olive fruit is nearly undetectable [15]. Within the *FAD2-2* gene in olives, structural variants are thought to influence the regulatory mechanisms associated with variations in the oleic and linoleic acid levels [16]. Furthermore, in chia, the amplification of the *ShSAD* gene family through tandem duplications is proposed to significantly contribute to the accumulation of high levels of ω -3 fatty acids [17].

A number of studies have documented the presence of various members of the FAD family across different plant species. For instance, the genomes of rice and chickpeas encompass 20 and 18 FAD genes, respectively [7,18]. In contrast, the *Camelina sativa* genome possesses 24 FAD genes [19], and the wheat genome contains 68 genes [20]. Despite many plant species having been the subject of genome-wide studies on this gene family, there remains a significant gap in the literature concerning a thorough evaluation of the FAD family's role in tree peony.

The tree peony, a decorative plant from China, has significant ornamental and medicinal properties and has been cultivated in China for over 1600 years [21]. Nowadays, the oil potential of tree peony seeds has garnered increasing interest, positioning them as a promising source of economically viable vegetable oil, particularly due to their rich concentrations of UFA (>90%) and ALA (>45%) [22]. The content and quality of oil in tree peony seeds can fluctuate considerably, influenced by species, cultivars, cultivation techniques, geographical locations, and individual plants [23–27]. Thus, it is essential to investigate the key enzymes and regulatory pathways involved in fatty acid biosynthesis in peony seeds, paving the way for the transgenic enhancement of cultivars that exhibit an improved oil content and quality.

Previous studies have primarily focused on identifying the fatty acid composition [28,29], the extraction process [30–32], and the mechanisms underlying ALA synthesis in wild species, including *P. rockii*, *P. lutea*, and *P. ostii*. Transcriptome and proteome sequencing have been employed to analyze several genes and transcription factors associated with lipid biosynthesis and accumulation in the developing seeds of tree peony, such as *PrFAD2* [33], *PrFAD3* [33,34], *PoFAD6* [33], *PoFAD8* [35], *PoSAD* [36], *PoPDAT* [37], *PoWRI1* [38], *PrNF-YC2* [39], *PrPDAT2* [40], and *PrDREB2D* [41]. However, the molecular mechanisms of synthesizing fatty acid (FA) across various cultivars through transcriptome sequencing have not yet been documented in tree peony.

Thus, we chose three tree peony cultivars to undergo high-throughput transcriptome sequencing to identify the potential candidate genes linked to FA biosynthesis. Furthermore, our investigation culminated in a thorough analysis encompassing various aspects, including the phylogenetic analysis of the genes, their structural characteristics, conserved motifs, chromosomal distribution, synteny, and the presence of *cis*-acting elements within the promoters of the *PoFAD* genes. Finally, we examined the expression profiles of these genes across different stages of seed development.

Together, the insights gleaned from this research pave the way for a deeper understanding of the molecular mechanisms underlying fatty acid biosynthesis, as well as the evolutionary dynamics and biological roles of the FAD gene family in tree peony. This knowledge will also be valuable for advancing genetic engineering efforts aimed at enhancing the oil content in tree peony.

2. Materials and Methods

2.1. Plant Material

This study utilized three tree peony varieties from distinct variety groups. The flowers of 'FD' ('LU NONG DAN No.1', Jiangnan variety group) are white and the fruits consist of eight carpels. In contrast, the flowers of 'CZR' (Zhongyuan variety group) and 'JL' (Japan variety group) are red, with both varieties containing five carpels. These varieties were introduced to Baihua Garden (35°16'23" N, 115°29'26" E) in Heze, China, where they were cultivated in open-air conditions under uniform environmental conditions for 7 years (Figure S1). Senior agronomist Mr. Wenhai Sun from Baihua Garden identified all the materials.

Each August, as the peony follicles matured, the color of the seeds within the pods transitioned from white to brown, ultimately becoming black when ripe. At this stage, mature peony seeds were manually collected from three individual plants, serving as three biological replicates. A portion of these mature seeds was snap-frozen in liquid nitrogen and stored at -80°C for RNA sequencing, while the remaining seeds were dried to a constant weight, ground into a powder (60-mesh), and stored at -4°C for fatty acid composition analysis.

2.2. Identification and Quantification of Major Fatty Acid Compositions

The fatty acid content in the seeds of the three different cultivars was measured using gas chromatography-mass spectrometry (GC7890/MS5975, Agilent, Santa Clara, CA, USA) following well-established analytical protocols [42]. The process began with a methyl esterification reaction, which is essential for transforming fatty acids into their corresponding fatty acid methyl esters (FAMES). To initiate this reaction, a 4 mL solution of 0.5 mol L^{-1} sodium hydroxide in methanol was added to 240 μL of peony seed oil. This mixture was then incubated in a water bath maintained at 60°C for approximately 30 min to ensure the complete dissolution of the components. Afterward, the solution was cooled to room temperature before adding 2 mL of a 5% H_2SO_4 -methanol solution. This new mixture was subsequently heated in a water bath at 60°C for 10 min to facilitate the esterification process. Finally, the mixture was allowed to cool, after which 2 mL of each n-hexane and saturated NaCl solution were added. The two-phase system resulting from this addition was allowed to separate, and the upper liquid phase was carefully collected. To ensure the removal of any residual water from this phase, it was treated with an appropriate quantity of anhydrous sodium sulfate and subsequently centrifuged. For the subsequent analysis, a precise volume of 0.9 mL from the upper liquid phase, along with 0.1 mL of an internal standard solution, was transferred into a sample vial. This mixture was filtered through a $0.22\text{ }\mu\text{m}$ filter membrane and subjected to GC-MS analysis on an HP-88 capillary column ($30\text{ m} \times 0.25\text{ mm}$, $0.20\text{ }\mu\text{m}$, Agilent).

The column temperature was initially set to 100°C for 2 min, then increased to 230°C at a rate of 15°C per minute and held for an additional 5 min. The injector temperature was maintained at 260°C for split injection (with a split ratio of 10:1) using a 1 μL injection volume. Helium was employed as the carrier gas, delivered at a flow rate of 1 mL per minute. The ionization potential of the mass-selective detector was 70 eV, and the scan range was from 30 to 450 amu, resulting in a scan time of 0.2 s.

Finally, the identification of fatty acids was achieved by searching a mass spectrum database (NIST 2011) and comparing the results with co-eluted components from a five-component FAME mix (Sigma-Aldrich, Saint Louis, MO, USA). A quantitative analysis was performed using a standard curve method with an internal standard, via the least

squares method. The five major FAs present in each sample were quantified in absolute terms through linear regression against their respective standards, with results reported as milligrams per gram of dry weight of the sample. Each sample underwent the analysis in triplicate.

2.3. RNA Sequencing and De Novo Assembly

We extracted the total RNA from the nine samples using RNA Extraction Kits (E.Z.N.A. HP Plant RNA Kit, Omega Bio-Tek, Norcross, GA, USA), ensuring the integrity of the samples was maintained throughout the process. The content and integrity of the extracted RNA were evaluated using an Agilent 2100 Bioanalyzer (RNA Nano Chip, Agilent, Santa Clara, CA, USA). Using mRNA as a template, random hexamer primers facilitated the synthesis of double-stranded cDNA, which was subsequently amplified via PCR. The resulting PCR product was purified with VAHTSTM DNA Clean Beads to generate the final library. The sample library was quality-controlled using the Agilent 2100 Bioanalyzer and an ABI StepOnePlus Real-Time PCR System (Thermo Fisher, Waltham, MA, USA). Ultimately, the generated libraries were sequenced using an Illumina HiSeq 2000 (San Diego, CA, USA) paired-end sequencing platform. Following sequencing, the raw reads generated were subjected to a filtration process using SOAPnuke (v1.5.2) to obtain clean reads. These clean reads were then assembled *de novo* with the Trinity software (v2.0.6). The Bowtie2 tool was employed to analyze the filtered reads acquired post-sequencing. Next, we utilized RSEM (v1.2.8) in order to estimate the transcript abundance.

2.4. Functional Annotation, GO, and KEGG Enrichment Analysis

We used several functional databases, including the Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), the non-redundant protein database (NR), the non-redundant nucleic acid sequence database (NT), and the Swiss-Prot protein sequence database to provide annotations for all unigenes. Additionally, Blast2GO and NR were employed to further annotate the GO terms.

2.5. Expression Analysis of Differentially Expressed Genes (DEGs)

The DESeq2 software was employed to assess the genes that were differentially expressed [43]. Reads per kilobase of transcript per million mapped reads served as the metric for the normalized levels of gene expression. We determined significant differences in gene expression by establishing a criterion whereby a p-value of less than 0.05, combined with an absolute logarithm of the fold change greater than 1 ($|\log_2 \text{ratio}| > 1$), served as our threshold for identifying noteworthy gene expression alterations. The differentially expressed genes underwent GO and KEGG enrichment analysis to classify their functionalities and biological pathways. A p-value was calculated for each gene set, and an FDR of less than 0.05 was utilized to ascertain the significance of the enrichment results.

2.6. Identification and Phylogenetic Analysis of PoFAD Gene Family

The genome sequence and annotated gff3 files for *P. ostii* were obtained from the China National Gene Bank (<https://ftp.cnbg.org/pub/CNSA/data5/CNP0003098/CNS0560369/CNA0050666/>) (accessed on 25 June 2024) [44]. Genomic data of *Arabidopsis thaliana* and *Vitis vinifera* were sourced from TAIR (<http://www.arabidopsis.org>) (accessed on 25 June 2024) and the Ensembl Plants database (<http://plants.ensembl.org>) (accessed on 25 June 2024), respectively. The FAD protein sequences from *A. thaliana* were downloaded and employed as queries in the BLATSP to search for *PoFADs*, incorporating an e-value threshold. Each putative *PoFAD* was confirmed by TBtools-II software v2.119, and the genes lacking a complete domain were excluded [45].

A phylogenetic tree was constructed using MEGA X software v10.2 for aligning multiple sequences, utilizing the neighbor-joining method and 1000 bootstrap replicates for support values [46]. Additionally, the tree visualization was conducted using the iTOL v4 web server [47].

2.7. Conserved Motif and Gene Structure of PoFAD Genes

The gene structure and motif analyses of the *PoFADs* gene family were facilitated by TBtools-II software v2.119 and MEME v5.1.0 (<http://meme-suite.org/tools/meme> (accessed on 28 June 2024)), respectively. The gene structure features were analyzed by searching for the *PoFAD* family genes in the gene annotation gff3 file. Visualization was performed with the ‘Visualize Gene Structure and Gene Location Visualize plugins’ in TBtools-II software v2.119.

2.8. Cis-Elements, Chromosome Location, and Collinearity Analysis of PoFAD Genes

TBtools-II software v2.119 was employed to arrange the upstream sequences (2000 bp) and analyze the collinearity relationships among *PoFAD* genes across different species. The structured data were then utilized to predict *cis*-elements via the Plant Care database.

2.9. Expression Pattern of PoFAD Genes from the Transcriptome Database

We downloaded RNA-seq data corresponding to each *PoFAD* gene from the *P. ostii* genome database (<https://db.cngb.org/search/Project/CNP0003098> (accessed on 25 June 2024)), including different growth stages (35, 49, 63, 77, 91, and 119 DAF) of tree peony seeds. Normalized gene expression data were used to generate a heatmap with TBtools-II software v2.119.

3. Results

3.1. Transcriptome De Novo Assembly and Functional Annotation

We selected three different tree peony cultivars for transcriptome sequencing to investigate the candidate genes underlying the fatty acid biosynthesis in tree peony seeds. The transcriptome yielded over 452 million raw reads from the nine samples. Our quality assessment indicated that the sequence data were of high integrity, with Q20 scores exceeding 96% and Q30 scores surpassing 91% (Table S1). After assembly and removing redundancy, 126,306 unigenes were obtained, yielding an N50 value of 1555 base pairs (bp) and an average length of 941 bp (Table S2).

The transcriptomes were mostly identified by conducting BLASTX searches on NR and SwissProt databases, and BLASTN searches on the Nucleotide Sequence Database (NT) using an E value cutoff of 10^{-5} . Among them, 63,473 unigenes (50.25%) were annotated by NR databases, 54,628 (43.25%) were matched to NT, 39,745 (31.47%) were annotated in Swiss-Prot, 37,970 (30.06%) were aligned to KEGG, and 25,318 (20.04%) were annotated in COG. In addition, 47,157 (37.34%) were annotated in GO. In summary, 53.47% of the total unigenes (67,542) were present in the databases (Table S3).

After NR annotation, the proportion of annotated unigenes across different species was evaluated. As illustrated in Table S2, the unigenes from *P. ostii* exhibited the highest similarity with *Vitis vinifera* (48.63%), succeeded by *Prunus persica* (11.79%), *Ricinus communis* (7.35%), and *Populus trichocarpa* (6.50%) (Table S4).

3.2. Differentially Expressed Genes (DEGs) and Functional Annotation in Three Varieties

To identify the DEGs of seeds in tree peony, two pairwise comparisons were conducted: FD vs. CZR and FD vs. JL. A total of 5433 differentially expressed genes were discovered between the FD and CZR groups, comprising 4197 that were upregulated and 1236 that were downregulated. Similarly, the comparison of the FD group with the JL group resulted in the identification of 7610 DEGs, of which 5178 were upregulated and 2432 were downregulated (Figure 1A). Additionally, cross-comparisons between the two groups revealed 2433 DEGs that were common to both comparisons. Notably, 3000 genes were uniquely expressed in the FD vs. CZR comparison, while 5177 genes were uniquely expressed in the FD vs. JL comparison (Figure 1B). The expression trends of the DEGs showed notable variations between the two groups, indicating distinct regulatory mechanisms involved in lipid biosynthesis.

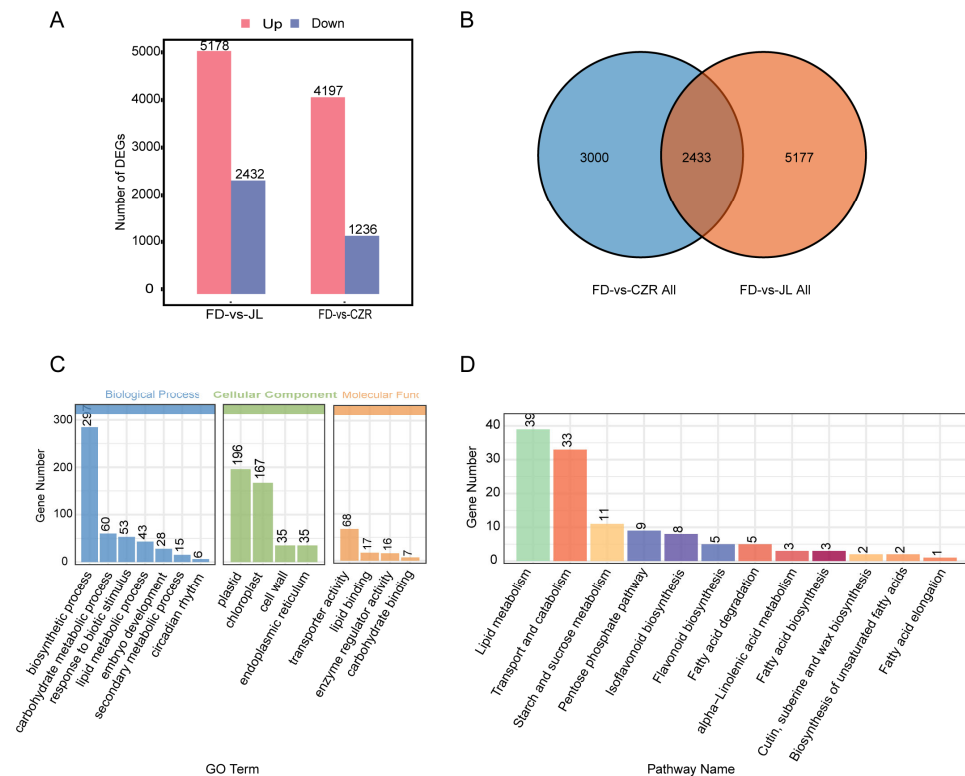


Figure 1. Acquisition and functional analysis of differentially expressed genes. **(A)** The number of differentially expressed genes in FD vs. CZR and FD vs. JL groups. The red represents upregulated genes, and the blue represents downregulated genes. **(B)** Venn diagrams of DEGs of cross-comparisons between FD vs. CZR and FD vs. JL groups. **(C)** The GO enrichment of 2433 significant DEGs. **(D)** The KEGG enrichment of 2433 significant DEGs.

The GO enrichment analysis of cross-comparisons revealed that 2433 DEGs were categorized into 15 functional subgroups. These DEGs were associated with lipid binding, carbohydrate binding, and enzyme regulatory activities within molecular functions, and were linked to components such as the cell wall, chloroplast, and endoplasmic reticulum within cellular components. Additionally, they corresponded with processes like the carbohydrate metabolism, secondary metabolism, response to biotic stimuli, and lipid metabolic processes within biological processes (Figure 1C, Table S5).

A further analysis using KEGG was carried out to investigate the functions of the genes. The DEGs from the FD vs. CZR and FD vs. JL groups and the cross-comparison group were linked to 203, 206, and 184 pathways, respectively. Notably, the DEGs identified through the cross-comparison were related to isoflavonoid biosynthesis (ko00943), the biosynthesis of other secondary metabolites (ko09110), plant hormone signal transduction (ko04075), and the carbohydrate metabolism (ko09101) (Figure 1D, Table S6).

3.3. Expression Level of DEGs Associated with FA Biosynthesis in Peony Seeds of Different Varieties

The KEGG functional annotation revealed that 2433 DEGs (identified through a cross-comparison) were linked to important pathways associated with the lipid metabolism, including lipid biosynthesis proteins (ko01004), FA biosynthesis (ko00061), FA degradation (ko00071), FA elongation (ko00062), and the alpha-linolenic acid metabolism (ko00592) (Table S7). An analysis of approximately 41 DEGs from a cross-comparison was performed based on the FPKM values derived from transcriptome sequencing. As shown in Figure 2, by comparing the FD group with the CZR and JL groups, most genes (32/41) related to fatty acid biosynthesis, including *FAD2* (CL2800.Contig1_All), *FAD6* (CL15098.Contig3_All,

Unigene11696_All), and SAD (CL10910.Contig2_All), were significantly upregulated in tree peony seed (Table S7).

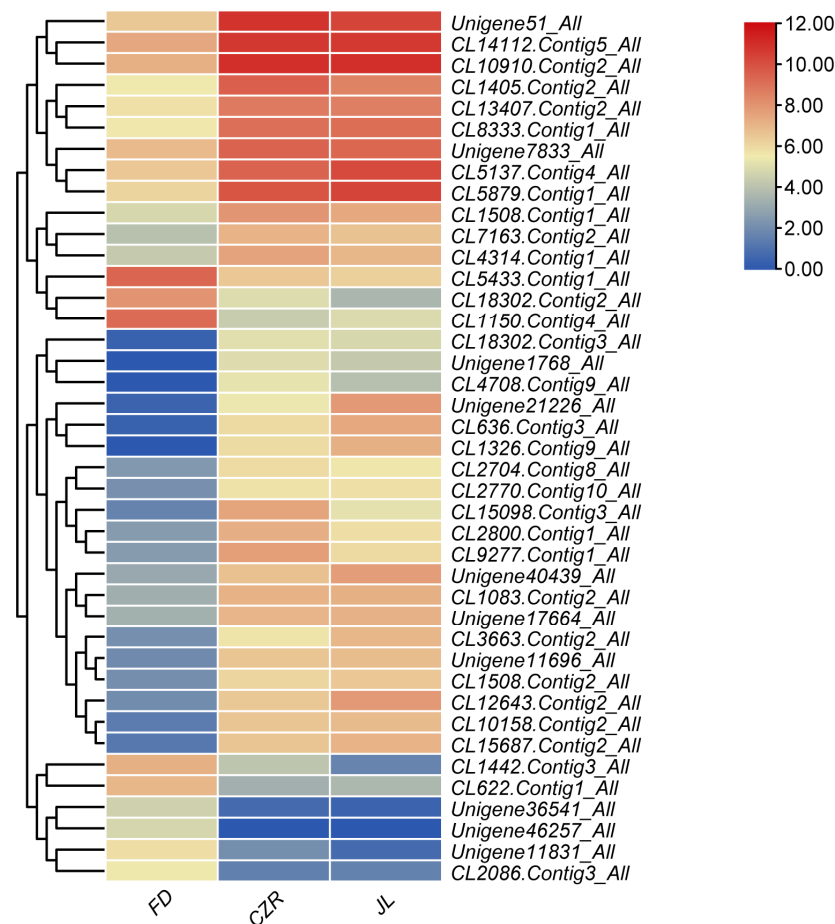


Figure 2. The heatmap showing the expression profiles of 41 DEGs related to fatty acid biosynthesis. The hierarchical clustering of genes is shown in the dendrogram on the side of the heatmap.

Nine genes related to the lipid metabolism (Unigene36541_All, CL18302.Contig2_All, Unigene11831_All, CL2086.Contig3_All, Unigene46257_All, CL5433.Contig1_All), lipid biosynthesis proteins (CL1442.Contig3_All), fatty acid degradation (CL1150.Contig4_All), and fatty acid biosynthesis (CL622.Contig1_All) were highly upregulated in the FD group (Figure 2).

3.4. Genome-Wide Analysis and Phylogenetic Tree of PoFAD Gene Family

The BLASTP method identified 24 PoFADs from the *P. ostii* genome. A thorough examination of the physical and chemical characteristics of the amino acids demonstrated a range between 150 and 450. The molecular weight distribution was between 17,542.67 Da and 51,455.85 Da. Additionally, the theoretical isoelectric points ranged from 5.09 to 9.62. The predictions regarding the subcellular localization of PoFADs suggested that these proteins are predominantly situated within the chloroplast, cytoplasm, endoplasmic reticulum, and plastid. These findings align well with the established traits of the FAD gene family, highlighting their functional significance in different cellular environments (Table S8).

We constructed a neighbor-joining (NJ) tree using the MEGA X software based on the alignment of 29 AtFADs in *Arabidopsis thaliana*, 18 VvFADs in *Vitis vinifera*, and 24 PoFADs in *P. ostii* to elucidate the phylogenetic relationships among 71 FADs in different species. The analysis of the phylogenetic tree revealed that all identified FAD genes can be categorized into six distinct groups: ADS, SLD, FAD3/FAD7/FAD8, FAD2/FAD6, FAD4, and FAB2

(Figure 3). However, the FAD genes from *P. ostii* were limited to four subfamilies, with neither the ADS nor the FAD4 members identified, suggesting a potential loss during the evolutionary history of the species.

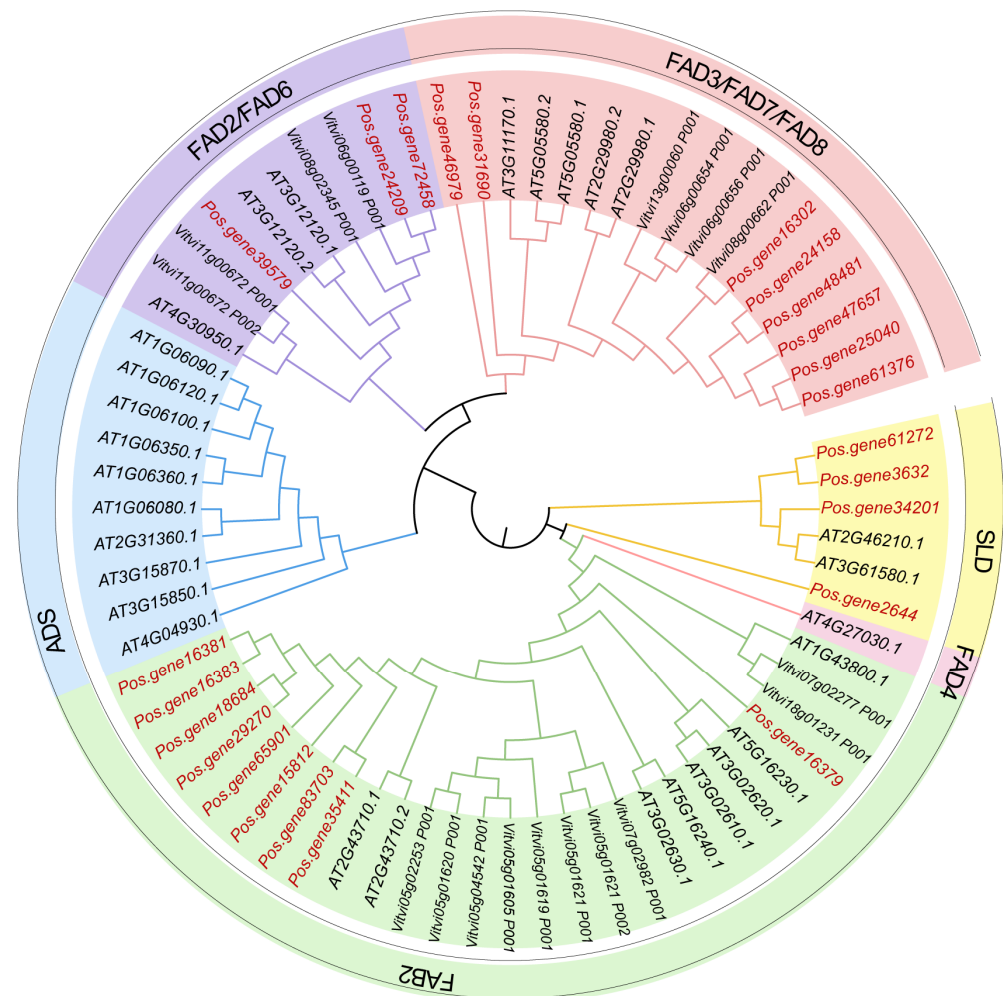


Figure 3. Phylogenetic tree of FAD gene family members in *A. thaliana*, *V. vinifera*, and *P. ostii*. Different colors were used to represent the six subfamilies, consisting of 29 AtFADs, 18 VvFADs, and 24 PoFADs. PoFAD genes of tree peony are marked in red.

Among the six subfamilies, FAB2 desaturase was the largest one, containing eight AtFAD genes, 10 VvFAD genes, and nine PoFAD genes. A notable separation was observed between the soluble FAB2 subfamily and the membrane-bound FAD group, indicating the functional differentiation among these groups. The omega-6 desaturase (FAD2/FAD6) contained three AtFAD genes, four VvFAD genes, and three PoFAD genes. Within the omega-3 desaturase subfamily, 17 gene members were identified. This subfamily has undergone several gene duplication events, particularly among the FAD8 genes (Pos.gene16302, Pos.gene25040, Pos.gene48481, Pos.gene61376, Pos.gene24158, Pos.gene31690, and Pos.gene46979), which has significantly contributed to the enlargement of the PoFAD gene family.

3.5. Conserved Motifs, Domain Prediction, Gene Structure, and Cis-Elements of PoFADs

The conserved motifs and domains in PoFAD proteins were employed using the MEME software v5.1.0 to better forecast the diversity and similarity of PoFAD genes. The analysis predicted and named 20 conserved motifs, labeled as motifs 1–20. All PoFAD genes in the FAB2 desaturase subfamily possessed 3–8 motifs, whereas those in the omega-3 desaturase

subfamily included 2–11 motifs, the front-end desaturase subfamily contained 2–5 motifs, and the omega-6 desaturase subfamily possessed 4–8 motifs (Figure 4A).

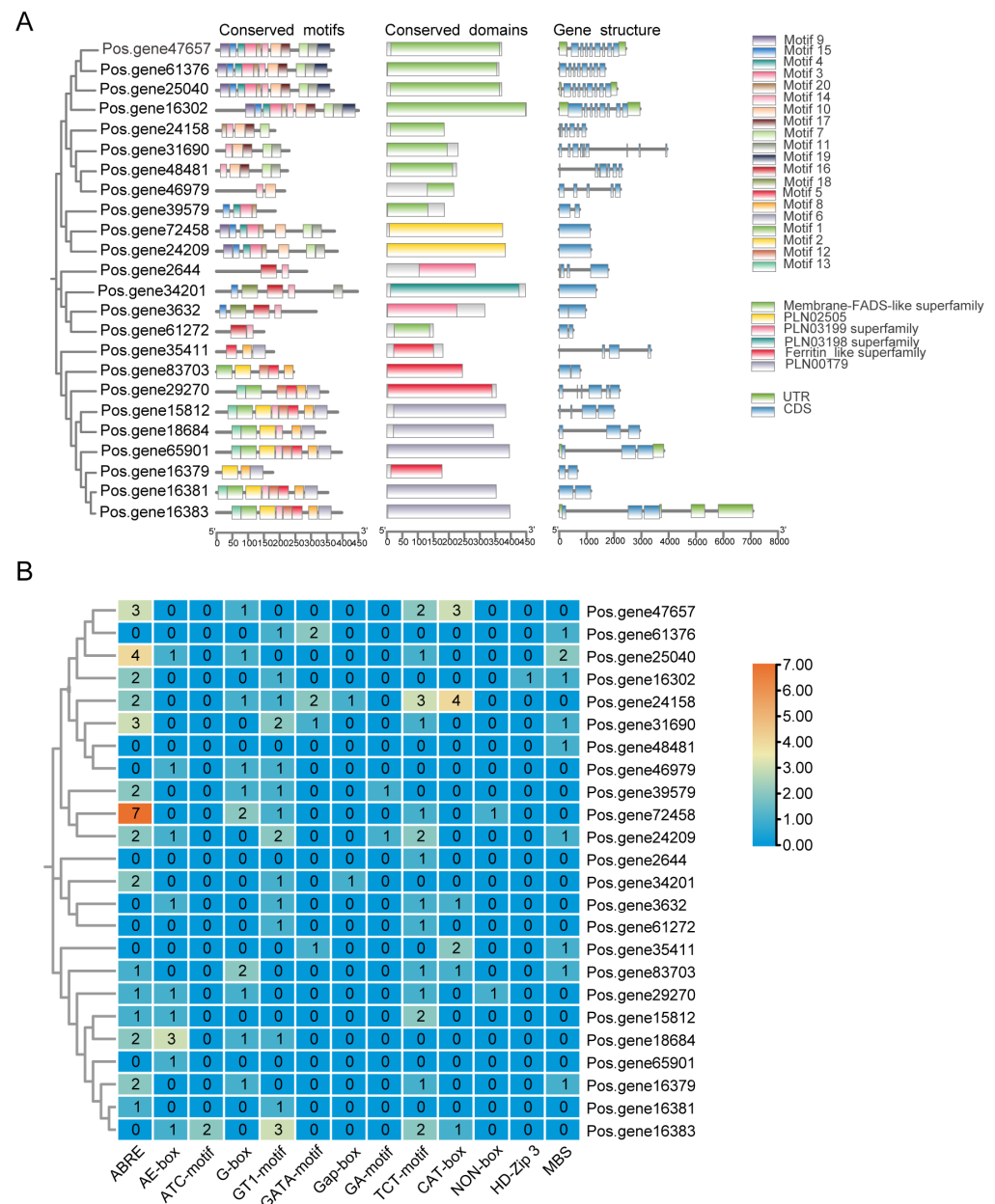


Figure 4. The gene structure, protein motif, domain, and *cis*-element analysis of *PoFAD* gene family members. (A) The gene structure, conserved motif, and domain of *PoFAD* gene family. (B) *Cis*-element analysis of *PoFAD* promoters (2000 bases upstream). The numbers in the colored boxes indicate the number of *cis*-elements in the *PoFAD* promoters.

When studying conserved domains, the research discovered the membrane-bound FADS-like superfamily, ferritin-like superfamily, and PLN02505, PLN03199, PLN03198, and PLN00179 superfamilies. This observation confirmed the conservation of the domain in different subfamilies. A detailed investigation into the introns and exons distribution across the 24 members of the *PoFAD* gene family revealed that the number of introns varied from zero to eight, while the exons ranged from one to nine (Figure 4B). The family members of the omega-3 desaturase had the most exons, all more than five. Several members belonging to the front-end desaturase and omega-6 desaturase families were found to possess only a single exon with no introns, consistent with earlier research findings [48].

(Pos.gene65901 and Pos.gene29270) and the rest were membrane-bound FAD genes. *P. ostii* and *V. vinifera* exhibited six colinear gene pairs, potentially indicating a close similarity between the two species.

3.7. Expression Pattern of PoFAD Gene Family during Seed Development

To analyze the temporal expression profiles of *PoFADs* throughout the developmental stages (35, 49, 63, 77, 91, and 119 DAF) of *P. ostii* seeds, the public RNA-seq data were extracted from the *P. ostii* genome database. Subsequently, the findings indicated that the *PoFADs* expression patterns can be categorized into three groups, indicating different regulatory functions at various stages of peony seed development (Figure 6).

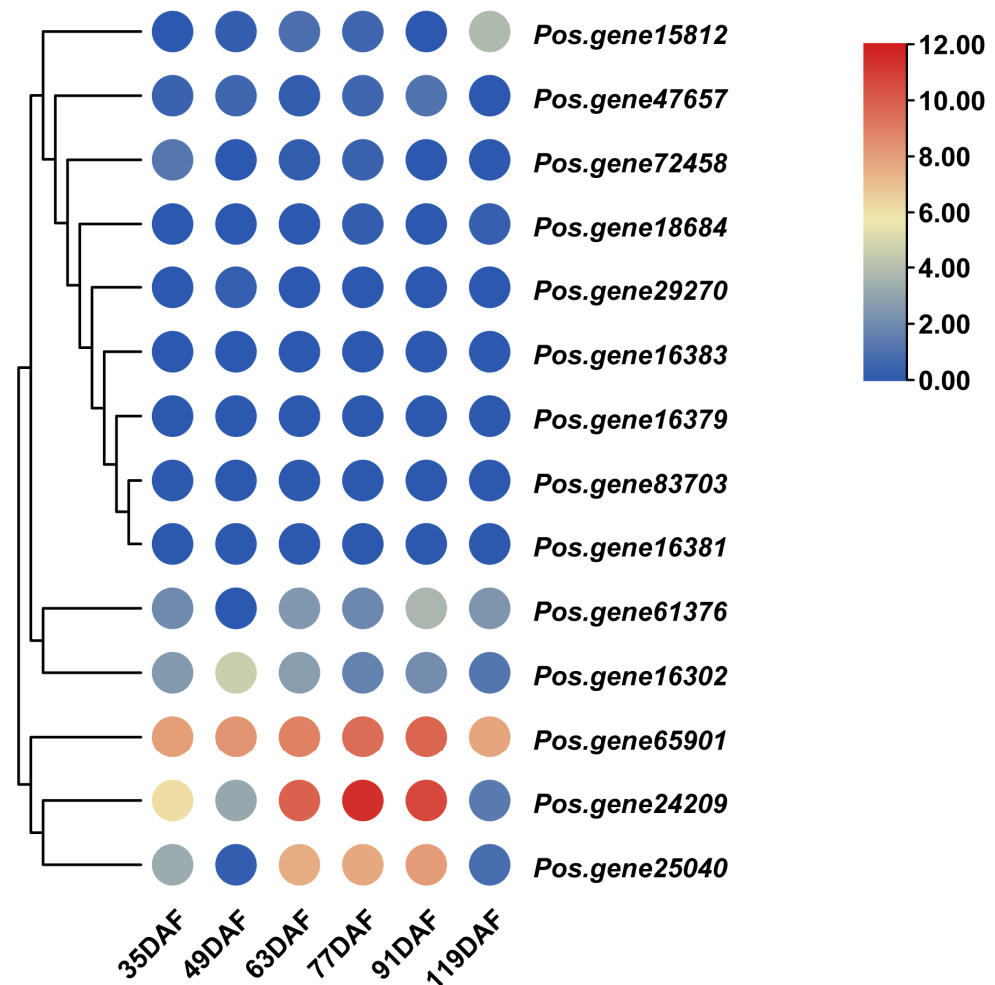


Figure 6. The expression profiles of *PoFAD* genes from RNA-seq of developing seeds of ‘Fengdan’ *P. ostii*. Seeds were collected at six time points during their development (35, 49, 63, 77, 91, and 119 DAF).

The first cluster comprised mostly genes belonging to the FAB2 subfamily, exhibiting relatively low expression in multiple developmental stages. Two *FAD8* genes with low expression levels, belonging to the omega-3 desaturase subfamily, were found in the second cluster. These genes were integral to the biosynthesis of ALA in peony seeds. The third cluster consisted of three genes: Pos.gene65901 (FAB2 subfamily), Pos.gene24209, and Pos.gene25040 (omega-6 desaturase subfamily), which were highly expressed, especially in the middle developmental stages (63, 77 and 91 DAF). The results indicated that *PoFADs* displayed unique patterns of expression in various subfamilies, with consistent expression patterns within each subfamily. The omega-3 desaturase and omega-6 desaturase subfami-

lies show relatively high gene expression, suggesting that these genes are essential for the biosynthesis of linolenic acid in peony seeds.

4. Discussion

Tree peony is recognized as a promising oil crop due to its high levels of unsaturated fatty acids, specifically linolenic acid and linoleic acid in seeds. Multiple research studies showed notable variations in the composition and quality of peony seed oil across various species and types. An examination of the fatty acid profiles across eleven species of *Paeonia* spp. demonstrated that alpha-linolenic acid (ALA) was the most abundant in the oils extracted from seven species within the subsection *Vaginatae*. Conversely, oleic acid was found to be the predominant fatty acid in two species classified in subsection *Delavayanae* [23]. Furthermore, a thorough examination revealed notable differences in oil yields and fatty acid profiles among 10 tree peony varieties. Our study discovered variations in the total fatty acid content, as well as in the linolenic acid and linoleic acid contents among three distinct peony varieties. The three varieties contained five main fatty acids, with linolenic acid having the highest content, followed by linoleic acid. These findings were inconsistent with other studies.

In recent years, some key structural genes associated with FA synthesis during peony seed development have been reported from transcriptomic, metabolomics, and proteomic studies. For instance, Zhang et al. reported that in association with a high ALA content in tree peony seeds, the genes *SAD*, *FAD2*, and *FAD3* were expressed more strongly in *P. rockii* when compared to *P. lutea* [49]. In addition, the comparison of gene expression profiles in two varieties with a varying ALA content validated the correlation between high levels of *PsFAD3* expression and the accumulation of ALA in seeds [50]. Research conducted by Li et al. indicated a strong correlation between the expression of *PoFAD2* and *PoFAD3* and the accumulation of linoleic acid (LA) and ALA in developing endosperms. In contrast, the expression of *PoFAD6* exhibited no significant variations throughout the course of seed development [33]. Moreover, the distribution changes of *PoFAD8* within cells have expanded its catalytic capabilities, which in turn enhances the ALA content in peony seeds [35]. *PrLPAAT4* and *PrPDAT2* play an important role in high-level α -linolenic acid accumulation tree peony seed development [40,51]. Our results showed that *FAD2* (CL2800.Contig1_All), *FAD6* (CL15098.Contig3_All, Unigene11696_All), and *SAD* (CL10910.Contig2_All) were significantly upregulated in FD compared with CZR and JL. This specific expression pattern may be intricately linked to the elevated levels of linolenic acid observed in FD and the high linoleic acid content in CZR and JL seed oils (Table S10).

In addition to the structural genes involved in FA biosynthesis mentioned above, several transcription factors like *PoABI5*, *PrNF-YC2*, *PrWRI1*, and *PrDREB2D* have been discovered, and their functions have been confirmed [34,38,39,52]. For example, overexpressing *PrWRI1* increased total FAs and also changed the FA composition. Furthermore, *PrDREB2D* enhanced its regulatory role over ALA accumulation by interacting with the cofactor *PrABF2b*, a binding factor responsive to abscisic acid (ABA) elements. In the future, we will select some transcription factors and perform functional verification to elucidate the molecular basis of FA biosynthesis.

The FAD genes encode enzymes that play a crucial role in the desaturation of fatty acids, significantly influencing both the content and the composition of these important biomolecules. Although previous studies have highlighted certain *FAD* genes, such as *PoFAD2*, *PoFAD3*, and *PoFAD8*, there has been a lack of a comprehensive genome-wide assessment of the FAD gene family, specifically in tree peony. The knowledge on diversity and functions of *PoFADs* in tree peony was also limited. We successfully identified 24 *PoFAD* genes in the genome of *P. ostii*, categorizing them into four distinct subfamilies. Notably, the first desaturase and the FAD4 subfamily were not detected, implying a potential loss during the evolutionary process in *P. ostii*. This observation aligned with previous investigations into the FAD gene families of barley [53], underscoring a potentially common pattern in the evolution of these species. Moreover, we found that the motif composition was significantly

different between the membrane-bound FAD and the soluble FAB2 subfamilies. The results indicated that the *PoFAD* family may have evolved in two distinct pathways in *P. ostii*.

The distribution of *PoFAD* genes across five chromosomes appeared to be random and uneven, with only six colinear gene pairs between *P. ostii* and *Vitis vinifera*. These results were in agreement with earlier research on the *PoGAox* gene family [54], which could be associated with the transposition of the long terminal repeat (LTR) and its large genome (12.28 Gb). Our investigation showed a significant presence of abscisic acid responsiveness, drought inducibility, and meristem-specific activation-related elements within the *PoFAD* promoters. It appears that *PoFAD* genes were essential for promoting growth and enhancing tolerance to abiotic stresses. In this article, the thorough analyses offer essential insights into the diversification of the structure and the functional roles of *PoFAD* genes, which can improve the quality of peony seed oil in the future.

5. Conclusions

In this study, we conducted the transcriptional analysis of three different tree peony varieties to distinguish the candidate genes related to FA biosynthesis. We identified 41 DEGs involved in fatty acid biosynthesis and lipid metabolism. We revealed that *PoFAD2*, *PoFAD6*, and *PoSAD* had a specific expression pattern, which might be associated with the elevated levels of ALA in FD and the higher OA content in CZR and JL seed oils. The phylogenetic analysis led to the classification of the 24 identified *PoFAD* genes into four subfamilies, which displayed relatively consistent exon–intron structures and conserved motifs within the same subfamily. The *cis*-element analysis pointed to the responsiveness of *PoFAD* expression to environmental factors, suggesting that these genes may be influenced by light, abscisic acid levels, and drought conditions. The close affinity in both species may be related to the six colinear gene pairs found between *P. ostii* and *V. vinifera*. The temporal expression profiles of *PoFAD* genes indicated similar expression patterns in the same subfamily. These findings significantly enhance our understanding of the molecular mechanisms governing fatty acid production in tree peony, elucidate the functional roles of the FAD gene family, and provide a solid foundation for future genetic manipulation efforts aimed at augmenting FA levels in tree peony.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy14102378/s1>, Figure S1: The blooming flowers of three different varieties (FD, CZR, and JL); Table S1: Statistics of raw reads and clean reads quality in three cultivars; Table S2: Statistics of assembled unigenes; Table S3: Summary of the annotation results of *P. ostii* seed unigenes; Table S4: The ratio of unigene annotation in various species; Table S5: The GO classification of DEGs; Table S6: The KEGG pathways of DEGs; Table S7: DEGs related to lipid biosynthesis in tree peony seed transcriptome; Table S8: Physical and chemical properties and subcellular localization of *PoFADs*; Table S9: The distribution of *cis*-elements in promoters of *PoFAD* gene family members; Table S10: Major fatty acids in seed oil of three tree peony varieties.

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Data Availability Statement: The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive in the National Genomics Data Center [55], the China National Center for Bioinformation/Beijing Institute of Genomics, and the Chinese Academy of Sciences (GSA: CRA017083) which are publicly accessible at <https://ngdc.cnbc.ac.cn/gsub/submit/gsa/subCRA027408/finishedOverview> (accessed on 15 June 2024).

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