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Characteristics of Oil Body Development and the Cloning and Expression Analysis of *PDAT* Genes in *Eucommia ulmoides*

Jian Zhong [1,2,†], Jun Qing [1,2,†], Chenlu Liu [1,3], Qi Wang [1,3], Hongyan Du [1,2], Panfeng Liu [1,2], Lanying Du [1,2], Lu Wang [1,2,*] and Qingxin Du [1,2,*]

1 Research Institute of Non-Timber Forestry Chinese Academy of Forestry (CAF), Zhengzhou 450003, China
2 Key Laboratory of Non-Timber Forest Germplasm Enhancement & Utilization of State Forestry and Grassland Administration, Zhengzhou 450003, China
3 Central South University of Forestry and Technology, Changsha 410004, China
* Correspondence: wanglu181716@163.com (L.W.); duqingxin20102325@163.com (Q.D.)
† These authors contributed equally to this work.

Abstract: Studies in multiple species have shown that phospholipid:diacylglycerol acyltransferase (*PDAT*) and oil bodies are important factors affecting plant oil accumulation. However, little information is available about the *PDAT* genes and oil bodies in *Eucommia ulmoides* Oliv. a high-quality oil tree. In this study, the oil bodies in *Eucommia ulmoides* seeds at different developmental stages were observed by frozen section technology combined with Nile red staining. It was found that there was a significant positive correlation between oil content and oil body number. In addition, seven *Eucommia ulmoides PDAT* (*EuPDAT*) genes were cloned from *Eucommia ulmoides* seeds based on genome data. They were assembled into three subgroups according to their classifications in Arabidopsis. *EuPDAT* genes in the same subgroup had similar gene structures and conserved motifs. Putative cis-element analysis of *EuPDAT* gene promoter regions uncovered numerous elements related to stress and plant hormones response. The expression patterns showed that *EuPDAT2* and *EuPDAT7* expression levels were positively related to oil content in different periods, and the correlation coefficients were 0.78 and 0.91, respectively. Our results suggest that oil body number and *EuPDAT* genes are important factors affecting oil accumulation in *E. ulmoides* seeds, and this work provides a theoretical reference for the directional cultivation of new high-oil-content *E. ulmoides* varieties.

Keywords: *Eucommia ulmoides* oil; *PDAT*; oil body; gene cloning; bioinformatics analysis

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1. Introduction

Plant oils play important roles in plant growth, human diet, and industrial development, and they are mainly stored in oil bodies in the form of triacylglycerols (TAGs) [1]. The oil body is mainly composed of TAGs, phospholipids, and oil body proteins, and it exists in plant seeds and pollen [2,3]. In recent years, there have been two main views on the relationship between oil bodies and plant oil content. One view is that during the process of plant growth and development, based on the mutual recognition of oil body proteins, small oil bodies fuse to form large oil bodies. According to this view, it is believed that the size of oil bodies is positively correlated with oil content [4,5]. Another view is that the oil content is mainly determined by the total cross-sectional area of the intracellular oil bodies but has no significant correlation with the size of intracellular oil bodies [6]. *Eucommia ulmoides* Oliv, as an important economic tree species, has excellent oil quality. Therefore, it is of great significance to study the correlation between oil content and the oil bodies of *E. ulmoides* for the development of the *E. ulmoides* oil industry.

At present, the Kennedy pathway is known as the main pathway of oil accumulation; it relies on acyl-CoA and synthesizes TAG mainly under the catalysis of glycerol-3-phosphate acyltransferase (*GPAT*) [7,8]. In addition, a subsidiary pathway for oil synthesis that is independent of acyl-CoA has been found. *PDAT* directly catalyzes the binding of

diacylglycerol (DAG) with phospholipids to produce TAG and lysophospholipids [9–13]. *PDAT* was first discovered by Voelker in yeast and was later studied in species including *Arabidopsis thaliana* (L.) *Heynh*, *Helianthus annuus* L., *Brassica napus* L., and *Camellia oleifera* Abel. It was found that there were differences in *PDAT* functions in different species [14,15]. When RNAi technology was used to knock down the *PDAT* gene in *Arabidopsis thaliana*, the oil content of *A. thaliana* seeds decreased by about 75%, and the embryos did not develop normally [9]. When the diacylglycerol acyl-transferase (*DGAT*) gene, the other key gene involved in TAG synthesis, was knocked down, the oil content of *A. thaliana* seeds decreased by about 30%, and the embryo development was also hindered [16,17]. Therefore, it is speculated that the *PDAT* gene and *DGAT* gene overlap in function in *A. thaliana* [18,19]. In flax, it has been demonstrated that the *PDAT* gene has a specific preference for linolenic acid substrate, which can not only improve the oil content of flax seeds, but can also improve the content of linolenic acid in oil [20]. The *PDAT* gene in *Linum usitatissimum* L was transferred to *A. thaliana*, and it was found that the linolenic acid content in *A. thaliana* oil was significantly increased [21,22]. The *PDAT* gene was cloned from *Ricinus communis* Mill, and it was found that its substrate preference contained phospholipids with castor acyl chains, which could significantly increase the content of hydroxyl fatty acids in oil. This further supports the conclusion that the *PDAT* gene in different species has different substrate preferences [16].

E. ulmoides is a unique economic tree species that has been utilized in China for more than 2000 years [23]. *E. ulmoides* seed oil is rich in unsaturated fatty acids, which are beneficial to the human body [24,25]. Among these acids, the alpha-linolenic acid content is as high as 65%, which is one of the highest linolenic acid contents found in plant oils at present. Alpha-linolenic acid is essential to the human body and can only be obtained through dietary intake. It can be converted into docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in the human body, which can effectively enhance intelligence and reduce the incidence of cerebral thrombosis, hypertension, myocardial infarction, and other diseases [26–30]. Therefore, *E. ulmoides* oil has great development and utilization value. However, the relationships between oil bodies, *PDAT* genes, and oil content have not been reported in *E. ulmoides*. Therefore, in this study, the oil bodies of *E. ulmoides* seeds at different growth stages were investigated. *PDAT* genes were identified and cloned from *E. ulmoides* seeds, and their gene structure, subcellular localization, and expression pattern were analyzed. These findings provide a theoretical reference for the directional cultivation of new varieties of *E. ulmoides* with high oil contents.

2. Materials and Methods

2.1. Plant Materials and Stress Treatment Methods

The leaves and bark of *E. ulmoides* were obtained from the Institute of Economic Forestry, Chinese Academy of Forestry, the forest planting base in Yuanyang County, China (34°55′–34°56′ N, 113°46′–113°47′ E) [31]. 'Huazhong 8' *E. ulmoides* were selected as typical representative plants, and samples were collected once per week. Fruit samples were collected from 60 days after fertilization (DAF) (when the seeds had fully formed) to 180 DAF (when the seeds had fully matured). Samples were collected a total of 18 times. After each sampling, the seeds were peeled quickly and divided into three sets. One set of seeds was frozen using liquid nitrogen and brought back to the laboratory for storage in a freezer at −80 °C. The other set of seeds was oven-dried at 65 °C to constant weight, then stored in a refrigerator at 4 °C for further testing. The last set of seeds was placed into a sampling tube, fixed with a formalin–glacial acetic acid 50% ethanol mixture (FAA fixative), and placed in a refrigerator at 4 °C for further testing [32].

2.2. Determination of E. ulmoides Oil

The Soxhlet extraction method was used to extract oil from *E. ulmoides* seeds. The dried seeds were crushed, and the extraction bottle (m1) and 10 g sample (m2) were weighed to an accuracy of 0.01 g. The samples were placed into a filter paper bag and set into a

Soxhlet extractor. Petroleum ether (boiling range 60–90 °C) was used as the extraction solvent for extraction at 80 °C for 8 h. After the extraction, the filter paper bag was taken out, the petroleum ether was removed by evaporation, and the sample was cooled to room temperature (26 °C). The total mass of the extraction bottle and the oil (m3) was weighed three times, and the average value was taken as the weight. The oil content was calculated as follows:

$$\text{Oil content} = (m3 - m1)/m2 \times 100\%. \quad (1)$$

2.3. Observation of *E. ulmoides* Seed Oil Bodies

Frozen sections were taken, and fluorescent Nile red dye staining and laser scanning confocal microscopy were used to observe the oil bodies of *E. ulmoides* seeds at different growth stages. The treated seeds were randomly selected and sliced using a frozen cutting machine (KD-III). The temperature of the blade and the cutting table was set to −20 °C, and the slice thickness was 6 μm. Slices were dyed in 5 ug/mL Nile red for 1 h. Then, 0.1 mol/L phosphate-buffered saline buffer was used to clean the slices three times, and finally, the 20-fold and 40-fold objective lenses of the laser confocal laser microscope (FV-3000) were used for observation, with an absorption wavelength of 552 nm [33].

2.4. Identification of the PDAT Gene Family in *E. ulmoides*

E. ulmoides genome data were downloaded from the Genome Warehouse (https://ngdc.cncb.ac.cn/gwh/Assembly/13/show) (PRJCA000677) (accessed on 4 May 2022). The *Arabidopsis PDAT* gene sequences were downloaded from TATR (https://www.arabidopsis.org/) [34] (accessed on 4 May 2022). Blast software (v. 2.2.20; NCBI, Altschul, Bethesda, MD, USA) was used to construct the *PDAT* protein sequence library in *Arabidopsis* and blast search the *E. ulmoides* protein sequences. The *PDAT* conserved domain (PF02450) HMM file was downloaded from the Pfam database (http://pfam.sanger.ac.uk/) (accessed on 4 May 2022), and the *E. ulmoides* protein sequences were searched by HMMER3.0. Nine candidate genes were preliminarily obtained, and two incomplete protein sequences were manually deleted. After online comparison with NCBI CDD (http://www.ncbi.nlm.nih.gov/cdd/) (accessed on 4 May 2022) and Pfam (https://pfam.xfam.org/) (accessed on 4 May 2022), it was determined that all seven sequences contained *PDAT* conserved domains. The *PDAT* gene cloning experiment was performed using *E. ulmoides* seeds as experimental materials to further detect the *PDAT* prediction genes. Primer software(v. 5.0; Premier Company, San Francisco, CA, USA) was used to design the primers. The primers are shown in Table 1. Total RNA was extracted using the RNAprep Pure Plant Kit (TIANGEN, Beijing, China) and reverse-transcribed into cDNA [35]. The polymerase chain reaction (PCR) cycling conditions were as follows: 94 °C for 3 min; 34 cycles of 94 °C for 10 s, 58 °C for 15 s, and 72 °C for 10 s; and final extension at 72 °C for 5 min. The target fragment was purified and ligated into the pEASY-T5-ZO vector. The recombinant plasmid was transformed into *Escherichia coli* DH5α cells, and the positive clones were sequenced.

2.5. Bioinformatics Analysis of PDAT Protein

A total of 42 *PDAT* protein sequences from *E. ulmoides*, *A. thaliana*, *Carya cathayensis*, *R. communis*, and *L. usitatissimum* were compared using clustalW, and the phylogenetic tree of the *PDAT* gene family was constructed using MEGA 7.0 maximum likelihood (ML) and 500 bootstrap tests (bootstraps = 500). Based on the genome annotation information of *E. ulmoides*, TBtools (v.1.098; CJ-Chen, China) was used to analyze the position and chromosome length of each *EuPDAT* gene on chromosomes. Based on the *EuPDAT* full-length sequences and CDS sequences of *E. ulmoides*, the GSDS (http://gsds.cbi.pku.edu.cn/) (accessed on 5 May 2022) online tool was used to draw a gene structure intron and exon map [35]. The MEME tool (http://meme.nbcr.net/meme/tools/meme) (accessed on 5 May 2022) was used to analyze the conserved domains of the *EuPDAT* protein. The parameters were set as follows: the number of repeats was any, the maximum number of

motifs was 10, and the optimal length of each motif was 6–30. ExPASy (https://web.expasy.org/protparam/) (accessed on 6 May 2022) was used to predict the protein isoelectric point and molecular weight. The online tool CELLO v. 2.5 (http://cello.life.nctu.edu.tw/) (accessed on 6 May 2022) was used to perform a subcellular localization prediction analysis of *EuPDAT*. The sequence 2000 bp upstream of the 5′ end of the *PDAT* gene was obtained from the *E. ulmoides* genome using TBtools and was submitted to the plantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (accessed on 6 May 2022) website for cis-acting element prediction. GSDS online tool was used to analyze the *EuPDAT* cis-acting elements. The STRING protein interaction database (http://string-db.org/) (accessed on 7 May 2022) was used to analyze the interaction network of seven *EuPDAT* proteins. The plant model was set to *A. thaliana* [36].

Table 1. Primers used in PCR analysis.

Name	Primer Sequence
EuPDAT1-F	ATGGGAATCAAAGTGGCAGTC
EuPDAT1-R	TCACCGTGAAACGTTAATCTGAG
EuPDAT2-F	ATGGCTTCTTCCTCTAAAAATTCTG
EuPDAT2-R	TTACACATAATGCAAGAGGAGATCA
EuPDAT3-F	ATGGCTTCTTCAGTTCTTCGGTT
EuPDAT3-R	TCACAACTGAATATTCAATCTCTCCG
EuPDAT4-F	ATGTCGATGTTGAGGCGGAG
EuPDAT4-R	TCAAGCTTTCCCCATACTATTTACA
EuPDAT5-F	ATGCTCGGGGGATGCTGTT
EuPDAT5-R	TTAGAGAACTCGGGAGCTTTCCTC
EuPDAT6-F	ATGGCGCTGATTCGAAGAAG
EuPDAT6-R	CTAAAAGTCTCTTCCTTTGTACTGCA
EuPDAT7-F	ATGGACCTTGATCTATGGATGTTG
EuPDAT7-R	TCATATGATAGATGGTCCCTTACCC

2.6. RNA Extraction and Quantitative Reverse Transcription PCR Assay

The EZgene Plant Easy Spin RNA Miniprep Kit (TIANGEN, Beijing, China) was used to extract total RNA from *E. ulmoides* seeds at different growth stages. Reverse transcription of RNA into cDNA was performed according to the novoprotein E047-01B reverse transcription kit instructions. The primers used were designed using Primer 5.0, and actin was used as the reference gene [28]. The primers are shown in Table 2. Expression analysis was performed using ChamQ Universal SYBR qPCR Master Mix fluorescent dye in a DLAB Precision 96 fluorescence quantitative PCR instrument. The reaction program was as follows: 95 °C for 5 min, 95 °C for 10 s, 60 °C for 10 s, 72 °C for 10 s, and 75 °C for 5 s, for a total of 40 cycles. The program was repeated three times. SPSS (v.23.0; Norman H. Nie, Chicago, IL, USA) was used to analyze the significant differences.

Table 2. Primers used in qRT-PCR analysis.

Name	Primer Sequence
EuPDAT1-F	AGACCTCCGACGGTTTTTCC
EuPDAT1-R	GGTTCCTTTGGAGGACCGAG
EuPDAT2-F	GTGAGTTGCCTTGCGGATTC
EuPDAT2-R	CAACCTGCAGCCGTAGTACA
EuPDAT3-F	GAGAACACCAAAGCGGAGGA
EuPDAT3-R	GCGCCACGAATTTCATCTCC
EuPDAT4-F	GGACTCCGCAAAGAAAGGGA
EuPDAT4-R	GTCTCGGTCAGCTCGGTAAG
EuPDAT5-F	TTCCCGCATATCTGGAACCG
EuPDAT5-R	TAACCAGGCCTCCACCTTCT
EuPDAT6-F	CGCTATGGATCCCACACCTC

Table 2. *Cont.*

Name	Primer Sequence
EuPDAT6-R	CCGAGAGCGTCGATGAGAAA
EuPDAT7-F	AAAACGACTCCGATGCGACT
EuPDAT7-R	TCAGTGGCAGTGGACCAATC
Actin-F	TTGTTAGCAACTGGGATGATATGG
Actin-R	CAGGGTGTTCTTCAGGAGCAA

3. Results

3.1. Correlation Analysis between Oil Bodies and Oil Content

Seeds have the highest oil content of all *E. ulmoides* tissues. The observation of seeds (Figure 1) revealed that there were differences in the distribution, size, and quantity of oil bodies at different developmental stages. The oil bodies in the seeds were dyed red using Nile red dye and observed using a laser confocal microscope. As shown in Figure 2, there were clear oil bodies in the seeds at 60 DAF; these were small in volume and irregular in shape and were mainly distributed around the cell wall, with large gaps between the oil bodies (Figure 2A). With the development of seeds, the number of oil bodies gradually increased, and the shape gradually changed from irregular to spherical and ellipsoidal. The distribution of oil bodies began to spread from around the cell wall to the center of the cell, and the gaps between oil bodies gradually narrowed (Figure 2B,C). At 120 DAF, the number of oil bodies in seeds further increased, and the shapes were mostly spherical and ellipsoidal. The gap between oil bodies further narrowed, and the number of large oil bodies increased (Figure 2D). At 140 DAF, the number of oil bodies in seeds increased significantly and filled whole cells (Figure 2E). The number, size, and distribution of oil bodies in seeds at 180 DAF exhibited no significant change compared with those at 140 DAF (Figure 2F). This indicates that oil bodies are formed at the early stage of seed development. From 60 DAF to 140 DAF, the number, size, and distribution of oil bodies changed significantly, and these became stable after 140 DAF.

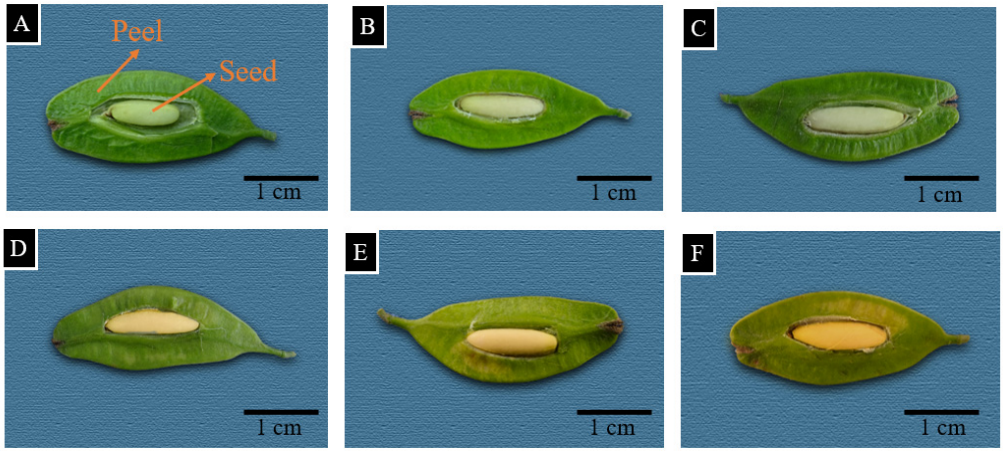

Figure 1. Seeds at different growth stages. (**A**–**F**): Seeds collected on 60, 74, 95, 122, 145, and 180 DAF, respectively.

In order to understand the correlation between the oil content and oil bodies during the development of seeds, this study used laser confocal microscopy at different stages to take pictures of seeds after staining, and three to five cells were randomly selected to obtain the diameter and number of oil bodies. There were some differences in the numbers and shapes of oil bodies in kernels at different developmental stages. The oil body diameters at different developmental stages ranged from 5 to 8 μm (Figure 3A). The number of oil bodies and oil content of *Eucommia ulmoides* seeds showed the same "S"-shaped change trend (Figure 3B,C). The correlation analysis showed that the oil content was significantly

positively correlated with the number of oil bodies, with a correlation coefficient of 0.94 (Figure 3D), but was not significantly correlated with the size of oil bodies. Therefore, it can be speculated that the oil content of *Eucommia ulmoides* seed is mainly affected by the oil body number during the development of *E. ulmoides* seeds but has no significant correlation with the size of oil bodies.

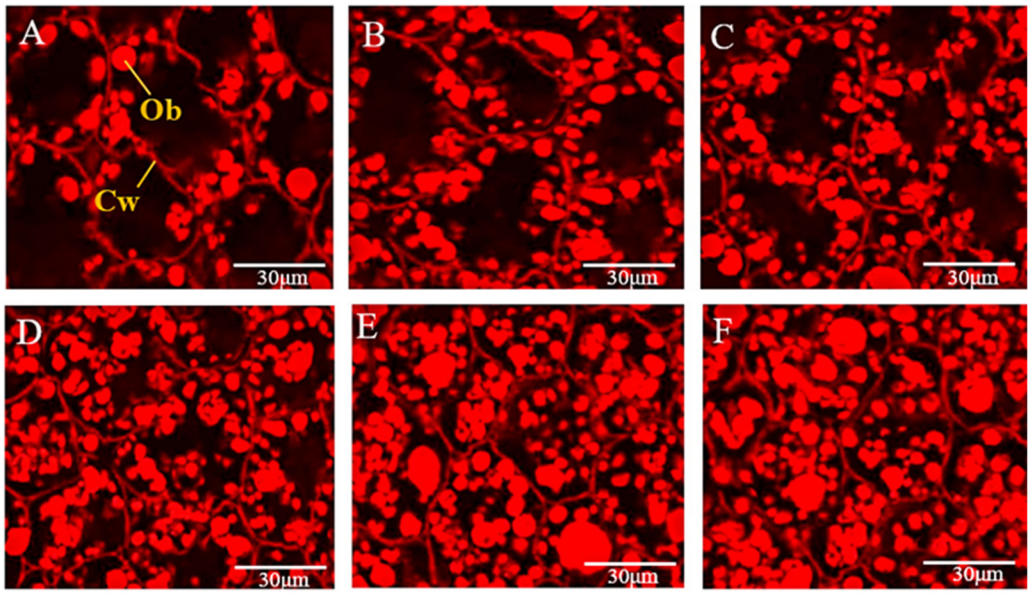

Figure 2. Laser confocal microscopy images of the seeds' different development periods. (**A**–**F**): Seeds collected on 60, 74, 95, 122, 145, and 180 DAF, respectively. Ob: Oil body; Cw: Cell wall.

3.2. Identification and Analysis of the Physicochemical Properties of EuPDAT Proteins in E. ulmoides

In this study, based on the whole genome data of *E. ulmoides*, seven target gene bands were obtained through PCR amplification. After sequencing, it was confirmed that the target gene was a *EuPDAT* gene. The longest CDS sequence was found in the *EuPDAT2* gene at 1178 bp, encoding 392 amino acids. The shortest CDS sequence was found in the *EuPDAT1* gene at 225 bp, encoding 75 amino acids (Table 3). The physicochemical properties of the *EuPDAT* proteins were analyzed using ExPasy (v.3.0; SIB, CHE). The results showed that the molecular weight of *EuPDAT* proteins ranged from 37.8 to 95.4 kDa; the theoretical isoelectric point was between 5.12 and 8.29, among which four theoretical isoelectric points were less than 7, indicating that most were alkaline proteins. The instability coefficient of *EuPDAT* proteins ranged from 31.28 to 49.23, among which five *EuPDAT* protein instability coefficients were greater than 40, indicating that most *EuPDAT* proteins were unstable proteins. The average hydrophobic coefficient was greater than 0, indicating that the *EuPDAT* proteins were hydrophobic proteins. The results of subcellular localization prediction showed that the *PDAT* proteins were mainly distributed in the plasma membrane, endoplasmic reticulum, and nucleus, which suggests that the different distributions of the *EuPDAT* proteins might be related to the diversity of their functions.

Table 3. Genome-wide characterization of *EuPDAT* proteins in *Eucommia ulmoides*.

Gene Name	Number of Amino Acids	Molecular Weight (kDa)	Theoretical pI	Instability Index	Aliphatic Index	Grand Average of Hydropathicity	Subcellular Location
EuPDAT 1	75	37.8	5.12	41.07	91.64	0.634	Plasma membrane
EuPDAT 2	392	95.4	8.29	45.40	80.55	0.721	Endoplasmic reticulum
EuPDAT 3	313	76.5	5.95	43.05	81.01	0.737	Plasma membrane
EuPDAT 4	96	86.8	7.56	37.02	77.94	0.846	Endoplasmic reticulum
EuPDAT 5	182	65.7	5.91	44.77	85.98	0.959	Plasma membrane
EuPDAT 6	138	81.4	5.59	49.23	70.47	0.781	Nucleus
EuPDAT 7	178	78.6	7.09	31.28	97.07	0.813	Endoplasmic reticulum

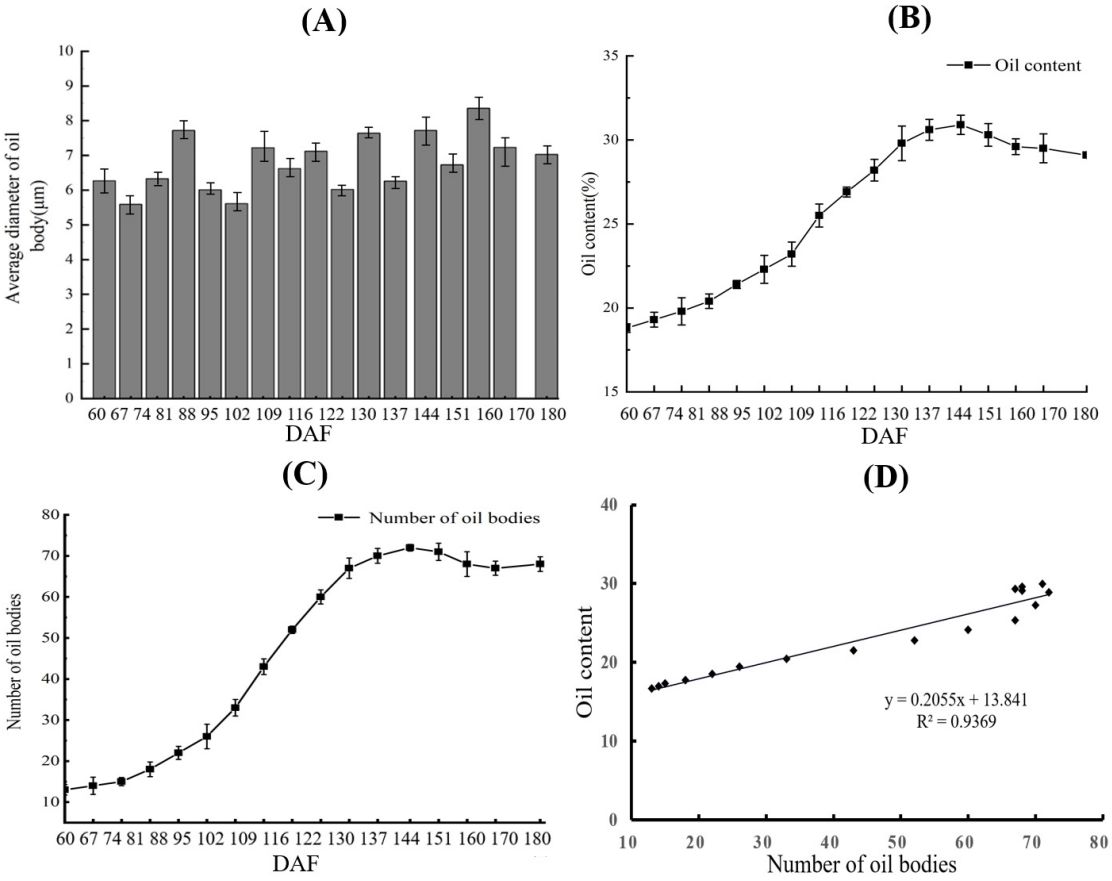

Figure 3. Variation characteristics of oil bodies and their correlation with oil content: (**A**) Changes in the oil body diameter of *E. ulmoides* seeds at different developmental stages; (**B**) Changes in the oil content of *E. ulmoides* seeds at different development stages; (**C**) Changes in the oil body number of *E. ulmoides* seeds at different development stages; (**D**) Correlation between the number of oil bodies and oil content of *E. ulmoides* seeds in different developmental stages.

3.3. Phylogenetic Tree, Gene Structure, and Conserved Motif Analysis of the EuPDAT Genes in E. ulmoides

To explore the evolutionary relationships among the *PDAT* proteins in *E. ulmoides*, *C. cathayensis*, *R. communis*, *L. usitatissimum*, and *Arabidopsis*, a phylogenetic tree was constructed using MEGA 7.0 based on the *PDAT* protein sequences (Figure 4). According to the classification of *PDAT* proteins in *Arabidopsis*, the seven *PDAT* proteins in *E. ulmoides* were divided into three subfamilies, namely, *PDAT* group I, group II, and group III. Among them, the group III subfamily had the smallest number of members, consisting of only *EuPDAT1*, while the other two groups (I and II) both contained three members. The phylogenetic tree analysis demonstrated that *PDAT* proteins in *Arabidopsis* and *E. ulmoides* share a high similarity.

The exon and intron structures of *EuPDAT* genes in *E. ulmoides* were constructed using GSDS (v.2.0; GUO An-Yuan, China). As shown in Figure 5A, the seven *EuPDAT* genes were divided into three groups. The *EuPDAT* genes within the same group had similar intron–exon gene structures, and different subfamilies of the *EuPDAT* structures showed various differences. The exon number of the *EuPDAT* in group I was about 8–11, group III contained the maximum number of exons (16), and group II contained about 10–14 exons (Figure 5B). The results indicate that the gene structures of *EuPDAT* in group II and III were more complex and diverse than those in group I. The online MEME program was used to analyze the conserved structures of *EuPDAT* proteins in *E. ulmoides*. A total of 10 distinct conserved motifs were found; motif 2 and motif 10 were found to encode the *PDAT* domain. As illustrated in Figure 5, most *PDAT* members within the same clade,

especially the most closely related members, usually shared common motif compositions (e.g., *EuPDAT4* and *EuPDAT7*), indicating potential functional similarities among *PDAT* proteins. Motif 3, Motif 4, Motif 5, Motif 6, Motif 7, and Motif 9 were unique to the members in Clade II, which may be important to the functions of unique *EuPDAT* III proteins. Taken together, these results suggest that there is functional similarity of the *EuPDAT* proteins in the same group, and the *EuPDAT* proteins in group II contain a greater variety of complex functions compared with proteins in groups I and III.

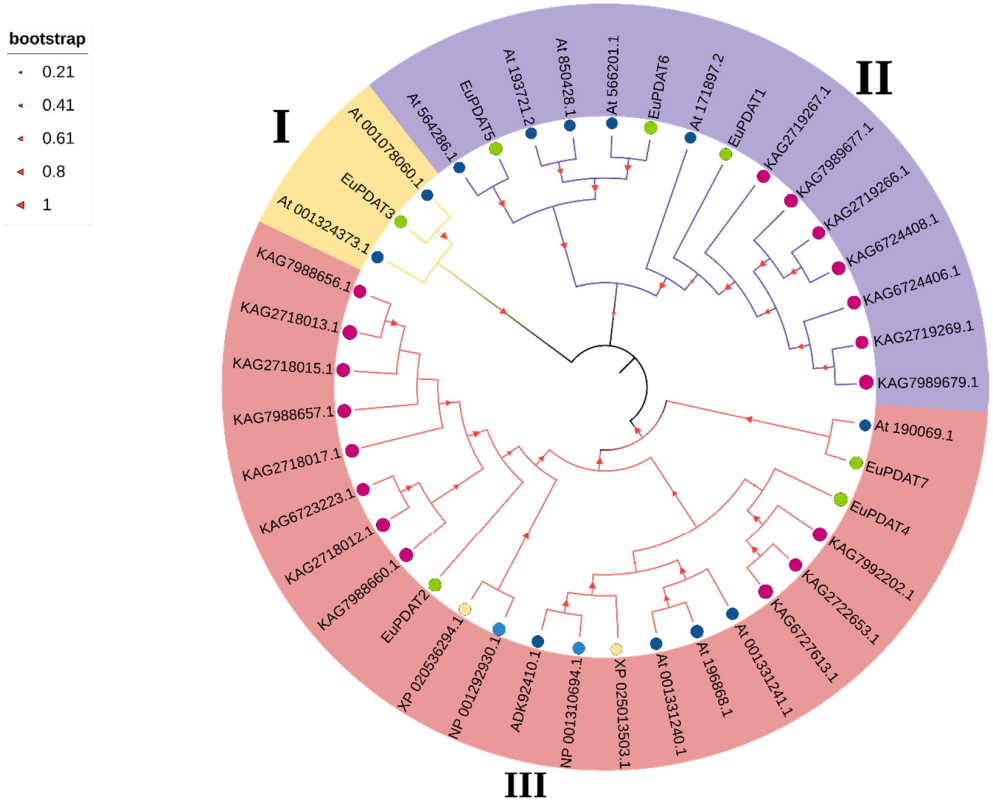

Figure 4. Unrooted phylogenetic tree representing relationships among the *PDAT* domains of *Eucommia ulmoides*, *Arabidopsis*, *Carya cathayensis*, *Ricinus communis*, and *Linum usitatissimum*. I, II and III represent three groups that are close according to genetic relationship, respectively. The different colored arcs indicate different groups (or subgroups) of *PDAT* domains. The green, yellow, burgundy, and blue circles represent *PDAT* domains from *E. ulmoides*, *Ricinus communis*, *Carya cathayensis*, and *Arabidopsis*, respectively. *PDAT* proteins from *E. ulmoides* with the prefix "Eu" indicate "*EuPDAT*". *PDAT*: phospholipid:diacylglycerol acyltransferase.

3.4. Chromosomal Locations and Putative Cis-Element Analysis of the EuPDAT Genes in E. ulmoides

The results of chromosome mapping showed that the seven *EuPDAT* genes were mapped onto six chromosomes (Figure 6). Among them, chromosome 3 contained the most genes (*EuPDAT2* and *EuPDAT3*), while the other five chromosomes contained only one gene each.

To identify the functions of the *EuPDAT* genes in response to stresses, the putative cis-elements related to stress responsiveness in the upstream 2000 bp promoter sequence for each *EuPDAT* gene were identified using PlantCARE (Figure 7). Several stress responsive elements, such as drought response, low-temperature response, defense, and stress response, and hormone responsive elements, including abscisic acid response, methyl jasmonate response, and salicylic acid response, were identified. As shown in Figure 7, multiple cis-elements were found in the promoters of all *EuPDAT* genes. The cis-element analysis suggests that *EuPDAT* genes play an important role in the response to various stresses.

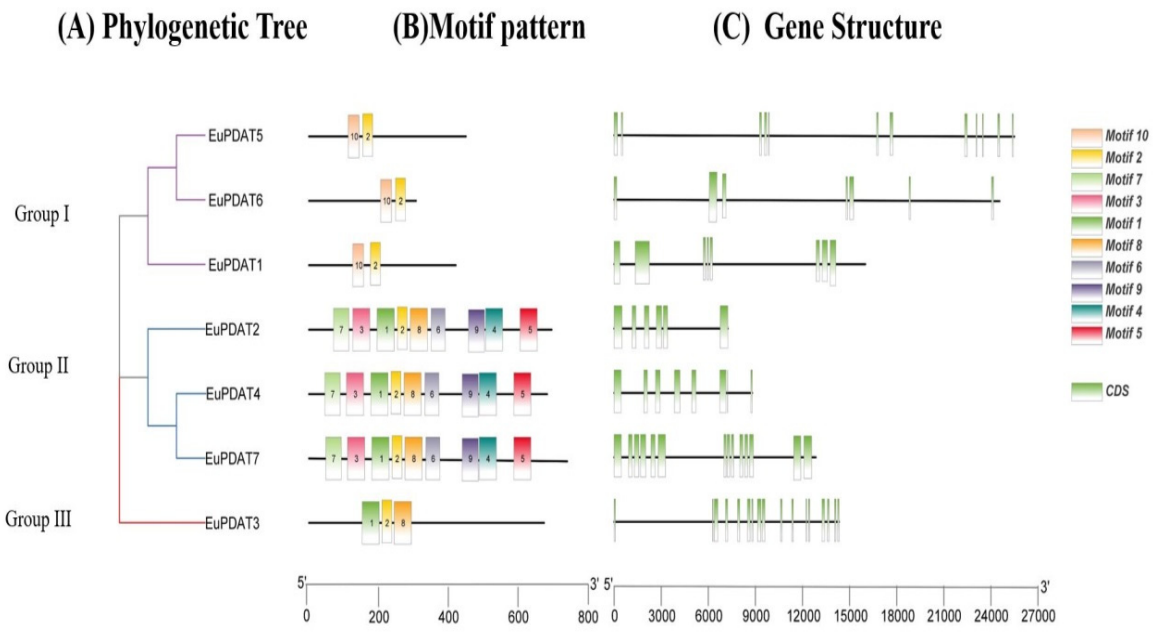

Figure 5. Phylogenetic relationships, gene structure, and architecture of conserved protein motifs in *PDAT* genes from *Eucommia ulmoides*. (**A**) The phylogenetic tree was constructed based on the full-length sequences of *E. ulmoides PDAT* proteins using MEGA (v.7.0; Mega Limited, Auckland, New Zealand). Cluster details are shown in different colors. (**B**) The motif composition of *E. ulmoides PDAT* proteins. Motifs 1–10 are shown in different colored boxes. The length of each protein can be estimated using the scale at the bottom. (**C**) Exon–intron structure of *E. ulmoides PDAT* genes. Green boxes indicate exons; black lines indicate introns. The number indicates the phases of the corresponding introns. *PDAT*: phospholipid:diacylglycerol acyltransferase.

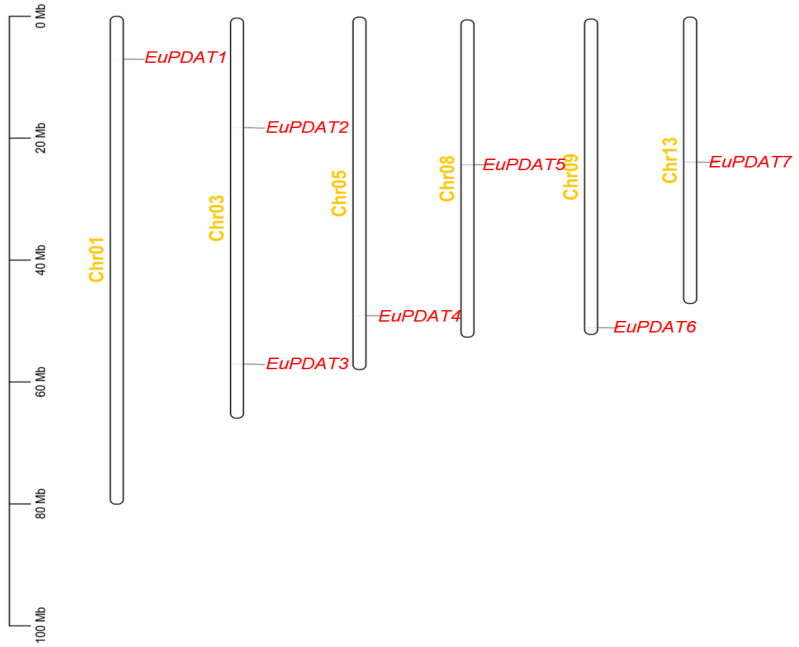

Figure 6. Genome locations of the seven *Eucommia ulmoides PDAT* genes on six chromosomes. The length of each chromosome can be estimated using the scale at the left. *PDAT*: phospholipid:diacylglycerol acyltransferase.

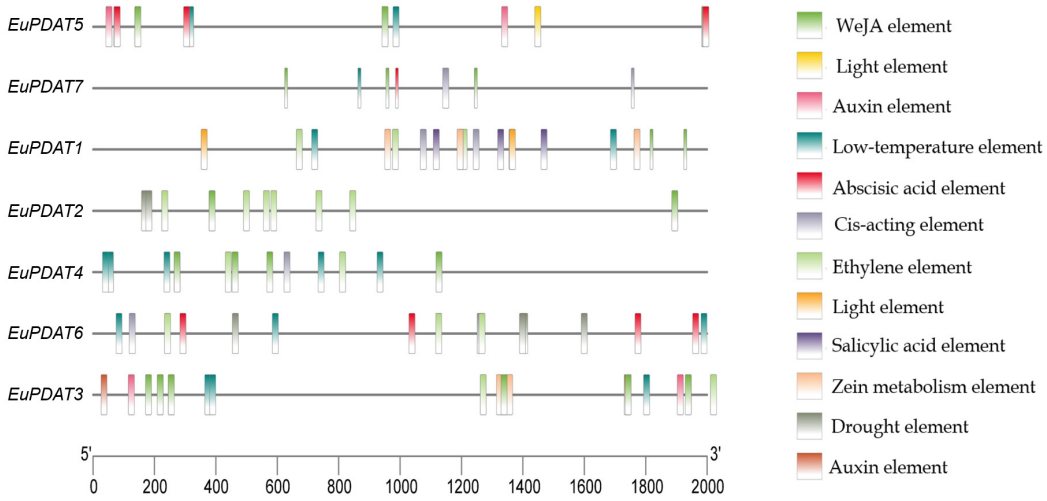

Figure 7. Putative cis-elements related to stress responsiveness in the promoter sequence for *EuPDAT*s. The scale represents the length of the upstream segment of the *EuPDAT*.

3.5. PDAT Protein Interaction Network Analysis of E. ulmoides

The interactions between proteins are the foundation for complex biological functions. Therefore, in this study, *A. thaliana* was used as the plant model, and the STRING protein interaction database was used to analyze the protein interaction network of the seven *PDAT* genes in *E. ulmoides* (Figure 8). The results showed that, with the exception of *EuPDAT2*, the other six proteins all interact. Among them, *EuPDAT3*, *EuPDAT5*, and *EuPDAT7* correspond to *AT4G19860*, *AT1G27480*, and *AT3G44830*, respectively, which are involved in the regulation of low temperature and drought in *A. thaliana*, suggesting that *PDAT* genes in *E. ulmoides* play an important role in oil synthesis and could also interact with and resist various abiotic stresses. Based on the fact that the *EuPDAT2* protein did not correspond to any protein in *A. thaliana* in the interaction network, it is speculated that *EuPDAT2* may be a unique protein in *E. ulmoides*.

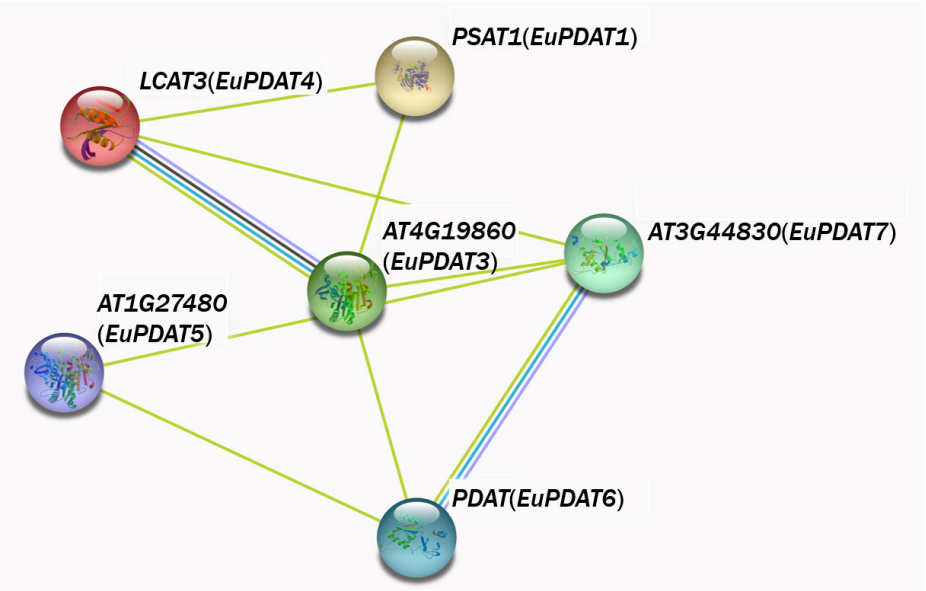

Figure 8. The predicted protein interaction network of the *EuPDAT* proteins based on orthologs in *Arabidopsis* using the STRING database.

3.6. Expression Profiles of EuPDAT Genes at Different Stages and Treatments

To explore the regulatory mechanism of *EuPDAT* genes in the oil synthesis process of *E. ulmoides* seeds, we selected nine periods in which the oil content changed significantly during the oil synthesis process and used real-time fluorescence quantitative PCR technology to analyze the expression levels of *EuPDAT* genes, with the first period used as the control (Figure 9). The results showed that the seven *EuPDAT* genes were differentially expressed in the nine periods. The expression of *EuPDAT1* decreased rapidly and then increased slowly with the passage of time, and the expression of *EuPDAT6* did not change significantly in each period. The expression of *EuPDAT2*, *EuPDAT4*, *EuPDAT5*, and *EuPDAT7* increased first and then decreased with the passage of time, and the expression levels were the highest at 140 DAF, which was consistent with the highest oil content period of the *E. ulmoides* seeds. The correlation analysis between the *EuPDAT* gene expression levels and the oil content at different stages (Figure 10) showed that *EuPDAT2*, *EuPDAT4,* and *EuPDAT7* were positively correlated with the oil content, and *EuPDAT2* and *EuPDAT7* were significantly positively correlated with the oil content. In summary, the oil synthesis of *E. ulmoides* is regulated by multiple *EuPDAT* genes, of which *EuPDAT2*, *EuPDAT4*, and *EuPDAT7* play a major regulatory role in seed oil synthesis.

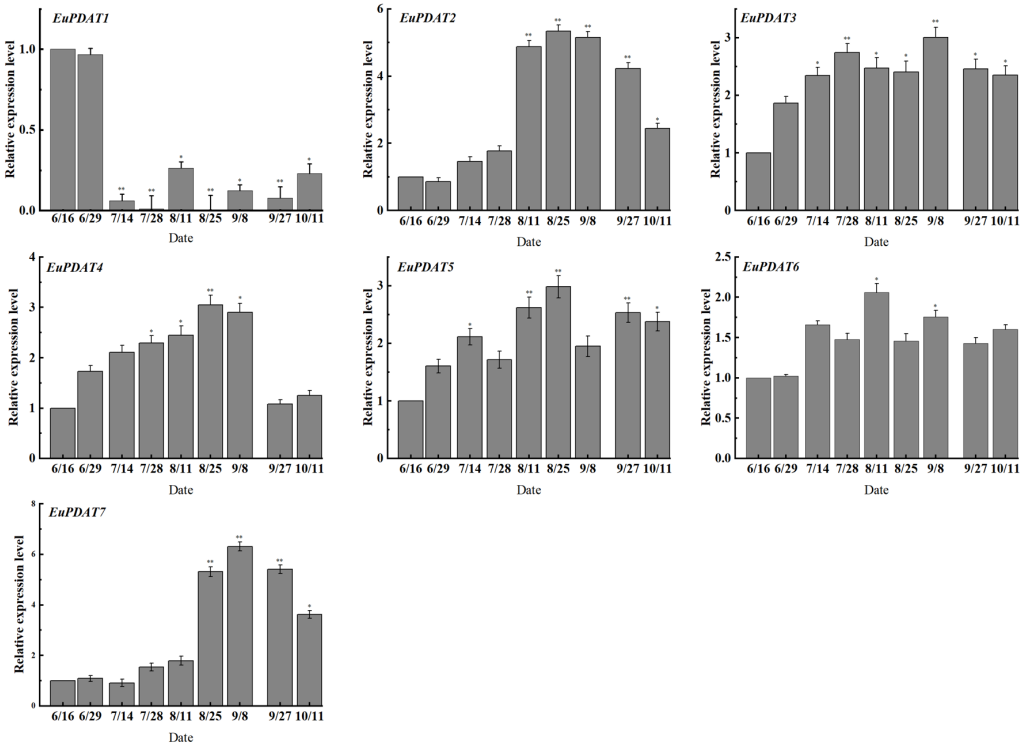

Figure 9. *EuPDAT* gene expression profiles in *Eucommia ulmoides* at different developmental stages. * and ** indicate significant differences at the $p < 0.05$ and $p < 0.01$ levels, respectively.

To explore the responses of *EuPDAT* genes under different stress conditions in *E. ulmoides*, we analyzed the expression profiles of the *EuPDAT* genes treated with methyl jasmonate (WeJA), ethylene, and low temperature. The qRT-PCR analysis showed that most of the *EuPDAT*s responded to these stresses in varying degrees. For WeJA stress (Figure 11), the expression trends of *EuPDAT1, EuPDAT4*, and *EuPDAT5* were increased continuously and reached a peak after 12 h of treatment. For ethylene stress (Figure 12), the expression trend of *EuPDAT1* was increased significantly, up to 13-fold after 12 h of treatment. For low-temperature stress (Figure 13), the expression trends of *EuPDAT1* and *EuPDAT4* were initially increased and then decreased, and they reached a peak after 8 h of treatment. In addition, the expression patterns of *EuPDAT2*, *EuPDAT5*, and *EuPDAT7* were

consistent with each other. These various expression results imply that *EuPDAT* genes may be complex and diverse in their response to ethylene, WeJA, and low-temperature stresses.

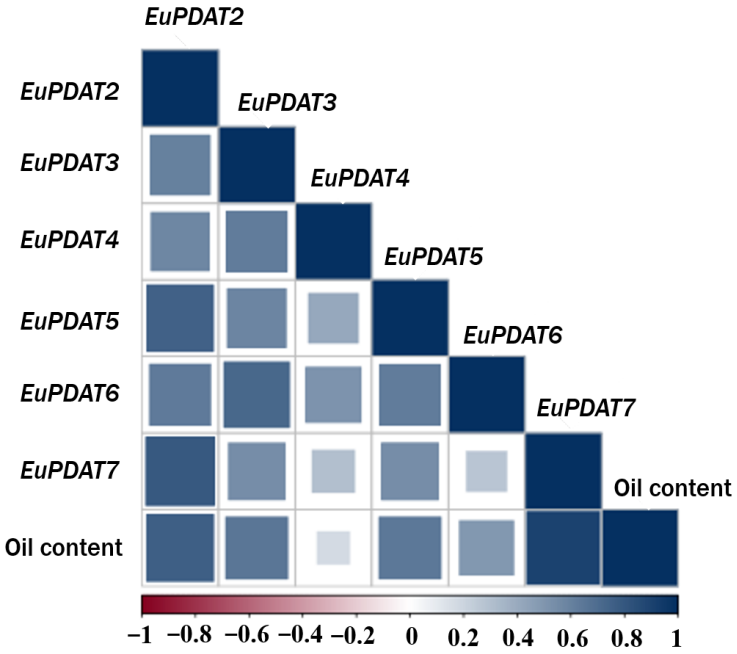

Figure 10. Correlations between *EuPDAT* gene expression and oil content. The deeper the blue color, the more significant the correlation.

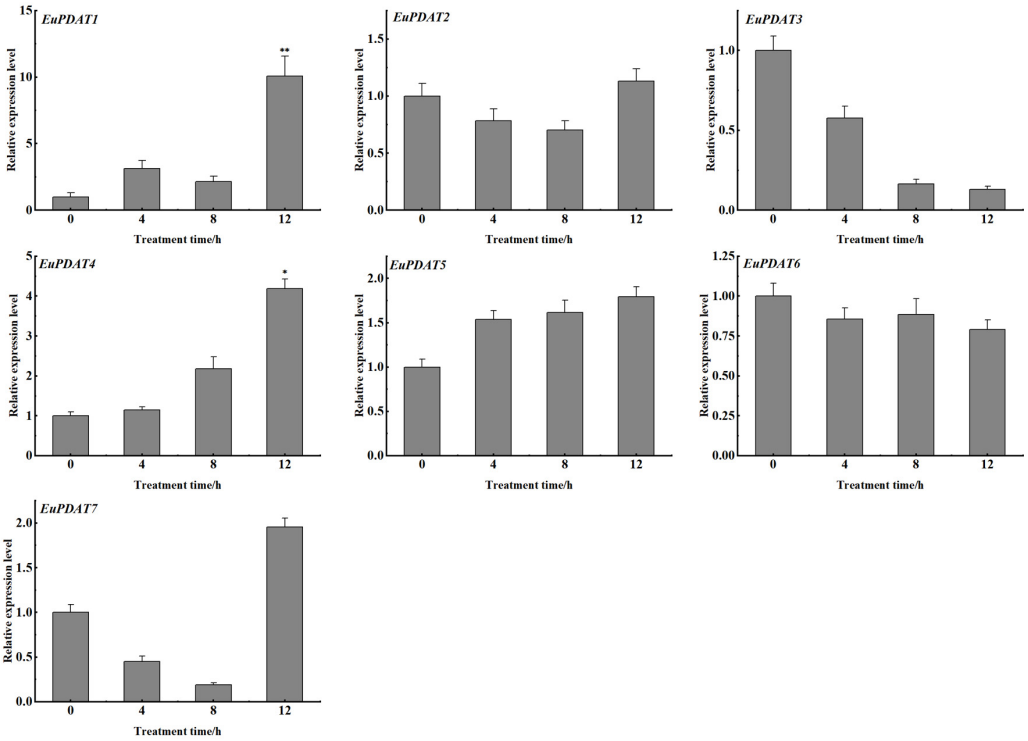

Figure 11. Expression profiles of *EuPDAT* genes under WeJA stress conditions. * and ** indicate significant differences at the $p < 0.05$ and $p < 0.01$ levels, respectively.

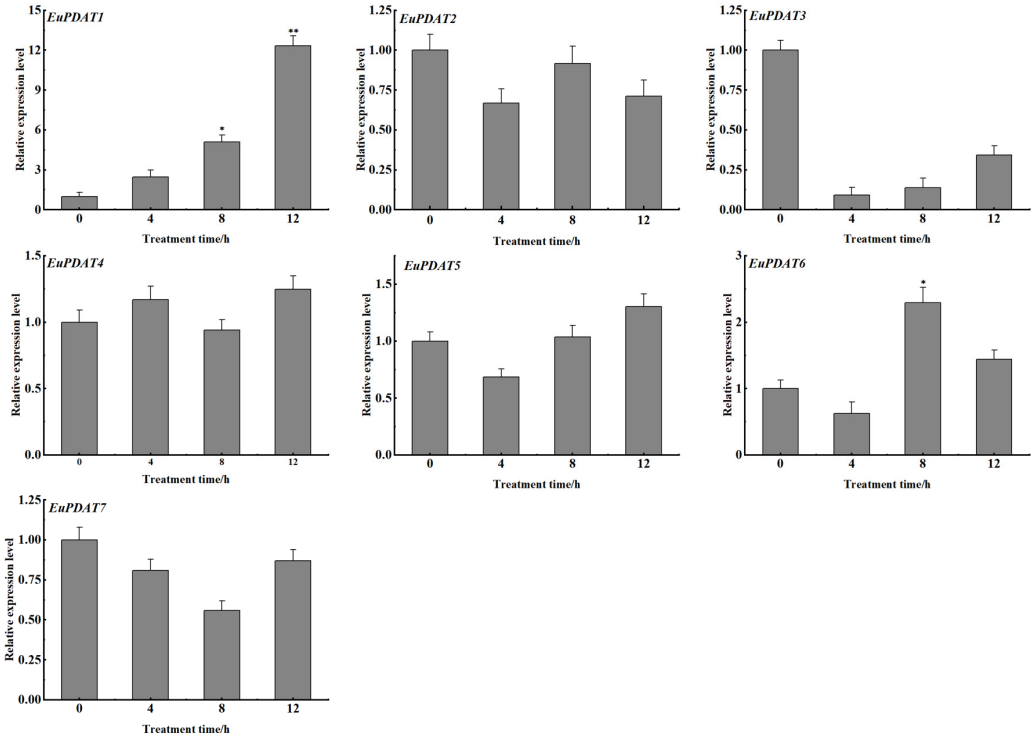

Figure 12. Expression profiles of *EuPDAT* genes under ethylene stress conditions. * and ** indicate significant differences at the $p < 0.05$ and $p < 0.01$ levels, respectively.

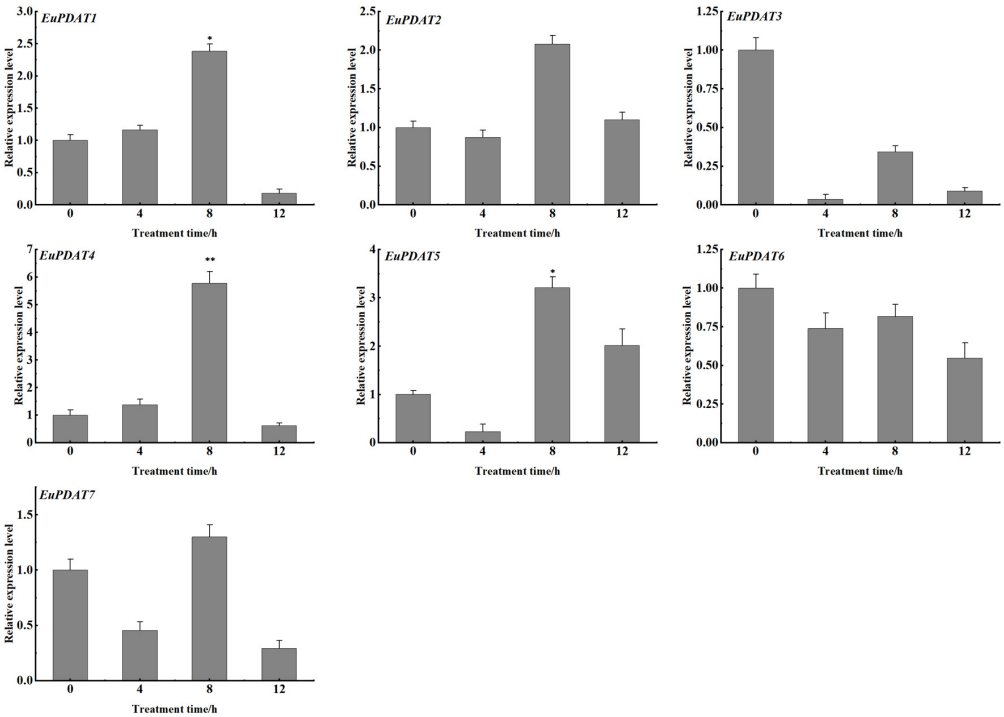

Figure 13. Expression profiles of *EuPDAT* genes under low-temperature stress conditions. * and ** indicate significant differences at the $p < 0.05$ and $p < 0.01$ levels, respectively.

4. Discussion

Lipid metabolism is not only one of the basic metabolic pathways that sustains plant life but also an important source of energy for human health and development [37,38]. The oil body is the smallest organelle in plants, and it is mainly composed of triacylglyc-

erol (TAG), phospholipid (PL) and oil body proteins. Therefore, studying the dynamic changes to oil bodies in *E. ulmoides* seeds is helpful to understanding the regulation of oil accumulation and degradation during seed development [32,39]. The diameter of oil bodies in the seeds of *E. ulmoides* ranged from 5 to 8 μm. With the development of seeds, the number of oil bodies gradually increased and then slowly decreased, and the oil body distribution gradually gathered from the cell wall to the center of the cell. Compared with common oil-bearing crops, such as *Arachis hypogaea* (1–3 μm), *Juglans regia* (1–4 μm), *B. napus* (0.5–2 μm), and *C. oleifera* (2–4 μm), *E. ulmoides* had the same change trend in oil body number and distribution, but there were great differences in oil body diameter, indicating that the morphological diversity of oil bodies in different species may be an important reason for their differing oil contents [4,40,41]. Heneen found that large oil bodies were formed by the fusion of small oil bodies, which indicated that oil content was positively correlated with oil body size [42]. Dong found that the number and cross-sectional area of oil bodies were positively correlated with oil content in *B. napus* [6]. In this study, frozen sections, Nile red staining, and laser confocal microscopy were combined for the first time to analyze the correlation between the oil content and oil bodies in *E. ulmoides*. The results showed that the number of oil bodies was positively correlated with the oil content, but the size of oil bodies was not correlated with the oil content. These findings further indicate that the correlation between oil bodies and oil content is related to species.

The *PDAT* gene, as one of the key genes regulating oil synthesis in plants, was first discovered by Dahlqvist in *H. annuus* and *R. communis*. At present, it has been studied in species including *Arabidopsis*, *C. oleifera*, *A. hypogaea*, and *L. usitatissimum* [18,20,43,44]. In this study, seven *PDAT* genes were cloned from the seeds of *E. ulmoides* based on the whole genome data. Subcellular localization prediction showed that *EuPDAT* genes were mainly located in the plasma membrane and endoplasmic reticulum. This is consistent with the conclusion that TAG is mainly synthesized in the plasma membrane and endoplasmic reticulum [19,45]. Multiple sequence alignment showed that *EuPDAT* genes had high homology with the *PDAT* genes of *C. oleifera*, *C. cathayensis*, and *L. usitatissimum*, and they all belonged to woody oil tree species. It is speculated that *EuPDAT* genes may have functional mechanisms in regulating TAG synthesis that are similar to those of *PDAT* genes in these species. The *EuPDAT* family in *E. ulmoides* was divided into three subfamilies according to the relatedness of these genes with homologs in *Arabidopsis*, in line with earlier studies in other species, such as *H. pluvialis*, poplar, *A. hypogaea*, and *C. oleifera* [46,47]. The gene structure and conserved motif analysis showed that *EuPDAT* genes had structural similarity in the same group, and subfamilies exhibited various differences. This suggests that *EuPDAT* genes have a variety of functions. Recent studies have found that the *PDAT* gene not only plays an important role in regulating TAG synthesis, but also participates in various abiotic and biotic stress responses in plants [9,22]. In *Arabidopsis*, it was found that the *PDAT* gene enhanced heat tolerance by regulating TAG synthesis. Overexpression of the *PDAT* gene increased seedling survival by about 50% under high temperature [16,48]. Studies in *Chlorophyta* showed that *MiPDAT* accelerated the conversion of membrane lipids to TAG under nitrogen deficiency stress, thereby reducing the damage to plants [21]. This study found that *EuPDAT* genes had different degrees of response to low temperature, ethylene, and methyl jasmonate treatment. Among these *EuPDAT* genes, the expression levels of *EuPDAT1*, *EuPDAT4*, and *EuPDAT5* were obviously induced by almost all stress; in particular, the expression of *EuPDAT1* was upregulated more than 13-fold after the ethylene treatment, showing significantly higher expression than the other genes. These results indicate that *EuPDAT* genes not only play an important role in the synthesis of plant oil, but also play an important role in the process of biotic and abiotic stress.

To explore the regulating role of *EuPDAT* genes in oil synthesis by *E. ulmoides*, the oil of *E. ulmoides* in different periods was obtained by Soxhlet extraction, and the expression levels of EuPDAT genes in different periods were determined by qRT-PCR. The results showed that the variation trends of the expression levels of *EuPDAT2*, *EuPDAT3*, *EuPDAT5*, and *EuPDAT7* were correlated with oil content, and the variation trends of the expression

levels of *EuPDAT2* and *EuPDAT7* were significantly correlated with oil content. It is speculated that oil synthesis in *E. ulmoides* is regulated by multiple genes, with *EuPDAT2* and *EuPDAT7* playing major regulatory roles and other *EuPDAT* members possibly mainly involved in other physiological processes. The substrate-specific preference of the *PDAT* gene was observed in *Arabidopsis*, *C. oleifera*, flax, and other species, resulting in significant differences in the fatty acid composition of various species. The content of linolenic acid in *E. ulmoides* oil is about 65%, which is relatively high. However, whether the *PDAT* gene also exhibits substrate preference needs to be further explored. The quality of *E. ulmoides* oil is good, but the low oil content seriously restricts the development of *E. ulmoides* oil industrialization [49–51]. Therefore, the correlations between oil body characteristics, oil content, and the *PDAT* gene, which plays an important regulatory role in the process of oil synthesis, were analyzed in this study. The aim of this study was to clarify the correlation between morphological changes to oil bodies and oil content, and to investigate the regulatory effect of *EuPDAT* genes on oil synthesis. Taken together, these findings can not only provide a reference for further exploring the law of oil accumulation in *Eucommia ulmoides*, but also provide a theoretical basis for directional cultivation of new varieties of *Eucommia ulmoides* with high oil content.

5. Conclusions

There was a positive correlation between the oil content and oil body number in *E. ulmoides* seeds at different developmental stages. The *PDAT* gene family of *E. ulmoides* contains seven members and can be divided into three subgroups according to the classification in *Arabidopsis*. *EuPDAT*s in the same subgroup had similar gene structures and conserved protein motifs. The *EuPDAT* genes, which are mainly distributed in the endoplasmic reticulum and plasma membrane, play an important role in the lipid synthesis pathway and participate in a variety of abiotic stress response processes. The expression levels of *EuPDAT2*, *EuPDAT4*, and *EuPDAT7* at different stages were significantly positively correlated with oil content. It is speculated that *EuPDAT2*, *EuPDAT4*, and *EuPDAT7* are the main regulatory genes in oil synthesis in *E. ulmoides*. This study can provide a theoretical reference for further exploration of the correlation between oil body characteristics and oil content and the regulatory role of the *PDAT* gene in the synthesis of plant oils.

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