Transcriptome Analysis of *Acer truncatum* Seeds Reveals Candidate Genes Related to Oil Biosynthesis and Fatty Acid Metabolism

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ABSTRACT. *Acer truncatum* seeds are an excellent source of beneficial natural compounds, including high levels of unsaturated fatty acids (UFAs), that promote health. Recently, *A. truncatum* has emerged as an oil crop. Therefore, the transcriptomes of *A. truncatum* seeds at 70, 85, 100, 115, 145, 180 days after flowering (DAF) were analyzed to gain a better understanding of the transcriptional and translational regulation of seed development and oil biosynthesis. A total of 28,438 genes were identified, and 3069/2636, 3288/3438, 1319/2750, and 5724/5815 upregulated/downregulated genes were identified when comparing different samples with 85 DAF seeds. Sixteen lipid metabolism pathways with 754 differentially expressed genes (DEGs) were identified, including 34 DEGs associated with UFA biosynthesis. A phylogenetic analysis revealed that six putative fatty acid desaturase (*FAD*) genes clustered into five *FAD* groups. A quantitative real-time polymerase chain reaction analysis indicated that the temporal expression patterns of oil biosynthesis genes and transcription factors were largely similar to the RNA sequencing results. The results of this study will enhance the current understanding of oil metabolism in *A. truncatum* seeds and allow new methods of improving oil quality and seed yield in the future.

Acer truncatum is a versatile oil-producing woody tree. It is well-known for its colorful leaves and high value as an ornamental, medicinal, and oil plant (Ma et al., 2005, 2020; Yang et al., 2017). In 2011, the seed oil was approved as a New Resource Food by the National Health and Family Planning Commission of the People's Republic of China, with potentially important implications for the fields of food and medicine. Previous studies of *A. truncatum* have mainly focused on its seed oil (Chen et al., 2017; Liang et al., 2019; Wang et al., 2018) that contains UFAs, including oleic acid (C18:1), linoleic acid (C18:2), α -linolenic acid (C18:3), and nervonic acid (C24:1) (Liu et al., 2003; Wang et al., 2006). In particular, the proportions of C18:1 and C18:2 are higher than those in other edible oils, including peanut (*Arachis hypogaea*), rapeseed (*Brassica napus*), and sunflower (*Helianthus annuus*) oils (Zhao et al., 2006). In addition, *A. truncatum* seeds are rich in phytosterols, polysaccharides, and flavonoids (Hu et al., 2017; Liang et al., 2019). Because of the potential value of *A. truncatum* seeds as a source of “healthy” oil, it is important to understand seed development and oil biosynthesis in *A. truncatum* to identify strategies for increasing the oil content and quality through breeding and management practices.

Although some researchers have reported the seed oil and UFA contents, there is limited information regarding the regulatory mechanisms controlling seed development and oil biosynthesis. RNA sequencing (RNA-seq) is a powerful tool used to study global gene expression and identify genes that are functionally important under different physiological conditions or developmental stages (Alotaibi et al., 2020; Wang et al., 2019). Recently, an RNA-seq analysis has been used to examine the regulatory mechanisms associated with seed development, fatty acid (FA) biosynthesis, and UFA accumulation in some woody oilseed crops, such as tree peony [*Paeonia* section *Moutan* (Liang et al., 2019)], camelina [*Camelina sativa* (Mudalkar et al., 2014)], olive [*Olea europaea* (Muñoz-Mérida et al., 2013)], and physic nut [*Jatropha curcas* (Grover et al., 2014)]. However, such research of *A. truncatum* is limited. The first comprehensive study characterizing the gene expression in *A. truncatum* seeds was conducted by Wang et al. (2018). They compared the transcriptomes of two genotypes of *A. truncatum* seeds and identified candidate genes that influenced the very-long-chain fatty acid (VLCFA) accumulation. They identified 10 (differentially expressed genes) DEGs that were potentially involved in VLCFA synthesis. However, they only studied two genotypes of *A. truncatum* seeds. No studies have performed a transcriptome analysis of developing *A. truncatum* seeds with a focus on the biosynthesis of FAs and triacylglycerol (TAG). Oil accumulates in *A. truncatum* seeds as the seed develops, which takes $\approx$ 6 months from pollination to seed maturity. In addition, seed morphology and the levels of chemical compounds vary greatly at different developmental stages, and the expressions of genes related to FA metabolism vary (Wang et al., 2019). The temporal and spatial characteristics of the transcriptome, how FA and TAG biosynthesis pathways are regulated, and

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how these pathways affect the oil content, composition, and accumulation during seed development are unclear. Therefore, it is necessary to explore the transcriptome of developing seeds to gain a better understanding of the regulation of lipid metabolism.

In this study, the FA content and composition in developing seeds were analyzed and a time-series analysis of transcriptomic data was performed to reveal DEGs in six phases of *A. truncatum* seed development. This study aimed to assess the transcriptional profiles and identify the main functional genes related to FA and TAG biosynthesis at five time points. Our systematic identification of the core biological processes involved in oil synthesis and the newly identified transcription factors (TFs) will help us to further explore the molecular regulatory mechanisms of oil biosynthesis and metabolism in developing *A. truncatum* seeds.

Materials Methods

PLANT MATERIALS. *A. truncatum* plants were grown in the Jiangsu Province Aceraceae Germplasm Repository (Lishui District, Nanjing, China, lat. 31°65'N, long. 119°02'E) under natural conditions. Seeds were collected at six time points during their development (70, 85, 100, 115, 145, and 180 DAF) from three individuals of “Lishui” provenance. Seeds collected from the same individuals were used for RNA sequencing and oil content measurements. The samples were frozen in liquid nitrogen immediately following collection and stored at -80 °C until further use.

LIPID ANALYSIS. Fifty seeds from each developmental stage were weighed and dried at 65 °C for 72 h. The seed oil was extracted with petroleum ether (30 to 60 °C), and the distillation temperature was kept at 65 °C for 24 h. Following Gao (2006), 0.2 mL oil was dissolved in 2 mL benzene/petroleum ether (1:1, v/v) and then mixed with 2 mL KOH-CH₃OH (0.4 mol·L⁻¹) to generate FA methyl esters. The resulting FAs were analyzed using the gas chromatography-mass spectrometry (GC-MS) method as described by Jiao et al. (2015). The amounts of FAs were calculated by combining the peak areas of the FA methyl esters according to standards (Zhang et al., 2018).

RNA EXTRACTION, LIBRARY CONSTRUCTION, AND SEQUENCING. Total RNA was extracted from developing seeds at 70, 85, 100, 115, 145, and 180 DAF from three plants (three biological repeats) using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. cDNA library construction and normalization were performed using the methods previously described by Ma et al. (2020). The 18 cDNA libraries were sequenced on an Illumina HiSeq X10 (Illumina, San Diego, CA) and 150-bp paired-end reads were generated. The paired-end reads that remained after quality control (low-quality reads containing >10% unknown bases or 50% low-quality bases) were mapped to our *A. truncatum* reference genome (Ma et al., 2020) using HISTA2 version 2.0.4 (Kim et al., 2015).

DIFFERENTIAL EXPRESSION ANALYSIS AND FUNCTIONAL ANALYSIS. The DEGs was performed with the Deseq2 package (Wang et al., 2010) using a model based on the negative binomial distribution [$P < 0.05$ (P was adjusted using Benjamini and Hochberg's approach)] with the 85 DAF sample serving as the control. All DEGs were mapped to gene ontology (GO) terms (Ashburner et al., 2000) to conduct the GO enrichment analysis. The hypergeometric test (Mao et al.,

2015) was used to identify significantly enriched GO terms, and the probability was adjusted by Bonferroni correction ($P < 0.05$) (Kirk, 2006). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway-related database was used to perform the DEG pathway enrichment analysis (Kanehisa et al., 2008).

PHYLOGENETIC ANALYSIS OF *A. TRUNCATUM* FATTY DESATURASE GENES. The expression patterns of six annotated *A. truncatum* fatty desaturase (*FAD*) genes were displayed as a heat map according to log₁₀ [fragments per kilobase per million (FPKM) +1] values. A phylogenetic analysis was performed using the maximum likelihood method with MEGA 5.2 (Tamura et al., 2011). Bootstrap tests were performed with 1000 replicates for statistical reliability. *FAD* genes of *Arabidopsis thaliana*, *Sesamum indicum*, *Glycine max*, and *O. europaea* were downloaded from NCBI.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION ANALYSIS OF GENES INVOLVED IN OIL BIOSYNTHESIS. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to validate the results of the RNA-seq analysis using 16 differentially expressed genes and five TFs involved in FA biosynthesis and metabolism. Specific primers were designed with Primer5 (Supplemental Table 1). Total RNAs were extracted as described previously. The first-strand cDNA was reverse-transcribed from 1 µg total RNA with M-MLV reverse transcriptase (Promega, Madison, WI). The qRT-PCR was performed using SYBR Premix ExTaq™ (TaKaRa Bio, Kusatsu, Japan) on a Step One Plus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). There were three biological replicates for each time point from three different trees (nested with three technical replicates). The relative gene expression levels were normalized to the internal control *Actin* (Wang et al., 2018) using the 2^{-ΔΔT} method (Livak and Schmittgen, 2001).

Results

MAJOR FA CONTENT AND COMPOSITION DIFFERENCES IN DEVELOPING SEEDS. To obtain a time series of oil content variation during seed development, *A. truncatum* seeds at 70, 85, 100, 115, 145, and 180 DAF were selected for oil content and composition measurements. The morphology of developing seeds at six different time points is shown in Fig. 1. The seeds maintained a low growth rate at the early stage (70–85 DAF), and the kernels rapidly swelled after 85 DAF. Five dominant components were observed in developing seeds, including palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and α-linolenic acid (C18:3). As shown in Fig. 1, no UFAs were detected at 70 DAF. At 85 DAF, only C18:1 and C18:2 were detected, and the proportions were 0.46% and 0.63%, respectively. The UFA proportions rapidly increased from 85 to 115 DAF. By 115 DAF, C16:0 and C18:0 had increased to 4.89% and 2.68%, respectively, and C18:1, C18:2, and C18:3 increased to 20.93%, 30.21%, and 0.85%, respectively. Therefore, the results showed that 85 DAF was the key time point for FA synthesis. Overall, the content variation of the major components from 115 to 180 DAF was not significant, thus providing a platform to elucidate how FAs are biosynthesized in *A. truncatum* seeds.

RNA SEQUENCING AND TRANSCRIPTOME ASSEMBLY. Eighteen libraries were sequenced from seeds collected at six stages of

Fig. 1. Changes in fatty composition during *Acer truncatum* seed development. The x-axis represents days after flowering and the y-axis shows the percentage of different oil components.

development to provide a profile of the global transcriptome of *A. truncatum* seeds. A total of 129.56 Gb of data with an average of 49,335,115 raw sequencing reads and 47,987,471 clean reads per sample were obtained after the removal of poly-N sequences and low-quality reads. The clean reads were submitted to the Sequence Read Archive (SRA) under study accession number SRS5367121 and biosample accession number SAMN12389479. The average ratio of clean reads to raw reads was 97.27%. The clean reads were mapped to the *A. truncatum* genome we sequenced previously (Ma et al., 2020), with an average mapping percentage of 90.39% per sample. Overall, 73.71% to 84.08% of the reads were uniquely matched, whereas 6.80% to 12.83% of reads mapped to multiple positions (Supplemental Table 2). A total of 28,438 genes were identified.

DIFFERENTIAL EXPRESSION ANALYSIS AND CLUSTERING OF DEGs. A differential expression analysis was conducted to characterize the temporal changes in global gene expression. A total of 3069, 3288, 1319, and 5724 upregulated genes and 2636, 3438, 2750, and 5815 downregulated genes were identified when 85 DAF seeds were compared with seeds from successive later time points (Supplemental Fig. 1A). The numbers of upregulated and downregulated genes were similar at different time points except for 145 vs. 85 DAF. The four comparisons with the 85 DAF sample were grouped into two main clusters based on the patterns of differential gene expressions: 85 to 100 + 100 to 115 and 115 to 145 + 145 to 180 DAF (Supplemental Fig. 1B). The clustered time points suggested that *A. truncatum* seeds were at similar developmental stages at the time points in those two clusters.

GENE ONTOLOGY FUNCTIONAL CLASSIFICATION AND ENRICHMENT ANALYSIS OF DEGs. GO functional annotation was performed for the DEGs identified during the four comparisons with the 85 DAF sample (Supplemental Fig. 2). The 33 functional groups in the biological process, cellular component,

and molecular function categories were classified according to the GO annotations of the DEGs. The four comparisons exhibited similar patterns of GO functional classification; however, they differed in enrichment classifications (Supplemental Fig. 2). The “compound biosynthetic process” and “primary metabolic biosynthetic process” categories were enriched in the 100 vs. 85 DAF and 115 vs. 85 DAF comparisons, including the terms “nitrogen compound metabolic process,” “regulation of cellular metabolic process,” “regulation of primary metabolism,” and “RNA biosynthetic process,” indicating that crude fat and various FAs were rapidly being synthesized (as shown in Fig. 1, the levels of various FAs peaked in the 115 DAF samples). “Cell part,” “organelle,” and “chromatin” were the main enriched terms in the cellular component category. Notably, in the 180 vs. 85 DAF sample (when desiccation occurs and the levels of FAs start to decrease), the “response to stress” and “degradation complex” (peptidase, hydrolase activity) terms were enriched.

KEGG FUNCTIONAL CLASSIFICATION AND PATHWAY ENRICHMENT ANALYSIS OF DEGs. To identify the major biological pathways active in developing *A. truncatum* seeds, the DEGs were mapped to the reference pathways in KEGG. The DEGs were assigned to 24 KEGG terms under the six primary categories, including cellular processes, environmental information processing, genetic information processing, metabolism, and organismal systems (Supplemental Fig. 3). To elucidate the enriched biological functions during the four comparisons, an enrichment analysis of the 280 pathways to which the DEGs were mapped was performed (Supplemental Table 3). The results showed that ribosome processing and protein processing in the endoplasmic reticulum pathways were mainly enriched in the 100 DAF sample, when seeds were rapidly developing. Flavonoid biosynthesis and FA biosynthesis were also becoming significantly enriched during this period (Supplemental Fig. 4). The biosynthesis of secondary metabolites was significantly enriched after 115 DAF. Interestingly, in 115 vs. 85 DAF comparisons, 15 and 4 DEGs were significantly enriched in α -linolenic acid and the linoleic acid metabolism pathway, respectively. This is consistent with fact that the UFA proportions rapidly increased from 85 to 115 DAF. In addition, the DEGs identified in the present study mapped to 16 of the 17 pathways for lipid metabolism (Supplemental Table 4). Specifically, 754 DEGs were associated with FA metabolism, 34 were associated with arachidonic acid metabolism, 34 were associated with UFA biosynthesis, 42 were associated with cutin, suberin, and wax biosynthesis, 44 were associated with FA biosynthesis, 42 were associated with FA degradation, 45 were associated with FA elongation, and 20 were associated with linoleic acid metabolism. Six FADs were identified and differentially expressed at different stages of seed

Fig. 2. Putative fatty acid desaturase (FAD) genes identified in *Acer truncatum* seeds. (A) Heat map showing relative expression levels of three individuals of 'Lishui' provenance at different seed development stages. (B) Phylogenetic tree depicting the relationships of *Acer truncatum* (Atru) FADs with other species: *Arabidopsis thaliana* (Atha), *Sesamum indicum* (Sin), *Olea europaea* (Oeur), and *Glycine max* (Gmax). The groups of FAD proteins are designated according to Dong et al. (2016). The heat map is displayed according to log₁₀ [fragments per kilobase per million (FPKM) + 1] values.

development (Fig. 2A), including two FAD2, one FAD3, one FAD5, one FAD6, and one FAD7/8 genes based on the phylogenetic analysis of *A. thaliana*. Interestingly, FAD4 was not found in *A. truncatum*, unlike in the other selected species (Fig. 2B). The DEGs related to lipid metabolism at different time points of *A. truncatum* seed development can provide important information about the key stages of oil accumulation and the mechanism regulating UFA synthesis.

QRT-PCR ANALYSIS OF OIL BIOSYNTHESIS GENES DURING THE FIVE TIME POINTS OF *A. TRUNCATUM* SEED DEVELOPMENT. To validate the reliability of the RNA-seq results, 16 genes related to FA biosynthesis were selected for qRT-PCR validation (Fig. 3). The results indicated that *ACC*, *FATA1*, *FATA2*, and *FATB* were highly expressed at 85 DAF, and that *SAD1*, *FAD2*, and *FAD5* were highly expressed from 85 to 100 DAF. In addition, *PDAT* and *DGAT* are the key genes involved in triglyceride synthesis; we found that they were mainly highly expressed at 180 DAF. In general, the qRT-PCR results for oil biosynthesis genes, except *SAD3* and *FAD6*, displayed a high degree of consistency with the RNA-seq results. These results confirmed the reliability of the RNA-seq data obtained during our study.

IDENTIFICATION AND EXPRESSION PROFILING OF FATTY ACID TAG BIOSYNTHESIS GENES. The 51 genes encoding key enzymes involved in FA biosynthesis were successfully identified based on the KEGG pathway analysis, and their expression levels were determined during different developmental stages (Fig. 4). The oil synthesis pathway in *A. truncatum* was constructed based on the results of the present and previous studies (Liao et al., 2018; Wang et al., 2019). In plants, the de novo synthesis of FAs occurs first in the plastid, with acetyl-CoA carboxylase (ACCase) catalyzing the biosynthesis of malonyl-CoA from

acetyl-CoA. ACCase catalyzes the rate-limiting step in FA biosynthesis. Its four subunits are biotin carboxylase (BC), carboxyl transferase α (CT- α), carboxyl transferase β (CT- β), and biotin carboxyl carrier protein (BCCP). All subunits except CT- β were highly expressed during *A. truncatum* seed development. Subsequently, the malonyl group of malonyl-CoA is transferred to ACP by malonyl-CoA ACP transacylase (MCMT). Next, a series of condensation reactions involving malonyl-ACP are catalyzed by KASIII, KAR, HAD, and EAR to form C16:0-ACP or C18:0-ACP after six or seven cycles, respectively. Additionally, the C16:0-ACP or C18:0-ACP can be further catalyzed by *SAD* to generate C16:1-ACP or C18:1-ACP; three *SAD* genes were identified and highly expressed, especially *SAD1* (Figs. 3 and 4). Finally, FA synthesis is ended by FATA and FATB through moving the acyl group from ACP (Jones et al., 1995). After this, LACS on the outer membrane of the plastid esterifies free FAs to generate an acyl-CoA pool (Rawsthorne, 2002; Wang et al., 2012). Some acyl-CoA enter the phosphatidylcholine pool, whereas other acyl-CoA are combined with glycerol-3-phosphate to form TAG. In the phosphatidylcholine pool, PC-C18:1 is converted to PC-C18:2 by FAD2, which is then converted to PC-C18:3 by FAD3. The C18:1 and C18:2 FAs together account for 52% of the total FA content in *A. truncatum*. Previous studies have demonstrated that the accumulation of C18:2 is correlated with the expression of FAD2/FAD6, whereas FAD3/FAD7/FAD8 expression contributes to the synthesis of C18:3 (Wang et al., 2019; Yin et al., 2018; Zhang et al., 2019). *FAD2* and *FAD3* exhibited higher expressions than the other FAD genes (Figs. 3 and 4), suggesting that they have an important role in UFA biosynthesis.

Fig. 3. Quantitative real-time polymerase chain reaction (qRT-PCR) validation of oil biosynthesis genes at five time points of *Acer truncatum* seed development. The relative expression levels of genes were normalized with the internal reference gene *Actin* (Wang et al., 2018). Three biological replicates (nested with three technical replicates) are represented by the error bars ($P < 0.05$).

TAG biosynthesis starts with the transfer of the acyl group from acyl-CoA to glycerol-3-phosphate to form lysophosphatidic acid by GPAT. In this study, nine GPAT genes were identified, but most of them were barely expressed; only two

were relatively highly expressed during seed development. Subsequently, lysophosphatidic acid is dephosphorylated by LPAT and PAP to sn-1,2-diacylglycerol (DAG) (Dahlqvist et al., 2000; Lung and Weselake, 2006). The biosynthesis of

Fig. 4. The pathways of fatty acid biosynthesis. The icons next to the key enzymes represent the relative expression levels of the transcripts at 85, 100, 115, 145, and 180 d after flowering (DAF). The heat map is displayed according to $\log_{10} [\text{fragments per kilobase per million (FPKM)} + 1]$ values. PDHC = pyruvate dehydrogenase complex; ACCase = acetyl-CoA carboxylase; MCMT = malonyl-CoA-ACP transacylase; KASI/II/III = ketoacyl-ACP synthase I/II/III; KAR = ketoacyl-ACP reductase; HAD = hydroxyacyl-ACP dehydrase; EAR = enoyl-ACP reductase; SAD = stearyl-ACP desaturase; FAD2/6 = oleate desaturase; FAD3/7/8 = linoleate desaturase; FATA/B = acyl-ACP thioesterase A/B; PCH = palmitoyl-CoA hydrolase; LACS = long-chain acyl-CoA synthetase; LPCAT = lysophosphatidylcholine acyltransferase; GK = glycerol kinase; GPAT = glycerol-3-phosphate acyltransferase; LPAAT = 1-acylglycerol-3-phosphate acyltransferase; PAP = phosphatidic acid phosphatase; DGAT = diacylglycerol acyltransferase; PDAT = phospholipid:diacylglycerol acyltransferase.

TAG is ended by DGAT, which is a rate-limiting enzyme in the Kennedy pathway (Liao et al., 2018). The qRT-PCR results showed that *DGAT* had the highest expression at 180 DAF (Fig. 3). This indicated that TAG assembly and accumulation through the glycolysis pathway occurred in seeds at ≈ 180 DAF.

IDENTIFICATION OF TRANSCRIPTION FACTORS INVOLVED IN OIL BIOSYNTHESIS AND DEPOSITION. TFs have important roles in transcription and can modulate gene expression as regulators (Riechmann et al., 2000). However, little is known about the transcriptional regulation of lipid biosynthesis in *A. truncatum* seeds. In this study, a total of 1049 TFs were identified during the seed oil accumulation stages using iTAK software (Zheng et al., 2016). The top six TF families in terms of sequence abundance were bHLH (59), HB (51), MYB-related (48), NAC (39), bZIP (32), and B3 (30) domain proteins. In previous studies, several TFs were shown to have important roles in seed

oil biosynthesis, including *wrinkled1* [*WRI1* (Ji et al., 2018)], *leafy cotyledon1* [*LEC1* (Mu et al., 2008; Shen et al., 2010)], *fusca3* [*FUS3* (Zhang et al., 2016)], and *Abcisic acid insensitive3* [*ABI3* (Rohde et al., 2010)]. They were also identified among our transcripts, including one *WRI1*, two *LEC1*, one *FUS3*, and one *ABI3*. To further determine the expression differences of the TFs, these five TFs were selected for qRT-PCR analysis during the five seed development stages (Fig. 5). The results showed that their expression tendencies were consistent with the RNA-seq results. However, the expressions of the two homologous *LEC1* genes differed greatly. The expression of *LEC1* was significantly higher than that of *LEC1.1*. In short, the expression trends of *WRI1* and *LEC1.1* increased and those of *FUS3* and *ABI3* decreased. This might have been due to tissue differences or specific regulatory differences in the expression patterns. Further studies will be

Fig. 5. Quantitative real-time polymerase chain reaction (qRT-PCR) validation of fusca3 (*FUS3*), leafy cotyledon1 (*LEC1*), leafy cotyledon1 (*LEC1.1*), Absciscic acid insensitive3 (*ABI3*), and wrinkled1 (*WR11*) at five time points of *Acer truncatum* seed development. The relative expression levels of genes were normalized with the internal reference gene *Actin* (Wang et al., 2018). Three biological replicates (nested with three technical replicates) are represented by the error bars.

needed to elucidate the regulatory mechanisms of the TFs involved in oil accumulation in *A. truncatum* seeds. In summary, our results provide some valuable clues to help understand the molecular mechanisms of FA and lipid biosynthesis.

Discussion

DYNAMIC CHANGES IN GENE EXPRESSION DURING SEED DEVELOPMENT. *A. truncatum* is an important woody tree species that produces seeds with high levels of UFAs (Ma et al., 2020). Studying the key stages of seed development and the molecular mechanism of oil synthesis is a critical step in improving the quantity and quality of *A. truncatum* seed oil. In a previous study, two genotypes of *A. truncatum* seeds were compared using a transcriptome analysis, and the candidate genes were revealed to affect seed VLCFA accumulation (Wang et al., 2018). During this study, we obtained comprehensive genomic data characterizing gene expressions at six time points of seed development in the *A. truncatum* tree. A total of 28,438 genes were identified. The oil component results showed that 85 DAF was the key time point for FA synthesis during seed development. Therefore, we conducted a differential expression analysis by comparing other time points with 85 DAF. The results revealed that the numbers of upregulated and downregulated genes were similar at the different time points, except for 145 vs. 85 DAF. More importantly, some DEGs were associated with FA metabolism, including UFA biosynthesis, suberin and wax biosynthesis, FA biosynthesis, FA degradation, and FA elongation (34, 42, 44, 42, and 45 DEGs, respectively). In addition, we identified 20 DEGs associated with linoleic acid metabolism. The current study provides large-scale transcriptomics data for future research of lipid metabolism.

KEY GENES OF *A. TRUNCATUM* FATTY ACID BIOSYNTHESIS AND OIL ACCUMULATION. Oil synthesis mainly includes de novo FA

biosynthesis, TAG assembly, and oil body formation in oil seed plants (Liu et al., 2013). In general, these genes are clearly part of the main pathways of FA and oil biosynthesis (Liao et al., 2018; Wan et al., 2016; Wang et al., 2019; Wu et al., 2020). However, the numbers and expression patterns of key genes vary from species to species during the process of seed development. In this study, we identified the key genes of FA biosynthesis and oil accumulation in *A. truncatum* seeds (Fig. 4). *A. truncatum* is rich in oleic acid (C18:1) and linoleic acid (C18:2), which account for 20.93% and 30.21% of its seed oil, respectively. Some studies have reported that C18:1-ACP is catalyzed by *SAD* from C18:0-ACP (Liao et al., 2018; Liu et al., 2019; Wang et al., 2019). We identified three *SAD*-homologous genes. Although *SAD1* showed the highest FPKM values, its expression trend decreased from 85 DAF. Previous studies have reported that members of the FAD family have important roles in the biosynthesis of linoleic acid and linolenic acid (Alotaibi et al., 2020; Liao et al., 2018; Liu et al., 2019). In *A. truncatum*, six *FAD* genes were identified, which is less than that in *A. thaliana* (15), *S. indicum* (7), *O. europaea* (8), and *G. max* (15). Transgenic *A. thaliana* seeds overexpressing *FAD2* and *FAD3* from *Paeonia suffruticosa* contained more linolenic acid than wild-type seeds (Yin et al., 2018; Zhang et al., 2019). Based on the qRT-PCR and RNA-seq analyses, *FAD2/6* were highly expressed and *FAD3/7/8* showed low expression. A previous study indicated that *FAD3/7/8* are ω -3 desaturases that insert a double bond in linoleic acid (18:2) to synthesize linolenic acid (C18:3) (Wang et al., 2019). Furthermore, they are primarily responsible for the (18:3) content of seeds (Hernández et al., 2016). In *A. truncatum*, the highest content of C18:3 was only 0.92% at 145 DAF. Therefore, this may be the main reason for the high linoleic and low linolenic acid contents in *A. truncatum*.

TAG is stored in oil bodies surrounded by a lipid monolayer and is associated with oil body membrane proteins (Frandsen et al., 2001). Fan et al. (2013) reported that overexpression of *PDAT* stimulated FA and TAG synthesis, and that coexpression of *PDAT* with oleosin increased the dry weight of triacylglycerol by up to 6.4%. Additionally, *DGAT* works in coordination with *PDAT* to enhance TAG assembly in seeds (Hong and Seo, 2018). Two *PDAT* genes and one *DGAT* gene were identified in *A. truncatum*. These genes have the potential to increase the FA content and TAG synthesis in *A. truncatum* in the future.

TRANSCRIPTION FACTORS INVOLVED IN OIL ACCUMULATION. Regulation of FA biosynthesis has been shown to occur at multiple levels, among which transcriptional regulation is considered a major means of regulating the pathway (Millar et al., 2000). Previous studies showed that *LEC1*, *WRI1*, *FUS3*, and *ABI3* are key regulators of FA biosynthesis (Baud et al., 2007; Mu et al., 2008). For example, overexpression of maize (*Zea mays*) *LEC1* (*ZmLEC1*) and *WRI1* (*ZmWRI1*) increased oil accumulation by $\approx 48\%$ in maize seeds (Shen et al., 2010). Transient expression of a *Ricinus communis* *WRI1* (*RcWRI1*) splicing isoform in *Nicotiana tabacum* leaves upregulated the expression of *WRI1* target genes and increased the oil content by 4.3-fold to 4.9-fold compared with controls (Ji et al., 2018). The *ABI3* could promote oleosin gene expression in *B. napus* (Crowe et al., 2000). In general, the regulatory mechanism of FA metabolism is complicated and may need several TFs to work together. To validate the expression differences in the identified TFs, we analyzed their expression trends at different seed development stages by using qRT-PCR. The results showed that the expression trends of *WRI1* and *LEC1.1* increased during seed development, but that *FUS3* expression decreased from 85 to 180 DAF. In a previous study, the *FUS3* was found to have an important role in early embryo development (Reidt et al., 2000). This may be the reason for its high expression during early seed development. These results provide some valuable clues to understanding the molecular mechanism of FA and lipid biosynthesis. However, further studies are needed to decipher the regulatory roles of TFs in *A. truncatum*, including identifying the binding motifs of the TFs that interact with the promoters of genes involved in oil biosynthesis.

Conclusions

In this study, oil accumulation during seed development showed an “S” pattern from 70 to 180 DAF; 85 DAF was the key time point for FA biosynthesis, when the main FA components were detected. To further understand the basic molecular mechanisms of lipid biosynthesis and metabolism, RNA-seq data were generated from *A. truncatum* seeds at six developmental stages. GO and KEGG pathway enrichment analyses revealed that the DEGs were involved in biological processes such as FA and TAG biosynthesis. In addition, genes encoding key enzymes and TFs involved in the biosynthesis and metabolism of FAs and TAG were successfully identified. This is the first comprehensive transcriptomic analysis of the metabolic pathways of oleic acid, linoleic acid, linolenic acid, and TAG biosynthesis. The results will serve as an important foundation for more deeply exploring the regulatory mechanism of oil accumulation in this species and may provide a reference for other oil crops.

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Supplemental Fig. 1. Differentially expressed genes (DEGs) identified in *Acer truncatum* seeds at various development stages: (A) quantity of DEGs and (B) cluster analysis of DEGs. The heat map is displayed according to $\log_{10}$ [fragments per kilobase per million (FPKM) + 1] values. DAF = days after flowering.

100 vs 85 DAF

Supplemental Fig. 2. Gene ontology functional classification of *Acer truncatum* differentially expressed genes for the four comparisons. DAF = days after flowering.

145 vs 85 DAF

Supplemental Fig. 2. Cont.

180 vs 85 DAF

Supplemental Fig. 2. Cont.

Supplemental Fig. 2. Cont.

KEGG Classification

Supplemental Fig. 3. Histogram of clusters of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for the differentially expressed genes in different *Acer truncatum* seed developmental stages. The horizontal axis represents the percentage of genes and the vertical axis shows the names of the KEGG clusters: (A) cellular processes; (B) environmental information processing; (C) genetic information processing; (D) metabolism; and (E) organismal systems.

Supplemental Fig. 4. Results of the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of *Acer truncatum* differentially expressed genes for the four comparisons. DAF = days after flowering.

Supplemental Fig. 4. Cont.

Supplemental Table 1. Primers (5'–3') used in the quantitative real-time polymerase chain reaction (qRT-PCR) to validate the reliability of the RNA-seq results.

Gene	Forward primer	Reverse primer
ACC	TGAAGGTATTTCCGCTATTT	TCCACTTGCCACTACTATCC
FATA1	TCCTGCTTGGAGTGATGTGG	TTTCTGAAGTCGCCTGGTGT
FATA2	TCCTGCTTGGAGTGATGT	GTCGCCTGGTGTCTTGAT
FATB	GAACTCTGACCCTATTGTGG	ATCCAGCCAATGTATTTTCAC
SAD1	ACCACCATTCTCCACCGTCAG	TTGCCAGCATTTATCTACCG
SAD2	CAATGGCCTCCACTCTTCGT	TTATCCTCAGCCCAACCCTC
SAD3	GGCCTCCACTCTTCGCTCCA	CCTCCGCCCAATCCTCCATA
FAD2	TGGTCCCTTATTTCTCGTGG	AATTTCTGCCTTGGTCTTGG
FAD2	TGACCCTTACGATTACACTTAC	AATAACGGCACTCCATACAC
FAD3	CCGAGGAAAGGAATGGAGTT	GTGAGGGATTGAGGGAAGA
FAD5	TACCACTCCACCGACCAAAAC	TATTCGCTTCTGCTTCAACT
FAD6	ATGGTTGGGCTATCACTTCT	CTCAATCCAACGAGGGTAAT
FAD7/8	CCCGAGTGAGATGAAAGATG	CAAGTAAGTCACGAAATCCAG
PDAT1	CAGTTGCTTCTACTAGGTGGT	AACAGGTGTCCACGCACGAC
PDAT2	CGGCGACGACATACAGGAAA	AGCAACCACCATATCGAGCA
DGAT	AAGAGGATTTGTTTCGCATAG	GCATTGGTTGTTTATAGGGT
ABI3	GAAGTCCGATACAGATGAAGG	GATGCCGTAGAAGACAAAGC
LEC1	TTGATGACTACATTGAGCCGTTGA	GCAGCAGGTGCGAAAGAAGC
LEC1.1	CTCACTTGGAGCACAATAAC	CTGCCATTACCATTCTCATC
FUS3	GACCAAACGGCTACTAATACG	GACTGGTAACAGCAGCGACA
WR11	AGGCGAAACAAGGAGATAGT	TTGGGATGATTGATAGAGGG
Actin	ATCATGTTTGAGACCTTCAACAC	GATCTTCATGAGGTAGTCAGTCAGGT

Supplemental Table 2. Summary of sequencing data of *Acer truncatum* seeds and alignment information of clean reads.

Sample ^z	Raw reads	Clean reads	Clean bases	Clean data rate (%)	Total mapped reads (%)	Unique matches (%)	Multi-position matches (%)
70DAF_1	51,614,678	50,548,700	7.58G	97.93%	90.32	83.52	6.80
70DAF_2	37,144,834	36,363,230	5.45G	97.90%	89.71	81.79	7.93
70DAF_3	46,343,392	45,391,246	6.81G	97.95%	89.45	81.52	7.93
85DAF_1	56,718,166	54,937,224	8.24G	96.86%	91.01	83.84	7.17
85DAF_2	49,957,250	48,619,534	7.29G	97.32%	91.16	84.08	7.07
85DAF_3	57,092,212	55,301,938	8.3G	96.86%	90.92	83.46	7.46
100DAF_1	49,137,600	47,956,846	7.19G	97.60%	90.62	83.31	7.31
100DAF_2	60,755,562	59,280,166	8.89G	97.57%	90.61	83.23	7.38
100DAF_3	51,269,214	49,835,732	7.48G	97.20%	90.88	83.05	7.82
115DAF_1	46,211,774	45,051,936	6.76G	97.49%	89.84	82.44	7.40
115DAF_2	46,399,842	45,279,334	6.79G	97.59%	90.36	82.90	7.46
115DAF_3	49,317,264	48,307,076	7.25G	97.95%	91.24	83.86	7.39
145DAF_1	40,162,964	38,493,262	5.77G	95.84%	90.20	77.37	12.83
145DAF_2	50,623,568	48,784,840	7.32G	96.37%	91.35	78.85	12.50
145DAF_3	49,329,602	47,496,066	7.12G	96.28%	85.36	73.71	11.65
180DAF_1	54,005,776	52,803,466	7.92G	97.77%	90.89	82.14	8.75
180DAF_2	42,914,112	41,795,622	6.27G	97.39%	91.57	83.09	8.48
180DAF_3	49,034,272	47,528,266	7.13G	96.93%	91.45	82.39	9.06

^zDAF = days after flowering.

Supplemental Table 3. Significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of differentially expressed genes in *Acer truncatum* seeds from different developmental periods.

Pathway	Genes (no.)	P
100 vs. 85 DAF ^z		
Biosynthesis of secondary metabolites	252	0.583423118
Ribosome	122	0.004531798
Protein processing in endoplasmic reticulum	90	0.000159454
Plant hormone signal transduction	74	0.333131831
Carbon metabolism	65	0.401327625
Flavonoid biosynthesis	12	0.024871355
Biosynthesis of amino acids	59	0.783457098
Fatty acid biosynthesis	17	0.058908918
Fatty acid elongation	7	0.682132256
Ether lipid metabolism	7	0.522889655
Sesquiterpenoid and triterpenoid biosynthesis	1	0.995839534
115 vs. 85 DAF		
Biosynthesis of secondary metabolites	345	0.025954254
Protein processing in endoplasmic reticulum	90	0.014229807
Carbon metabolism	84	0.195709832
Plant hormone signal transduction	84	0.501917605
Ribosome	80	0.997662321
Biosynthesis of amino acids	79	0.50474039
Flavonoid biosynthesis	17	0.003249311
Starch and sucrose metabolism	57	0.568025609
Fatty acid biosynthesis	22	0.022698982
Unsaturated fatty acid biosynthesis	17	0.099071687
Alpha-linolenic acid metabolism	15	0.139017983
Fatty acid elongation	12	0.302619961
Linoleic acid metabolism	4	0.367364226
145 vs. 85 DAF		
Protein processing in endoplasmic reticulum	45	0.036950766
Plant hormone signal transduction	48	0.160450493
Biosynthesis of secondary metabolites	175	0.024215476
Carbon metabolism	29	0.900982643
Flavonoid biosynthesis	7	0.063414814
Brassinosteroid biosynthesis	6	0.005756841
Fatty acid elongation	7	0.220137827
Biosynthesis of unsaturated fatty acids	6	0.473282401
Fatty acid biosynthesis	5	0.715980947
180 vs. 85 DAF		
Biosynthesis of secondary metabolites	568	0.011740226
Biosynthesis of amino acids	132	0.455449777
Plant hormone signal transduction	130	0.729465793
Carbon metabolism	125	0.480568449
Protein processing in endoplasmic reticulum	111	0.550702943
Ribosome	99	0.999999998
Unsaturated fatty acid biosynthesis	26	0.109515596
Fatty acid degradation	28	0.121109584
Fatty acid elongation	18	0.37856479
Ether lipid metabolism	12	0.668666136

^zDAF = days after flowering.

Supplemental Table 4. All genes associated with lipid metabolism identified in the transcriptome.

Lipid metabolism pathway	Genes with pathway annotation (no.)	Pathway
Arachidonic acid metabolism	34	ko00590
Biosynthesis of unsaturated fatty acids	34	ko01040
Cutin, suberine, and wax biosynthesis	42	ko00073
Ether lipid metabolism	34	ko00565
Fatty acid biosynthesis	44	ko00061
Fatty acid degradation	42	ko00071
Fatty acid elongation	45	ko00062
Glycerolipid metabolism	117	ko00561
Glycerophospholipid metabolism	116	ko00564
Linoleic acid metabolism	20	ko00591
Primary bile acid biosynthesis	1	ko00120
Sphingolipid metabolism	80	ko00600
Steroid biosynthesis	37	ko00100
Steroid hormone biosynthesis	46	ko00140
Synthesis and degradation of ketone bodies	6	ko00072
Alpha-linolenic acid metabolism	56	ko00592