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COMPARATIVE TRANSCRIPTOMIC ANALYSES OF HIGH AND LOW OLEIC ACID CONTENT SUNFLOWER (*HELIANTHUS ANNUUS* L.) SEED DEVELOPMENT

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Abstract

Helianthus annuus L. seeds possess high nutritional value and more stable oleic acid oil. Initial analysis of sunflower seed for oleic acid content during seed development revealed rapid oleic acid accumulation by 17 days after flowering (DAF), with high oleic acid levels persisting until after 27 DAF. Subsequent transcriptome sequencing analysis of high ('L-1-OL-1') and low ('86-1') oleic acid oil-producing sunflower cultivars enabled gene expression profile comparisons. Further analysis on differentially expressed genes (DEGs) in seed from different group at 17 and 27 days revealed 5971 (2751 up, 3220 down), 4906 (2518 up, 2388 down), 12761 (5807 up, 6954 down), and 9883 (3803 up, 6080 down) DEGs for D8617d vs D8627d, D8617d vs L17d, D8627d vs L27d, and L17d vs L27d, respectively. DEGs annotation detected the key fatty acid biosynthesis genes and transcription factors with potential roles in lipid metabolism. Furthermore, quantitative real time PCR (RT-PCR） results of 12 DEGs involved in fatty acid metabolism were positively correlated with their expression patterns using RNA-Seq implying a good reliability of results produced by this method. The results in this research may pave theoretical basis for researching molecular mechanisms of sunflower lipid metabolism.

Key words: *Helianthus annuus* L., Lipid metabolism, Oleic acid, Seed development, Transcriptomes.

Introduction

Sunflower (*Helianthus annuus* L.) is reported to be a major pivotal oil crop, cultivated globally. In 2018, sunflower seed production as an edible oil crop approached about 51.95 million tons worldwide, second only to that of rapeseed canola (*Brassica napus* L. and *Brassica campestris* L.) and soybean (*Glycine max* L. Merr.) (FAO, 2020). Sunflower seed oil is rich in unsaturated fatty acids and provides high nutritional value for food consumption. Indeed, the polyunsaturated fatty acid (linoleic acid) content of standard cultivated sunflower oil is high (approaching 50%~70%), although its monounsaturated fatty acid (oleic acid) content is low. From a nutritional standpoint, a diet rich in oleic acids can reduce blood plasma cholesterol by decreasing levels of unhealthy low-density lipoprotein without decreasing beneficial high-density lipoprotein levels (Mounts *et al*., 1988), the latter of which may reduce the risk of coronary heart disease (Grundy, 1986). Moreover, as compared to low oleic acid oil, high oleic acid oil has another advantage for its superior oxidative stability that protects the oil from chemical breakdown during frying, refining processes, and storage (Zhou *et al*., 2018). Consequently, sunflower breeders aim to develop sunflower cultivars that produce high oleic acid oil-containing seed.

Toward the goal of improving seed oil content, elucidation of key regulatory mechanisms involved in seed oil production would facilitate development of sunflower varieties with relevant traits for marker-assisted selection or genetic manipulation. More specifically, to improve the quality of sunflower seed oil would likely require identification of pivotal factors/genes involved in the biosynthesis pathways of fatty acid (FA). Although researchers have already characterized that sunflower contains some known enzymes encoded by a few genes involved in FA biosynthesis, such as enoyl-[ACP]-reductase (*ENR*) (Irene *et al*., 2015), long-chain acyl-coenzyme A synthetases (*LACS*) (Aznar-Moreno *et al*., 2013), β-ketoacyl-acyl carrier protein synthase III (*KAS III*) (González-Mellado *et al*., 2010), β-hydroxyacyl-[ACP] dehydratase (*HAD*) (Irene *et al*., 2016), and delta-12 desaturase (*FAD2*) (Dar *et al*., 2017), very few other relevant genes have been functionally characterized. Moreover, molecular regulatory mechanisms underlying sunflower seed FA biosynthesis and metabolism remains to be un-intensively deep discovered due to the limited genetic resources.

During seed development, processes leading to the formation of oils of other major storage compounds (e.g., starches and proteins) are influenced by many physiological events that are, in turn, governed by a regulatory mosaic of gene expression-modulating procedures (Weber *et al*., 2005). It is thus of great importance to comprehensively measure transcript abundance under various conditions in order to clarify molecular mechanisms responsible for oil accumulation in developing seeds. To date, many researchers focused on the transcriptional profiling studies of sunflower under stress, such as high salinity (Wang *et al*., 2017), herbicides (Gil *et al*., 2018), verticillium wilt (Guo *et al*., 2017), drought (Liang *et al*., 2017), and powdery mildew (Sujatha *et al*., 2018). However, investigations focus on sunflower seed oil content and quality are less, especially transcriptome-based analyses linking seed developmental processes to oleic acid content (OAC).

Currently, scientists firstly monitored seed OAC variations during different developmental stages then compared results obtained for high OA oil cultivar 'L-1-OL-1' and low oleic acid oil cultivar '86-1'. Next, we performed high-throughput sequencing of RNA samples isolated during two key seeds at different growth stage identified as relevant to cultivar-based OAC differences. Subsequently, we selected numerous differentially expressed genes (DEGs) through pairwise comparative analysis. Afterwards, we predicted the possible functions of these selected genes by Gene Ontology (GO) and

Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, which subsequently led to discovery of several DEGs that are involved in lipid metabolism. Results in this research revealed theoretical basis for the future elucidation of molecular mechanisms that regulate sunflower lipid metabolism. Moreover, DEGs and additional information revealed herein will likely aid future efforts to improve sunflower seed oil quality using genetic engineering technology.

Materials and Methods

Plant materials: Sunflower maintainer line 'L-1-OL-1', with high OAC seed (87.65%), and maintainer line '86-1', with low OAC seed (18.61%), were planted in the experimental field of the Industrial Crops Institute, Heilongjiang Academy of Agricultural Sciences (Harbin, China) and cultivated under natural conditions. The experimental blooming plants were observed at the same time point every day. Flowering plants were tagged, and flowering dates were recorded. Seeds at 7, 12, 17, 20, 22, 24, 27, 30, 32, and 37 days after flowering (DAF) were harvested from the outermost 3 rings of flower discs and subjected to OAC determinations. The results led to further characterization of seeds collected from 'L-1-OL-1' and '86-1' at 17 DAF and 27 DAF via comparative transcriptome analysis. The tested biological plants were collected in three replicates at every stage of growth. All the above-mentioned samples were frozen immediately in liquid nitrogen after collection. Samples were stored at −80°C till RNA extraction.

Determination of OAC: We measured the OAC values of seed using gas chromatography system (Model 7890A, Agilent Technologies, USA) at the Oil Crops Research Institute (China Academy of Agricultural Sciences, Beijing, China). The percentages of total FA content were selected to represent OAC value.

Isolation, library construction and sequencing analysis of RNA: Isolation: Total RNA was isolated from each collected sample by an RNAprep Pure Plant Kit (TianGen, China) according to the manufacturer. Then, the collected total RNA was digested with DNase I (included in the kit) to remove DNA. Afterwards, the quality and integrity of isolated total RNA were assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, USA). For RNA preparation, the total RNA (1 μg/sample) was chosen as input. Library construction: the isolated RNA samples from seeds collected at two different stages of growth (17, 27 DAF) from 'L-1-OL-1' and '86-1' cultivars were selected for the construction of RNA-Seq library. Following the instructions of NEBNext® Ultra™ RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA), we generated the RNA sequence library, and then, gave index codes to link the obtained sequences to represent their respective samples. Described in brief, poly-T oligo-conjugated magnetic beads was introduced to purify the mRNA, and then, NEBNext® First Strand Synthesis Reaction Buffer (5×) was chosen to fragment the obtained mRNA treated with divalent cations at an elevated temperature. Afterwards, random hexamer primers and M-MuLV Reverse Transcriptase and DNA Polymerase I and RNase H were chosen to synthesis the first-strand cDNA and the second-strand cDNA, respectively. By using exonuclease/ polymerase. The remaining overhangs were converted into blunt ends. Later, we adenylated the 3′ ends of DNA fragments to obtain the NEBNext adaptors with hairpin loop structures, and then ligated them for hybridization use. AMPure XP System (Beckman Coulter, Beverly, MA, USA) was chosen to purify the library fragments (cDNA) for the purpose of preferentially select cDNA fragments in 240 bp length. Next, 3 μL of USER™ Enzyme (NEB, USA) was added into the selected adaptor-ligated cDNA followed by a 37°C incubation for 15 min then a 5-min incubation at 95°C. Subsequent, PCR was performed with NEB Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer then purified the produced PCR products by AMPure XP System. Agilent 2100 Bioanalyzer System was chosen to evaluate the library quality. The cBot Cluster Generation System and TruSeq PE Cluster Kit v4-cBot-HS (Illumina) were chosen to cluster the index-coded samples. Illumina Novaseq 6000 platform was chosen to analyze the sequence details of library once the cluster was generated, and the paired-end reads were finally produced.

Data processing and analysis: In-house Perl scripts were chosen to select clean data through removal of reads containing adapter and/or poly-N sequences and low-quality reads among raw data. Concurrently, we assessed the levels of Q20, Q30, GC-content, and sequence duplication for the obtained clean data to produce the high quality of clean data, which was chosen for the subsequent experiments. Clean reads of high quality were then mapped to the reference genome sequence (https:// www.ncbi.nlm.nih.gov/genome/?term=Helianthus+annuus%5Borgn%5D). Only reads with a perfect match or one mismatch relative to the reference genome were further analyzed and annotated, sequences alignment to the reference genome using HISAT2 (Kim *et al*., 2015) . Gene expression levels were estimated and expressed as fragments per kilobase of transcript per million fragments mapped (FPKM). Differential expression analysis was performed using DESeq2 (Love *et al*., 2014). Benjamini-Hochberg approach was introduced to adjust the P-value < 0.01 for the purpose of controlling the false discovery rate (FDR). |log2 fold change (FC)| ≥ 2 and FDR < 0.01 were used as threshold settings in order to grade significance of differential expression results. For prediction of possible functions and pathway enrichment of DEGs under whole seed transcriptome condition, GO annotations using Blast2GO (Conesa *et al*., 2005) by the GOseq R package based on a Wallenius non-central hyper-geometric distribution (Young *et al*., 2010) and KEGG enrichment analysis using KOBAS (Mao *et al*., 2005) based on hyper-geometric tests. To detect transcription factors (TFs) within the sunflower seed transcriptome, all genes were searched against the plant TF database PlantTFDB (http://planttfdb.gao-lab.org/blast.php) using BLAST tool with a cut-off of $1e^{-5}$. Based on the average FPKM of genes across RNA-Seq library replicates, we performed heat map analysis of expression quantity clustering results using the clustering heat map drawing tool on the platform BMK Cloud (www.biocloud.net).

Validation of DEGs by quantitative RT (qRT)-PCR: We conducted the qRT-PCR analysis to validate the predicted results of RNA-Seq analysis of DEGs involved in FA metabolism. Described in brief, isolation of total RNA was described in section 'Isolation, library construction and sequence analysis of RNA'. Total RNA was extracted using a RNAprep Pure Plant Kit (TIANGEN) and samples were treated with DNase I (included in the kit) to remove DNA. First-strand cDNA was synthesized using ReverTra Ace^{TM} qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan) then qRT-PCR was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan) on a Roche LightCycler® 480 II System (Roche, Switzerland). Each reaction mixture (20-µl total volume) contained 10 µl of SYBR Green Taq, 0.6 µl of each primer (10 µM), 1.2 µl of cDNA template, and 7.6 µl of ddH_2O. Amplification conditions were as follows: 98°C for 2 min (proceeds with 45 cycles) of 98°C for 10 s, 58°C for 10 s, and 68°C 30 s. Dissolution curves were analyzed after completion of PCR reactions. Primer 5.0 was chosen for the design of primers for the targets (Supplemental Table 1). Samples were processed with 3 repeats and relative gene expression levels were normalized to the internal control *β-actin* (AF282624) after they were assessed by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Results

Determination of relevant developmental periods for transcriptome sequencing: To determine relative degree of variation in OAC during seed development of cultivars with high and low oleic acid oil-containing seed, samples collected from sunflower seeds at 7, 12, 17, 20, 22, 24, 27, 30, 32, and 37 DAF were chosen for OAC assessment (Fig. 1). In developing seeds of 'L-1-OL-1', OAC increased significantly from 26.2% in seeds at 7 DAF to 65.8% in seeds at 20 DAF and then changed slightly from 20 to 27 DAF, with only a slight change from 27 to 37 DAF (68.6%-71.8%) that culminated in the highest level observed at 37 DAF. In developing seeds of '86-1', oleic acid was also increased dramatically from 7.6% in 7 DAF seeds to 27.5% in 20 DAF seeds, reached highest level at 22 DAF (28.4%), then slightly decreased from 22 to 27 DAF and thereafter remained relatively steady through 37 DAF (23.7%-21.5%). Based on abovementioned oleic acid variation trends for both cultivars, 17 DAF was identified as a time point of rapid oleic acid accumulation in sunflower seeds, while 27 DAF was found to be a relatively stable period for OAC. Thus, transcriptome sequencing was conducted using seed samples collected at 17 DAF and 27 DAF.

Transcriptome sequencing of sunflower seeds: We chose RNA-Seq sequencing to select the DEGs in high/low oleic acid seed. Altogether, 12 cDNA libraries were finally obtained from biological triplicate RNA samples isolated from seeds obtained at 2 different time points during the growth (17 DAF and 27 DAF) of 'L-1-OL-1' and '86-1' (hereafter denoted L17d, D8617d, L27d, and D8627d). Supplemental Table 2 displayed the total 86.68 Gb of clean data that were received after the stringent quality control and data filtering. Each library produced clean data ranging in total sequence length from 5.93 to 9.19 Gb, with Q30 base percentages all above 91.24% and GC percentages ranging between 45.34%~48.35%. Further deep map analysis against sample genome revealed that the total number of mapped reads exited from 37.7 to 57.4 million/library, with mapped ratios ranging from 88.11% to 94.87%/library, and the total number of unique mapped reads ranged from 33.2 to 54.9 million/library (Supplemental Table 3). These results indicated that sequencing data were of suitable quality for further analyses.

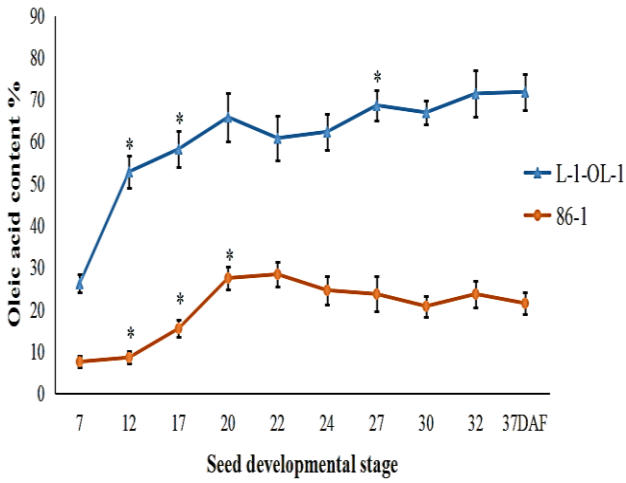

Fig. 1. Patterns of variation of seed OAC at different developmental stages of 'L-1-OL-1' and '86-1'. Seeds at 7, 12, 17, 20, 22, 24, 27, 30, 32, and 37 DAF (days after flowering) were harvested for oleic acid measurements. Mean contents of oleic acid are presented with standard errors bars for three biological replicates. Asterisks indicate that OAC during this period significantly differs from that of the previous period according to t-tests at $p<0.05$.

Comparison of gene expression at 17 and 27 DAF in developing seeds of high and low oleic acid oil sunflower cultivars: We sorted the data to obtain DEGs with $|\log2\ FC| \geq 2$ and FDR ≤ 0.01 for each comparison group. Totally, 5971 (2751 up, 3220 down), 4906 (2518 up, 2388 down), 12761 (5807 up, 6954 down), and 9883 genes (3803 up, 6080 down) were differentially expressed in D8617d vs D8627d, D8617d vs L17d, D8627d vs L27d, and L17d vs L27d pairwise comparisons, respectively (Fig. 2A). Most DEGs were obtained for D8627d vs L27d, while fewest DEGs were obtained for D8617d vs L17d. Interestingly, we observed that the total lowly expressed genes were more than the total highly expressed genes for D8617d vs D8627d, D8627d vs L27d, and L17d vs L27d comparisons. (Fig. 2B-E) showed general trends of gene expression levels and fold changes for each pairwise comparison. From these results, 357 DEGs were shared across all four pairwise comparisons, D8617d vs L17d and D8627d vs L27d pairwise comparisons shared 3036 DEGs, and D8617d vs D8627d and L17d vs L27d comparisons shared 2076 DEGs (Fig. 2F).

Functional categorization of DEGs: The possible functions and the potential involved pathways of the selected DEGs of two seed developmental stages between high and low oleic acid oil cultivars were predicted using GO and KEGG assays. Supplemental Table 4 listed the predicted functional categories of DEGs identified in all four pairwise comparisons were categorized into 52 sub-

groups. Data in (Fig. 3) revealed the basic number of DEGs in each category, and also displayed DEGs and all genes identified in the sunflower seed transcriptome sequence results. It was observed that 3 biological processes (cellular process, metabolic process, and single-organism process) were significantly gathered, while 2 molecular functions (catalytic activity and binding) were dramatically gathered in all four pairwise comparisons.

With regard to KEGG pathways, the greatest enrichment of DEGs was mainly found in ribosome (D8617d vs D8627d), sulfur metabolism (D8617d vs L17d, L17d vs L27d), and fatty acid biosynthesis (D8627d vs L27d) (Supplemental Fig. 1). KEGG pathways associated with the highest numbers of DEGs were carbon metabolism and biosynthesis of amino acids (D8617d vs L17d, D8627d vs L27d, L17d vs L27d); however, the metabolic pathway containing the highest number of DEGs was ribosome, as detected in the pairwise comparison D8617d vs D8627d (Supplemental Table 5). Meanwhile, KEGG pathway analysis revealed pathways related to FA metabolism (FA biosynthesis, FA elongation, and biosynthesis of unsaturated FA, etc.). Number of DEGs involved in these metabolic pathways and their proportions of total DEG numbers is shown in Table 1.

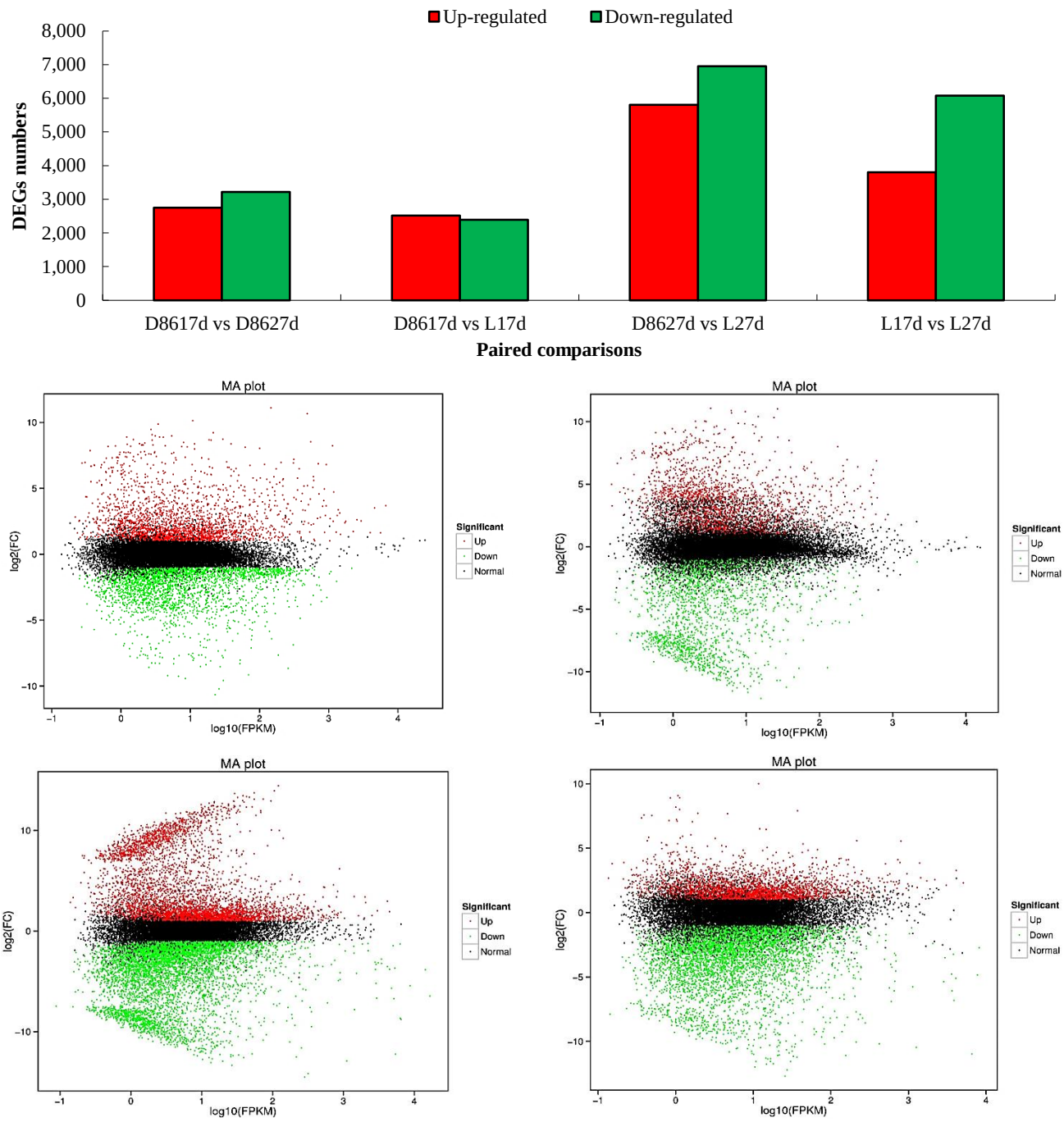

Fig. 2. Analysis of differentially expressed genes (DEGs). A: Total selected DEGs in four paired comparisons (D8617d vs D8627d, D8617d vs L17d, D8627d vs L27d, and L17d vs L27d); B-E: Distribution of DEGs based on $\log_{10}$ (FPKM) values in four pairwise comparisons (B: D8617d vs D8627d; C: D8617d vs L17d; D: D8627d vs L27d; E: L17d vs L27d). Red dots represent the up-regulated genes while green dots represent down-regulated genes; F: Venn diagram showing DEGs detected in the four pairwise comparisons.

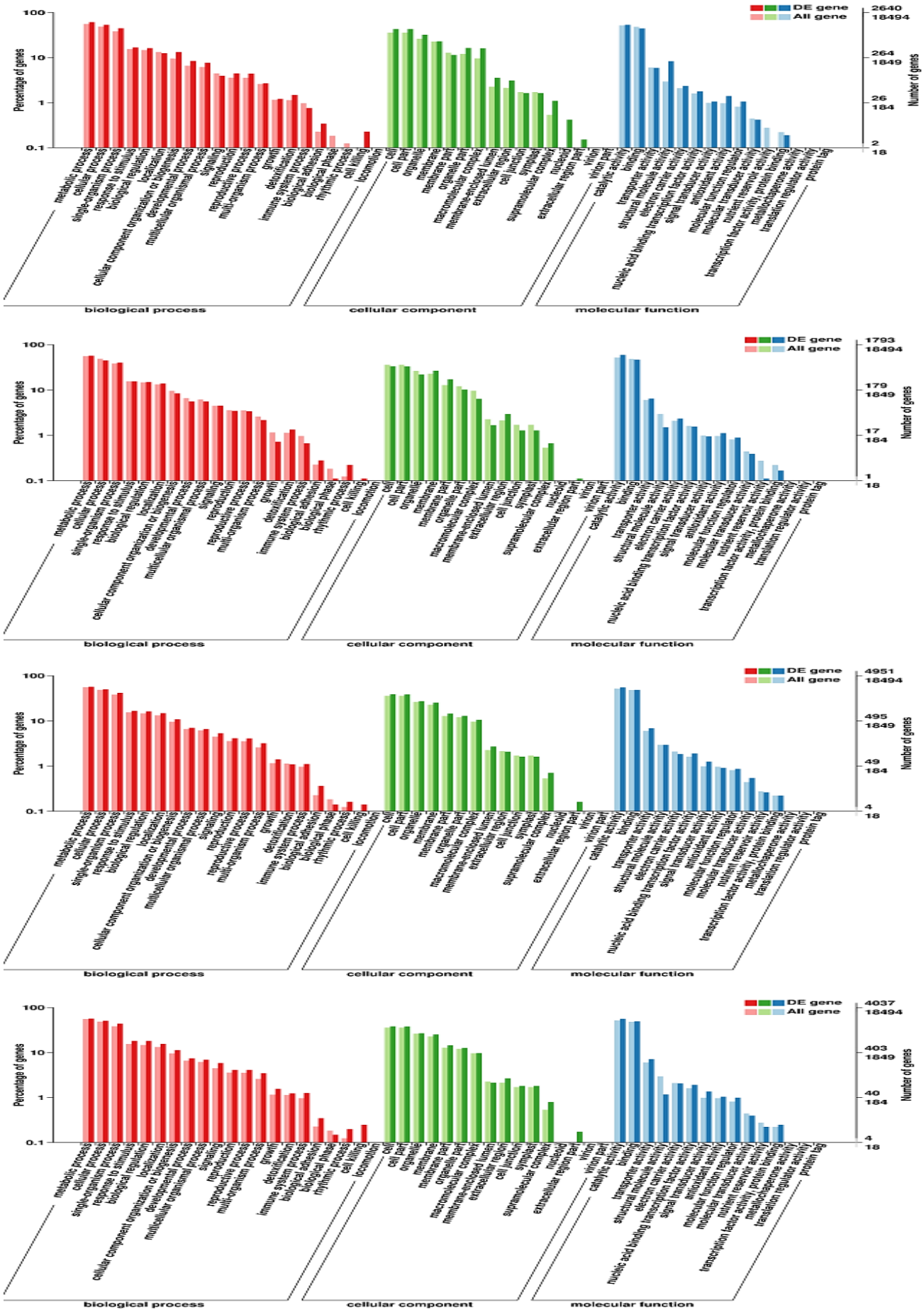

Fig. 3. Function analysis of the selected DEGs (A: D8617d vs D8627d; B: D8617d vs L17d; C: D8627d vs L27d; D: L17d vs L27d).

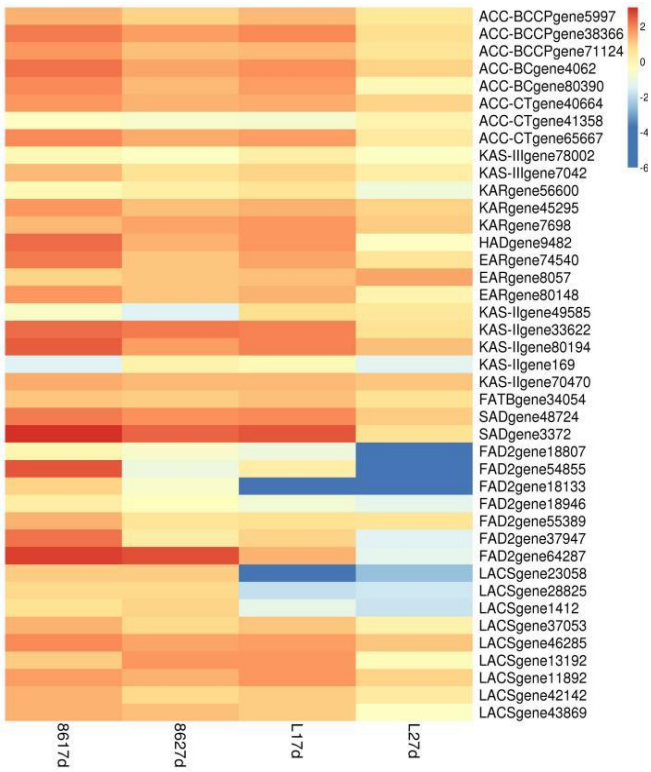

Fig. 4. Transcript abundance of DEGs involved in FA biosynthesis during seed development in '86-1' and 'L-1-OL-1'. Mean $\log_{10}$ FPKM values depicted using different color scales based on legends shown in boxes. 8617d: '86-1' at 17 DAF; 8627d: '86-1' at 27 DAF; L17d: 'L-1-OL-1' at 17 DAF; L27d: 'L-1-OL-1' at 27 DAF.

Identification and expression pattern of DEGs involved in FA biosynthesis: We successfully identified 41 genes that could encode key enzymes participated in FA biosynthesis after KEGG analysis (Fig. 4). Comparison results on expression values for the above selected genes among the four sample groups were listed in Supplemental Table 6. Next, identities of these genes were related to current knowledge of FA biosynthetic pathways. In plant, acetyl-CoA (ACC) was catalyzed by ACC carboxylase (ACCase) to produce malonyl-CoA. Heteromeric ACCase consisted of four subunits: carboxyl transferase (α-CT and β-CT), biotin carboxylase, and biotin carboxyl carrier protein. Notably, 8 DEGs encoding ACC, or its subunits were found in our RNA-Seq data. Except for 41358 (ACC-CT), expression level of the other 7 DEGs (5997, 38366, 71124, 4062, 80390, 40664, and 65667) in 8627d and L27d were lower than in 8617d and L17d, while expression levels of 41358 were low in all four sample groups. Other DEGs were found to be linked to a known FA pathway whereby malonyl-ACP accesses a four-step elongation cycle and thereby catalyzed by a round of enzymes, for instance, 3-ketoacyl-ACP (KA) synthase III (KASIII), KA reductase (KAR), 3-hydroxyacyl-ACP dehydratase (HAD), enoyl-ACP reductase (EAR), and 3-ketoacyl-ACP synthase I (KASI). Here, we identified 2 DEGs (78002, 7042) encoding KASIII, 3 DEGs (56600, 45295, 7698) encoding KAR, 1 DEG (9482) encoding HAD, and 3 DEGs (74540, 8057, 80148) encoding EAR. Most of these genes exhibited higher expression levels at 17 DAF than at 27 DAF in both '86-1' and 'L-1-OL-1' except for 8057, 56600, and 7698. The generated C16:0-ACP is known to be deeply elongated to C18:0-ACP by KASII after several cycles, followed by desaturation to form C18:1-ACP by 18:0-ACP desaturase (SAD). Here, 5 DEGs (49585, 33622, 80194, 169, 70470) encoding KASII and 2 DEGs (48724, 3372) encoding SAD were identified, with all DEGs exhibiting decreasing expression from 17 DAF to 27 DAF in both '86-1' and 'L-1-OL-1' (with the exception of 169 in the D8617d vs D8627d group); by contrast, expression levels of *SAD* in '86-1' were observed to be significantly higher than corresponding levels in 'L-1-OL-1' during the same period (17 DAF or 27 DAF). In addition, DEGs were also annotated to known pathways, for instance, in which the nascent C16:0-ACP or C18:0-ACP could release as free saturated FAs catalyzed by fatty acyl-ACP thioesterase B (FATB) or which C18:1-ACP could hydrolyze to unsaturated FAs catalyzed by FATA. Here, we identified one DEG encoding FATB (34054) but no DEG encoding FATA, with expression of 34054 in the L27d sample group was significantly lower than corresponding levels found in the other three comparison groups. Meanwhile, additional DEGs were found to be associated with known processes that generated free FAs in plastids through esterification of COA during biosynthesis process of triacylglycerol (TAG) or via long-chain acyl-COA (LAC) synthesis (LACS) at the plastid envelope. Interestingly, expression levels of eight DEGs (23058, 28825, 1412, 37053, 46285, 11892, 42142, 43869) encoding LACS pathway enzymes in '86-1' were significantly higher than corresponding levels observed for 'L-1-OL-1' for the same timepoint (17 DAF or 27 DAF). Intriguingly, one DEG for LACS (13192) exhibited an opposite trend of transcriptional change in the D8617d vs L17d group compared to the other three comparison groups. Ultimately, significantly higher LACS activity in '86-1' at 17 DAF may lead to greater trends of C18:0 COA, C18:1 COA, or C16:0-COA towards the biosynthesis of TAG in that cultivar compared to 'L-1-OL-1'.

Finally, another biosynthesis process of polyunsaturated FA was also discovered to be correlated with the other DEGs. Here, seven DEGs (64287, 18807, 54855, 18133, 18946, 55389, 37947) encoding FAD2 were identified; although *FAD3* and *FAD7* genes expressed differentially, none of them matched any DEG obtained here, while no *FAD6* genes were found to be expressed. Nevertheless, expression levels of *FAD2* genes in '86-1' were significantly higher than corresponding levels found in 'L-1-OL-1' during the same developmental stage (particularly at 17 DAF). Importantly, this result highlights the negative correlation observed between OA accumulation and *FAD2* expression; consequently, relatively greater FAD2 desaturase activity in '86-1' relative to that found in 'L-1-OL-1' might lead to promotion of C18:1 to C18:2 conversion followed by greater C18:2 accumulation. Ultimately, this scenario might explain why '86-1' seed possessed relatively lower C18:1 content than content found in 'L-1-OL-1' seed. Nonetheless, gene expression in the same tissues of 'L-1-OL-1' and '86-1' revealed that *FAD2* expression levels at 17 DAF significantly exceeded *FAD2* expression levels at 27 DAF, demonstrating that more C18:1 than C18:2 was formed at 17 DAF than at 27 DAF.

Table 1. Statistics of DEGs in fatty acid metabolism-related pathways.

Metabolic pathway	D8617d vs D8627d	D8617d vs L17d	D8627d vs L27d	L17d vs L27d
Fatty acid biosynthesis	25 (1.94%)	11 (1.29%)	44 (1.81%)	33 (1.70%)
Fatty acid metabolism	46 (3.57%)	25 (2.93%)	60 (2.47%)	50 (2.57%)
Fatty acid elongation	5 (0.39%)	6 (0.70%)	12 (0.49%)	11 (0.57%)
Fatty acid degradation	19 (1.48%)	17 (2.00%)	27 (1.11%)	20 (1.03%)
Biosynthesis of unsaturated fatty acids	23 (1.79%)	10 (1.17%)	15 (0.62%)	17 (0.87%)
Alpha-Linolenic acid metabolism	18 (1.40%)	19 (2.23%)	23 (0.95%)	26 (1.34%)
Linoleic acid metabolism	9 (0.70%)	6 (0.70%)	9 (0.37%)	10 (0.51%)
Arachidonic acid metabolism	7 (0.54%)	3 (0.35%)	5 (0.21%)	8 (0.41%)

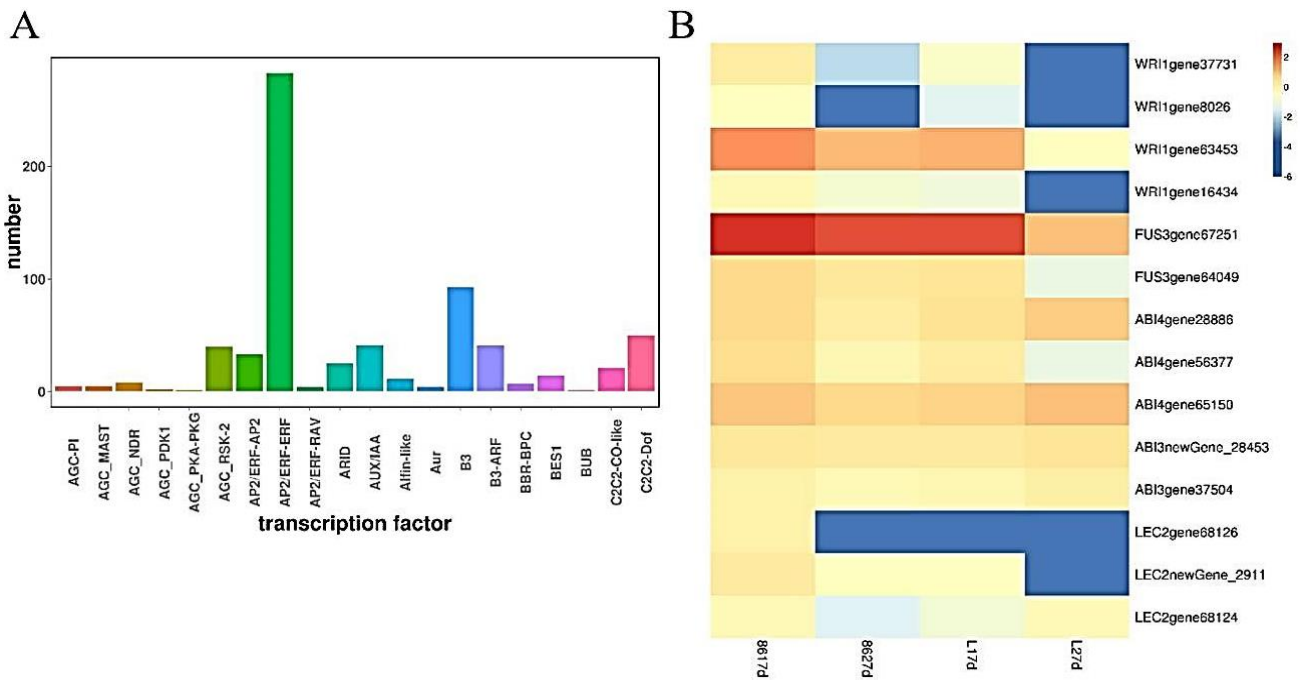

Fig. 5. Identification of transcription factors (TFs) A. Distribution of numbers of TFs in each identified TF family; B. Transcript abundance of TFs involved in lipid metabolism during seed development for '86-1' and 'L-1-OL-1'. Mean $\log_{10}$ FPKM values depicted using different color scales shown in the boxes. 8617d: '86-1' at 17 DAF; 8627d: '86-1' at 27 DAF; L17d: 'L-1-OL-1' at 17 DAF; L27d: 'L-1-OL-1' at 27 DAF.

Identification and expression profiling of TFs involved in lipid metabolism: In the Plant TF Database, we identified TFs in the sunflower seed transcriptome; the top three TF families were identified as AP2/ERF, B3, and C2H2-Dof in terms of sequence abundance (Fig. 5A). Subsequently, we focused on some certain TFs with possible roles in lipid metabolism and deeply explored their transcriptional patterns (Fig. 5B; Supplemental Table 6). WRINKLED1 (*WRI1*), a member of APETALA2/ ETHYLENE RESPONSE FACTOR (AP2/ERF) family, is recognized to be the major factor in regulating oil synthesis of plant. We detected four *WRI1* transcripts in our sunflower seed transcriptome whereby expression levels of these transcripts (37731, 8026, 63453, 16434) in '86-1' exceeded their corresponding levels in 'L-1-OL-1' at each developmental stage. Meanwhile, expression levels at 17 DAF in samples of both cultivars exceeded their corresponding levels at 27 DAF. In addition, we also identified lipid-metabolism-related TFs, including FUSCA3 (*FUS3*), ABSCISIC ACID INSENSITIVE3 (*ABI3*), *ABI4*, and LEAFY COTYLEDON1 (*LEC2*). *FUS3* (67251 and 64049) shared similar expression profiles with *WRI1* transcripts. Except for one *ABI4* transcript (56377), all *ABI3* (28453 and 37504) and *ABI4* (28886 and 65150) exhibited skyscraping expression levels in the L27d sample, followed by 8617d, L17d, and 8627d expression levels ranked from next highest to lowest. Meanwhile, expression levels of three *LEC2* (68126, 2911, and 68124) were low in all four samples, although levels of expression were relatively highest in the 8617d sample.

Validation of transcriptome data by qRT-PCR: We chose qRT-PCR to assess the expression situations of DEGs selected after the RNA-Seq analysis. A total of 12 genes involved in FA metabolism were selected: LAC synthetase (*ACSL*: gene1412), microsomal omega-6 FA desaturase (*FAD2*: gene64287, gene54855, gene55389), 3-oxoacyl-ACP reductase (*fabG*: gene24919, gene7698, gene76007), acetyl-CoA acyltransferase 1 (*ACAA1*: gene2190), 3-oxoacyl-ACP synthase I (*KASI*: gene80194), very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase (*HACD*: gene62919), acyl-ACP desaturase (*FAB2*: gene48724), and very-long-chain enoyl-CoA reductase (*TER*: gene64935). RNAs sampled from seeds of 'L-1-OL-1' and '86-1' at 17 DAF and 27 DAF were used as templates. Correlation analysis displayed that there was a positive correlation between qRT-PCR and RNA-Seq of the selected 12 DEGs (Fig. 6), thus confirming the expression of 12 DEGs in RNA-Seq.

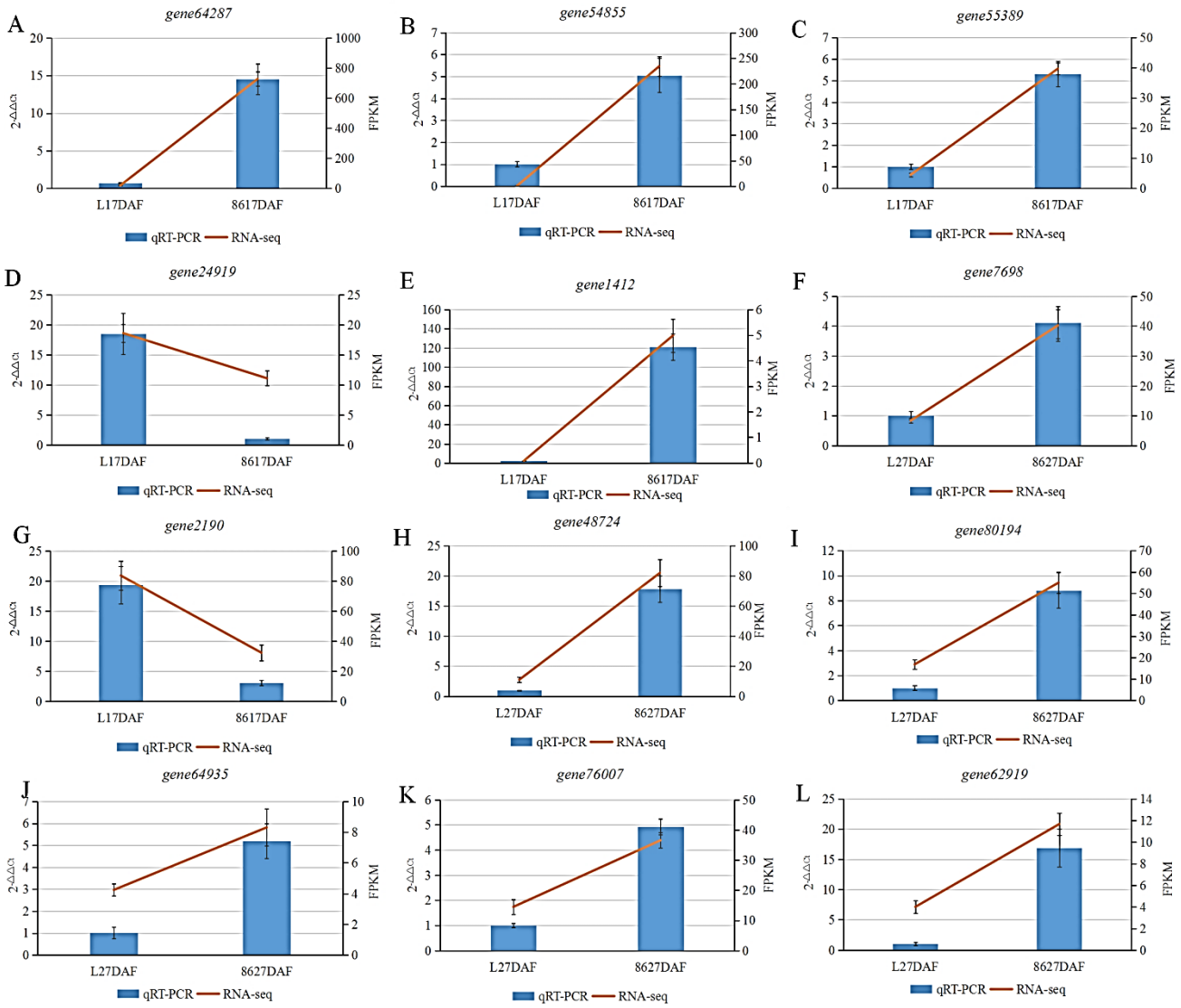

Fig. 6. Gene expression profiles of the 12 selected genes involved in seed development. Values represent the mean ± SD (standard deviation) of three replicates.

Discussion

It is important to improve the understanding of the regulatory models/mechanisms participated in the process of seed development for devising the strategies on improving the seed nutritional quality and oil content of sunflower. Toward this end, transcriptome studies of developing seeds conducted via RNA-Seq may possibly pave positive prints on gene expression networks behind the seed development. Indeed, recent evidence provides us directions that RNA-Seq has already been widely used in research of investigating gene expressions of seed development in many kinds of plants such as rice (Xue *et al*., 2012), soybean (Jones & Vodkin, 2013), chickpea (Pradhan *et al*., 2014), Pongamia (Huang *et al.,* 2018), perilla (Liao *et al*., 2018), and wheat (Rangan *et al*., 2017). Although sunflower is an oil crop that is rich in unsaturated fatty acids, especially linoleic acid, few investigations have been conducted of gene expression and key genes participated in unsaturated FA synthesis during sunflower seed development. Therefore, such studies are needed, especially those involving transcriptome analysis of developing sunflower seeds, prompting this study. However, transcriptome profiles of developing sunflower seeds of high oleic acid oil-producing 'J9' and lower oleic acid oil-producing 'NK244' were determined and compared at 20 days post-pollination using RNA-Seq, yielding 5447 DEGs between genotypes (Yu *et al*., 2018). By contrast, here we selected two critical oleic acid accumulation periods for transcriptome sequencing for the purpose of transcriptomes comparison of seeds obtained from high and low oleic acid sunflower cultivars during different developmental stages. Notably, the high sequence coverage obtained in this research ensured a large extent of sequence assembly accuracy and yielded high-quality expression profiling results.

Importantly, our KEGG analyses revealed numerous DEGs encoding key enzymes involved in FA metabolism, such as *LACS* (Jessen *et al*., 2015),very-long-chain 3-oxoacyl-CoA reductase (*KAR*) (Islam *et al*., 2019), hydroxyacyl-ACP dehydratase (*HAD)* (Liu *et al*., 2019), and 3-oxoacyl-ACP synthase III (*KASIII*) (Yu *et al*., 2015), and others. Moreover, our results revealed several DEGs that might influence OAC of plant seeds to some extent,

including FA ACP thioesterase B (*FATB*) (Ozseyhan *et al*., 2018), delta-12 desaturase (*FAD2*) (Bera *et al*., 2018), and stearoyl-ACP 9-desaturase (*SAD*) (Huang *et al*., 2016). All of these findings may pave theoretical basis for the near coming explorations of molecular regulatory patterns associated with oleic acid (OA) synthesis and metabolism. Previous evidence revealed that a deep desaturation of C18:1 in separate pathways within plastids and the endoplasmic reticulum (ER) determined the achievement of the process of polyunsaturated FA. There were reports that C18:1 acyl groups were possibly be brought into phosphatidylcholine (PC) in the ER, and then might be sequentially desaturated by microsomal FA desaturases 2 (FAD2) and omega-3 FAD3 to form C18:2 and C18:3. An alternative to the two ways, chloroplast omega-6 FAD6 and FAD7 were together responsible for converting C18:1 to C18:2 and C18:3 (Huang *et al*., 2018). Currently, *FAD2* is recognized to a pivotal gene in controlling linoleic acid content, OAC, and the ratio between linoleic acid ratio and OA in plants (Long *et al*., 2018). Notably, depression of *FAD2* in oil crops could dramatically suppress synthesis of linoleic acid, significantly increased OAC, and thereby improved oil nutritional and economic value (Yang *et al*., 2017). Thus, our findings may enhance understanding of sunflower oleic acid formation mechanisms for use in improving oil quality via breeding and germplasm-based innovative measures.

Gang's study demonstrated the pivotal regulatory functions of transcription factors in modulating gene expression during gene transcription (Gang *et al*., 2019). The AP2/ERF transcription factors are a large group of factors that are mainly found in plants. Numerous reports have documented that the AP2/ERF type TFs were important regulators involved in plant growth and development, such as grain development, seed/fruit development, and ripening (Feng *et al*., 2020). AP2 plays important and manipulated roles in controlling both seed mass and seed yield. The factor of AP2 was very important in seed development and yield, including seed size and seed weight in Arabidopsis and also determined the accumulation of seed oil and protein (Jofuku *et al*., 2005; Sharma *et al*., 2017). AP2/ERF TFs were associated with seed development and metabolism in the tropical tree *Bixa orellana* L. and maize (Soares *et al*., 2011; Wang *et al*., 2010). In this study, plenty of members in AP2/ERF family were found in the TFs identified in the sunflower seed transcriptome, which indicates that AP2/ERF TFs play an important role in sunflower seed development. So far, the basic transcriptional regulatory models in lipid metabolism in sunflower seeds remain undiscovered. This study revealed that analysis on transcriptome level provided possibilities to identify putative TF expression patterns in developing seeds possessing different OAC levels. The WRI1 transcription factor protein, a member of the Apetala2 ethylene response element binding factor (AP2/EREB) domain, regulates expression of several enzymes participated in FA biosynthesis pathways in the process of seed development (Baud *et al*., 2007). Durrett and his colleague discovered that the highly expressed *WRI1* in *A. thaliana* or maize enhanced the content of TAG in vegetative tissues and seeds (Durrett *et al*., 2011). FUSCA3 (FUS3) is another lipid-metabolism-related transcription factor (Roscoe *et al*., 2015). Here, *WRI1* transcript levels in '86-1' exceeded levels in 'L-1-OL-1' at each developmental stage, while *FUS3* expression patterns mirrored *WRI1* expression patterns as evidence for an association between *WRI1* and *FUS3* expression and sunflower seed OAC. As upstream TFs, *LEC1* and *LEC2* have putative roles in regulating several genes among the processes of condensation, desaturation during glycerolipid biosynthesis, and chain elongation(Mu *et al*., 2008). The expression levels of three *LEC2* transcripts were low in all four samples, possibly reflecting the regulatory differences for *LEC2* expression patterns at tissue/species specificity angle that had been reported previously; *GmABI3* enhances the seed-specific TAG and long-chain FA biosynthesis in soybean (Manan & Zhao, 2020), while *ABI4* represses lipid breakdown by increasing expression of *DGAT1*, which is recognized to be the key enzyme catalyzing the rate-determining step among biosynthesis of TAG (Yang *et al*., 2011). Here *ABI3* and *ABI4* transcripts were also detected in FA biosynthesis. To sum up, all findings in this research might pave theoretical knowledge in illustrating the potential existed molecular mechanisms underlying FA and lipids biosynthesis in sunflower.

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Availability of Data

The datasets supporting the conclusions of this article have been deposited in the NCBI GEO database under accession numbers GSE164103 (https://www.ncbi.nlm.nih.gov/ geo/query/acc.cgi?acc=GSE164103).

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