

Integrated transcriptomic and metabolomic analyses reveals anthocyanin biosynthesis in leaf coloration of quinoa (*Chenopodium quinoa* Willd.)

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1 **Integrated transcriptomic and metabolomic analyses reveals**
2 **anthocyanin biosynthesis in leaf coloration of quinoa**
3 **(*Chenopodium quinoa* Willd.)**

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14 **Abstract:** Quinoa leaves demonstrate a diverse array of colors, offering a potential
15 enhancement to landscape aesthetics and the development of leisure-oriented sightseeing
16 agriculture in semi-arid regions. This study utilized combined transcriptomic and metabolomic
17 analyses to investigate the mechanisms underlying anthocyanin synthesis in both emerald green
18 and pink quinoa leaves. Differentially expressed genes (DEGs) and differentially accumulated
19 metabolites (DAMs) were analyzed between two different germplasms at different periods.
20 Several key genes, including *4CL*, *C3'H*, *HCT*, *CHS*, *CHI*, *ANR*, *CYP75B1*, *UGT79B1*, *FG3*,
21 *FG2*, *CYP73A*, *MYC2*, *bHLH14*, *HY5*, and *TGA*, were identified in anthocyanin biosynthesis

using DEG analysis. GO enrichment analysis revealed seven closely related GO Terms. KEGG enrichment analysis identified six metabolic pathways that were significantly associated with anthocyanin biosynthesis, with flavonoid biosynthesis (ko00941) emerging as the most pivotal. Metabolomic analysis confirmed cyanidin 3-O-(3'',6''-O-dimalonyl glucoside) and naringenin as key DAMs in pink leaves. Combined transcriptomic and metabolomic analyses indicated that both the flavonoid biosynthesis pathway (ko00941) and anthocyanin biosynthesis pathway (ko00942) were involved in the anthocyanin biosynthesis pathway. Ten DEGs, including *PAL*, *CHI*, *CYP75B1*, *F3H*, *FG3*, *CYP73A*, *HCT*, *C3'H*, *4CL*, and *CHS*, were verified through qRT-PCR, with the results across the nine comparison groups consistent with that from transcriptomic sequencing. These findings provide a foundation for elucidating the molecular regulatory mechanisms governing flavonoid synthesis in quinoa leaves.

Keywords: Quinoa, Anthocyanin, Transcriptomics, Metabolomics, qRT-PCR

Background:

Quinoa (*Chenopodium quinoa* Willd., $2n=4x=36$), recognized as the "king of food", "nutritional gold", "food of the future", and "super food", originated in the Andes mountains of South America^[1]. Quinoa, which has been cultivated in South America for more than 7,000 years, served as a staple food crop in Chile, Ecuador, Bolivia, Argentina and Brazil. Since its introduction in the 1980s, quinoa cultivation in China has increased to 300,000 acres. This remarkable grain has garnered global attention because of its exceptional nutritional profile, health benefits, and robust resistance to adverse environments^[2]. Quinoa has immense potential in various sectors, including food, medicine, livestock feed, and industry^[3]. It is abundant in essential nutrients, such as vitamins, polyphenols, flavonoids, saponins, phytosterols, and other

bioactive compounds, offering high protein content, rich dietary fiber, easy digestibility, and a low predicted glycemic index^[4].

Quinoa, esteemed for its high ornamental value, serves a dual purpose as both a food crop and a contributor to landscape greening, enhancing the development of leisure and sightseeing agriculture^[5]. Its leaves exhibit a vibrant array of colors, including pink, green, yellow, purple, red, and orange, creating an aesthetically ideal landscape^[6]. The diversity in leaf coloration is closely related to anthocyanin biosynthesis, which is a crucial factor in determining the quality of quinoa tea products. Anthocyanins, a type of flavonoid, are widespread in various plant leaves, fruits, and other tissues and organs and are known for their potent antioxidant properties^{[7],[8]}. They are soluble in water, methanol, and other solvents^[9] and can inhibit cancer cell growth^[10], inflammation, and obesity^[11], while also promoting vascular health, preserving eyesight, and contributing to beauty care^[12].

The synthesis and metabolism of anthocyanins in quinoa is a complex, yet well-defined biosynthetic pathway. Phenylalanine (PHE) serves as a precursor for synthesizing dihydroflavonol (DHK) via a series of enzymatic reactions involving phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone 3-hydroxylase (F3H). Subsequently, pelargonidin, cyanidin, and delphinidin are synthesized via three distinct metabolic branching pathways. Pelargonidin is produced through dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS), and flavonoid 3-glucosyltransferase (UFGT) in the first branching pathway. Cyanidin is synthesized by flavonoid 3'-hydroxylase (F3'H), DFR, ANS, and UFGT in the second branching pathway. Delphinidin is formed by flavonoid 3'-5' hydroxylase (F3'-5'H),

66 ANS, DFR, and UFGT in the third branching pathway. Liu Y et al.^[13] conducted an
 67 investigation on the structural genes of purple Chinese cabbage (*Brassica rapa*). Their study
 68 revealed significant up-regulation of key genes including *PAL*, *C4H*, *4CL*, *CHS*, *CHI*, *F3H*,
 69 *F3'H*, *FLS*, *DFR*, and *ANS* across all three growth stages (cotyledon, seedling, and large-leaf
 70 stages). Xiang et al.^[14] examined the differential expression of genes related to anthocyanin
 71 synthesis in purple tea tree buds (*Camellia sinensis*), identifying an up-regulation of *PAL*, *C4H*,
 72 *CHS*, *CHI*, *F3H*, *F3'H*, *F3'-5'H*, *DFR*, and *ANS* key enzyme genes. Additionally, Yu M et al.^[15]
 73 established a positive correlation between *PAL* activity and anthocyanin content in grapes (*Vitis*).
 74 In addition to these structural genes, several regulatory genes can affect the anthocyanin
 75 synthesis pathway. These genes operate indirectly by acting as transcription factors (TFs),
 76 thereby modulating the expression of structural genes. At the transcriptional level, the TF
 77 family governing anthocyanin biosynthesis predominantly comprises the v-myb avian
 78 myeloblastosis viral oncogene homolog (MYB), basic helix-loop-helix (bHLH), and WD40,
 79 which form complexes that directly bind to the promoters of anthocyanin biosynthesis structural
 80 genes^[16]. Nesi N^[17] and Baudry A et al.^[18] demonstrated that TESTA 2 (TT2) (MYB) can
 81 regulate late biosynthesis genes by binding to TT8 (bHLH42) and TTG1 (WD40), resulting in
 82 seed coat pigmentation. In petunia (*Petunia hybrida*) petal cells, anthocyanin biosynthesis is
 83 also regulated by the MYB-bHLH-WD40 complex, although certain MYB factors such as
 84 *AtMYB12* and *VvMYB1* can regulate flavonoid biosynthesis without bHLH and WD40
 85 cofactors^{[19],[20]}. In addition, the bZIP TF family promotes anthocyanin accumulation. An JP et
 86 al.^[21] discovered that the bZIP TF *MdbZIP44* can regulate abscisic acid and promote
 87 anthocyanin accumulation in apples (*Malus pumila*). Additionally, Liu CC et al.^[22] reported that

the bZIP TF *HY5* mediates *CRY1a* to induce anthocyanin biosynthesis in tomatoes (*Solanum lycopersicum* L).

The molecular regulatory mechanisms governing leaf coloration in quinoa remain unexplored. In this study, germplasm materials exhibiting emerald and pink leaf hues were selected from the germplasm resources of Quinoa at the Science and Technology Innovation Service Center in Hebei Province, China. Employing an integrated approach of transcriptomics and metabolomics, DEGs and DAMs were investigated for their association with anthocyanin biosynthesis in different leaf colors. The results of this study are anticipated to provide pivotal insights for comprehensive exploration of the molecular mechanisms underlying anthocyanin biosynthesis in quinoa.

1. Materials and Methods

1.1 Plant materials

The examined quinoa varieties, M202 and M146, displayed emerald green and pink leaves, respectively. These varieties were cultivated at the Zhangbei Experimental Station of Hebei Agricultural University on May 25, 2021 and May 26, 2022, with row spacing of 40 cm and plant spacing of 18 cm.

Leaf characteristics of M202 and M146 were recorded from 20 to 110 d of growth, with observations at 10-d intervals. The leaves from the middle of each plant were selected for evaluation. Three distinct stages were identified: color change initiation (70 days after germination, 70 DAG), partial color change (90 DAG), and full color change (110 DAG). The stages were labeled as N1, F1, N2, F2, and N3, F3 for M202 and M146, respectively, representing the initial, elongation, and maturity stages of leaf development. Each sample,

weighing approximately 1–3 g, underwent to three biological replicates. Samples were sealed in plastic bags with 5–10 grains of silica gel and stored at -80°C.

1.2 Identification of leaf color in quinoa

Samples from stages N1, N2, N3, F1, F2, and F3 were quantified using a chromatic meter (CR-10plus, Japan) in triplicate. “L” represents black and white value, “+” and “-” represent partial white and duskiness; “a” represents the red-green value, “+” and “-” represent reddish and green, respectively; “b” indicates the yellow-blue value, “+” and “-” indicate yellowish and bluish, respectively. Based on the criteria established for N1 and F1, difference calculations ($\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$) were conducted using SPSS26. When $\Delta E \leq 2$, the color difference is imperceptible to the naked eye; when $2 < \Delta E \leq 6$, the color difference can be discerned but is not pronounced; and when $\Delta E > 6$, the human eye can easily distinguish the color difference.

1.3 Quantification of total anthocyanin in quinoa leaves

The anthocyanin content was assessed using the method described by Serrano^[23]. Each 0.5 g sample (N1, F1, N2, F2, N3, and F3) was ground into powder in 0.1 mol/L hydrochloric methanol solution. The mixture was then shielded from light in tin foil and maintained at 4°C until the tissue had a white appearance. Subsequently, centrifugation at 10000 g for 10 min was performed, and the absorbance of the supernatant was measured at 530 nm and 657 nm. The difference in anthocyanin absorbance at 530 nm was calculated using the equation $A_{530} - 0.25 \times A_{657}$. This value was normalized to the fresh weight to represent the total anthocyanin content.

$$\text{Anthocyanin content (OD/g)} = (A_{530} - 0.25 \times A_{657}) / m$$

132 *A530*: Absorption value of anthocyanin at 530nm

133 *A657*: Absorption value of anthocyanin at 657nm

134 *m*: weight of sample (unit: g)

135 **1.4 Transcriptomic sequencing and DEG analysis**

136 RNA was extracted from the samples using the TRIzol precipitation method in Rio DC^[24].
137 Subsequently, a cDNA library was prepared. The samples were sequenced on the Illumina
138 (<http://www.illumina.com>) platform with three biological replicates. The basic group mass
139 values for the raw data were determined using Sequencing by Synthesis (SBS) techniques.
140 High-quality clean data were obtained by removing reads containing joints, reads with an N
141 ratio exceeding 10%, and reads with both a mass value $Q \leq 10$ and a base number surpassing 50%
142 of the entire read.

143 High-quality clean data were compared to the tetraploid
144 ([https://www.ncbi.nlm.nih.gov/genome/?term=txid63459\[orgn\]](https://www.ncbi.nlm.nih.gov/genome/?term=txid63459[orgn])) quinoa reference genome
145 using an efficient HISAT2^[25] comparison system. Subsequently, StringTie^[26] was employed for
146 the assembly and quantification of the reads, generating the initial transcript. This transcript
147 was further compared with various databases, including Nr (<ftp://ftp.ncbi.nih.gov/blast/db/>),
148 Swiss-Prot (<http://www.uniprot.org/>), GO (<http://www.geneontology.org/>), and KEGG ([http://](http://www.genome.jp/kegg/)
149 www.genome.jp/kegg/) using BLAST. Annotation information was obtained by employing the
150 HMMER^[27] software in conjunction with the Pfam database.

151 The transcript expression levels were normalized for accurate representation. StringTie^[28]
152 employed Fragments Per Kilobase Million (FPKM) as a metric to quantify transcript or gene
153 expression levels using a maximum traffic algorithm. DESeq2^[29] software facilitated the

analysis of DEGs. DEGs were selected based on the following criteria: $|\log_2(\text{FC})| \geq 1$ and False Discovery Rate (FDR) < 0.05 , with Fold Change (FC) indicating differences in gene expression levels. DEGs were classified as up-regulated ($\log_2(\text{FC}) > 1$) or down-regulated ($\log_2(\text{FC}) < -1$). R software was used to generate volcano plots, visually representing the DEG statistics for each comparison group compared to the FDR values. Gene overlap analyses were conducted for the selected DEGs and Venn diagrams were constructed to illustrate the unique and shared genes among the comparison groups.

1.4.3 GO and KEGG enrichment analyses of DEGs

GO enrichment analysis was conducted using the GO database (<http://www.geneontology.org/>), which includes three distinct ontologies: molecular function (MF), cellular component (CC), and biological process (BP)^[30]. The DEGs were systematically associated with terms in the GO database to calculate the number of genes within each term. This facilitated the generation of a catalog comprising genes linked to specific GO functions along with the statistical number of genes. Hypergeometric tests were employed to identify the significant enrichment of GO terms within the DEGs in contrast to the entire genome background. In KEGG enrichment analysis, pathways were treated as the units of analysis. The hypergeometric test was applied to identify pathways exhibiting significant enrichment in the DEGs relative to the background gene set. For both GO and KEGG analyses, pathways were considered significantly enriched if they yielded a q-value (Benjamini -- Hochberg-corrected p-value) of less than 0.05.

1.5 Metabolomic analysis

Metabolites were analyzed using liquid chromatography-tandem mass spectrometry (LC-

MS/MS) for both qualitative and quantitative assessments. Original data from the mass spectrometry analysis underwent baseline filtering. Peak identification, filtering, and alignment of each metabolite were performed using the XCMS package (v3.3.2) in R. This process yielded a data matrix of the mass-to-charge ratio (m/z), retention time, and peak intensity. Metabolite annotations were determined by referencing public mass spectrometry databases such as HMDB (<http://www.hmdb.ca/>), Massbank (<http://www.massbank.jp/>), LipidMaps (<http://www.lipidmaps.org>), and METLIN^[31] (<http://metlin.scripps.edu/index.php>) .

All detected metabolites were annotated using the MetWare database. Metabolomic data were subjected to multivariate statistical analysis using MetaboAnalyst software (version 5.0). Unsupervised dimension reduction and principal component analysis were performed using the R package (<http://www.r-project.org/>) to visualize distinctions among the sample groups. Additionally, the orthogonal projection to latent structures-discriminant analysis (OPLS-DA) model was rigorously validated using cross-validation and permutation tests. Score and permutation plots were generated to illustrate the differences between comparison groups. Variable importance in projection (VIP) scores were used to describe the overall contribution of each variable to the model. The criteria for identifying DAMs were set at $VIP > 1$, $|\log_2(FC)| \geq 1$, and $P < 0.05$.

1.6 Integrated transcriptomic and metabolomic analyses

Transcriptomic and metabolomic data were used to determine the differences in gene expression and metabolite levels across the samples. DEGs identified from the transcriptome and DAMs identified from the metabolome were subjected to joint analysis using the Pearson correlation coefficient (PCC) method. The threshold of $PCC \geq 0.6$ and $P < 0.05$ was applied for

this analysis. $PCC \geq 0.6$ indicates a significant correlation between DEGs and DAMs. These DEGs and DAMs were annotated using the KEGG database to determine the common KEGG pathways. Network diagrams depicting the Pearson correlation coefficient $|r| > 0.6$ between DEG expression and DAM abundance were generated using the Cytoscape software.

1.7 Analysis of differentially expressed TFs

The selected DEGs were subjected to BLAST comparisons with the quinoa genome database and were subsequently combined with the PlantTFDB database (<http://planttfdb.cbi.pku.edu.cn/>) to identify differentially expressed TF genes. These selected TFs, along with their genes associated with anthocyanin biosynthesis, were further analyzed. The expression patterns of these differentially expressed TFs were analyzed according to the FPKM values from the transcriptomic data.

1.8 qRT-PCR verification

RNA was extracted and subjected to reverse transcription to synthesize cDNA for qRT-PCR verification. The reaction conditions comprised denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 15 s for a total of 40 cycles. Using *ACT7* as an internal reference, primers were designed using Premier5.0 software based on the CDS sequence corresponding to the target gene in the sequence library. Relative expression levels were determined using the $2^{-\Delta\Delta C_t}$ method^[32]. Statistical significance of the relative expression data was assessed using a *t*-test with three biological replicates.

2. Results and analysis

2.1 Phenotypic observation of leaf color in quinoa

The growth changes in quinoa leaves, spanning from 20 to 110 d, and the phenotypes at

maturity were recorded (Figure 1A). Leaves at 70, 90, and 110 d represented the stages of color transition, half coloration, and full coloration, respectively. The colorimeter readings for M202 revealed L values between 38 and 44, a values between -12 and -5, and b values between 15 and 22 (Figure 1B). Moreover, M146 exhibited L values between 42 and 49, a values between 13 and 19, and b values between 4 and 8. Both M202 and M146 displayed darkening of leaf color as the developmental period was extended. The ΔE values, indicating color differences, increased with leaf growth. When N1 was the reference, ΔE values for N2 and N3 were 3.13 and 6.87, respectively. Similarly, using F1 as the standard, ΔE values for F2 and F3 were 2.87 and 5.95, respectively.

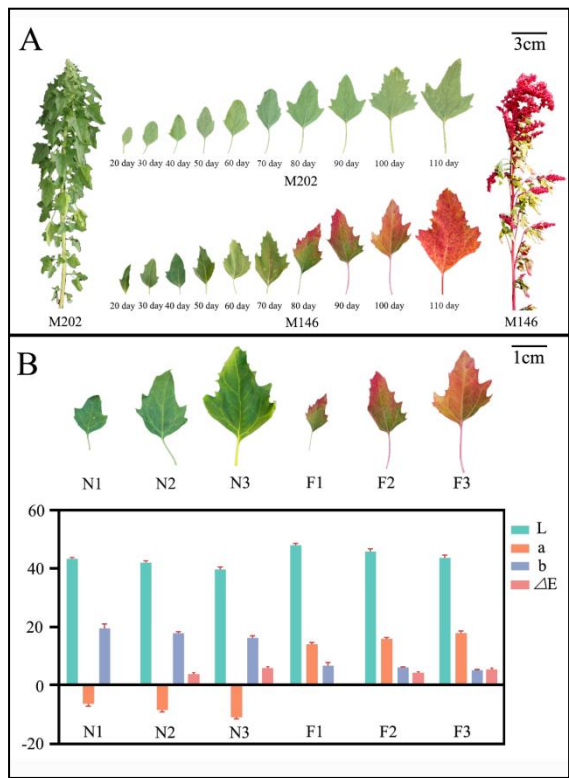


Figure 1. Different stages of leaf development and values of chromatism between two quinoa varieties. A: Growth changes of quinoa leaves from 20 to 110 d and plant phenotypes at maturity B: Color difference values (L, a, b, and ΔE) for six samples.

2.2 Quantification of total anthocyanin content

Quantification analysis revealed a progressive increase in the total anthocyanin content in quinoa leaves as they matured. Specifically, at stages N1, N2, N3, F1, F2, and F3, the total anthocyanin content in the leaves was 1.57, 1.93, 2.11, 1.52, 2.50, and 3.00 OG/g, respectively (Figure 2).

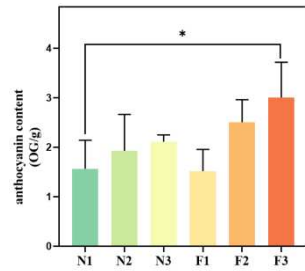


Figure 2. Changes in anthocyanin content among different quinoa varieties and leaf developmental periods. The vertical axis represents anthocyanin content (OG/g), the abscissa indicates the name of each sample, and * indicates statistically significant differences ($p < 0.05$).

2.3 Transcriptomic analysis and screening of DEGs

Eighteen cDNA libraries were generated from the leaf samples at stages N1, F1, N2, F2, N3, and F3. Transcriptomic sequences were obtained after the removal of adapters and low-quality reads. A total of 140.52 Gb of high-quality clean data were obtained, with a Q20 base composition percentage exceeding 97.52% and a Q30 base composition percentage exceeding 92.96%. The GC content measured greater than 45.15% (Table 1).

Table 1. Analysis of transcriptomic sequencing quality

sample	Raw Reads	Clean data(bp)	N(%)	Q20%	Q30%	GC(%)
N1	8587572800	8521089852	0%	98.17%	94.50%	45.38%
N2	8737575700	8665021391	0%	97.96%	94.04%	45.66%

N3	7028937300	6972622440	0%	97.52%	92.96%	45.15%
F1	6908289000	6853592286	0%	97.68%	93.37%	46.18%
F2	7840968600	7787675930	0%	98.02%	94.15%	45.62%
F3	8860698200	8796222315	0%	97.84%	93.73%	45.16%

249 DEGs were identified using the criteria of $|\log_2(\text{FC})| \geq 1$ and $\text{FDR} < 0.05$, in nine pairwise
 250 comparisons: N1vsN2, N1vsN3, N2vsN3, F1vsF2, F1vsF3, F2vsF3, N1vsF1, N2vsF2, and
 251 N3vsF3. The total DEGs for each comparison were 4560, 12563, 4546, 6060, 9479, 8432, 8475,
 252 4661, and 7519, respectively. Among these, up-regulated genes numbered 2602, 7620, 1994,
 253 2781, 5054, 5148, 3591, 2470, and 3480, while down-regulated genes totaled 1958, 4943, 2552,
 254 3279, 4425, 3284, 4884, 2191, and 4039, respectively (Figure 3). Gene overlap analysis
 255 revealed distinct sets of genes for each of the nine comparison groups, with 100, 2206, 89, 315,
 256 990, 853, 923, 246, and 731 genes identified as unique to their respective groups (Figure 4).
 257 Additionally, 33 DEGs were shared across all nine comparison groups. None of these genes
 258 was significantly associated with anthocyanin biosynthesis according to gene annotations,
 259 suggesting their involvement in other metabolic pathways in quinoa leaves.
 260

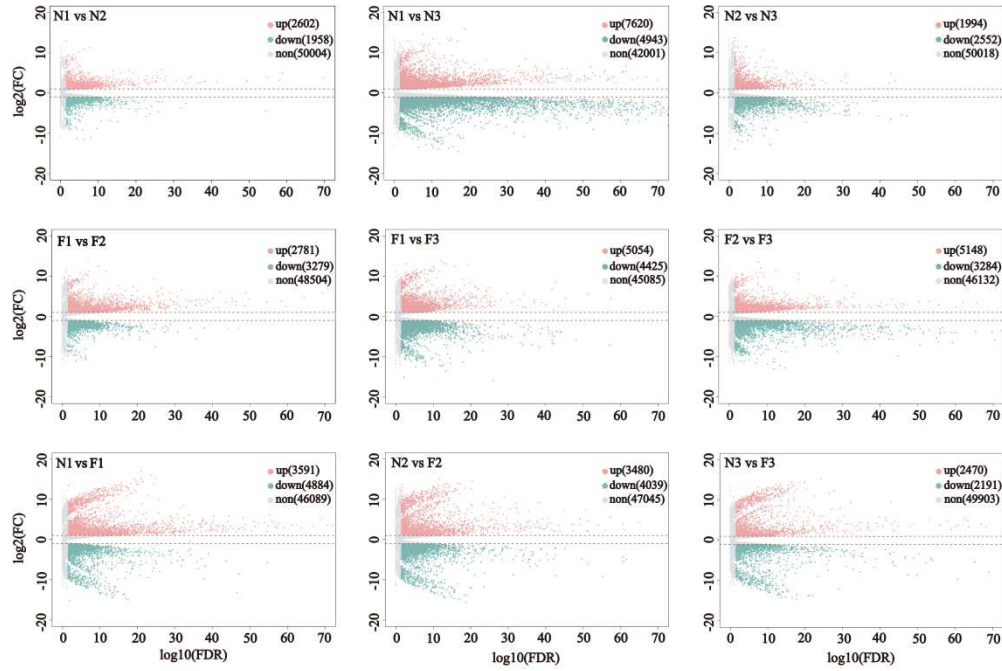


Figure 3. M-versus-A plot (MA) diagrams of DEGs in the nine comparison groups. ‘Up’ represents up-regulated expression, ‘down’ represents down-regulated expression, and ‘non’ represents indifferent genes.

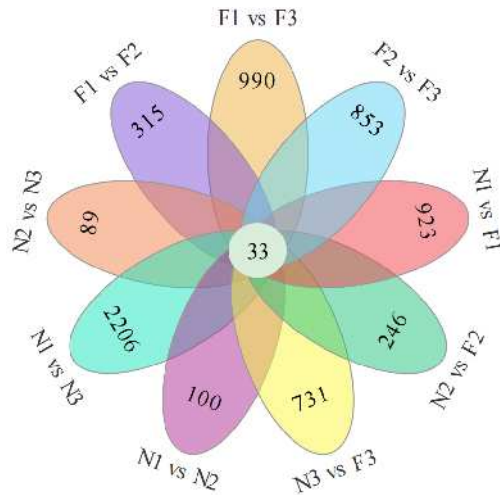


Figure 4. Venn diagram of DEGs in the nine comparison groups. The Venn diagram represents the overlapping DEGS among the nine comparison groups, and the number represents the unique number of DEGS in each comparison group.

2.4 GO and KEGG enrichment analyses

GO enrichment analysis revealed the enrichment of GO terms in various comparisons.

Specifically, in N1vsN2, N1vsN3, N2vsN3, F1vsF2, F1vsF3, N1vsF1, N2vsF2, and N3vsF3, the number of enriched DEGs in GO terms were 1489, 2107, 1421, 1660, 1857, 1797, 1815, 1436, and 1705, respectively. These enrichments encompassed biological processes (BP) with 1035, 1397, 962, 1148, 1251, 1249, 1235, 993, and 1136 DEGs; molecular functions (MF) involving 324, 504, 333, 372, 431, 389, 410, 314, and 403 DEGs; and cellular components (CC) comprising 130, 206, 126, 140, 175, 159, 170, 129, and 166 DEGs (Figure 5).

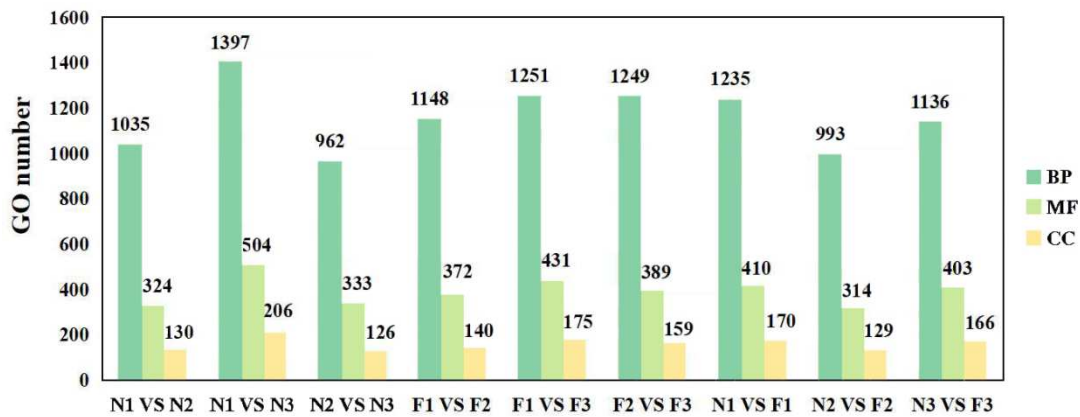


Figure 5. Statistical diagram of GO terms enriched for each comparison group. Note: The vertical axis represents the number of GO terms enriched in each comparison group, and the horizontal axis represents each comparison group. The yellow, green, and blue columns represent the BP, MF, and CC components, respectively.

Annotation analysis revealed that seven GO terms were closely associated with anthocyanin biosynthesis, primarily within BP and MF categories. For BP, the relevant GO terms included GO:0009812 (flavonoid metabolic process), GO:0010468 (regulation of gene expression), GO:0051553 (flavone biosynthetic process), GO:0009813 (flavonoid biosynthetic process), and GO:0009411 (response to UV). In the MF category, GO:0043169 (cation binding) and GO:0016703 (oxidoreductase activity) were identified. Furthermore, the differential expression of TFs *WRKY22*, *HY5*, and *TGA* was enriched in the GO:0010468 term (Figure 6).

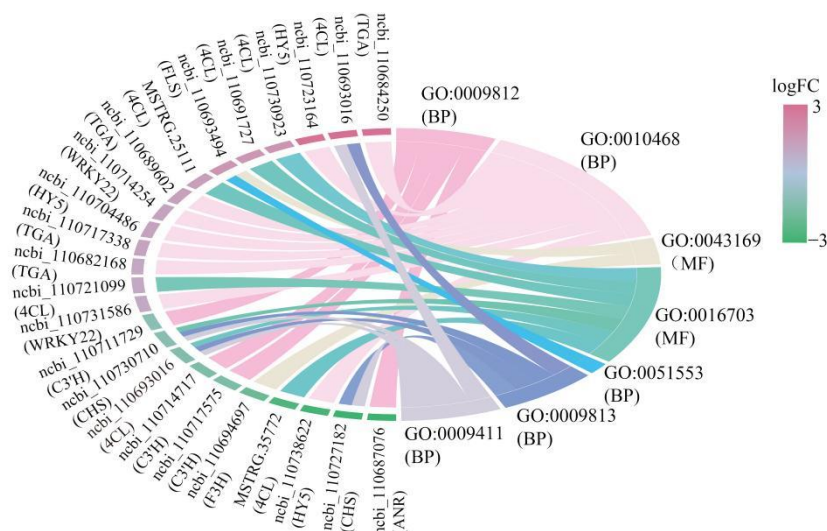


Figure 6. Chordal graph of DEGs enriched in GO analysis of quinoa leaves. GO:0009812, GO:0010468, GO:0043169, GO:0016703, GO:0051553, GO:0009813, and GO:0009411 represent the flavonoid metabolic process, regulation of gene expression, cation binding, oxidoreductase activity, flavone biosynthetic process, flavonoid biosynthetic process, and response to UV, respectively. GO:0043169 and GO:0016703 are MFs (molecular function), and the remaining GO terms are BPs (biological process). KEGG enrichment analyses in various comparisons (N1vsN2, N1vsN3, N2vsN3, F1vsF2, F1vsF3, N1vsF1, N2vsF2, and N3vsF3) revealed the involvement of a total of 121, 133, 124, 125, 132, 131, 128, 124, and 131 significantly different metabolic pathways, respectively. Among these, six pathways were notably associated with anthocyanin biosynthesis: phenylalanine metabolism (ko00360), phenylpropanoid biosynthesis (ko00940), flavonoid biosynthesis (ko00941), plant hormone signal transduction (ko04075), flavone and flavonol biosynthesis (ko00944), and circadian rhythm-plant (ko04712). Phenylpropanoid biosynthesis exhibited the highest gene enrichment in all comparisons, with 62, 120, 83, 72, 114, 100, 74, 52, and 111 genes, respectively. The highest enrichment factors for flavonoid biosynthesis were 0.056, 0.222, 0.222, 0.167, 0.222, 0.333, 0.167, 0.278, and 0.444, respectively (Figure 7).

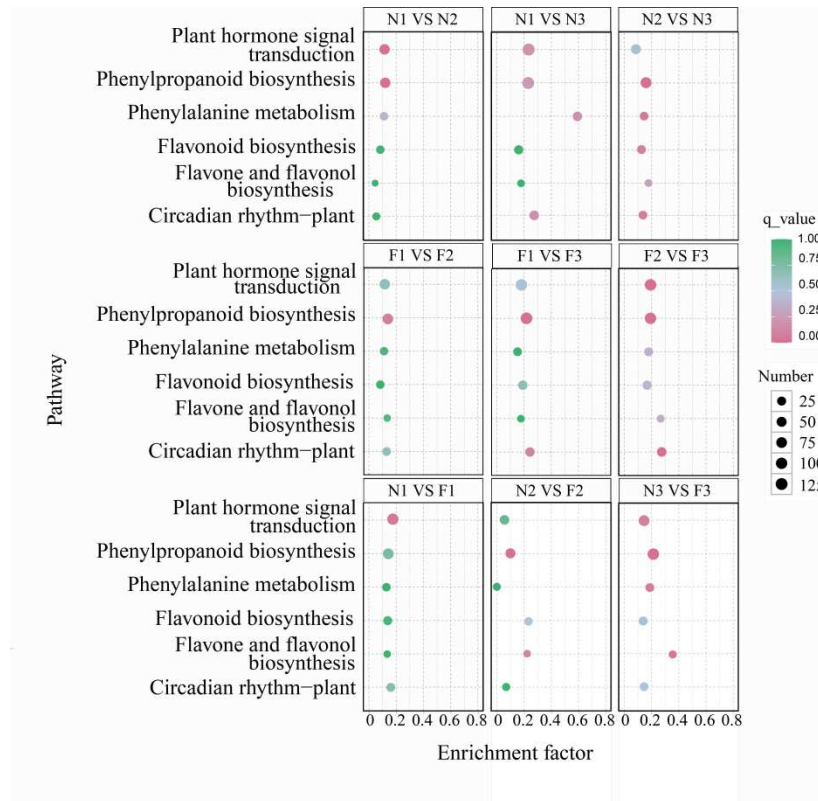


Figure 7. Bubble diagram of DEGs based on KEGG enrichment analysis. Note: The vertical axis represents the metabolic pathways obtained by KEGG enrichment analysis, and the horizontal axis represents enrichment factors. Source: www.kegg.jp/kegg/kegg1.html

The structural genes identified in the nine comparison groups included *4CL*, caffeoyl-CoA O-methyltransferase (*C-COA*), hydroxy cinnamoyl transferase (*HCT*), *CHS*, *CHI*, anthocyanidin reductase (*ANR*), Flavonoid 3' monooxygenase (*CYP75B1*), anthocyanidin 3-O-glucoside-2"-O-xylosyltransferase (*UGT79B1*), flavonol-3-O-glucoside (*FG3*), flavonol-3-O-glucoside L-rhamnosyltransferase (*FG2*), and trans-cinnamate 4-monooxygenase (*CYP73A*). Furthermore, TFs screened through KEGG enrichment included *MYC2* and *bHLH14* of the bHLH family, and *HY5* and *TGA* of the bZIP family, which were enriched in plant hormone signal transduction (ko04075), circadian rhythm-plant (ko04712), and MAPK signaling pathway-plant (ko04016).

2.5 Metabolomic analysis

Metabolomics analysis indicated that there were 6, 6, 4, 6, 6, 3, 6, 3, and 5 DAMs in N1vsN2, N1vsN3, N2vsN3, F1vsF2, F1vsF3, F2vsF3, N1vsF1, N2vsF2, and N3vsF3, respectively. Notably, cyanidin, delphinidin, pelargonidin, and their derivatives were identified in DAMs (Figure 8). KEGG enrichment analysis indicated that the anthocyanin biosynthesis pathway (ko00942) was enriched. Analysis of log₂(FC) values highlighted pelargonidin 3-O-(6-caffeoyl-beta-D-glucoside), delphinidin 3-(6-p-coumaroyl) glucoside, and cyanidin 3-rutinoside 5-glucoside as highly expressed in M146, indicating their crucial role in anthocyanin biosynthesis and the subsequent increase in total anthocyanin content. The concentration of cyanidin 3-O-(3'',6''-O-dimalonyl glucoside) exhibited a more consistent increase in M146 than in M202 during the same period. This suggested that cyanidin 3-glucoside plays a pivotal role as a key metabolite in the generation of pink leaves. Additionally, cyanidin 3-O-rutinoside was up-regulated during F1 and F3, and leucocyanidin was up-regulated during F1 and F2, implying its involvement in pink quinoa leaf formation. Naringenin displayed initial differential expression in the N3 and F3 stages, suggesting its significance in later stages of pink quinoa leaf development. The analysis of metabolite content between M146 and M202 revealed higher levels of both anthocyanin category and content in pink quinoa leaves than in emerald green quinoa leaves. This suggests that quinoa tea products derived from pink quinoa leaves in the F3 period exhibited the highest anthocyanin content with superior nutritional value.

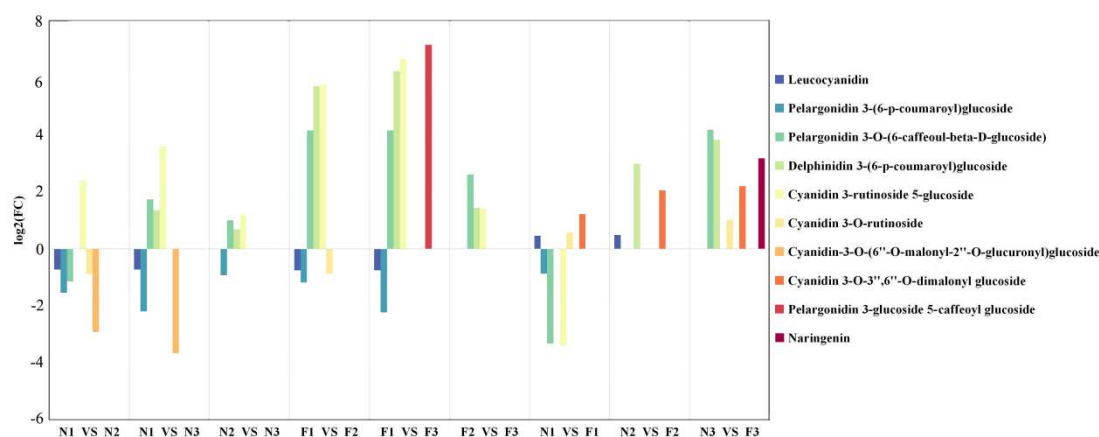


Figure 8. Expression regulation of DAMs in different comparison groups. This legend presents the number of DAMs, their difference multiples ($\log_2FC$), and the name of the key DAMs in the nine comparison groups.

2.6 Integrated transcriptomic and metabolomic analyses

Integrated transcriptomic and metabolomic analysis revealed significant correlations between DEGs and DAMs within the phenylpropanoid biosynthesis (ko00940), flavonoid biosynthesis pathway (ko00941), and anthocyanin biosynthesis pathway (ko00942) (Figure 9). The correlation network plot illustrated eight positive correlations (*4CL*, *C-COA*, *CYP73A*, and *UGT79B1* positively correlated with Leucocyanidin; *UGT79B1* positively correlated with pelargonidin 3-(6-p-coumaroyl) glucoside, pelargonidin 3-O-(6-caffeoyl-beta-D-glucoside), cyanidin-3-O-(6''-O-malonyl-2''-O-glucuronyl) glucoside, and cyanidin 3-O-rutinoside) and five negative correlations (*CHS*, *HCT*, *FLS*, and *C3'H* negatively correlated with leucocyanidin; *UGT79B1* negatively correlated with cyanidin 3-rutinoside 5-glucoside) within the N1 vs N2 comparison group. The correlation network plot illustrated fifteen positive correlations (*4CL*, *FLS*, *PAL*, *CHS*, *HCT*, *C3'H*, *C-COA*, *CHI*, *CYP73A*, *CYP75B1*, *UGT79B1* and *F3H* positively correlated with Leucocyanidin; *UGT79B1* positively correlated with Pelargonidin 3-(6-p-coumaroyl) glucoside, Pelargonidin 3-O-(6-caffeoyl-beta-D-glucoside) and Cyanidin-3-O-(6''-

354 O-malonyl-2''-O-glucuronyl) glucoside) and two negative correlations (*UGT79B1* negatively
 355 correlated with Cyanidin 3-rutinoside 5-glucoside and Delphinidin 3-(6-p-coumaroyl) glucosid)
 356 within the N1 vs N3 comparison group. The correlation network plot illustrated one positive
 357 correlations (*UGT79B1* positively correlated with Pelargonidin 3-(6-p-coumaroyl) glucoside)
 358 and three negative correlations (*UGT79B1* negatively correlated with Pelargonidin 3-O-(6-
 359 caffeoyl-beta-D-glucoside), Cyanidin 3-rutinoside 5-glucoside and Delphinidin 3-(6-p-
 360 coumaroyl) glucoside)) with in the N2 vs N3 comparison group. The correlation network plot
 361 illustrated eight positive correlations (*C-COA*, *HCT*, *C3'H*, *4CL*, and *UGT79B1* positively
 362 correlated with leucocyanidin; *UGT79B1* positively correlated with Cyanidin 3-O-rutinoside,
 363 Pelargonidin 3-(6-p-coumaroyl) glucoside and Pelargonidin 3-O-(6-caffeoyl-beta-D-glucoside))
 364 and four negative correlations (*ANR* and *CHS* negatively correlated with leucocyanidin;
 365 *UGT79B1* negatively correlated with Delphinidin 3-(6-p-coumaroyl) glucoside and Cyanidin
 366 3-rutinoside 5-glucoside) with in the F1 vs F2 comparison group. The correlation network plot
 367 illustrated twelve positive correlations (*ANR*, *FLS*, *CYP75B1*, *F3H*, *CHI*, *C3'H*, *HCT*, *CHS*,
 368 *4CL* and *UGT79B1* positively correlated with leucocyanidin; *UGT79B1* positively correlated
 369 with Pelargonidin 3-(6-p-coumaroyl) glucoside and Pelargonidin 3-O-(6-caffeoyl-beta-D-
 370 glucoside)) and four negative correlations (*C-COA* negatively correlated with leucocyanidin;
 371 *UGT79B1* negatively correlated with Delphinidin 3-(6-p-coumaroyl) glucoside, Cyanidin 3-
 372 rutinoside 5-glucoside and Pelargonidin 3-glucoside 5-caffeoyl glucoside) with in the F1 vs F3
 373 comparison group. The correlation network plot illustrated two positive correlations (*UGT79B1*
 374 positively correlated with Delphinidin 3-(6-p-coumaroyl) glucoside and Cyanidin 3-rutinoside
 375 5-glucoside) and one negative correlations (*UGT79B1* negatively correlated with Pelargonidin

3-O-(6-caffeoyl-beta-D-glucoside)) with in the F2 vs F3 comparison group. The correlation network plot illustrated eight positive correlations (*FLS*, *C-COA*, *C3'H*, *HCT*, *4CL* and *UGT79B1* positively correlated with leucocyanidin; *UGT79B1* positively correlated with Pelargonidin 3-O-(6-caffeoyl-beta-D-glucoside) and Cyanidin 3-rutinoside 5-glucoside) and two negative correlations (*F3H* and *CYP73A* negatively correlated with leucocyanidin) with in the N1 vs F1 comparison group. The correlation network plot illustrated five positive correlations (*CHS*, *C-COA*, *CHI*, *CYP75B1* and *FLS* positively correlated with leucocyanidin) and six negative correlations (*HCT*, *CYP73A*, *F3H* and *UGT79B1* negatively correlated with leucocyanidin; *UGT79B1* negatively correlated with Delphinidin 3-(6-p-coumaroyl) glucoside and Cyanidin 3-O-(3'',6''-O-dimalonyl glucoside)) with in the N2 vs F2 comparison group. The correlation network plot illustrated eleven positive correlations (*C-COA*, *C3'H*, *HCT*, *CHS*, *CYP73A*, *4CL* and *UGT79B1* positively correlated with leucocyanidin; *UGT79B1* positively correlated with Pelargonidin 3-O-(6-caffeoyl-beta-D-glucoside), Cyanidin 3-O-(3'',6''-O-dimalonyl glucoside), Cyanidin 3-O-rutinoside and Delphinidin 3-(6-p-coumaroyl) glucoside) and one negative correlations (*CHI* negatively correlated with leucocyanidin) with in the N3vsF3 comparison group.

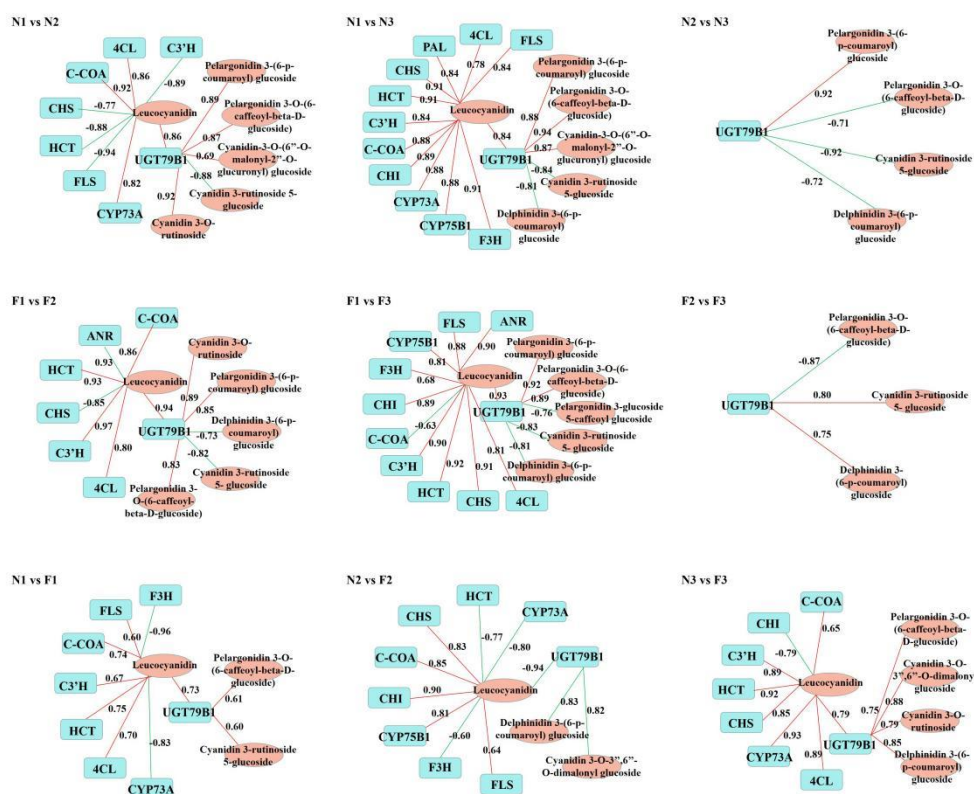


Figure 9. Correlation network diagram of differentially expressed genes and metabolites related to anthocyanin biosynthesis. The red line indicates a positive correlation between the two. The green line indicates a negative correlation between the two. The ellipses are marked as related differential metabolites, and the rectangles are marked as related differential genes. The numbers represent the coefficients of correlation between the DEGs and DAMs.

2.7 Analysis of transcription factors

In the TF analysis, 2645 DEGs spanning 55 TF families were identified. Notably, the bHLH, bZIP, and WRKY TF families, which are involved in anthocyanin regulation, were highlighted. Specifically, the bHLH and bZIP TF families exhibited significant enrichment of 201 and 91 DEGs, respectively. The WRKY TF family was enriched in 100 DEGs (Figure 10). Key TFs included *MYC2* and *bHLH14* of the bHLH family, *HY5* and *TGA* of the bZIP family, and *WRKY24* and *WRKY46* of the WRKY family. These TFs were found to be expressed to varying

degrees during quinoa leaf growth, and they were associated with four metabolic pathways:
 circadian rhythm-plant (ko04712), plant hormone signal transduction (ko04075), MAPK
 signaling pathway-plant (ko04016), and plant-pathogen interaction (ko04626) (Table 2).

Table 2. FPKM values of transcription factors in each sample

TF family	Gene name	Gene ID	N1	N2	N3	F1	F2	F3
bHLH	MYC2	LOC110730184	295.61	192.72	109.71	64.59	226.65	34.29
		LOC110732543	124.63	66.28	37.12	18.19	100.99	8.33
	bHLH14	LOC110732803	2.68	4.59	9.27	3.9	9.29	4.07
		LOC110731636	1.81	1.69	1.02	6.23	4.73	0.26
		LOC110714364	0.81	0.94	0.47	8.16	1.35	1.50
bZIP	HY5	LOC110704486	15.40	17.28	7.15	10.00	6.12	22.26
		LOC110723164	16.71	22.41	7.46	9.42	4.05	25.51
	TGA	LOC110682168	14.23	30.90	28.00	17.05	27.68	40.03
WRKY	WRKY24	LOC110684199	211.06	190.1	129.80	30.1	184.48	21.81
		LOC110684669	161.95	172.14	142.52	70.67	156.59	19.85
		LOC110698105	9.56	23.79	42.28	10.49	57.38	7.47
		LOC110730454	11.47	27.42	47.98	9.85	57.75	7.95
	WRKY46	LOC110701898	4.67	7.41	10.54	6.95	6.54	12.57

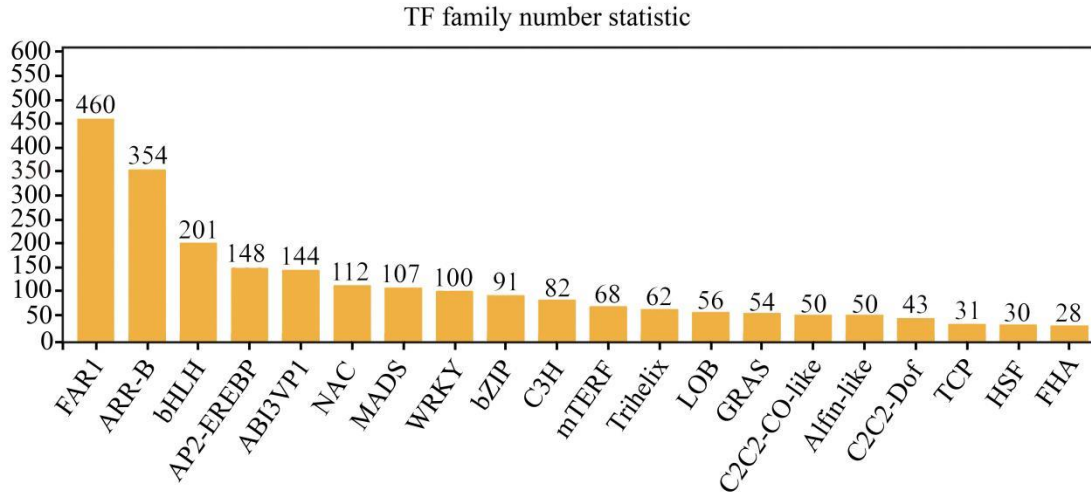


Figure 10. Number of the top 20 TF families. The top 20 TF families with the highest numbers of differential genes were enriched. The horizontal coordinates indicate the names of the TF families, and the vertical coordinates indicate the number of differential genes enriched by each TF family.

2.8 qRT-PCR verification of DEGs

Ten DEGs across the six enriched pathways were selected for validation via qRT-PCR. In the N1vsN2 comparison, five genes (*PAL*, *CHI*, *CYP75B1*, *F3H*, and *FG3*) were identified. For N1vsN3, six genes (*PAL*, *CHI*, *CYP75B1*, *F3H*, *FG3*, and *CYP73A*) were identified. In the N2vsN3 comparison, seven genes (*PAL*, *CHI*, *F3H*, *FG3*, *HCT*, *C3'H*, and *CYP73A*) are identified in N2vsN3) were validated. Focusing on the F1vsF2 comparison, four genes (*4CL*, *CHS*, *HCT*, and *CYP75B1*) were detected. In the F1vsF3 comparison, five genes (*PAL*, *CHS*, *4CL*, *FG3* and *C3'H*) were identified. In the F2vsF3 comparison, seven genes (*4CL*, *CHI*, *CHS*, *F3H*, *C3'H*, *CYP75B1*, and *CYP73A*) were verified. Turning to N1vsF1, two genes (*PAL* and *4CL*) were identified. For N2vsF2, seven genes (*PAL*, *CHI*, *F3H*, *HCT*, *C3'H*, *CYP75B1*, and *CYP73A*) were verified. Lastly, in N3vsF3, eight genes (*PAL*, *4CL*, *CHI*, *F3H*, *FG3*, *HCT*, *C3'H*, and *CYP73A*) were validated. The validation results were consistent with the trends observed in the transcriptome data (Figure 11).

3.Discussion

3.1 Phenotypic analysis of quinoa leaves

Phenotypic observations and analysis of anthocyanin content revealed higher levels in pink quinoa leaves than in emerald green leaves across all growth stages. This trend was consistent with findings in ornamental kale^[33], purple and green perilla leaves^[34], and quinoa grains^[35], indicating a close relationship between the phenotype and anthocyanin content. Color difference analysis showed that ΔE values between adjacent periods were all below 4, indicating imperceptible color changes to the naked eye. However, at 70 DAG (N1, F1) and 110 DAG (N3, F3), the ΔE values exceeded 6, indicating noticeable color changes. Previous studies have demonstrated the accumulation of anthocyanins during senescence^[36]. According to the anthocyanin content measurements in this study, as quinoa leaves matured after discoloration, there was a progressive increase in anthocyanin content, resulting in a slight deepening of color at 110 DAG. This trend in anthocyanin content variation was consistent with the observed color difference.

3.2 Analysis of key DEGs

4CL is a pivotal regulator upstream of the flavonoid metabolic pathway, which regulates anthocyanin and flavonoid synthesis. *4CL* exhibits notably varied substrate preferences and specificities^[37]. *CHI* is the second key enzyme in flavonoid synthesis, and its up-regulation can increase the synthesis of flavonoid secondary metabolites in plants and catalyze intramolecular cyclization^[38]. Zhang Kaimin et al.^[39] demonstrated a positive correlation between *4CL* expression and flavonoid content in medlar. Overexpression of *CHI* in licorice hairy roots significantly increased the flavonoid levels^[40]. Additionally, *CHI* can participate in flavonoid

anabolism in *Arabidopsis*, increasing the flavonoid content^[41]. In this study, *4CL* expression exhibited a progressive increase during each growth period, which was consistent with the change in total anthocyanin content. This is consistent with previous studies indicating a positive correlation between *4CL* and anthocyanin content. Remarkably, *4CL* expression was significantly higher at the F3 stage than at the F2 stage, implying its potential to promote pink pigment formation. Conversely, *CHI* expression displayed a consistent reduction across all periods, positively correlating with *bHLH14*. We speculated that bHLH14 regulates *CHI* during the discoloration phase, jointly contributing to anthocyanin synthesis, although the precise regulatory mechanisms require further investigation.

Fukusaki et al.^[42] employed RNAi to suppress *CHS* in *Torenia lybrida*, resulting in transgenic plants with white and grey flowers. Similar successes have been documented in the leaves of *Gerbera hybrida*^[43], *Petunia*^[44], and *Gentian corolla*^[45]. These results consistently indicated a positive correlation between *CHS* expression and anthocyanin content. In this study, *CHS* expression was consistently up-regulated across all stages, with a particularly notable increase in the N3 and F3 stages. This trend was consistent with the alterations in total anthocyanin content, suggesting the involvement of *CHS* in anthocyanin accumulation and reaffirming its positive correlation with anthocyanin levels, which is consistent with the results of previous studies. Hinderer et al.^[46] demonstrated that *CHS* activity in carrots was inhibited by naringin and chalcone, influenced by downstream flavonoids in the metabolic pathway. In this study, the N3vsF3 comparison group displayed notable differential naringin expression for the first time. Moreover, *CHS* expression in the F3 period was significantly lower than that in the N3 period, implying that the increased naringin expression in quinoa leaves also exerted inhibitory effects

on *CHS* activity.

PAL predominantly facilitates the catalysis of trans-cinnamic acid production from L-phenylalanine ammonia, serving as the pivotal entry- and rate-limiting enzyme within the phenylpropane pathway in plants^[47]. Li Yunping et al.^[48] reported a positive association between *PAL* activity and anthocyanin content in purple sweet potato. *PAL* enhances anthocyanin biosynthesis in the stems, leaves, and flowers of transgenic tobacco plants^[49]. In this study, M202 leaves exhibited negligible *PAL* expression, whereas the difference in *PAL* expression gradually decreased in M146. Significantly higher expression was observed in the F3 stage than in the N3 stage, indicating that *PAL* may contribute to the persistent pink hue in the quinoa leaves. Notably, no significant correlation was identified between *PAL* expression and anthocyanin content, which deviates from the results of previous studies. This suggests that *PAL* potentially co-regulates anthocyanin synthesis along with other genes, necessitating further exploration of the precise regulatory mechanisms involved.

F3H plays a pivotal role in catalyzing the hydroxylation of flavonoids, which is a crucial step in anthocyanin biosynthesis^[50]. Studies on alfalfa and carnations^{[51],[52]} have indicated that reduced *F3H* expression can lead to deeper coloration. In this study, both transcriptomic analysis and qRT-PCR validation consistently demonstrated a gradual decrease in *F3H* expression across distinct leaf growth periods within the same quinoa variety. This trend was consistent with the results of color differentiation and quantification of anthocyanin content in quinoa leaves. The results of this study are consistent with those of previous studies in this field.

Hydroxycinnamoyltransferase (HCT) has been identified as a key regulator of anthocyanin biosynthesis in quinoa leaves. HCT facilitates the conversion of 4-coumaroyl CoA into

caffeyl-CoA. In addition, Liu Y^[53] elucidated the branching pathway of anthocyanin biosynthesis by inducing concurrent alterations in anthocyanin, chlorophyll, and carotenoid contents, resulting in the emergence of green spots within ornamental kale exhibiting pink leaves. In this way, *HCT* transforms 4-coumaroyl CoA into caffeoyl-CoA, which subsequently undergoes synthesis through *CHS*, *F3H*, *DFR*, and *ANS* to yield anthocyanins. This established anthocyanin synthesis pathway is consistent with our current findings. Studies have indicated that silencing *HCT* not only suppresses lignin biosynthesis but also leads to activation of *CHS*, resulting in the accumulation of various flavonol glycosides and acylated anthocyanins^[54]. Li Jiawei et al.^[55] speculated that *HCT* exhibited substrate preference. Transcriptomics data in this study indicated an up-regulation of *HCT* during the N2 period, followed by a down-regulation in the F2 period, another down-regulation in the N3 period, and a significant up-regulation in the F3 period. This suggests that in M146, there was a propensity towards the formation of caffeoyl-CoA, subsequently leading to the generation of a series of anthocyanins.

3.3 Analysis of key DAMs

The flavonoid biosynthesis pathway (ko00941) and anthocyanin biosynthesis pathway (ko00942) were identified as pivotal routes in anthocyanin biosynthesis, as indicated by the analysis of anthocyanin-related metabolites mapped to the KEGG database. Metabolites within these pathways affect quinoa leaf pigmentation. Notably, naringenin, which is derived from naringenin chalcone through the action of *CHI*, is a crucial intermediary in anthocyanin synthesis. Motallebi M et al.^[56] demonstrated significance of naringin as a polyphenolic flavonoid compound with notable pharmacological effects in plants. This study revealed a significant differential expression of naringin during the N3 and F3 periods, suggesting its

pivotal role in the transition from green to pink pigmentation. Furthermore, although no substantial alterations were observed in cyanidin 3-O-(3",6"-O-dimalonyl glucoside) across different periods within the same variety, distinct variations were observed between different varieties during the same period. This indicated its potential as a key metabolite in the formation of pink pigmentation. Yifan Yan et al.^[57] demonstrated a direct correlation between changes in blueberry surface color and anthocyanin concentration. In this study, we observed significant differences in the content of pelargonidin, delphinidin, and cyanidin derivatives within quinoa leaves. This suggests a potential association between the color transformation of quinoa leaves and the levels of these derivatives.

3.4 Analysis of TFs

In this study, six pivotal TFs related to anthocyanin biosynthesis were identified: *MYC2* and *bHLH14* from the bHLH family; *HY5* and *TGA* from the bZIP family; and *WRKY24* and *WRKY46* from the WRKY family. Based on transcriptomic-metabolomic data, an anthocyanin biosynthesis pathway specific to quinoa leaf coloration was elucidated (Figure 12a). Pearson's coefficient analysis was used to determine correlations between 13 differentially expressed TFs and 18 DEGs (Figure 12b). The results highlighted a positive correlation between *bHLH14* and *CHS*, *CHI* and *FLS*. This suggests that it may bind to the promoters of these structural genes, thereby indirectly regulating anthocyanin synthesis, which plays a crucial role in anthocyanin biosynthesis regulation. Moreover, *TGA* exhibited a positive correlation with *4CL*, whereas *WRKY46* demonstrated positive correlations with *4CL*, *C-COA*, and *CHS*, indicating their potential as promoters of these structural genes. Conversely, *MYC2* was negatively correlated with *C-COA*, *HY5* was negatively correlated with *CYP75B1*, and *WRKY24* was negatively

genes, and metabolites in quinoa leaves was achieved through a combined transcriptomics-metabolomics approach. (1) Metabolomic analysis identified cyanidin 3-O-(3'',6''-O-dimalonyl glucoside) and naringenin as key metabolites contributing to the distinctive coloration of pink quinoa leaves. (2) Critical structural genes (*PAL*, *4CL*, *CHS*, *CHI*, *HCT*, *CYP75B1*, and *F3H*) within the flavonoid biosynthesis pathway (ko00941) and *UGT79B1* in the anthocyanin biosynthesis pathway (ko00942) were pivotal for anthocyanin accumulation. Among these, *4CL*, *F3H*, and *HCT* emerged as central structural genes governing pink leaf coloration. (3) The analysis of TFs enriched in the transcriptomics indicated that bHLH14 regulated *CHS*, *CHI*, and *FLS*; WRKY24 regulated *C-COA* and *FG3*; *WRKY46* regulated *4CL*, *C-COA*, and *CHS*; *MYC2* regulated *C-COA*; *TGA* regulated *4CL*; and *HY5* regulated *CYP75B1*.

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List of abbreviations

English name	abbreviation
differentially expressed genes	DEGs
differentially accumulated metabolites	DAMs
transcription factors	TFs
v-myb avian myeloblastosis viral oncogene homolog	MYB
basic helix-loop-helix	bHLH
70 days after germination	70 DAG
90 days after germination	90 DAG
110 days after germination	110 DAG
Kyoto encyclopedia of genes and genomes	KEGG
Gene ontology	GO
Fragments Per Kilobase per Million	FPKM
Fold change	FC
Polymerase chain reaction	PCR
Real-time quantitative PCR	qRT-PCR
Code sequence	CDS
False discovery rate	FDR

Declarations

Ethics approval and consent to participate

Not applicable. All methods were carried out in accordance with relevant guidelines and regulations.

Consent to publish

Not applicable.

Availability of data and materials

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below:
<https://www.ncbi.nlm.nih.gov/>, PRJNA986269.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author's contributions

HW and WL conceived and designed the research. JW and MW performed the measurement of anthocyanin. JW, YL and CL planted the materials and conducted field management. XS and JL does the transcriptomics data analysis. XL and XY does the metabolomics data analysis. MZ wrote the manuscript. GM revised the manuscript. All authors contributed to the article and approved the submitted version.

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