

# Transcriptomic and microstructural analyses in *Liriodendron tulipifera* Linn. reveal candidate genes involved in nectary development and nectar secretion

Huanhuan Liu

Nanjing Forestry University

Jikai Ma

Nanjing Forestry University

Huogen Li (✉ [hgli@njfu.edu.cn](mailto:hgli@njfu.edu.cn))

Nanjing Forestry University <https://orcid.org/0000-0001-6455-9346>

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## Research article

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# Abstract

**Background** Nectar is a major flower attractant and reward for insects for pollination. *Liriodendron*, a genus of the Magnoliaceae family, has only two relict species, *L. chinense* and *L. tulipifera*, that are considered “basal angiosperms” according to plant evolutionary history. The flowers of *Liriodendron* plants are insect pollinated and secrete nectar to attract pollinators. To date, the morphology and anatomy of the nectary, the mechanism of nectar secretion and the molecular mechanism involved in nectary development in *Liriodendron* remain poorly understood. **Methods** In this study, we examined the nectary surface cells and the change in starch in *L. tulipifera* by using scanning electron microscopy and periodic acid-Schiff techniques to select definitive samples for next research. Transcriptome sequencing was performed on the top and middle parts of the immature nectary and the middle parts of the mature nectary and the postsecreted nectary in *L. tulipifera*. We evaluated the expression profiles of 21 DEGs that were closely related to nectary development and nectar secretion for real-time quantitative PCR analysis. **Results** The *L. tulipifera* nectary is a starch-storing nectary and is located in the top and middle parts of *L. tulipifera* petals. After analyzing the RNA-seq data, we obtained 115.26 Gb clean data in 12 libraries and mapped the results to the *L. chinense* reference genome with 71.02%-79.77% efficiency. In total, 26,955 DEGs were identified by analyzing six different pairwise comparisons. The flavonoid biosynthesis, phenylpropanoid biosynthesis, anthocyanin biosynthesis and starch and sucrose metabolism pathways were enriched and related to nectar secretion and pigment change. We identified 56 transcription factor families, and members of the TCP, Trihelix, C2H2, ERF, and MADS families changed dynamically during nectary development. Moreover, to further verify the accuracy of the RNA-seq results, we validated the expression profiles of 21 candidate genes. **Conclusions** We evaluated the nectary development and secretion process comprehensively and identified many related candidate genes in *L. tulipifera*. These findings suggest that the nectary may play important roles in flavonoid synthesis and petal color presentation.

## Background

The original attractant for insects to insect-pollinated plants was pollens; however, nectar, an inexpensive foodstuff, was generated as an alternative to pollen, and then, the nectary was formed[1]. Owing to its diversity in position and structure, the nectary is classified into different categories[2]. According to their position, nectaries can be classified into two types: floral and extrafloral nectaries. *Pteridium aquilinum* is an ancient extrafloral plant with nectaries on its fronds[3-5]. According to their structure, nectaries can be divided into two groups: structural and nonstructural nectaries[6, 7]. The structure of the most angiosperms nectary contains three major components: epidermis, specialized parenchyma and vascular bundle[8, 9]. During nectary development and nectar secretion in most plants, starch grains accumulate and then degrade. Therefore, according to whether starch undergoes regular dynamic changes, the nectary can also be divided into two groupings: starch-storing nectary and non-starch-storing nectary[10]. Thus far, the anatomy and taxonomy of nectaries have been comprehensively studied, and substantial

achievements have been obtained. However, research on the biochemistry and molecular biology of the nectary is delayed.

Currently, research is focused more on the molecular mechanism of the floral nectary rather than the anatomy and taxonomy of the floral nectary. Several studies on model plants have found a key gene regulating nectary development: *CRABS CLAW* (*CRC*). *CRC* is a transcription factor (TF) containing a zinc finger and a helix-loop-helix domain and is mostly expressed in the mutant and wild phenotype of *Arabidopsis thaliana* carpels and nectaries[11]. *CRC* is negatively regulated by the A and B organ identity genes and is regulated independent of C class genes according to research of ectopic carpels of floral homeotic mutants[11]. Remarkably, the *crc-1* and *crc-2* mutants both lack nectaries. Baum et al. proposed that the nectary is independent of any floral organ identity gene in *A. thaliana* but is restricted to the “third whorl” domain: *LEAFY* (*LFY*) and *UNUSUAL FLORAL ORGANS* (*UFO*)[12]. *CRC* is conservatively expressed in the nectaries of several core eudicot plants and is required for nectary development in both rosids and asterids, but *CRC* expression is not found in the nectary of basal eudicot plants[2]. In addition, *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2* can also promote nectary development. Interestingly, the nectary is present but abnormal in the *bop1/bop2* double mutant, while in the *bop1/bop2/crc* triple mutant lacks a nectary[13]. The *Arabidopsis* nectary transcriptome was analyzed, and *CRC*, *AGL5/SHATTERPROOF2* (*SHP2*), *AGL1/SHP1*, *AGAMOUS* (*AG*) and *APETALA2* (*AP2*) showed nectary-enriched expression profiles[14]. The *CELL WALL INVERTASE4* (*CWINV4*) gene is highly expressed in the nectary and is required for nectar secretion, but the *cwinv4* mutant cannot produce nectar in *Arabidopsis*[15]. The TF *MYB305* is strongly expressed in the ornamental tobacco nectary and regulates the expression of flavonoid metabolic and nectar proteins[16]. When *MYB305* is knocked down, starch accumulation and nectar production are reduced significantly in the tobacco nectary[17]. The other *MYB* TF, such as *MYB21* and *MYB24*, regulate *Arabidopsis* nectary development and function and volatile sesquiterpene production by cooperation with jasmonate synthesis pathways[18, 19]. *PIN6* and *MYB57* are nectary-enriched genes, and the former is positively regulated during nectar production and required for a proper auxin response in *Arabidopsis*[20].

Although research on nectary development and nectar secretion has made achievements in herbaceous plants, research in this field in woody plants still require exploration and developed. *Liriodendron* species are insect-pollinated and secrete nectar to attract pollinators. As “basal angiosperms”, Magnoliaceae plants occupy a critical evolutionary position, and a genus of Magnoliaceae, *Liriodendron*, has only two relict species: *L. chinense* and *L. tulipifera*[21-26]. The nectar production of *L. tulipifera*, a nectariferous plant, is greater than that of *L. chinense*[27, 28]. The nectary in *L. tulipifera* is located in petals. Furthermore, 42 nectar proteins have been identified[28]. However, the exact morphology and anatomy of the structure during floral nectary development, the mechanism of nectar secretion, and the molecular mechanism involved in the development of nectary and nectar secretion are unclear. In this study, ultrastructural observations of the nectary and the dynamic changes in starch in *L. tulipifera* were examined by scanning electron microscopy (SEM) and the periodic acid-Schiff techniques (PAS) to select definitive samples for further research. Then, high-throughput sequencing was performed to identify the

genes and pathways potentially involved in the process of nectary development and nectar secretion in *L. tulipifera*.

## Methods

### Plant materials

All plant materials were collected from 26-year-old trees in a provenance trial plantation of *Liriodendron* located in Zhenjiang, Jiangsu Province (119°13'20"E, 32°7'8"N). The plantation was established in 1994 and contained 12 provenances of *L. chinense* and 5 provenances of *L. tulipifera*[29]. One *L. tulipifera* tree (seed source from Georgia, USA) was used as the sample plant to collect petals at intervals of 3 to 7 days from February to May in 2018.

### Observation of dynamic changes in nectary surface morphology and starch

We collected petals from stage 1 to stage 6. Each petal was divided into three parts according to its color and then fixed in formalin-acetic acid-alcohol (FAA) solution for SEM and PAS techniques. The fixed material was dehydrated in an ethanol series, dried to the CO<sub>2</sub> critical point by an Emitech K850 critical point dryer (Emitech, Canterbury, UK), gold plated by an Edwards E-1010 ion sputter golden coater (Hitachi, Tokyo, Japan) and observed with an FEI Quanta 200 SEM (FEI, Eindhoven, the Netherlands). Some fixed petals were dehydrated in an ethanol series, transparentized in a xylene series, infiltrated in paraffin, and embedded in paraffin wax. The samples sliced into several 3-4 µm thick slices with a Leica RM2145 rotary microtome (Leica Microsystems, Wetzlar, Germany) and stained via the PAS technique following the manufacturer's instructions (Solarbio, Beijing, China). Then, the samples were sealed with neutral balata and observed with an Axioscope A1 fluorescence microscope (Carl Zeiss, Jena, Germany).

### RNA extraction, cDNA library construction, Illumina sequencing

Based on the above results of anatomy observation and PAS staining, four different samples of petals were prepared from *L. tulipifera*: immature nectary (green-white color, middle section, stage 2), immature nectary (green, top section, stage 2), mature nectary (yellow color, middle section, stage 3), and postsecretory nectary (orange-red, middle section, stage 5) (Table 1). The samples were quickly frozen in liquid nitrogen and stored at -80 °C in a refrigerator. Total RNA was extracted using the RNAPrep Pure Kit (Tiangen, China) following the manufacturer's instructions. RNA quality was checked using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Sequences of the cDNA libraries were performed on the Illumina Nova platform (California, USA) with a 125-bp paired-end read length at Biomarker Technologies (Beijing, China).

### Data analysis for RNA-seq experiments

The raw reads were cleaned by removing adapter sequences and low-quality reads to obtain clean data[30]. Clean data were mapped to the *L. chinense* genome sequence (NCBI, Bio Project,

PRJNA418360) with HISAT2 (v2.0.5)[31, 32]. The NR, Swiss-Prot, Gene Ontology (GO), Cluster Orthologous Groups (COG), Eukaryotic Orthologous Groups (KOG), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Pfam databases were used[33-39].

### Identification, annotation and enrichment analysis of differentially expressed genes

To identify differentially expressed genes (DEG) related to the development of the floral nectary, we mapped clean reads to the transcriptome assemblies for each sample using RNA-seq by expectation maximization (RSEM) and for two different samples by the DESeq method[40, 41]. A false discovery rate (FDR) of 0.01 and a fold change (FC) of 2 were chosen as the threshold for DEG identification. Then, the identified DEGs were used for GO and COG classification and KEGG pathway enrichment analysis.

### Real-time quantitative PCR validation

To verify the RNA-sequencing results, we evaluated the expression profiles of 22 DEGs that were closely related to nectary development and nectar secretion and chose *LcActin97* as a reference gene for real-time quantitative PCR analysis. All primers for RT-qPCR were designed by Oligo7 software and are listed in Supplementary Table S1. RT-qPCR was performed using the TB Green Premix Ex Taq Kit (Takara, Shiga, Japan) and run on an ABI StepOne plus thermal cycler (Applied Biosystems, California, USA). The relative expression of candidate DEGs was analyzed using the  $2^{-\Delta\Delta CT}$  method.

## Results

### Surface morphology of the *L. tulipifera* nectary

According to floral organ traits, we divided petals into six stages (Fig. 1). After dormancy, all floral organs began elongating (stage 1). One month later, the petal was over twice the size of the petal in stage 1, and the base was fleshy and white (stage 2). Each petal had three colors, with green on the top, yellow in the middle part, and white at base. At this site, the first flower of *L. tulipifera* appeared in April (stage 3). The blossoming stage lasted approximately 20 days (stage 4). During stage 4, the petal color changed from yellow to orange in the middle part. There was an abundance of nectar on the surface of petals from stage 3 to stage 4. As the petal transitioned to stage 5, the color of the middle part deepened, while nectar production declined. In the last stage, the petal began to fall, and whole floral development was completed (stage 6).

Then, to understand the process of nectar production and the difference among the three parts of the petals in *L. tulipifera*, the surface morphology of petals was observed via SEM. As shown in Fig. 2, we recorded changes in the epidermal cell morphology of the three parts and stomas from stage 1 to stage 6. The base part had only few stomas at stage 1, and the epidermal cell morphology was irregular. Then, there were no changes in the stomas and epidermal cells observed from stage 2 to stage 6 (Fig. 2). We speculated that the main function of the base part is to support the petals and transport nectar to the middle and the top parts rather than to secrete nectar. The epidermal cell morphologies and abundance

of stomas of the middle and top parts were similar, but the traits of the former were more evident than those of the latter in *L. tulipifera*. At stage 1, the epidermal cell morphology was flat and irregular, and the stomas were normal. At stage 2, the epidermal cell morphology became plump and polygonal, and the stomas were sunken slightly. From stage 3 to stage 5, the morphologies of the epidermal cells and stomas changed substantially. At stage 3, interestingly, the stomas were modified, open and full of a liquid, nectar, that was prepared for secretion. Next, we observed nectar, which was secreted from modified stomas with deep depressions. When stage 5 and stage 6 commenced, the stomas closed, degenerated and stopped secreting nectar. According to the stoma and epidermal cell morphology development, the nectary was mature at stage 3, and the nectar began to be secreted by modified stomas at stage 4. The epidermal cells were severely corrugated and ordered tightly. The modified stomas opened at stage 3 and closed at stage 5 and stage 6. At stage 6, the cell was flat again. From the above results, we found that the petal nectary is located in the middle and top parts of the petal and that the nectary secretes nectar at stage 3.

### **Dynamic changes in starch in *L. tulipifera***

To further investigate the mechanism by which nectar was secreted and whether the nectary was a starch-storing nectary in *L. tulipifera*, we measured the change in starch in the three petal parts during six stages using the PAS method. At stage 1, there were no starch grains in the whole petal (Fig. 3). Afterwards, the base part had visible starch grains, and the middle and top parts began to accumulate starch. Notably, there was an abundance of starch grains in the whole sepal at stage 3, especially in the base part. The number of starch grains in the base part was higher than in the middle part and the top part. At stage 4, the nectary still contained an abundance of starch grains. From stage 5 to stage 6, the starch grains decreased sharply. Although there were still a few starch grains in the middle and top parts, the starch grains were thoroughly degraded in the base part. Therefore, the nectary belongs to the starch-storing nectary in *L. tulipifera* because they undergo a growth and decline in starch. During stage 1 and stage 2, the nectary accumulated starch. Then, starch production reached a peak at stage 3 and stage 4, which indicated that the nectary was mature and that the starch was beginning to transform into nectar. These results are consistent with the morphological changes in the stomas. The changes in the surface morphology and starch in the nectary and the middle and the top parts from stage 2 to stage 5 are vital to nectary development, nectar secretion and color change. Therefore, these petal parts were subject to detailed investigations via RNA-seq analysis.

### **RNA-seq analysis of clean data in *L. tulipifera***

To explore the molecular mechanism of nectary development and nectar secretion and the difference between the middle and top parts of the petal in *L. tulipifera*, RNA-seq analysis was performed with the Illumina Nova platform with a 125-bp paired-end read length. Four samples were prepared from *L. tulipifera*: the middle and top parts at stage 2 (immature nectary) and the middle parts at stage 3 (mature nectary) and stage 5 (postsecretory nectary) (Table 1). RNA-Seq analysis was performed with three biological replicates for each sample, and 12 libraries were generated in total. After removing low quality

reads, 115.26 Gb clean data were obtained. The average number of reads per library was over 25 million, and a Q30 base was no less than 93.85% (Table 1). The RNA-seq reads were mapped with the *L. chinense* reference genome (NCBI, Bio Project, PRJNA418360) using HISAT2 with 71.02%-79.77% efficiency for each sample. In total, 24,072 genes were identified, and 21,853 (90.78%) genes were annotated by BLAST for the COG (7,961), GO (13,271), KEGG (8,986), Swiss-Prot (15,617), eggNOG (21,532), and NR (21,813) annotations. According to Fig. 4, the GO classification included biological process (30,021 genes), cellular component (12,643 genes), and molecular function (19,439 genes) (Fig. 4A). The COG classification was functionally classified into 25 COG categories, including transcription (1,207 genes, 15.16%), replication, recombination and repair (1,180 genes, 14.82%), and signal transduction (1,053 genes, 13.23%) (Fig. 4B). Furthermore, the annotated genes were enriched in 128 KEGG pathways. The top five enriched pathways were ribosome, plant-pathogen interaction, spliceosome, RNA transport and plant hormone signal transduction.

Table 1. Sample description and numbers of RNA-seq reads

| Developmental Stage | Sample Description                        | Sample ID | Total Reads | Mapped Reads       | %≥Q30  |
|---------------------|-------------------------------------------|-----------|-------------|--------------------|--------|
| Stage 2             | top; green; immature nectary              | S2T       | 56.2 M      | 42.7 M<br>(76.09%) | 94.60% |
|                     |                                           |           | 71.6 M      | 52.3 M<br>(73.06%) | 94.42% |
|                     |                                           |           | 53.3 M      | 37.8 M<br>(71.02%) | 94.28% |
| Stage 2             | middle; green-white; immature nectary     | S2M       | 81.2 M      | 62.6 M<br>(77.07%) | 94.72% |
|                     |                                           |           | 73.2 M      | 56.8 M<br>(77.53%) | 94.72% |
|                     |                                           |           | 69.5 M      | 51.1 M<br>(73.47%) | 94.57% |
| Stage 3             | middle; yellow; mature nectary            | S3M       | 72.8M       | 56.4 M<br>(77.52%) | 94.79% |
|                     |                                           |           | 66.8 M      | 51.9 M<br>(77.72%) | 94.55% |
|                     |                                           |           | 57.3 M      | 45.5 M<br>(79.32%) | 94.97% |
| Stage 5             | middle; orange-red; postsecretory nectary | S5M       | 56.8 M      | 43.9 M<br>(77.28%) | 94.90% |
|                     |                                           |           | 56.9 M      | 43.0 M<br>(75.58%) | 93.85% |
|                     |                                           |           | 58.0 M      | 46.3 M<br>(79.77%) | 94.69% |

Notes: M: millions

### Identification of DEGs in *L. tulipifera*

To identify DEGs between different samples, we first evaluated gene expression levels based on the threshold of fragments per kilobase of transcript per million mapped reads (FPKM) values using Cufflinks (v2.2.1) tool to measure gene expression distribution for each sample (Fig. 5A). Principal component analysis was also performed analyzed and revealed that the 12 samples could be distinctly assigned to four groups: S2T, S2M, S3M, and S5M (Fig. 5B). Additionally, we analyzed the expressed genes in the four groups, and the Venn diagram shows the distribution of specific genes (436, 390, 340, and 663 expressed genes in S2T, S2M, S3M, and S5M, respectively) and shared genes (13,622 expressed genes) (Fig. 5D).

Then, we considered Pearson’s correlation coefficient ( $R^2$ ) as an index to assess the biological variability correlation (Fig. 5D). As Fig. 5 shows, the replicate samples were strongly correlatedn.

Then, DEGs were identified using  $FC \geq 2$  and  $FDR < 0.01$  as criteria in each pairwise comparison. In total, RNA-seq data were divided into six different pairwise comparisons, and 26,955 DEGs were identified, including 12,556 upregulated genes and 14,399 downregulated genes (Table 2). The DEGs were annotated using the COG classification (10,401 DEGs, 38.53%), GO (16,696 DEGs, 61.94%), eggNOG (25,586 DEGs, 94.92%), NR (25,747 DEGs, 95.52%), KEGG pathway (10,207 DEGs, 37.87%) and Swiss-Prot (19,522 DEGs, 72.42%) (Table 3). The MA and volcano plots of each pairwise comparison clearly show the distribution of upregulated and downregulated genes and their FC (Fig. 6A, 6B). Considering the highly significant role of TFs during plant development and growth, we identified 56 major TF families by analyzing all annotated DEGs (Fig. 6C). The figure shows the top thirty TF families, mainly including Teosinte branched1/Cinnnata/proliferating cell factor (*TCP*, 2,261 DEGs), *MYB* (1,661 DEGs), *ERF* (1,281 DEGs), *NAM/ATAF1/CUC2* (*NAC*, 1,211 DEGs), *Trihelix* (1,126 DEGs), Basic helix-loop-helix (*bHLH*, 902 DEGs), and *MADS*(828 DEGs) TF families. Among them, the *TCP*, *ERF* and *NAC* TF families are plant-specific[42-44]. These TF families are widespread in many plant species and are involve in the control of plant development, senescence, abiotic and biotic stress responses, and floral bilateral symmetry. Noticeably, there are many genes encoding members of the *Trihelix* TF family, which has gained interest only in recent years. This is the first report of the *Trihelix* TF family in *L. tulipifera* at the transcriptome level.

Table 2. Numbers of DEGs in each developmental stage and tissues

|     | S2T               | S2M               | S3M               | S5M               |
|-----|-------------------|-------------------|-------------------|-------------------|
| S2T | —                 | 473+ <b>142</b>   | 2558+ <b>1802</b> | 3304+ <b>2732</b> |
| S2M | <b>473</b> +142   | —                 | 2704+ <b>2112</b> | 3645+ <b>3179</b> |
| S3M | <b>2558</b> +1802 | <b>2704</b> +2112 | —                 | 2046+ <b>2258</b> |
| S5M | <b>3304</b> +2732 | <b>3645</b> +3179 | <b>2046</b> +2258 | —                 |

Notes: Numbers in bold or in normal indicate the number of upregulated or downregulated genes when the sample in bold is compared with the sample in normal, respectively.

Table 3. Annotation of DEGs in six pairwise comparisons

| DEG Set    | DEG Number | COG    | GO     | KEGG   | NR     | Swiss-Prot | eggNOG |
|------------|------------|--------|--------|--------|--------|------------|--------|
| S3M_vs_S5M | 4,304      | 1,714  | 2,729  | 1,649  | 4,126  | 3,180      | 4,101  |
| S2M_vs_S2T | 615        | 243    | 435    | 251    | 587    | 488        | 587    |
| S2M_vs_S3M | 4,816      | 1,836  | 2,944  | 1,816  | 4,599  | 3,426      | 4,561  |
| S2M_vs_S5M | 6,824      | 2,588  | 4,138  | 2,578  | 6,495  | 4,885      | 6,452  |
| Total      | 26,955     | 10,401 | 16,696 | 10,207 | 25,747 | 19,522     | 25,586 |

### Analysis of DEGs involved in nectary development and nectar secretion in *L. tulipifera*

The nectary is located on the petal in *L. tulipifera* based on the above studies. It is very interesting that the color of the nectary undergoes evident changes during its development (Fig.1). Three pairwise comparisons were constructed to further analyze the regulatory mechanism and key genes involved in nectary development, nectar secretion and color change in *L. tulipifera*, and S2M was used as a control and compared with S2T, S3M and S5M, respectively. A Venn diagram representing the transcript levels for three pairwise comparisons was generated to further explore the number and distribution of overlapping genes among the three comparisons, (Fig. 7A). In S2M\_vs\_S2T, 615 DEGs were identified, with 473 upregulated genes and 142 downregulated genes; in S2M\_vs\_S3M, 4,816 DEGs were identified, with 2,112 upregulated genes and 2,704 downregulated genes; and in S2M\_vs\_S5M, 6,824 DEGs were identified, with 3,179 upregulated genes and 3,635 downregulated genes (Table 2, Fig. 7A). In addition, we analyzed the changes in TF family dynamics in the three comparisons during nectary development and nectar secretion (Fig. 7B). The results between S2M\_vs\_S3M and S2M\_vs\_S5M were similar. When S2M\_vs\_S2T was compared to other pairwise comparisons, the DEGs encoding the *TCP*, *Trihelix*, *C2H2*, *CAMTA* and *E2F/DPTF* families notably declined, and the DEGs of the *ERF*, *MADS*, *Golden2-like* (*G2-like*), and *Zinc Finger Homeodomain* (*ZF-HD*) TF families increased.

Table 4. Numbers of annotated DEGs in three pairwise comparisons

| DEG Set | Total | COG  | GO   | KEGG | NR   | Swiss-Prot | eggNOG |
|---------|-------|------|------|------|------|------------|--------|
| S2M_S2T | 587   | 243  | 435  | 251  | 587  | 488        | 587    |
| S2M_S3M | 4607  | 1836 | 2944 | 1816 | 4599 | 3426       | 4561   |
| S2M_S5M | 6499  | 2588 | 4138 | 2578 | 6495 | 4885       | 6452   |

The S2M\_vs\_S2T comparison had the lowest number of DEGs (in total 615 DEGs), and 95.45% (587 DEGs) of them were annotated. We analyzed the KEGG pathways of 415 upregulated and 135

downregulated annotated DEGs and chose the smallest 20 Q values to draw pathway enrichment factor scatter plots (Fig. 8). The upregulated genes were functionally assigned to 50 biological pathways with photosynthesis (24 DEGs, 5.78%), phenylpropanoid biosynthesis (22 DEGs, 5.30%), and carbon metabolism (18 DEGs, 4.34%) and the downregulated genes were mainly assigned to flavonoid biosynthesis (9 DEGs, 6.67%). S2T and S2M were the top and middle parts separately in the same petal at stage 2, so the pairwise comparison had fewer DEGs. Although S2T and S2M were in the same petal, the former was green, while the latter was green-white. The KEGG pathway analysis showed that the DEGs of S2T were significantly closely related to photosynthesis, which is consistent with the color. Considering that the DEGs of S2M were significantly enriched in flavonoid biosynthesis, we speculate that the difference between the color of the top and middle parts of the petal is related to flavonoids (Fig. 11).

Then, we also functionally analyzed the KEGG pathway of S2M\_vs\_S3M DEGs. There were 4,607 (95.66%) DEGs annotated with 2,021 upregulated and 2,586 downregulated genes. The upregulated genes were mainly classified as carbon metabolism (88 DEGs, 4.35%), biosynthesis of amino acids (62 DEGs, 3.07%), glycolysis/gluconeogenesis (42 DEGs, 2.08%) and terpenoid backbone biosynthesis (22 DEGs, 1.09%) (Fig. 9B). Thiamine metabolism, tryptophan metabolism and terpenoid backbone biosynthesis were significantly enriched by pathway enrichment factor analysis (Fig. 9A). The downregulated genes were mainly classified as phenylpropanoid biosynthesis (33 DEGs, 1.28%), plant hormone signal transduction (33 DEGs, 1.28%), starch and sucrose metabolism (31 DEGs, 1.20%) and flavonoid biosynthesis (13 DEGs, 0.50%) (Fig. 9D). According to starch metabolism during nectary development, the starch at stage 3 reached a peak. The S3M DEGs were significantly assigned to carbon metabolism, which is consistent with the PAS results. The terpenoid backbone biosynthesis pathway is also notable; we think the terpenoid backbone biosynthesis pathway may be related to floral volatiles, and the terpenoid is an essential ingredient of volatiles in *L. tulipifera* considering that the flower is prepared for anthesis at stage 3.

Moreover, we analyzed the annotated DEGs of the S2M\_vs\_S3M pairwise comparison. A total of 6,499 (95.24%) annotated DEGs containing 3,058 upregulated and 3,441 downregulated genes were identified. The upregulated genes were classified as carbon metabolism (92 DEGs, 3.01%), biosynthesis of amino acids (67 DEGs, 2.19%), starch and sucrose metabolism (43 DEGs, 1.41%) and phenylpropanoid biosynthesis (39 DEGs, 1.28%) (Fig. 10B). The enrichment factor of anthocyanin biosynthesis was more significant than that of other pathways (Fig. 10A). The downregulated genes were classified as starch and sucrose metabolism (47 DEGs, 1.37%), ribosome (47 DEGs, 1.37%), and carbon metabolism (40 DEGs, 1.16%) (Fig. 10D). S5M is an important stage for nectar secretion; therefore, the DEGs are involved in carbon, starch and sucrose metabolism. The middle part of the petals is orange-red at stage 5, and anthocyanin biosynthesis is highly significant; thus, anthocyanin may cause the difference between S2M and S5M (Fig. 11).

## RT-qPCR analysis of nectary-related genes

To calculate the accuracy of RNA-seq, we chose several DEGs related to nectary development and nectar secretion. Nectary development is primarily regulated by *CRABS CLAW-like* (*CRC*), *BLADE-ON-PETIOLE-like* (*BOP*), *MADS-box* family including *APETALA* (*AP*), *PISTILLATA* (*PI*), *AGAMOUS* (*AG*), and *SEPALLATA* (*SEP*), and nectar secretion is regulated by *SWEET-like*, *CELL WALL INVERTASE-like* (*CWINV*), *MYB-like*, Calreticulin (*CRT*), *Nectarin1/3*, and auxin efflux transporter *PIN-like* according to several studies of *Arabidopsis thaliana* and *Nicotiana tabacum*. We identified 21 DEGs by the functional prediction of annotated genes from the RNA-seq data, including *LtYABBY5*, *LtCRT*, *LtSEP1*, *LtAP2/3*, *LtAGL9*, *LtPI*, *LtSWEET1/3/4/16*, *LtPIN1a/b*, *LtPIN7*, *LtMYB2/34/86/305/306/330*, and *LtMYB1R1*. The 21 candidate genes were analyzed to verify the expression patterns of the RNA-seq data with RT-qPCR analysis. The expression profiles were analyzed by relative expression (RT-qPCR) and FPKM (RNA-seq) values. Then, the correlation between RNA-seq and RT-qPCR was measured by the scatter plot of log2-FC (Fig. 12A). The differential expression profile of DEGs was consistent between RNA-seq and RT-qPCR and showed a positive correlation coefficient ( $R^2 = 0.74952$ ,  $P < 0.01$ ) (Fig. 12B). Although 1 gene (4.76%, *LtPIN1b*) was significantly different in the expression profile, 16 genes (76.19%) displayed similar expression profiles when the RT-qPCR data were compared with the RNA-seq data. The MADS-box family mainly regulates the earlier development of floral organs in flowering plants; thus, we found that the *PI*, *AP2*, *AP3*, *AGL9* and *SEP1* genes show low expression levels in mature petals in *L. tulipifera*. Strikingly, the selected genes from the *MYB-like* and *SWEET-like* families exhibited high expression in S3M and S5M in *L. tulipifera*, especially the *MYB305* and *SWEET3* genes. These results suggest that these genes may involve in regulating nectar secretion in *L. tulipifera*.

## Discussion

### Nectary development and nectar secretion in *L. tulipifera*

The nectary is a multicellular glandular structure that may occur on vegetative or reproductive organs and secretes nectar and sweet solutions[45]. Research on nectary structure and classification has been performed for several species but is still lacking in *Liriodendron* species. *Liriodendron* species have low setting percentages and tend to be endangered, especially *L. chinense*. *L. tulipifera* has a higher setting percentage than *L. chinense*, which is closely related to the colorful petal and plentiful nectar. *L. tulipifera* is a nectariferous and insect-pollinated plant and its nectar is the major attractant for insects. Therefore, it is urgent to thoroughly understand the nectary classification, development and secretion process in *L. tulipifera* and explore the reason why nectar production has significant differences in *Liriodendron* plants. In this study, we evaluated the nectary surface cells, stomas, and starch metabolism in detail. The results show that the nectary in *L. tulipifera* is a floral nectary, petal nectary and starch-storing nectary. The nectary of several *Magnolia* species is a stigmatic nectary or carpel nectary, and *M. stellata* nectar secretion occurs by an epithelial ovarian nectary in which the epidermis of the entire carpel is involved[46, 47]. Considering the differences in the *Magnolia* and *Liriodendron* nectaries, we inferred that the nectaries of the different genera in Magnoliaceae may not be homologous or are subjected to differentiation or independent evolution[2, 48]. The floral nectaries of many species form a protrusion of the organ that

bears them, but we found that the nectary in *L. tulipifera* is flush with the surface[49, 50]. The nectary in *Coptis* and *Hydrastis* of Ranunculaceae, a member of basal eudicots, is located on the petal with flush with the surface, which is similar to the nectary in *L. tulipifera*[51]. In monocots, the nectary belongs to the septal nectary and is exclusive to this lineage, and in core eudicots, intrastaminal nectaries are common, followed sequentially by gynoeceal, extrastaminal, hypanthial, petal and sepal nectaries[48, 52]. Compared with the other nectary types, the petal nectary in *Liriodendron*, *Coptis* and *Hydrastis* may have relatively primitive characteristics.

With nectary development in *L. tulipifera*, its color also substantially changes from green to yellow to orange-red. Similarly, the tobacco nectary changes from lime green to yellow to bright orange[48]. Moreover, the starch content in the tobacco nectary increases during development and degrades prior to anthesis, which is also consistent with the trend in *L. tulipifera*[48]. Therefore, during future research on the molecular mechanism and genetic transformation of nectary development and nectar secretion, we can use tobacco as an assistant material rather than other species.

### **The vital genes involved in nectary development and nectar secretion in *L. tulipifera***

The nectary is a crucial medium for insects to assist plants in pollination. However, the molecular mechanism of nectary development and nectar secretion remains unclear and is a relatively new area of research. Only *CRC* and *BOP* have been identified to regulate nectary development, and *CWINV4*, *MYB* and *PIN6* have been shown to be related to nectar secretion[11, 13, 15, 17, 20]. Moreover, there are few studies on the molecular mechanism of nectary development and nectar secretion in *L. tulipifera*. Therefore, it is indispensable and urgent to conduct RNA-seq to provide significant insights into this field. Based on the stoma morphology and starch accumulation, the immature, mature and postsecreted nectary were chosen for RNA-seq analysis. In total, 26,955 DEGs and 56 TF families were identified, including *TCP*, *MYB*, *ERF*, *NAC*, *Trihelix*, *bHLH*, and *MADS* families. Combining previous studies and gene expression in RNA-seq analysis, we detected 21 DEGs via expression profiling with RT-qPCR. *CRC* is a vital and conserved gene for nectary development in core eudicot species, and no evidence of its expression in basal eudicot nectaries has been found[2]. During our RNA-seq analysis, we also did not find the expression of *CRC* in *L. tulipifera*, which is consistent with previous researches[2].

The most dramatic change in the ornamental tobacco nectary is the change in color during nectary development, which is consistent with the *L. tulipifera* nectary[48]. The color change from lime green to yellow to bright orange is related to the high concentration of  $\beta$ -carotene[53]. The color change among S2T, S2M, S3M and S5M is from green to yellow to orange-red in *L. tulipifera*. The pigment in plants mainly contains carotenoids, flavonoids and betalains[54]. The three pigments are responsible for different colors. Flavonoids are a group of secondary metabolites belonging to the class of phenylpropanoids, and anthocyanins are a class of flavonoids [54, 55]. By analyzing the KEGG pathway enrichment of DEGs, we found that a total of 55 genes were enriched in phenylpropanoid biosynthesis, flavonoid biosynthesis, terpenoid backbone biosynthesis and anthocyanin biosynthesis in S2T, S2M, S3M and S5M in *L. tulipifera* (Fig. 11). These findings suggest that flavonoids are responsible for the yellow

color in S2M and that orange-red color is caused by the accumulation of anthocyanin in S5M in *L. tulipifera*. Phenylpropanoid, flavonoid and anthocyanin biosynthesis and metabolism are important for pigment studies in *L. tulipifera*. We found the TF *MYB305* was strongly expressed in S5M in *L. tulipifera* via RT-qPCR analysis. *MYB305* is also highly expressed in the tobacco nectary and positively regulates flavonoid metabolic and nectar production[16, 17]. We speculate that *LtMYB305* involves nectary development and regulates flavonoid and nectar production in *L. tulipifera*. In future work, we will measure the anthocyanin content in different stages and identify *LtMYB305* function in nectary development, secretion and pigment.

The major component in nectar is sucrose, and the sucrose efflux is mediated by SWEET proteins as a key step for phloem transport[56]. *AtSWEET11,12* have been confirmed to be responsible for sucrose efflux and *SWEET9* is essential for nectar productions and function as an efflux transporter in *A. thaliana*, *Brassica rapa*, *Petunia hybrida* and *Nicotiana attenuate*[57, 58]. We analyzed the expression profiles of *LtSWEET1,3,4,16* by RT-qPCR. It is notable that *LtSWEET3* is highly expressed in S5M and S3M. The expression profile of *LtSWEET4* is similar to that of *LtSWEET3*, but compared with that of the former, the expression of the latter increases tenfold. The nectary morphology via SEM analysis indicates that the nectar is secreted in S3M and S5M. Moreover, by KEGG pathway analysis, carbon metabolism and the starch and sucrose metabolism pathways are primarily enriched in S3M and S5M. Combining these results, we suggest that *LtSWEET3,4* are related to nectar secretion in *L. tulipifera*. The TF *MYB21,24,57,305* are nectary-enriched genes in *Arabidopsis* and ornamental tobacco and regulate nectary development, flavonoid metabolic and nectar production[17, 18, 20]. *AtMYB1R1,2,34,86,305,306,330* are differentially expressed in the nectary. Among them, *AtMYB305* attracts our attention because its expression is far higher than that of other TFs. *AtMYB305* is mainly expressed in S3M and S5M, which is similar to *AtMYB2,306,330* and *LtSWEET3,4*. In summary, *SWEET* and *MYB* families are important for nectar secretion in *L. tulipifera*. These findings enrich the understanding of molecular networks and provide new insights into the mechanism of nectary development and nectar secretion in *L. tulipifera*. Next, we may focus on the function of the *SWEET*, *MYB*, and *YABBY* family genes to explore the molecular mechanism of nectary development and secretion in *L. tulipifera*, especially *CRC*, *SWEET3*, and *MYB305*.

## Conclusions

In this study, we recorded changes in surface cells, stomas and starch during nectary development by SEM and PAS methods in *L. tulipifera*. The floral nectary is located in the petal, is a starch-storing nectary and secretes nectar from stage 3 to stage 5. These findings not only expand our understanding of angiosperms nectaries but also provide strong evidence for RNA-seq analysis. We constructed 12 libraries and obtained 115.26 Gb clean mapped with the *L. chinense* reference genome with 71.02%-79.77% efficiency. According to the KEGG pathway analysis, the flavonoid, phenylpropanoid, and anthocyanin biosynthesis, and starch and sucrose metabolism pathways may be related to nectar secretion and pigment change. Additionally, we found that the *TCP*, *Trihelix*, *C2H2*, *ERF*, and *MADSTF* families undergo

dynamic changes during nectary development. The known *YABBY*, *SWEET* and *MYB* genes also have high expression in the *L. tulipifera* nectary according to RT-qPCR validation.

## Abbreviations

DEGs: Differentially expressed genes; RT-qPCR: real-time quantitative PCR; SEM: scanning electron microscopy; PAS: periodic acid-Schiff techniques; GO: Gene Ontology; COG: Cluster Orthologous Groups; KOG: Eukaryotic Orthologous Groups; KEGG: Kyoto Encyclopedia of Genes and Genomes; RSEM: RNA-seq by expectation maximization; FDR: false discovery rate; FC: fold change; FPKM: fragments per kilobase of transcript per million mapped reads; TFs: transcription factors.

## Declarations

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### Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

### Author's contributions

LHG and LHH conceived and designed the study; LHH performed the experiments; LHH and MJ analysed the data; LHH wrote the manuscript. LHG and MJ revised it. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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# Tables

Table 1. Sample description and numbers of RNA-seq reads

| Developmental Stage | Sample Description                        | Sample ID | Total Reads | Mapped Reads       | %≥Q30  |
|---------------------|-------------------------------------------|-----------|-------------|--------------------|--------|
| Stage 2             | top; green; immature nectary              | S2T       | 56.2 M      | 42.7 M<br>(76.09%) | 94.60% |
|                     |                                           |           | 71.6 M      | 52.3 M<br>(73.06%) | 94.42% |
|                     |                                           |           | 53.3 M      | 37.8 M<br>(71.02%) | 94.28% |
| Stage 2             | middle; green-white; immature nectary     | S2M       | 81.2 M      | 62.6 M<br>(77.07%) | 94.72% |
|                     |                                           |           | 73.2 M      | 56.8 M<br>(77.53%) | 94.72% |
|                     |                                           |           | 69.5 M      | 51.1 M<br>(73.47%) | 94.57% |
| Stage 3             | middle; yellow; mature nectary            | S3M       | 72.8M       | 56.4 M<br>(77.52%) | 94.79% |
|                     |                                           |           | 66.8 M      | 51.9 M<br>(77.72%) | 94.55% |
|                     |                                           |           | 57.3 M      | 45.5 M<br>(79.32%) | 94.97% |
| Stage 5             | middle; orange-red; postsecretory nectary | S5M       | 56.8 M      | 43.9 M<br>(77.28%) | 94.90% |
|                     |                                           |           | 56.9 M      | 43.0 M<br>(75.58%) | 93.85% |
|                     |                                           |           | 58.0 M      | 46.3 M<br>(79.77%) | 94.69% |

Notes: M: millions

Table 2. Numbers of DEGs in each developmental stage and tissues

|     | S2T               | S2M               | S3M               | S5M               |
|-----|-------------------|-------------------|-------------------|-------------------|
| S2T | ---               | 473+ <b>142</b>   | 2558+ <b>1802</b> | 3304+ <b>2732</b> |
| S2M | <b>473</b> +142   | ---               | 2704+ <b>2112</b> | 3645+ <b>3179</b> |
| S3M | <b>2558</b> +1802 | <b>2704</b> +2112 | ---               | 2046+ <b>2258</b> |
| S5M | <b>3304</b> +2732 | <b>3645</b> +3179 | <b>2046</b> +2258 | ---               |

Notes: Numbers in bold or in normal indicate the number of upregulated or downregulated genes when the sample in bold is compared with the sample in normal, respectively.

Table 3. Annotation of DEGs in six pairwise comparisons

| DEG Set    | DEG Number | COG    | GO     | KEGG   | NR     | Swiss-Prot | eggNOG |
|------------|------------|--------|--------|--------|--------|------------|--------|
| S2T_vs_S3M | 4,360      | 1,719  | 2,726  | 1,626  | 4,168  | 3,156      | 4,142  |
| S3M_vs_S5M | 4,304      | 1,714  | 2,729  | 1,649  | 4,126  | 3,180      | 4,101  |
| S2M_vs_S2T | 615        | 243    | 435    | 251    | 587    | 488        | 587    |
| S2M_vs_S3M | 4,816      | 1,836  | 2,944  | 1,816  | 4,599  | 3,426      | 4,561  |
| S2M_vs_S5M | 6,824      | 2,588  | 4,138  | 2,578  | 6,495  | 4,885      | 6,452  |
| S2T_vs_S5M | 6,036      | 2,301  | 3,724  | 2,287  | 5,772  | 4,387      | 5,743  |
| Total      | 26,955     | 10,401 | 16,696 | 10,207 | 25,747 | 19,522     | 25,586 |

Table 4. Numbers of annotated DEGs in three pairwise comparisons

| DEG Set | Total | COG  | GO   | KEGG | NR   | Swiss-Prot | eggNOG |
|---------|-------|------|------|------|------|------------|--------|
| S2M_S2T | 587   | 243  | 435  | 251  | 587  | 488        | 587    |
| S2M_S3M | 4607  | 1836 | 2944 | 1816 | 4599 | 3426       | 4561   |
| S2M_S5M | 6499  | 2588 | 4138 | 2578 | 6495 | 4885       | 6452   |

## Additional File

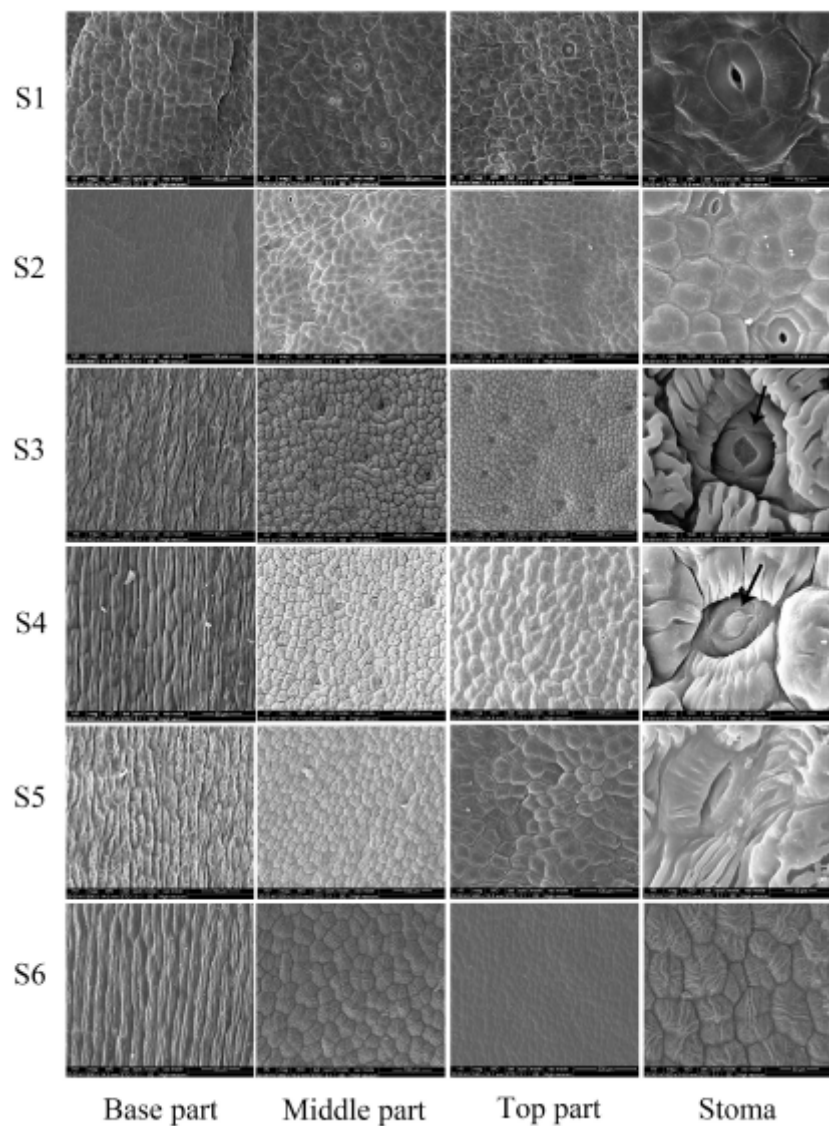
Additional file1: Table S1. List of 21 DEGs primers used for RT-qPCR.

## Figures



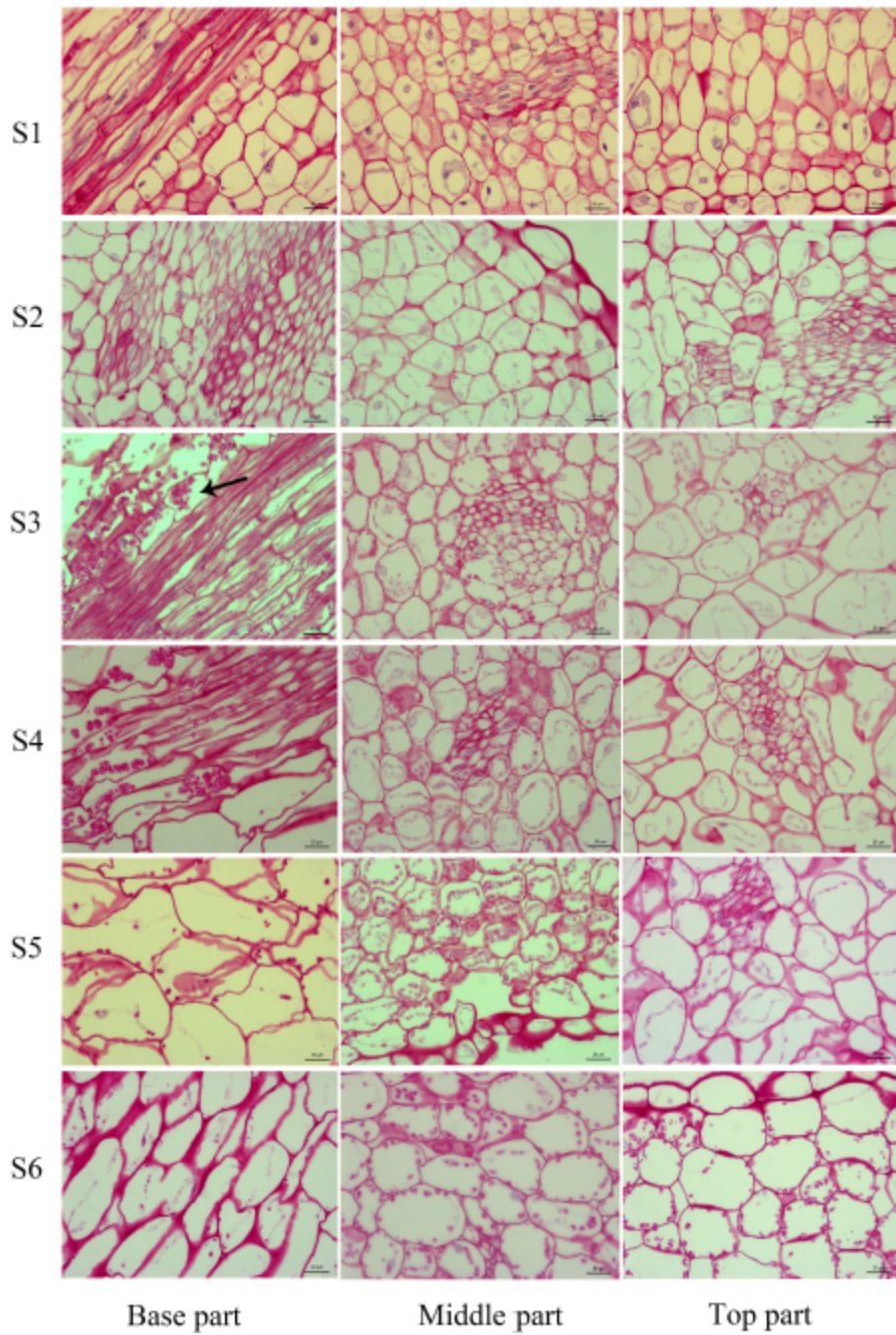
**Figure 1**

The development process of petals in *Liriodendron*. S1: stage1, all floral organs elongated; S2: stage2, the petal was over twice the size of the petal in stage 1 and the base was fleshy and white; S3: stage3, each petal present three colors obviously, with green on the top, yellow in the middle part, and white at base; S4: stage4, the petal color changed from yellow to orange in the middle part; S5: stage 5, the color of the middle part deepened; S6: stage 6, the petal fall; n: nectar; p: pollen.



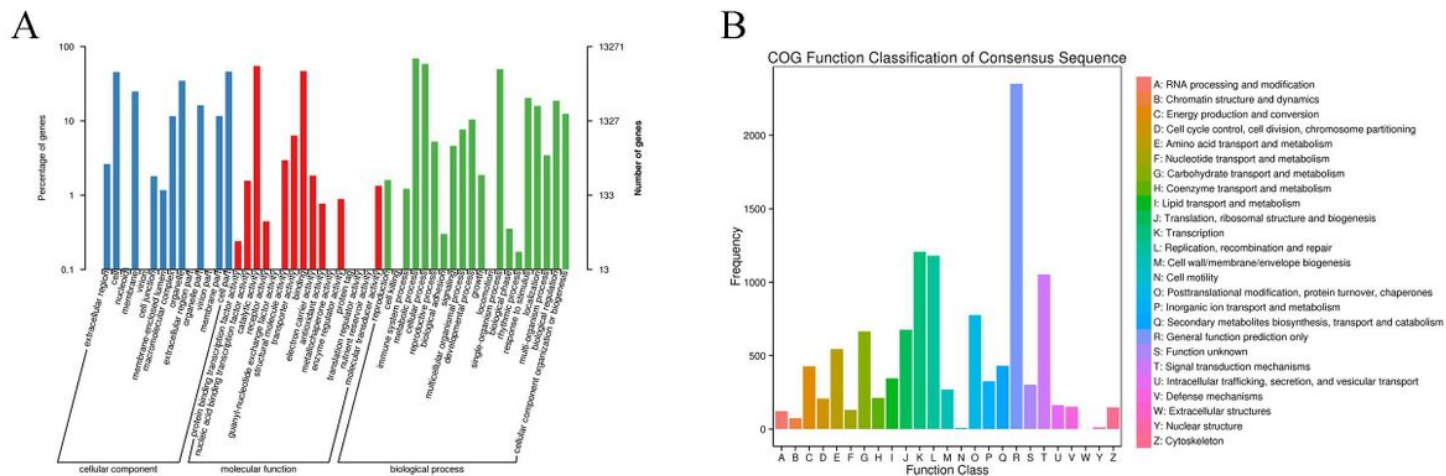
**Figure 2**

The epidermal cells of petals during flower development in *L. tulipifera*. The arrows mean secretions from the petal.



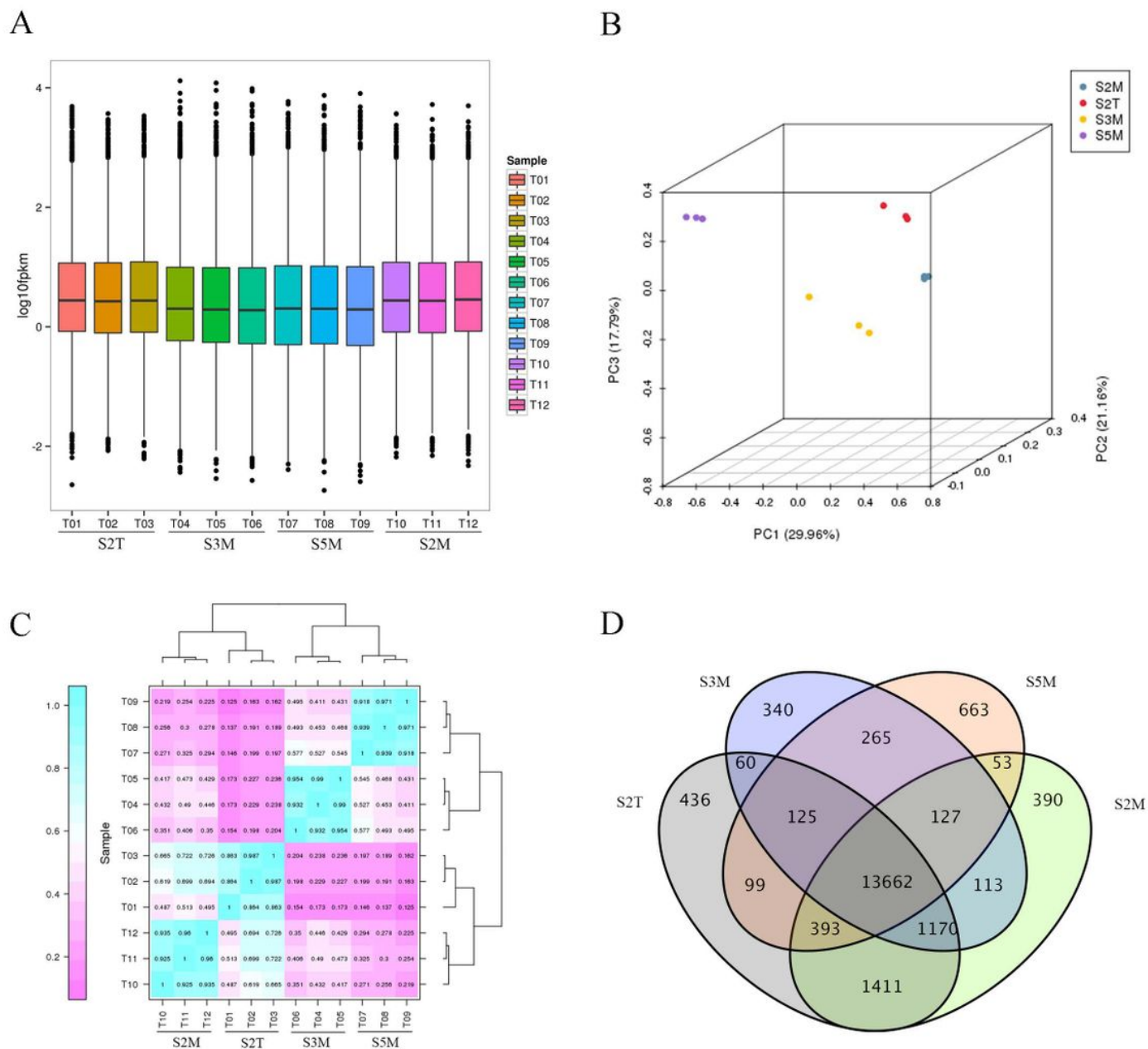
**Figure 3**

The dynamic changes in starch during flower development in *L. tulipifera*. The arrow means starch grain in the petal.



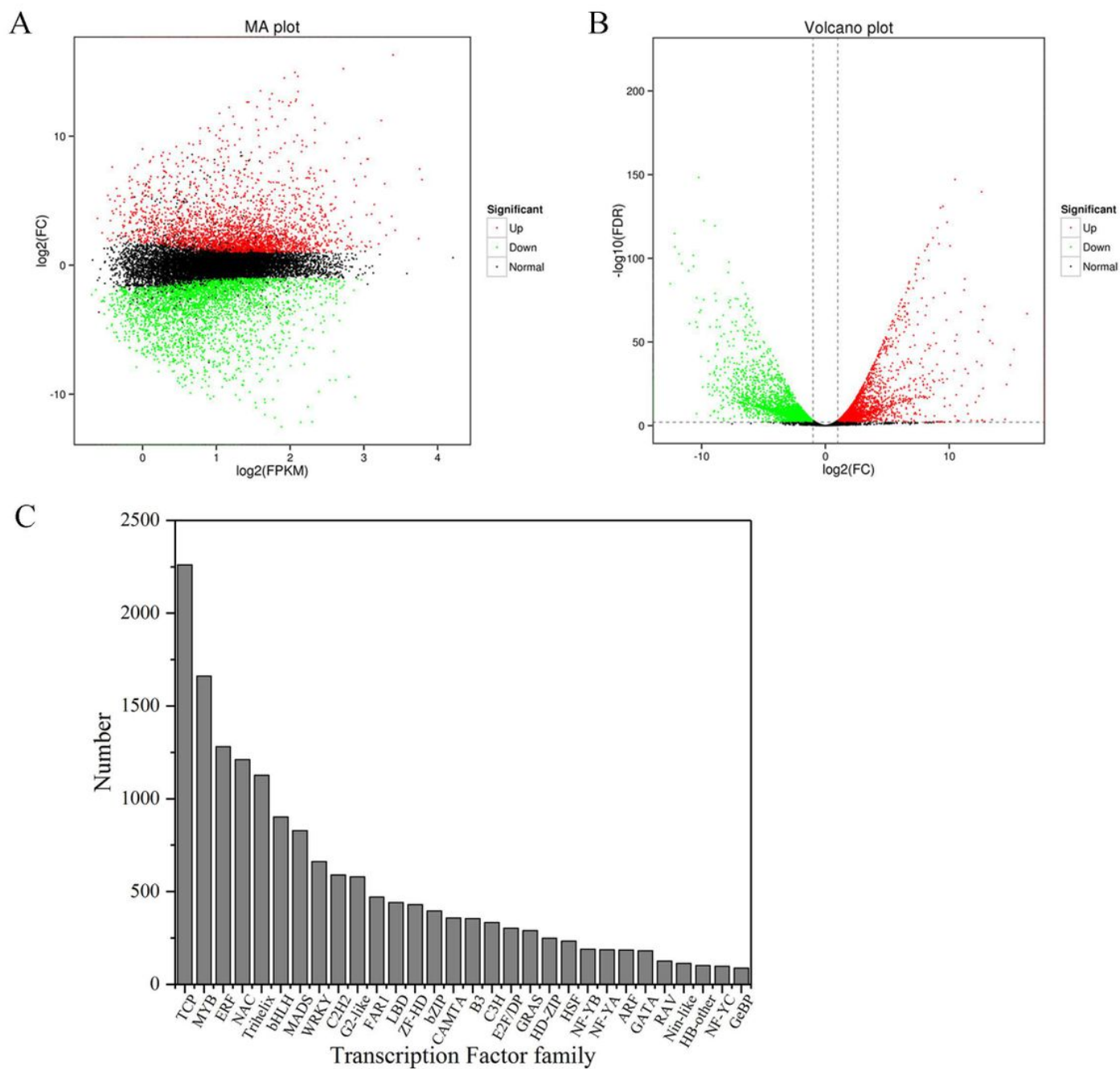
**Figure 4**

GO classification (A) and clusters of orthologous genes (B) of all identified genes.



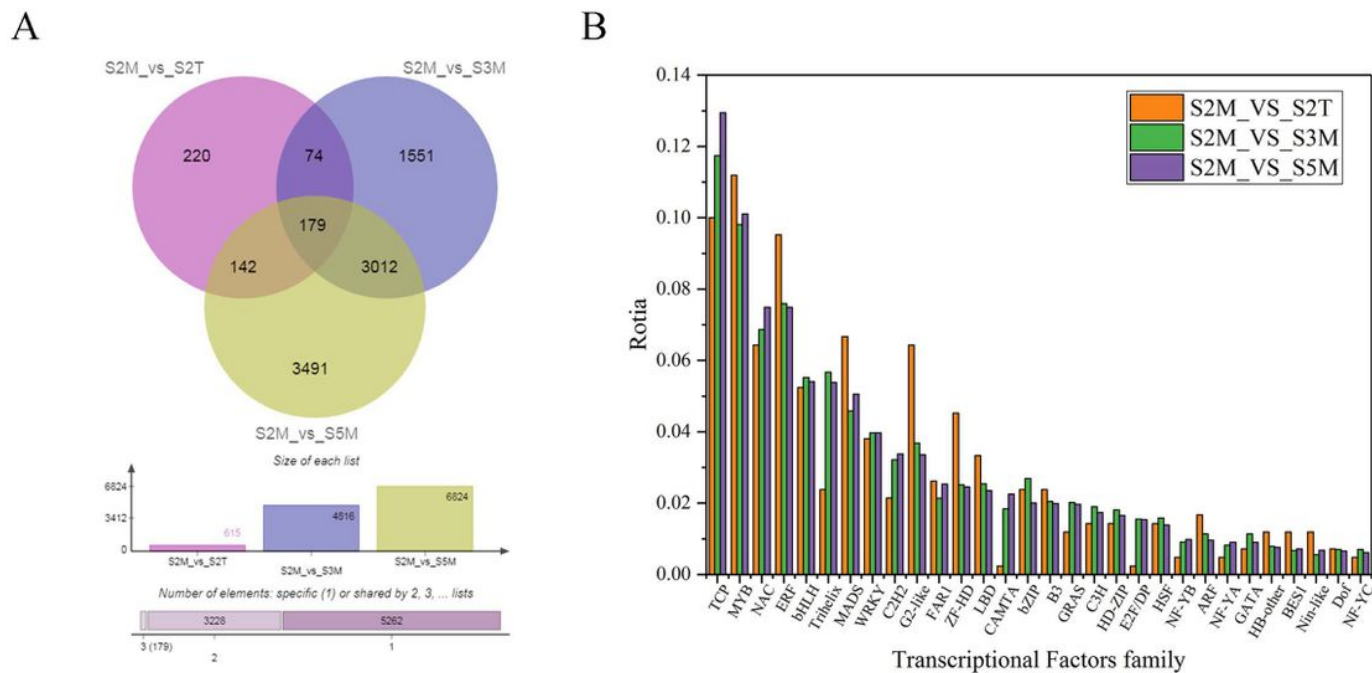
**Figure 5**

The analysis of the expressed genes. A: Boxplot of the gene expression distribution for each sample; B: Principal component analysis; C: Correlations between two samples; D: Venn diagram of four samples.



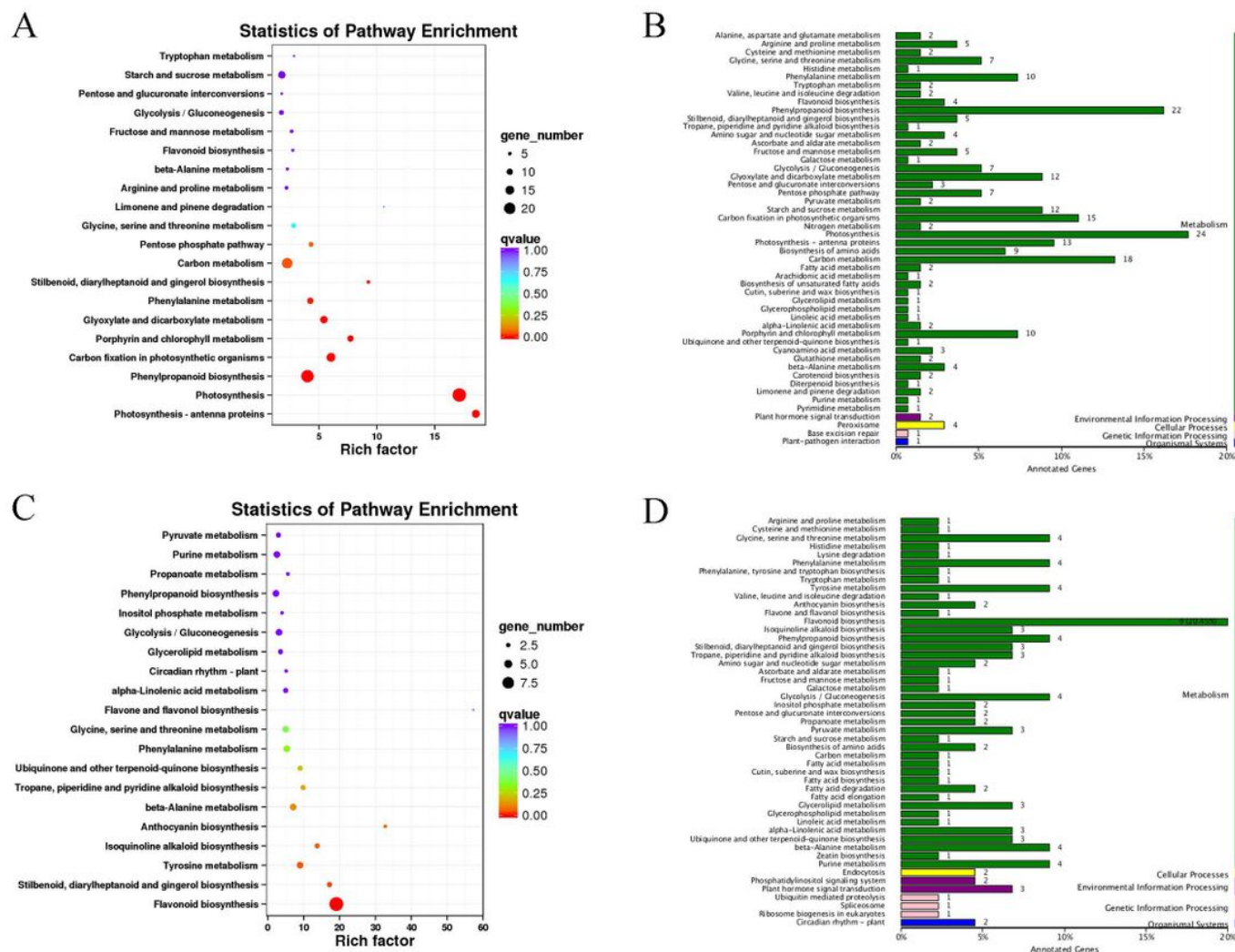
**Figure 6**

MA (A) and volcano plots (B) of RNA-seq data of the S2M\_vs\_S5M pairwise comparison; C: The top 30 TF families were identified among the annotated DEGs.



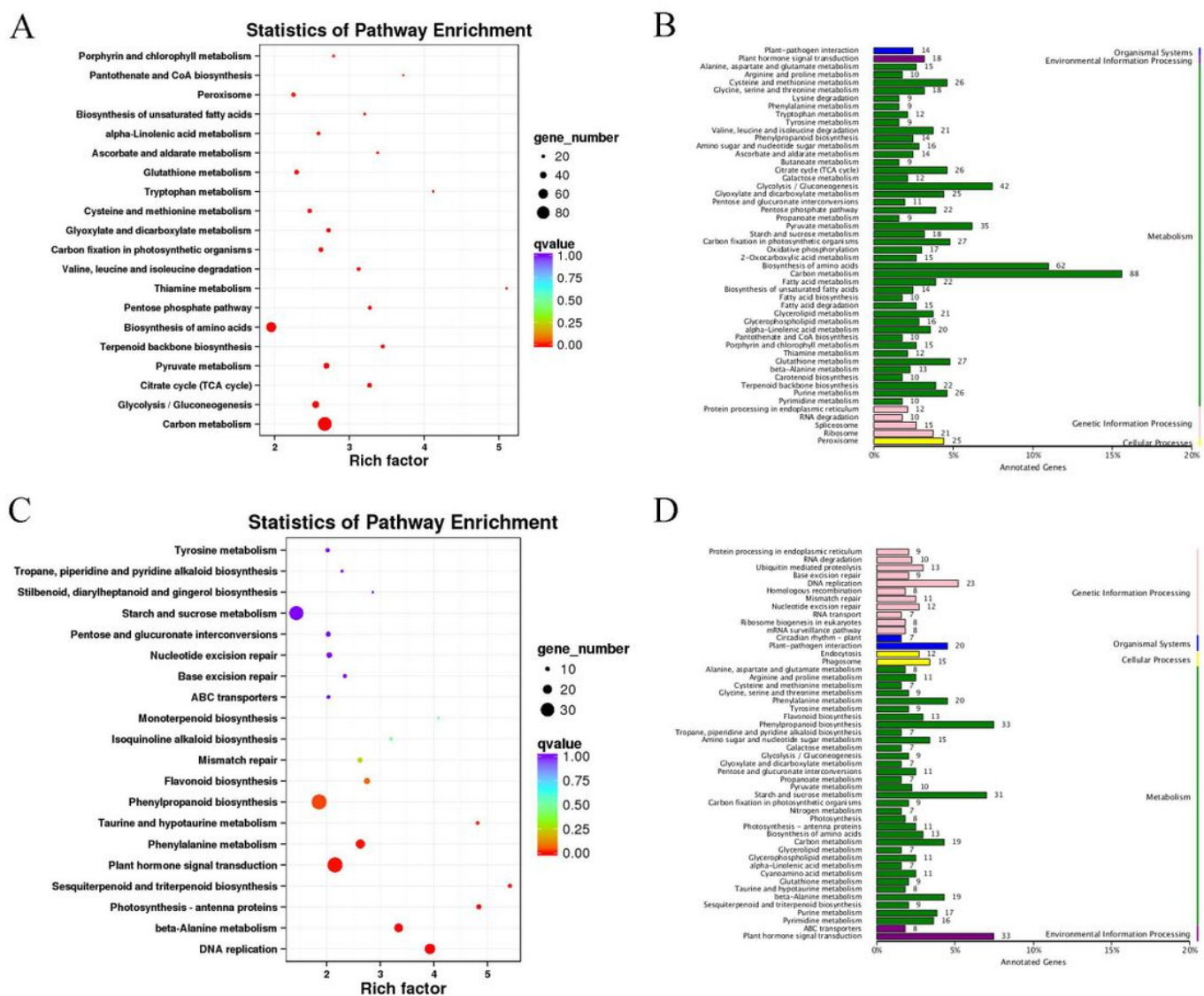
**Figure 7**

The analysis of DEGs and TF families among the three comparisons. A: Venn diagram showing the number and overlapping events of DEGs; B: The dynamic changes in TF families during nectary development.



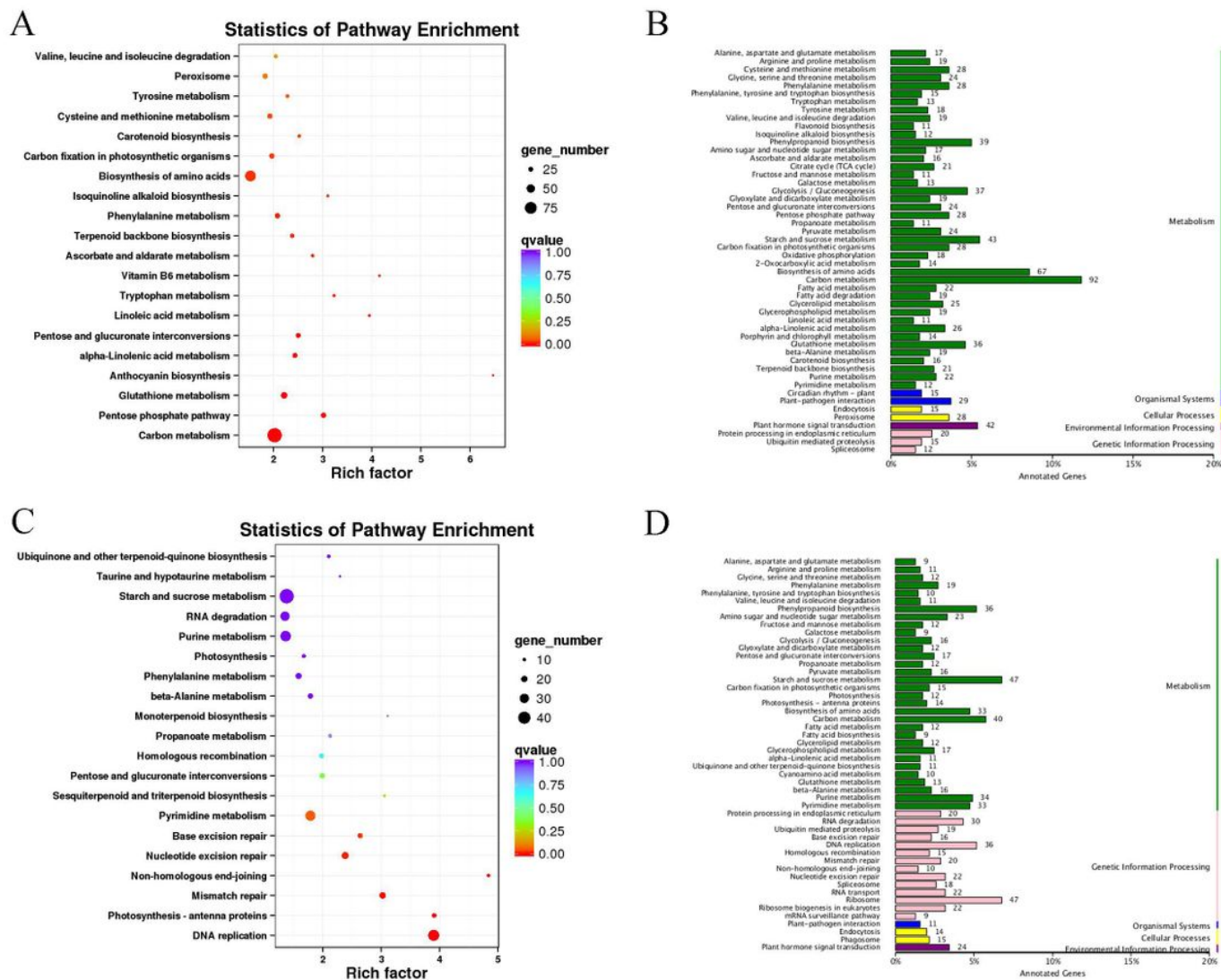
**Figure 8**

The KEGG pathway categories and enrichment factor analysis of S2M\_vs\_S2T DEGs. A, B: Upregulated genes; C, D: Downregulated genes.



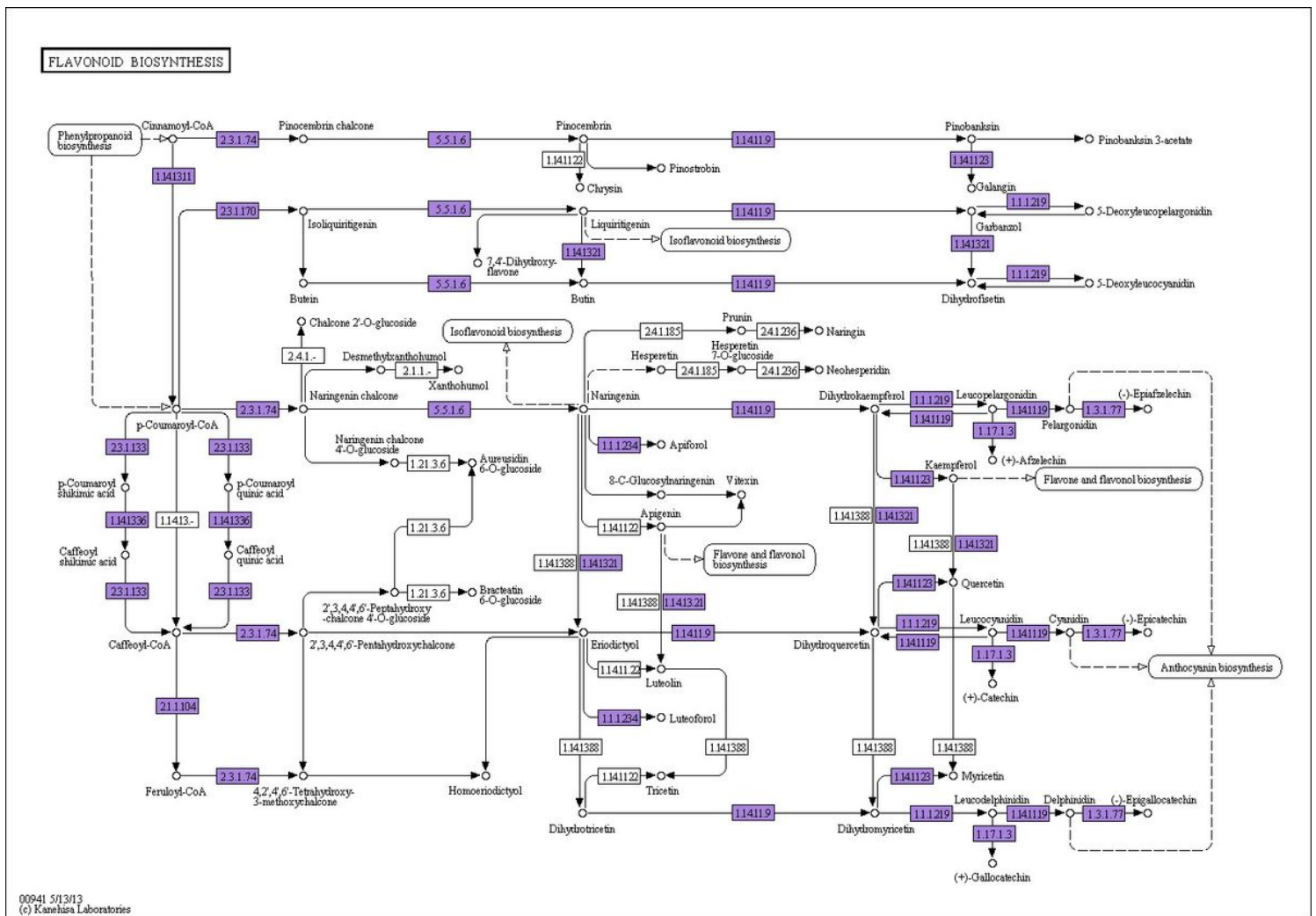
**Figure 9**

The KEGG pathway categories and enrichment factor analysis of S2M\_vs\_S3M DEGs. A, B: Upregulated genes; C, D: Downregulated genes.



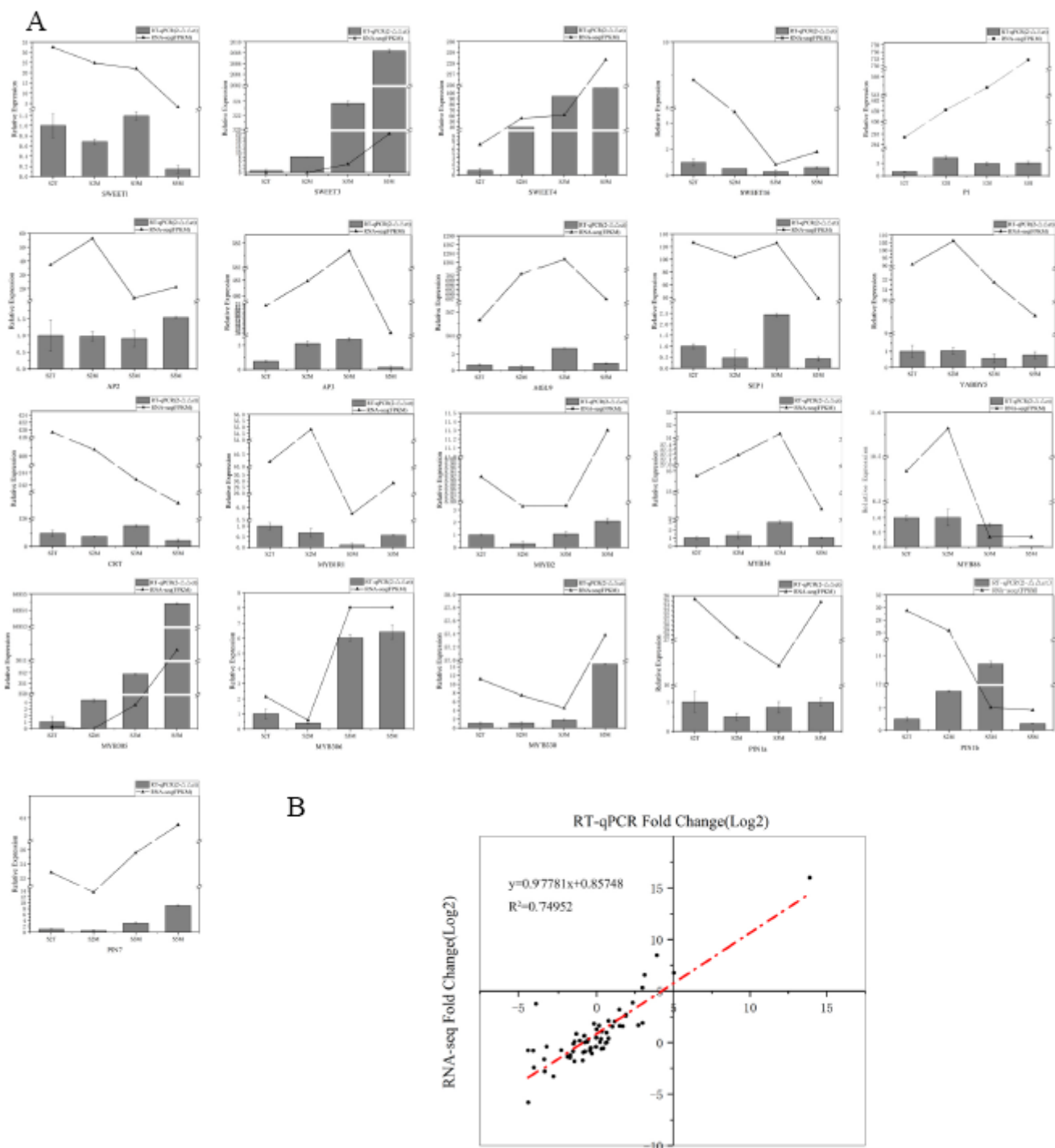
**Figure 10**

The KEGG pathway categories and enrichment factor analysis of S2M\_vs\_S5M DEGs. A, B: Upregulated genes; C, D: Downregulated genes.



**Figure 11**

There are 55 genes involved in flavonoid biosynthesis in *L. tulipifera*.



**Figure 12**

RT-qPCR validations of RNA-seq data. A: Expression profiling of 21 candidate genes involved in nectary development and nectar secretion in *L. tulipifera*; B: Scatter plot showing the correlation between RNA-seq and RT-qPCR.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1.docx](#)