Transcriptome and Metabolome Analysis Unveil Anthocyanin Metabolism in Pink and Red Testa of Peanut (Arachis hypogaea L.)

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Peanut (Arachis hypogaea L.) is an important source of oil and food around the world, and the testa color affects its appearance and commercial value. However, few studies focused on the mechanism of pigment formation in peanut testa. In this study, cultivars Shanhua 15 with pink testa and Zhonghua 12 with red testa were used as materials to perform the combined analysis of transcriptome and metabolome. A total of 198 flavonoid metabolites were detected, among which petunidin 3-O-glucoside and cyanidin O-acetylhexoside in Zhonghua 12 were 15.23 and 14.72 times higher than those of Shanhua 15 at the R7 stage, revealing the anthocyanins underlying the red testa. Transcriptome analysis showed that there were 6059 and 3153 differentially expressed genes between Shanhua 15 and Zhonghua 12 in different growth periods, respectively. These differentially expressed genes were significantly enriched in the flavonoid biosynthesis, biosynthesis of secondary metabolites, and metabolic pathways. Integrated analysis of transcriptome and metabolome indicated CHS gene (arahy.CM90T6), F3’H genes (arahy.8F7PE4 and arahy.K8H9R8), and DFR genes (arahy.LDV9QN and arahy.X8EVE3) may be the key functional genes controlling the formation of pink and red testa in peanut. Transcription factors MYB (arahy.A2IWKV, arahy.US2SKM, arahy.SJGE27, arahy.H8DJRL, and arahy.PR7AYB), bHLH (arahy.26781N, arahy.HM11VV, and arahy.MP3D3D), and WD40 (arahy.L6JJW9) in the biosynthetic pathway of anthocyanin were significantly upregulated in Zhonghua 12 which may be the key regulatory genes in testa pigment formation. This is a comprehensive analysis on flavonoid metabolites and related genes expression in peanut testa, providing reference for revealing the regulatory mechanism of pigment accumulation in peanut testa.

1. Introduction

Anthocyanin is a natural hydrosoluble pigment widely found in plant leaves, stems, flowers, fruits, and seeds. It is mainly synthesized on the surface of the endoplasmic reticulum and accumulated in vacuoles. Anthocyanins have a wide range of biological functions, making plant organs blue, pink, red, or purple [1]. In many plants, anthocyanins can protect them from drought stress, cold, ultraviolet radiation, and microorganisms. Besides, they make flowers and fruits colorful and attract animals and insects, thus promoting pollination and seed transmission [2–5]. In addition to the natural character, anthocyanins also have high nutritional value and antioxidant capacity. They can scavenge pathogenic free radicals in the human body [6], inhibit the oxidation of low-density lipids, and prevent cardiovascular and cerebrovascular diseases, cancer, and other chronic diseases [1, 7].

The biosynthesis of anthocyanins and other flavonoids consists of many enzymatic reactions [8], which can be divided into three steps. First, 4-coumaryl-CoA is synthesized from phenylalanine, which is catalyzed by phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumaryl-CoA ligase (4CL). These reactions are very common in the secondary metabolism of most
plants. Then, 4-coumaryl-CoA and malonyl-CoA are successively catalyzed by chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3'-hydroxylase (F3'H), flavanone 3'-hydroxylase (F3'H), and flavonoid 3',5'-hydroxylase (F3', 5'H) to synthesize dihydrokaempferol, which is a key step in the synthesis of flavonoids. Finally, dihydrokaempferol are synthesized by dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucosyl: flavonoid glucosyltransferase (UGFT) [9–12]. In addition, anthocyanin biosynthesis is regulated by a variety of transcription factors (TFs), including basic helix-loop-helix (bHLH), R2R3-MYB, WD40-repeat protein, NAC, WRKY, bZIP, and MADS-box [13–15]. Among them, transcription factors R2R3-MYB, bHLH, and WD40 can form MYB-bHLH-WD40 (MBW) complex and regulate the expression level of anthocyanin biosynthesis genes in most plants [16–18].

**2. Materials and Methods**

**2.1. Plant Materials.** Cultivars Shanhua 15 with pink testa and Zhonghua 12 with red testa were used as materials, and they were planted in the test field of the agricultural experimental station of Shandong Agricultural University (36.15’N, 117.15’E), Tai’an, China. According to a previous study, peanut pod development can be divided into nine stages, R1 to R9 [22]. In this study, seeds with identical size were selected from Shanhua 15 and Zhonghua 12 from R2 and R7 stages, respectively, and the testa was peeled off the seeds, frozen immediately in liquid nitrogen, and stored in a low-temperature refrigerator at -80°C for follow-up analysis. The peanut seeds begin to pigment deposition in testa within the R4 stage, and they are nearly mature with the almost completed coloration in the R7 stage; thus, testa samples of Shanhua 15 and Zhonghua 12 at R4 and R7 stage, named S4 and S7 and Z4 and Z7, respectively, were used for transcriptome and metabolome analysis.

**2.2. Total Anthocyanin Content Determination.** For extraction of anthocyanin, the method described by Rabino and Mancinelli [23] with some optimization was as followed. Testa samples were powdered in a mortar by adding liquid nitrogen; then, 0.1 g powder was mixed with 5 mL ethanol mixture (85:15 95% ethanol:1.5 M HCl, v/v) and stored overnight at 4°C. The mixture was centrifuged at 5000×g for 6 mins, and the supernatant containing anthocyanin was used to absorbance measurement at 530, 620, and 650 nm. Total anthocyanin content was calculated by the following formula: anthocyanin content (mmol g⁻¹ FW) = [(A1 – A2) – 0.1 × (A3 – A2)]/4.62 × 10⁴ × V/m × 106. A1, A2, and A3 represent the absorbance at 530, 620, and 650 nm, respectively, and V represents the extract volume, and m represents sample weight. The value 4.62 × 10⁴ represents the extinction coefficient of anthocyanin at 530 nm. Three replicates were analyzed for each sample.

**2.3. Flavonoids-Metabolites Detection and Multiple Reaction Monitoring (MRM).** Flavonoids-metabolites identification and quantification was carried out by Metware Biotechnology Co., Ltd. (Wuhan, China) using LC-ESI-MS/MS system (HPLC, Shim-pack UFLC Shimadzu CBM30A system, http://www.shimadzu.com.cn/; MS, Applied Biosystems 6500Q TRAP, http://www.appliedbiosystems.com.cn/). Detailed operation procedures were performed according to the methods described by Dong et al. [1]. Flavonoids-metabolites identification was adopted by Partial least square discriminant analysis (PLS-DA). The variable importance in projection (VIP) of the first principal component represents the contribution of the differential metabolites between two groups, and the differential metabolites were screened using thresholds of fold change (FC) ≥2 or ≤0.5 and VIP >1.

**2.4. Transcriptome Analysis.** Total RNA extraction, concentration, and cDNA library construction were performed according to methods described by Cao et al. [24]. With three biological replicates for each sample, a total of twelve libraries were constructed for samples S4, S7, Z4, and Z7. Then, the library products were sequenced on the HiSeq 4000 platform (Illumina, San Diego, CA, USA). After removing reads containing the adapter and low-quality sequences, the resulting high-quality clean data was mapped to the peanut reference genome (https://www.peanutbase.org/data/public/Arachis_hypogaea/).

The fragments per kb per million reads (FPKM) method was used to calculate gene expression [25]. Differential
expression analysis of testa samples was performed by the DEGseq R package, and the \( P \) values were adjusted using \( q \) values [26]. Differentially expressed genes (DEGs) were screened by the thresholds of \( \log_2 \text{(FC)} \geq 1 \) and False Discovery Rate (FDR) \( \leq 0.05 \). Multiple databases were used for gene function annotation, including Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), Clusters of Orthologous Groups (COG) of proteins database, NCBI non-redundant protein sequences (Nr), a manually annotated and reviewed protein sequence database Swiss-Prot, and database of TrEMBL that contains all translations of EMBL nucleotide sequence entries.

2.5. Validation of Transcriptome Profile. To validate gene expression results, nine flavonoid-related genes were randomly selected for quantitative real-time PCR (qRT-PCR) reactions, and the specific primers (Table S1) were designed by Primer Premier 5 software. The reaction was carried out according to the instructions of the SuperReal PreMix Plus kit (TIANGEN, Beijing, China), and the reaction system included 10 \( \mu \)L SYBR PreMix Plus, 1 \( \mu \)L each primer (10 \( \mu \)M), 2 \( \mu \)L cDNA template, and 6 \( \mu \)L RNase-free water. It was performed on the ABI 7500 PCR instrument (Applied Biosystems), with the following protocol: 95°C for 30 s, followed by 40 cycles at 95°C for 10 s, 55°C for 20 s, and 72.0°C for 20 s, and 75.0°C for 5 s. Peanut Actin gene was selected as the housekeeping gene [27], and relative gene expression was analyzed by the \( 2^{-\Delta\Delta C_{t}} \) method. Significant differences between samples were performed by standard deviation.

3. Results

3.1. Testa Anthocyanin Content Changes during Seed Development. As shown in Figure 1(a), the testa color of Shanhua 15 and Zhonghua 12 got deeper gradually from R2 to R7, especially in R7, when the seeds were colored completely and entered the mature stage. Testa of Shanhua 15 is pink, while it is red in Zhonghua 12. Anthocyanin content determination also showed that the total testa anthocyanin content increased gradually with the seed development and reached the highest level at the R7 stage, which were 0.026 mmol.g\(^{-1}\) and 0.087 mmol.g\(^{-1}\) in Shanhua 15 and Zhonghua 12, respectively (Figure 1(b)).
Figure 2: Continued.
anthocyanin content of Zhonghua 12 was significantly higher than that of Shanhua 15 at each stage \( (p < 0.01) \), with approximately three times higher on average (Figure 1(b)). Therefore, it is suggested that anthocyanin accumulation difference is cultivar specific and eating peanuts with red testa is more beneficial to health.
3.2. Testa Metabolites Differences between Shanhua15 and Zhonghua12.

In order to further study the composition of peanut testa, samples of Shanhua 15 (S4 and S7) and Zhonghua 12 (Z4 and Z7) testa at the R4 and R7 stage were used for flavonoid-related metabolite profiles analysis. A total of 198 flavonoid-related metabolites were detected in all samples (Table S2). Principal component analysis (PCA) showed that S4, S7, Z4, and Z7 were obviously separated in the PC1 × PC2 score chart (Figure 2(a)), and obvious differences were showed between both cultivars and growth periods (Figure 2(b)). The volcano plot also indicated that there were significant differences in metabolites content between the two varieties at R4 (Figure 2(c)) and R7 (Figure 2(d)) stages. The significantly changed metabolites (SCMs) could be divided into eight categories, including anthocyanins, proanthocyanidins, flavanone, flavone, flavonoid, flavonol, isoflavone, and polyphenol. Compared with S4, 68 SCMs were detected in Z4, 37 of which were upregulated and 31 were downregulated (Figure 2(e)), respectively, while 63 SCMs were identified in Z7 when comparing to those in S7, among which 34 SCMs were upregulated and 29 were downregulated, respectively (Figure 2(f)). The category with the most SCMs was flavanone, followed by flavonol.

Anthocyanin plays an important role in the coloration of seed coat and fruit skin [12, 21]. Eleven anthocyanins were identified from all samples. The content of cyanidin O-diacetyl-hexoside-O-glyceric acid in Z4 was 7.87 times higher than that in S4, while pelargonin content decreased significantly (Table S2). In Z7 testa, the contents of petunidin 3-O-glucoside and cyanidin O-acetylhexoside increased by 15.23 times and 14.72 times compared to S7, respectively (Table S2). The results suggested that the content of cyanidin O-diacetyl-hexoside-O-glyceric acid possibly leads to the color difference of peanut testa in the R4 stage, but petunidin 3-O-glucoside and cyanidin O-acetylhexoside possibly play an important role in the testa color formation in the R7 stage.

3.3. Transcriptome Analysis of Shanhua15 and Zhonghua12.

In order to further analyze the regulation mechanism of flavonoids and anthocyanin biosynthesis in the process of testa color formation, transcriptome sequencing was performed at the R4 and R7 stages using testa samples from Shanhua 15 and Zhonghua 12. With three biological replicates for each sample, twelve cDNA libraries were constructed and sequenced on the HiSeq4000 platform. The original sequencing data were stored in the NCBI Short Read Archive database (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra_sub/sub.cgi) with the BioSample accession number SRP271546 (BioProject ID: PRJNA638812). After removing the adaptors and low-quality reads, 57140101, 59405717, 56610333, and 48707327 clean reads were obtained from S4, S7, Z4, and Z7 libraries, respectively. Q20 value, proportion of nucleotides with quality value >20, was above 97% (Table S3), indicating that the RNA-Seq results were of high quality and suitable for follow-up analysis. Finally, 51299 genes were subsequently assembled from the four libraries.

Taking log2 (FC) ≥1 and FDR ≤0.05 as the thresholds, differentially expressed genes (DEGs) were screened out. A total of 3,266 and 1,536 upregulated genes and 2,793 and 1,617 downregulated genes were identified in S4-vs-Z4 and S7-vs-Z7, respectively. For one cultivar, 836 upregulated and 2,927 downregulated genes were detected in S4-vs-S7, while 2,652 upregulated and 5,406 downregulated genes were detected in Z4-vs-Z7, respectively (Figure 3(a)). Venn diagram of all DEGs detected in four compared groups, S4-vs-

![Figure 3: Functional annotation and classification of differentially expressed genes between R4 and R7 pod stages of Shanhua15 and Zhonghua12.](image)
Z4, S7-vs-Z7, S4-vs-S7, and Z4-vs-Z7, displayed that 125 common DEGs could be detected in each group (Figure 3(b)).

In the S7-vs-Z7 group, ontology analysis showed that 7198, 3104, and 7284 genes were assigned to cell component, molecular functional, and biological process class, respectively (Figure S1). Based on enrichment results, 58 DEGs were enriched in the secondary metabolite biosynthetic process (GO:0044550), including 32 upregulated and 26 downregulated genes, and it was the largest group (Table S4). From COG annotation, 1781 DEGs in the S7-vs-Z7 group were classified into 25 categories, and the largest class was the general functional cluster prediction only (318 genes, 17.85%), followed by signaling mechanism (219 genes, 12.30%), secondary metabolites biosynthesis, transport, and catabolism (156 genes, 8.76%) (Figure S2).

The enriched metabolic pathways analysis showed 1349 DEGs from the S4-vs-Z4 group were enriched into 280 KEGG pathways, while 679 DEGs from S7-vs-Z7 were enriched into 268 pathways. Five categories, cellular processes, environmental information processing, genetic information processing, metabolism, and organismal systems, were revealed from KEGG classification analysis. Pathways of biosynthesis of secondary metabolites, flavonoid, and cell cycle-yeast were significantly changed at the R4 stage, while circadian rhythm-plant, biosynthesis of flavonoid, secondary metabolites, and phenylpropanoid were the significantly changed pathways at the R7 stage (Table 1).

| No. | Pathway                               | DEGs with pathway annotation | All genes with pathway annotation | P value     | Corrected P value | Pathway ID |
|-----|--------------------------------------|------------------------------|-----------------------------------|-------------|------------------|------------|
| S4 vs. Z4                                    |                             |                               |           |                 |             |
| 1   | Biosynthesis of secondary metabolites | 377                          | 2759                              | 2.7E-09     | 7.5E-07          | ko01110    |
| 2   | Flavonoid biosynthesis                | 43                           | 162                               | 6.8E-09     | 1.9E-06          | ko00941    |
| 3   | Cell cycle-yeast                      | 38                           | 152                               | 2.8E-07     | 7.8E-05          | ko04111    |
| S7 vs. Z7                                    |                             |                               |           |                 |             |
| 1   | Flavonoid biosynthesis                | 36                           | 162                               | 4.7E-13     | 1.3E-10          | ko00941    |
| 2   | Circadian rhythm-plant                | 36                           | 162                               | 4.7E-13     | 1.3E-10          | ko04712    |
| 3   | Biosynthesis of secondary metabolites | 213                          | 2759                              | 6.1E-10     | 1.6E-07          | ko01110    |
| 4   | Phenylpropanoid biosynthesis          | 50                           | 452                               | 6.1E-07     | 0.00016          | ko00940    |
| S4 vs. S7                                    |                             |                               |           |                 |             |
| 1   | Metabolic pathways                    | 503                          | 4863                              | 5.9E-17     | 1.6E-14          | ko01100    |
| 2   | Biosynthesis of secondary metabolites | 314                          | 2759                              | 1.4E-11     | 3.8E-09          | ko01110    |
| 3   | Flavonoid biosynthesis                | 38                           | 162                               | 4.9E-10     | 1.3E-07          | ko00941    |
| Z4 vs. Z7                                    |                             |                               |           |                 |             |
| 1   | Biosynthesis of secondary metabolites | 551                          | 2759                              | 0           | 0                | ko01110    |
| 2   | Metabolic pathways                    | 837                          | 4863                              | 9.6E-10     | 2.8E-07          | ko01100    |
| 3   | Photosynthesis-antenna proteins       | 18                           | 28                                | 3.1E-09     | 9.1E-07          | ko00196    |

S4, Shanhua15 R4 pod period; Z4, Zhonghua12 R4 pod period; S7, Shanhua15 R7 pod period; Z7, Zhonghua12 R7 pod period. Significant pathways were identified by corrected P ≤ 0.01.

Z4, S7-vs-Z7, S4-vs-S7, and Z4-vs-Z7, displayed that 125 common DEGs could be detected in each group (Figure 3(b)).

In the S7-vs-Z7 group, ontology analysis showed that 7198, 3104, and 7284 genes were assigned to cell component, molecular functional, and biological process class, respectively (Figure S1). Based on enrichment results, 58 DEGs were enriched in the secondary metabolite biosynthetic process (GO:0044550), including 32 upregulated and 26 downregulated genes, and it was the largest group (Table S4). From COG annotation, 1781 DEGs in the S7-vs-Z7 group were classified into 25 categories, and the largest class was the general functional cluster prediction only (318 genes, 17.85%), followed by signaling mechanism (219 genes, 12.30%), secondary metabolites biosynthesis, transport, and catabolism (156 genes, 8.76%) (Figure S2).

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3.4 Differential Expression Genes Related to Flavonoid and Anthocyanin Biosynthesis. As shown in Table S2, a large number of flavonoids were detected in the testa of Shanhua15 and Zhonghua12. We finally rearranged 8 major compounds of flavonoids (Figures 2(c) and 2(f)). A total of 34 DEGs detected in S4-vs-Z4 were associated with flavonoid and anthocyanin biosynthesis, and 16 related DEGs were detected in S7-vs-Z7 (Table S5). Compared to Shanhua15, 24 upregulated and 10 downregulated genes were identified in Zhonghua12 at the R4 stage, indicating that flavonoids and anthocyanidins biosynthesis pathways were enhanced by upregulated gene
expression in Zhonghua12 testa. Genes PAL, C4H, CHS, F3H, F3’H, DFR, ANS, ANR, LAR, and FLS were all significantly upregulated in Zhonghua12. Only five 4CL genes (arahy.NR92KK, arahy.RB4KER, arahy.SN4H17, arahy.TQ54GF, and arahy.3E4P6E) were downregulated. Interestingly, many related genes had no significant difference in expression at the R7 stage, including genes C4H, CHI, F3H, ANS, ANR, and LAR. Nine genes were detected to be upregulated at the R7 stage, including CHS, F3’H, DFR, FLS, FNS, and four 4CL genes (Table S5). Among these upregulated genes, CHS (arahy.CM90T6) was upregulated by 4.97-fold, and it catalyzes the transformation of p-coumaryl-coA to both naringenin chalcone and isoliquiritigenin.

Then, isoliquiritigenin is finally catalyzed to formononetin 7-O-glucoside, and this may be the reason for its high accumulation in Z7 testa of Zhonghua12. The F3’H genes (arahy.8F7PE4 and arahy.K8H9R8) catalyze the conversion of dihydrotricetin to dihydromyricetin and finally to petunidin 3-O-glucoside, and they have high expression in Z4 testa. The DFR enzyme catalyzes different types of substrates to synthesize leucodelphinidin and leucocyanidin, and the corresponding genes (arahy.LDV9QN and arahy.X8EVF3) showed 7.48- and 1.71-fold increments in Z4 compared to S4 (Figure 4, Table S5).

3.5. Transcription Factors Involved in Flavonoids and Anthocyanin Biosynthesis. Except for functional genes, transcription factors (TFs) play an important role in regulating

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**Figure 4:** Transcript profiling of metabolite, Z7/S7. Heat map represents changes in transcripts in flavonoid and anthocyanin biosynthetic. Dots marked with red background represent increased abundances of metabolites. PAL: phenylalanine ammonia-lyase; CHI: cinnamic acid 4-hydroxylase; 4CL: 4-coumarate CoA ligase; CHS: chalcone synthase; CHI: chalcone isomerase; F3’H: flavanoid 3’-hydroxylase; DFR: dihydroflavonol 4-reductase; ANS: anthocyanidin synthase; FLS: flavonol synthesis; FNS: flavone synthase; LAR: leucocyanidin reductase; ANR: anthocyanin reductase; UFGT: UDP glucose-flavonoid 3-O-glucosyl-transferase; MT: methyltransferase.
most metabolic pathways. Studies have shown that the biosynthesis of flavonoid and anthocyanin is regulated by three types of TFs, including R2R3-MYB factor, basic helix-loop-helix (bHLH) proteins, and WD40 proteins [18]. Compared with Shanhua15, 425 and 237 TFs genes were found to be significantly changed in Zhonghua12 testa at R4 and R7 stages, respectively. Meanwhile, compared with R4, 354 differentially expressed TFs genes were detected in Shanhua15 at the R7 stage and 671 for Zhonghua12 (Table 2, Table S6).

MYB and bHLH have been confirmed to play important roles in regulating the expression of genes involved in flavonoid and anthocyanin biosynthesis in many plants [28–30]. In the S4-vs-Z4 group, 51 MYB TF genes were significantly changed, among which 35 genes were upregulated and 16 were downregulated in Zhonghua12. In the S7-vs-Z7 group, 30 MYB TF genes were differentially expressed in Zhonghua12 at the R7 stage and 671 for Zhonghua12 (Table 2, Table S6).

Table 2: Differentially expressed transcription factors in the testa of R4 and R7 stages of Shanhua15 and Zhonghua12 peanuts.

| Comparison group | Gene name | Number of DEGs | Upregulated DEGs | Downregulated DEGs | Biological functions |
|------------------|-----------|----------------|------------------|--------------------|---------------------|
| S4 vs. S7        | MYB       | 46             | 10               | 36                 | Cell development and anthocyanin pathway |
|                  | bHLH      | 37             | 8                | 29                 | Plant development and substance metabolism |
|                  | Other TFs | 262            | 67               | 195                |                     |
|                  | In total  | 345            | 85               | 260                |                     |
| S7 vs. Z7        | MYB       | 30             | 18               | 12                 | Cell development and anthocyanin pathway |
|                  | bHLH      | 29             | 10               | 19                 | Plant development and substance metabolism |
|                  | Other TFs | 178            | 85               | 93                 |                     |
|                  | In total  | 237            | 113              | 124                |                     |
| S4 vs. Z4        | MYB       | 51             | 35               | 16                 | Cell development and anthocyanin pathway |
|                  | bHLH      | 41             | 24               | 17                 | Plant development and substance metabolism |
|                  | Other TFs | 333            | 157              | 176                |                     |
|                  | In total  | 425            | 216              | 209                |                     |
| Z4 vs. Z7        | MYB       | 70             | 15               | 55                 | Cell development and anthocyanin pathway |
|                  | bHLH      | 59             | 15               | 44                 | Plant development and substance metabolism |
|                  | Other TFs | 542            | 207              | 335                |                     |
|                  | In total  | 671            | 237              | 434                |                     |

S4, Shanhua15 R4 pod period; S4, Zhonghua12 R4 pod period; S7, Shanhua15 R7 pod period; Z7, Zhonghua12 R7 pod period. Differentially expressed genes were identified by log2 (fold change) ≥1 and FDR ≤0.05.

Table: Differentially expressed transcription factors in the testa of R4 and R7 stages of Shanhua15 and Zhonghua12 peanuts.
Figure 5: Continued.
indicated MYB forms a ternary complex with bHLH and WD40 to activate genes related to anthocyanin biosynthesis.

3.6. qRT-PCR Validation of Gene Expression. A total of nine DEGs (Table S1) involved in flavonoid biosynthesis, seven functional genes, and two TF genes were randomly selected for qRT-PCR validation. As shown in Figure 6, the relative gene expression of nine test genes was all consistent with the RNA-seq results; thus, we believe that the transcriptome data is of high accuracy and reliability.

4. Discussion

Peanut contains many important nutrients, and the testa color affects its nutritional and commercial value. The anti-oxidant capacity of peanut testa is much higher than that of cotyledons [31]. For example, proanthocyanidin B is widely distributed in peanut testa, and it is a novel allosteric AKT inhibitor, which not only has antioxidant and anti-inflammatory properties but also has a strong antitumor efficacy [32]. In recent years, consumers prefer peanuts with dark testa due to their health benefits from the higher content of anthocyanins and other flavonoids [33]. In addition, aflatoxin resistance is also related to the biosynthesis pathway of flavonoids and phenylpropyl [34]. At present, the testa of most peanut varieties on the market is pink, and some varieties are red. Previous studies on peanut testa pigmentation were limited to genome and transcriptome level, for example, the differences of testa transcriptome in three growth stages of pink peanut varieties were analyzed by RNA-Seq [21]. In this paper, the combined analysis of transcriptome and metabolome provided a new approach to reveal the molecular mechanism of pigment formation in peanut testa.

The anthocyanin content and composition are important factors for different colors of plant tissues or organs. We detected the total anthocyanin content of testa of two peanut varieties during seed development, and the results showed that the total anthocyanin content of two varieties both increased from R2 to R7 stage, suggesting that testa pigments gradually accumulated with seed development. Moreover, the total anthocyanin content of red testa variety Zhonghua 12 was significantly higher than that of pink testa variety Shanhua 15 (Figure 1). Anthocyanins are considered to be the most important pigments in most plants [35]. However, previous studies have shown that different plants have different anthocyanins for pigmentation. During the process of red pigment formation in jujube peel, delphinidin, malvidin 3-O-glucoside, and delphinidin 3-O-glucoside were considered to be the key anthocyanins [36], but cyanidin O-malonylhexoside was the first anthocyanin identified in purple mutant figure [12]. When comparing different asparagus varieties, peonidin, cyanidin, and their glycoside derivatives were the major anthocyanins [1]. The strong
The red color of longan was due to the abundant contents of cyanidin 3-O-glucoside, cyanidin 3-O-6″-malonyl-glucoside, and cyanidin O-syringic acid found in its pericarp [37]. In order to clarify the pigmentation mechanism of peanut testa and analyze the metabolite basis of the difference between pink and red testa, the composition and content of testa metabolites at the R4 and R7 stage of Shanhua 15 and Zhonghua 12 were determined using the UPLC-MS/MS-based approach. The metabolome analysis showed that a total of 198 flavonoid-related SCMs were identified, and the main pigment components of peanut red testa were petunidin 3-O-glucoside and cyanidin O-acetylhexoside. This study further illustrates the primary anthocyanins that affect seed color in plant are petunidin derivatives and cyanidin derivatives.

Flavone plays an important role in the yellow pigment formation of many plants [38, 39]. Interestingly, the contents of tricin O-malonylhexoside, naringenin C-hexoside, and apigenin 7-O-glucoside were extremely higher in the Shanhua 15 S7 sample, and they were all over 1.00 + E04 times higher than that of Zhonghua 12 Z7, especially for tricin O-malonylhexoside, which was more than 1.00 + E06 times higher. Then, we noticed that the endotesta of Shanhua 15 was yellow, while it was white in Zhonghua 12, suggesting that high content of Tricin O-malonylhexoside, naringenin C-hexoside, and apigenin 7-O-glucoside maybe give a yellow coloration in peanut endotesta. In this study, changes of many other metabolites in peanut testa were also investigated, many of which were not directly related to testa pigmentation, but correlated with human health. For example, formononetin 7-O-glucoside (Ononin) can inhibit the proliferation of tumor cells and promote apoptosis and reduce cell invasion and migration [40], and its content was significantly higher in Zhonghua 12 testa with more than 2000-fold than Shanhua 15. Besides, persicoside content was very high at both the R4 and R7 stages of Zhonghua 12 testa, and it has potential radical scavenging activity [41]. To sum up, peanut with red testa contains more ingredients that are beneficial to human health.

Based on the identification of SCMs, the gene expression profile of peanut testa was performed between two varieties at R4 and R7 stages. Compared with Shanhua 15 S4 and S7, thousands of significant DEGs were identified in Zhonghua 12, and many of them were enriched in the secondary
metabolite biosynthetic process and flavonoid biosynthesis pathway through GO and KEGG analysis, suggesting that these genes are likely to be related to pigmentation in pink and red testa. The pigment of peanut testa accumulated gradually with seed development, and the coloring was basically completed at the R7 stage. It was found that the genes involved in flavonoid and anthocyanin biosynthesis, PAL, C4H, CHS, F3H, F3′H, DFR, ANS, ANR, LAR, and FLS, were significantly upregulated in Zhonghua12 testa at the R4 stage, but most of them were not significantly changed at the R7 stage. These results suggested that changes in gene expression occurred much earlier than phenotypic changes. In the early stage of peanut seed development, although the seed coat has not been completely stained, the genes related to flavonoid biosynthesis have been actively expressed, similar to studies on blueberry and pear that genes involved in anthocyanin and flavonoid synthesis were also highly expressed in the early stages of fruit development [42, 43]. In addition, affected by the active expression of related genes, a large number of flavonoid molecules have been accumulated in the white-period of jujube peel, and with the fruit ripening, the early actively expressed genes gradually become silenced [36]. Therefore, genes PAL, C4H, F3H, F3′H, DFR, ANS, ANR, LAR, and FLS are likely to play important roles in the early anthocyanin accumulation. Besides, CHS has been verified to be related to the red coloration in crabapple varieties [44], and it was significantly inhibited in white-flowered mustards, while other genes in the anthocyanin biosynthesis pathway were not significantly different from colored individuals [45]. The high expression of CHS was also related to higher anthocyanin content in peach, and the results were also verified in 30 peach varieties [24]. In our study, CHS (araphy. CM90T6) had higher expression in Zhonghua12 and was upregulated by 4.97-fold in Z7, indicating that the CHS gene may be related to testa pigmentation.

Figure 7: Prediction new model of red peanut testa formation. The red label indicated upregulated genes or metabolites. CHS: chalcone synthase; F3′H: flavonoid 3′-hydroxylase; DFR: dihydroflavonol 4-reductase.
arahy. K8H9R8) have a high expression level in Z4 testa, which catalyze the conversion of dihydroticretin to dihydro- 
myricetin or dihydrokaempferol to dihydroquercetin. The 
DFR enzyme can catalyze different types of substrates to 
synthesize leucodelphinidin and leucocyanidin. DFR genes 
(arahy. LDV9QN and arahy. X8EVF3) showed 7.48- and 
1.71-fold increments in Zhonghua12 compared to Shan-
hua15 in the R4 period. These DEGs may be the critical 
factor for the high accumulation of dihydromyricetin, cyanidin 
O-acetylhexoside, and petunidin 3-O-glucoside in Zhon-
ghua12 (Figure 7).

In addition to those functional genes, TFs have also been 
identified to play a critical role in the regulation of anthocy-
nin biosynthesis, including MYB, bHLH, and WD40 [44, 
48]. For example, MdMYB1 and MdMYB3 were the main 
regulators for anthocyanin biosynthesis and fruit coloring 
in apple [49, 50], and PpMYB10 and PpMYB114 regulate 
anthocyanin biosynthesis in pear [38]. In peanut, an 
R2R3-MYB gene was identified previously, regulating pur-
ple testa formation [19]. In the present study, many MYBs 
were detected to be differentially expressed between Shan-
hua 15 and Zhonghua 12 (Table S5). Among them, five 
MYB genes were annotated in seed coat development, 
anthocyanin regulatory, and flavonol biosynthetic process. 
The expression of all five genes was highly upregulated at 
the R4 stage in Zhonghua12. Protein sequence comparison 
revealed that the genes clustered together with GmMYB58, 
AtMYB5, and GmMYB12b, which have a major role in 
regulating testa developmental process in Glycine max and 
Arabidopsis thaliana [51–52]. The MYBs had similar 
expression patterns to structural genes, and their 
expression trends were closely related to the contents of 
petunidin 3-O-glucoside and cyanidin O-acetylhexoside. 
We also identified three differentially expressed bHLH 
(arahy.26781N, arahy.HM11YV, and arahy.MP3D3D) and 
WD40 (arahy.L6fJW9) transcription factor genes that may 
be involved in anthocyanin biosynthesis in peanut testa. 
The expression patterns of MYB, bHLH, and WD40 was 
consistent with flavonoid synthesis DEGs (Figure 7). This 
implies that control of the biosynthesis of the pigments in 
peanut testa may occur through transcriptional regulation 
by the MBW complex. The detailed functional analysis 
needs further verification.

5. Conclusions

In summary, flavonoid metabolites and related genes in pink 
and red testa of peanut were identified through combined 
transcriptome and metabolome analysis. The accumulation 
of petunidin 3-O-glucoside and cyanidin O-acetylhexoside 
in the late stage of seed development is the main reason for 
the red testa appearance. In addition, the genes PAL, C4H, 
CHS, F3H, F3′H, DFR, ANS, LAR, and ANR in the flavonoid 
biosynthesis pathway were highly expressed in the early 
stage, playing an important role in anthocyanin accumula-
tion. CHS gene arahy.CM90T6, F3′H genes (arahy. 8F7PE4 
and arahy. K8H9R8), and DFR genes (arahy. LDV9QN and 
arahy. X8EVF3) may be the key functional genes controlling 
the formation of pink and red testa in peanut. Transcript-
omic analysis indicated MYB, bHLH, and WD40 in the 
biosynthetic pathway of anthocyanin may be the key regu-
latory genes in pink and red pigment formation. This is a 
comprehensive analysis on flavonoid metabolites and 
related genes expression in peanut testa, providing a refer-
ence for revealing the regulatory mechanism of pigment 
accumulation in peanut testa.

Data Availability

The data used to support the findings of this study are 
included within the article. The data used to support the find-
ings of this study are included within the supplementary 
information files.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors’ Contributions

Fengzhen Liu and Yongshan Wan conceived of the idea and 
supervised the project. Qiqin Xue and Xiurong Zhang as 
principal authors were responsible for processing experi-
ments and wrote the manuscript. Hui Yang, Huadong Li, 
and Yuying Lv performed the experiments and analyzed 
the data. Kun Zhang and Yongguang Liu participated in the 
data analysis. All authors have carefully read and approved 
the manuscript. Qiqin Xue and Xiurong Zhang contributed 
equally to this work.

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Supplementary Materials

Supplementary Figure 1: GO classification of differentially 
expressed genes of R7 stage of Shanhuax 15 and Zhonghua12 
testa. Supplementary Figure 2: COG functional classification 
of differentially expressed genes of R4 and R7 stages of 
Shanhuax 15 and Zhonghua12 testa. S4, Shanhuax 15 R4 pod 
period; Z4, Zhonghua12 R4 pod period; S7, Shanhuax 15 R7 
pod period; Z7, Zhonghua12 R7 pod period. Supplementary 
Table 1: primers for qRT-PCR verification. Supplementary 
Table 2: significantly changed metabolites between Shan-
huax 15 and Zhonghua12 testa. Supplementary Table 3: 
summary of the sequencing and de novo assembly. Supple-
mental Table 4: statistics of GO Enrichment in the testa of 
R4 and R7 stages of Shanhuax 15 and Zhonghua12 
peanut. Supplementary Table 5: DEGs of flavonoid and
anthocyanin biosynthesis in the testa of R4 and R7 stages of Shanhua15 and Zhonghua12 peanut. Supplementary Table 6: differentially expressed transcription factors in the testa of R4 and R7 stages of Shanhua15 and Zhonghua12 peanut. (Supplementary Materials)

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