scientific reports

Check for updates

OPEN

Analysis of rabbiteye blueberry metabolomes and transcriptomes reveals mechanisms underlying potassium-induced anthocyanin production

Ting Yan, Zejun Song, Boping Yu, Qian Li & Delu Wang✉

Environmental factors play an important role in anthocyanin biosynthesis, and potassium, an essential nutrient for blueberry growth, can act as an enzyme activator. However, few reports exist on the transcriptional and anthocyanin metabolic changes in blueberries regulated by potassium. The results indicated that potassium treatment significantly increased the contents of malvidin, petunidin, and delphinidin in blueberry fruits and accelerated early color development, particularly favoring the accumulation of darker pigments such as malvidin, petunidin, and delphinidin when applied at the young fruit stage. Transcriptome analysis identified 102 glucose metabolism-related genes and 12 differential potassium transport genes potentially involved in potassium-mediated anthocyanin synthesis and accumulation, with *AKT1* and *KUP* potassium transporters being upregulated under potassium fertilization. In the anthocyanin biosynthesis pathway, 13 genes, including *UFGT*, *F3H*, *CHI*, *HCT*, *C12RT1*, *DFR*, *and F3′5′H*, were closely linked to flavonoid and anthocyanin metabolite synthesis regulated by potassium. Furthermore, potassium treatment markedly enhanced the activities of key enzymes, F3H, F3′5′H, and UFGT, in the anthocyanin synthesis pathway of blueberry fruits. Overall, these findings elucidate the influence of potassium application timing on anthocyanin synthesis and provide valuable insights into the molecular mechanisms governing anthocyanin biosynthesis in blueberries.

Keywords Blueberry, Potassium, Anthocyanin biosynthesis, Transcriptome, Metabolite

Blueberries (genus *Vaccinium*, family Ericaceae) are deciduous shrubs widely cultivated worldwide for their unique flavor and rich nutritional content[1,2]. Blueberries are one of the most nutrient-rich berries, containing fiber, vitamins, and antioxidants, primarily due to flavonoids such as flavonols, anthocyanins, and proanthocyanidins[3,4]. The anthocyanins in nature are edible, water-soluble pigments that also benefit human health[5]. Some of these benefits are lowering the sudden metabolic load of high-energy foods[6], protecting retinal pigment epithelial cells from damage caused by light[7], delaying brain cell aging and preventing neurodegenerative diseases[8,9], controlling inflammatory response, and improving glycolipid metabolism. It is known that there are more than 550 anthocyanins[10], but blueberries mostly contain six of them: Cyanidin, Pelargonidin, Peonidin, Delphinidin, Petunidin, and Malvidin[11]. Anthocyanins are anthocyanidins bound to one or more sugar molecules. This glycosylation gives it greater stability in plants and enables it to perform its color rendering function[12]. Anthocyanins are synthesized through the phenylpropane metabolic pathway in flavonoid biosynthesis, regulated by genes encoding several key enzymes[13], cinnamate 4-hydroxylase (*C4H)*, 4-coumarate-CoA ligase (*4CL*), chalcone isomerase (*CHI*), chalcone synthase (*CHS*), flavanone 3-hydroxylase (*F3H*), flavonoid 3'- monooxygenase (*F3'H*), flavonoid 3', 5'hydroxylase (*F3'5'H*), dihydroflavonol 4-reductase (*DFR*), phenylalanine ammonialyase (*PAL*), anthocyanidin synthase (*ANS*), and UDP-glucose flavonoid glucosyltransferase (*UFGT*)[14–16].

Anthocyanin biosynthesis is regulated by various environmental and growth variables, including temperature, light, nutrients, and plant hormones[17–20]. Environmental changes result in fluctuations in anthocyanin levels. After being exposed to light and treated with potassium (K^+), the pseudobulbs of *Dendrobium officinale* showed obvious purple color, and the content of anthocyanins increased significantly[21]. The anthocyanin content in

College of Forestry, Guizhou University, Guiyang 550025, China. ✉email: deluwang23@aliyun.com

grapes sprayed with potassium sulfate or potassium phosphate fertilizer was significantly higher than in the control group[22]. As an enzyme activator in plants, K^+ is essential for sustaining proper physiological metabolism[23]. However, potassium is easily lost in the soil. Due to the rapid growth of plants and heavy fruit load, the demand for potassium is high, making it crucial to supplement potassium fertilizer during fruit development[24]. Previous studies have shown that potassium can significantly increase fruit anthocyanin content[25,26], but how potassium affects the synthesis of blueberry anthocyanin has not been reported.

In recent years, metabolomics has been increasingly combined with transcriptomics. Based on multifunctional "omics" data, combined analyses have proven to be more effective for elucidating plant developmental biology and studying the signaling pathways and mechanisms of pigment accumulation in many plants[27]. To study blueberry fruit anthocyanin accumulation under potassium modulation and elucidate the regulatory mechanism of biosynthesis, transcriptome and metabolomic analysis were performed. A theoretical reference for the involvement of nutritional components in blueberry fruit anthocyanin production and accumulation was found by identifying potassium-regulated genes and metabolites.

Materials and methods

Plant materials

Seven-year-old rabbiteye blueberry 'Powderblue' (*Menditoo* × *Tifblue*) were cultivated in the nursery of Forestry College of Guizhou University. The cultivation region is located at 106°27′-106°52′ E and 26°11′-26°34′ N. The nursery and blueberries are owned by the Forestry College of Guizhou University. One seedling was planted per pot, with analytically pure K_2SO_4 used at a dose of 10 g per plant. Each treatment was replicated three times, with six plants per replicate. Potassium fertilizer was dissolved in 400 ml of water and applied during the flowering stage (KH) and young fruit stage (YG), while the control (CK) received an equal volume of water. A randomized block design was used, and other management practices were consistent across treatments. Ninety days after flowering, the upper layer fruits were selected, and 12 to 18 fruits were sampled for each replicate (Fig. 1). Following sampling, the fruits were frozen in liquid nitrogen and kept at -80 °C for subsequent experiments.

Determination of enzyme activity in blueberry

Enzyme-linked immunosorbent assay (ELISA) and the double-antibody one-step sandwich method were used to measure the activities of enzymes F3H and F3'5'H involved in anthocyanin glycoside synthesis[28]. The tissue was washed with pre-chilled PBS (0.01 M, pH 7.4), weighed 0.1 g, and thereafter underwent cutting. The cut tissue was pulverized with an equivalent amount of PBS (using a weight-to-volume ratio of 1:9), subjected to centrifugation at 5000×g for 10 min, and the resulting liquid portion was collected for analysis. The enzyme activity of UFGT was determined by spectrophotometry, and the flavonosyltransferase (UFGT) kit produced by Jiangsu Grace Biotechnology Limited Liability Company was used for detection.

Fig. 1. (**A**) CK, no fertilizer treatment; (**B**) KH-10, 10 g/ plant of K_2SO_4 applied at flowering stage; (**C**) YG-10, 10 g/plant of K_2SO_4 applied at young fruit stage.

Metabolomic analysis

After vacuum freeze-drying, the sample was ground for 1.5 min using a grinder at 30 Hz. Fifty milligrams of powder were weighed, and 1.2 mL of 70% methanol was used for overnight extraction at 4 °C, followed by centrifugation at 12,000 g for 3 min. The supernatant was filtered through a microporous filter membrane (0.22 μm pore size) and stored in a sample vial. The sample was then used for UPLC-MS/MS (Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry) analysis.

The system for data acquisition mainly includes ultra-high-performance liquid chromatography (UPLC) (ExionLC™ AD) and tandem mass spectrometry (MS/MS) (Applied Biosystems 4500 QTRAP)[29]. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were employed to examine the data collected from nine samples, which comprised three samples with three replicates each. Fold change and VIP values from the OPLS-DA model were combined to screen for differential metabolites. The screening criteria were: metabolites with a fold change ≥ 1 or ≤ 0.5 and VIP ≥ 1 were considered significantly different. The metabolites that differ were subsequently linked to the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp) database for a thorough analysis of significant enrichment and to identify the primary pathways that are enriched[30].

Construction of a complementary DNA (cDNA) library and RNA sequencing

Total RNA was extracted from blueberry by Trizol method[31]. RNA libraries for RNA-seq were prepared using NEBNext®UltraTM RNA Library Prep Kit following the manufacturer's protocols. RNA-Seq data were assembled and analyzed by Metware Biotechnology Co., Ltd. (Wuhan, China). The Illumina sequencing platform was employed for the construction and sequencing of the cDNA library. Clean data were acquired by filtering the raw data, and sequence alignment was conducted with a designated reference genome (https://phytozome-next.Jgi.doe.gov/info/Vdarrowii_v1_2)[32]. Quality control of raw data was performed using Fastp[33]. The reference genome was utilized for comparison against clean reads with HISAT2 software, and transcripts were constructed from the reads through the use of StringTie[34]. FPKM was used as the index of transcription or gene expression level[35], and DESeq2 was used to analyze differential expression among the sample groups[36,37]. The screening condition of differential genes was |log2Fold Change| >=1, and FDR < 0.05. GO and KEGG analyses of DEG were performed using the Clusterprofiler R software package, with $P < 0.05$ as the significance threshold for GO and KEGG enrichment[38].

Quantitative real-time PCR analysis

To validate the transcriptome data more thoroughly, qRT-PCR analysis was conducted on the selected differential genes, and the sequences of the primers can be found in Supplementary Table 1. The total RNA extracted was utilized as a template for the reverse transcription of cDNA, following the guidelines provided with the PrimeScript™ RT reagent Kit. The main instrument used was the Applied Biosystems fluorescence quantitative PCR detection system. The internal reference gene was Bilberry GAPDH (AY123769), and the amplification system consisted of 20μL containing 1μL cDNA, 2 μL forward and reverse primers, 10μL reaction MIX, and 7μL ddH2O. The reaction procedure was as follows: Predenaturation at 95 °C for 30 s, denaturation at 95 °C for 10 s, annealing at 55 °C for 20 s, extension at 72 °C for 20 s, cycling 40 times, followed by 75 °C for 5 s and 95 °C for 5 s. After the completion of the reaction, the analysis of the fluorescence change curve and the melting curve was carried out. The relative gene expression was determined utilizing the $2^{-\Delta\Delta CT}$ method[39].

Statistical analysis

In this study, all experiments were repeated three times, and data were expressed as mean ± standard deviation (SD). SPSS 26.0 software was used to estimate significant difference between treatment by ANOVA ($P < 0.05$). Origin 9.0 was used to create bar charts, and TBtools software was used to create and analyze heat maps.

Results

Effect of potassium on anthocyanin metabolism in blueberry fruit

The results of PCA and OPLS-DA demonstrated significant differences in metabolites between the sample groups, confirming the reliability and accuracy of subsequent metabolomic analyses (Supplementary Fig. 1). Metabolites exhibiting differences between the three treatments were analyzed by mapping the identified differential metabolites to the KEGG database, which provided comprehensive pathway information (Fig. 2A, B, and C). Notably, the metabolites that differed between KH-10 and CK were primarily associated with the biosynthetic processes of plant secondary metabolites, such as flavonoids, flavonols, and isoflavonoids. Additionally, the metabolites that varied among KH-10, YG-10, and CK showed significant enrichment in the biosynthesis pathways of anthocyanins.

In comparison to CK, a total of 163 distinct metabolites were identified in the anthocyanin synthesis pathway of blueberries under potassium regulation across various fertilization treatments (Fig. 3A). For subsequent cluster analysis among the three treatments, 25 anthocyanins were identified (Fig. 3B). Notably, the control group exhibited the fewest types of anthocyanins, with only four identified. These are pelargonidin-3-O-rutinoside, delphinidin-3-O-(6"-O-caffeoyl) glucoside, luteolinidin, cyanidin-3-O-sambubioside-[Cyanidin – 3-O-(2"-O-xylosyl) glucoside], and their expression level was low in fertilization treatments. The content of five anthocyanins, malvidin-3,5-di-O-glucoside (Malvin), malvidin-3-O-glucoside, delphinidin-3-0-(6"-O-feruloyl) glucoside, peonidin-3-O-glucoside, and peonidin-3-O-arabinoside, was higher in KH-10, being 13.48-23253.67 times higher than in CK and 1.23–2.26 times higher than in YG-10. There were nine types of anthocyanins in YG-10, which were 5.25-214.54 times higher than in CK and 1.47–2.91 times higher than in KH-10. Delphinidin-3-O-(6"-O-p-coumaroyl)glucoside, peonidin-3-O-(6"-O-p-coumaroyl)glucoside, petunidin-3-O-(6"-O-p-coumaroyl)glucoside, petunidin-3-O-(6"-O-acetyl)glucoside, malvidin-3-O-(6"-O-p-

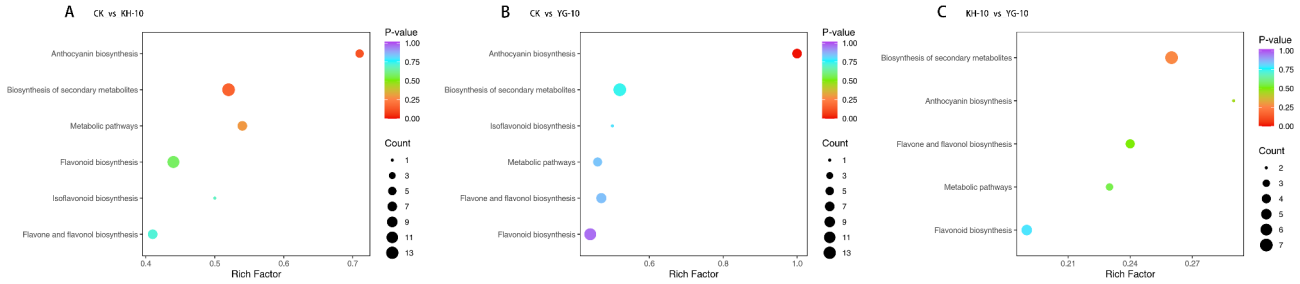

Fig. 2. KEGG enrichment maps of different metabolites in different groups. (**A**) (**B**) (**C**) represents the comparison of CK_vs_KH-10, CK_vs_YG-10, and KH-10_vs_YG-10 corresponding to different treatments, respectively. The dots in the KEGG enrichment maps indicate the number of different metabolites, with the color scale from red to purple indicating *P*-value levels.

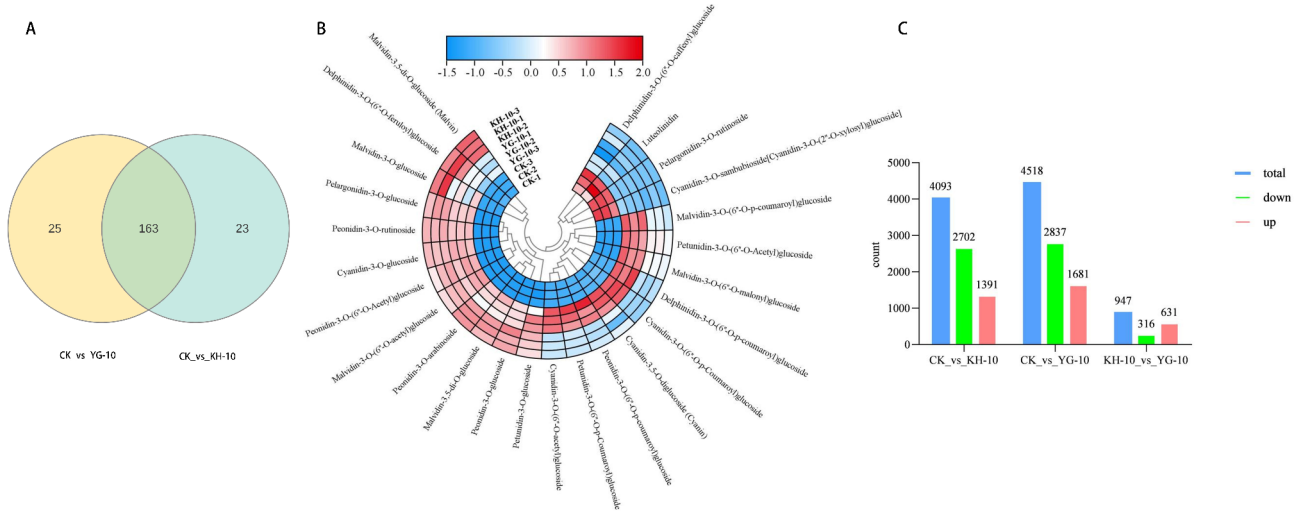

Fig. 3. Venn diagram of metabolite differences between samples (**A**) and a cluster heat map of relative contents of anthocyanin metabolites (**B**) and statistical histogram of differentially expressed genes in fruits under different potassium levels (**C**). In the cluster heat map (**B**), red indicates high relative metabolite content, while blue indicates low relative metabolite content.

coumaroyl)glucoside, malvidin-3-O-(6"-O-malonyl)glucoside, cyanidin-3,5-O-diglucoside (Cyanin), cyanidin-3-O-(6"-O-p-coumaroyl)glucoside, and cyanidin-3-O-(6"-O-acetyl)glucoside.

The application of potassium fertilizer during the flowering and young fruit stages significantly enhanced the levels of peonidin-3-O-rutinoside, peonidin-3-O-(6"-O-acetyl) glucose, pelargonidin-3-O-glucose, cyanidin-3-O-glucose, malvidin-3-O-(6"-O-acetyl) glucose, and petunidin-3-O-glucose. These findings suggest that these six anthocyanins may be involved in the coloration process of blueberry fruits under potassium regulation, with their increased accumulation potentially contributing to the observed color development.

Sequencing and gene functional annotation

To ensure data quality, the raw data were filtered before conducting bioinformatics analysis. Fastp was used for quality control of raw reads, filtering low-quality data, resulting in a total of 397,664,760 clean reads (Supplementary Table 2). Following filtration, the guanine-cytosine (GC) content in every sample exceeded 46%, with average Q20 and Q30 values of 97.79% and 93.67%, respectively. The results showed that 79.51-81.03% of clean reads were successfully mapped to the reference genome. The unique match rate was above 77.18%, indicating that the sequencing results were of high quality and suitable for further analysis. A new gene's sequence was obtained from the genome, and diamond software was employed to compare it with sequences from the KEGG, GO, NR, Swiss Prot, TrEMBL, KOG, and Pfam databases to achieve annotated results, utilizing alignment criteria with an E-value of 1e-5. (Supplementary Table 3). The findings indicated that the majority of novel genes were recorded in the TrEMBL database, totaling 4,219 sequences. In comparison, the NR and GO databases had 3,855 and 3,004 unique genes annotated, respectively.

GO enrichment and KEGG pathway analysis of DEGs

A differential expression analysis was performed between the sample groups utilizing DESeq2, whereby differentially expressed genes were identified based on the criteria of |log2Fold Change| >= 1 and FDR < 0.05 (Supplementary Fig. 2). The findings revealed that there were 3010 DEGs common to the CK_vs_KH-10 and CK_vs_YG-10 subgroups, with a cumulative total of 4093 genes identified in the CK_vs_KH-10 comparison, showcasing significant expression variations between these two sample sets. Among these, 2702 genes exhibited higher expression levels in CK compared to lower levels in KH-10. In the CK_vs_YG-10 analysis, the total count of differential genes reached 4518, which was the highest among all comparative analyses, including 1681 genes that were up-regulated and 2837 genes that were down-regulated. The KH-10_vs_YG-10 comparison revealed the fewest DEGs, totaling only 946, indicating a difference in potassium application between the flowering stage and the young fruit stage (Fig. 3C).

A total of 8,813 differentially expressed genes were annotated with GO terms across various sample combinations. Among these, 5,638 genes showed enrichment in the biological process category, making it the most represented category. Furthermore, 175 GO terms that were significantly enriched ($p < 0.05$) were linked to the DEGs, which included 115 secondary terms in the biological process category, 38 in the molecular function category, and 22 in the cellular component category (Supplementary Table 4). The enriched GO terms for DEGs within the biological process, cellular component, and molecular function categories cover essential genes associated with anthocyanoside and sugar synthesis. These include terms like "flavonoid biosynthetic process, GO:0046148," "carbohydrate catabolic process, GO:0016052," "pigment metabolic process, GO:0042440," "cellular glucan metabolic process, GO:0006073," "glucan metabolic process, GO:0044042," and "glucosyltransferase activity, GO:0046527" (Fig. 4A, B and C). The differential genes displayed significant enrichment in the flavonoid anabolic pathway and glucan metabolic process, indicating a possible connection between glucan metabolism and flavonoid synthesis.

KEGG enrichment (Fig. 4D, E, and F) revealed 51 DEGs linked to the phenylpropane biosynthesis pathway, 26 with the flavonoid biosynthesis pathway, and 72 with the sugar metabolism pathway in CK_vs_KH-10. In CK_vs_YG-10, 47 DEGs were linked to the phenylpropane biosynthesis pathway, 27 to the flavonoid biosynthesis pathway, and 83 to the sugar metabolism pathway. Comparison of fertilization periods (KH-10_vs_YG-10) showed 15 DEGs in the phenylpropane biosynthesis pathway, 4 in the flavonoid biosynthesis pathway, and 6 in the sugar metabolism pathway. Anthocyanidins, being a type of flavonoid, are closely related to the flavonoid synthesis pathway. Supplementary Table 5 shows that 34 DEGs are associated with the flavonoid synthesis pathway. Notably, 14 of these genes were up-regulated in fruits treated with potassium during the flowering stage, while another set of 14 genes exhibited up-regulation during the young fruiting stage in comparison to CK. Ten genes were found to be involved with enzymes in the anthocyanin synthesis pathway, including F3H, CHS, CHI, ANR (anthocyanidin reductase), DFR, F3'5'H, and LAR (leucoanthocyanidin reductase). This suggests that these enzyme activities are closely related to anthocyanin synthesis under potassium influence.

In the phenylpropane synthesis pathway (Supplementary Table 6), 67 genes were associated with this pathway, with 16 genes up-regulated in fruits across all fertilization periods under potassium regulation. These genes were linked to Prx (peroxidase), CCoAOMT (caffeoyl-CoA O-methyltransferase), PAL, REF1 (coniferyl-aldehyde dehydrogenase), HCT (shikimate O-hydroxycinnamoyltransferase), COMT (caffeic acid 3-O-methyltransferase/acetylserotonin O-methyltransferase), 4CL, and UGT (coniferyl-alcohol glucosyltransferase). Four genes were up-regulated only at the flowering stage when potassium was applied, and two genes were significantly up-regulated at the YG stage under potassium application compared to CK and the flowering stage. PAL and 4CL are initiating enzymes of flavonoid biosynthesis, catalyzing the synthesis of phenylalanine and providing precursors for the flavonoid biosynthesis pathway. CoAOMT, HCT, and CHS generate dihydroflavonoids in different ways, which are precursors for the production of anthocyanins and other related compounds.

In the comprehensive analysis, 102 genes in the carbohydrate metabolism pathway (Supplementary Table 7) were found to be related to sugar metabolism. Among these, 24 genes were up-regulated in fruit across all fertilization periods under potassium regulation. These genes were associated with SUS (sucrose synthase), malQ (4-alpha-glucanotransferase), HK (hexokinase), UGP2 (UTP-glucose-1-phosphate uridylyltransferase), glgA (starch synthase), glgC (glucose-1-phosphate adenylyltransferase), otsB (trehalose 6-phosphate phosphatase), AMY (alpha-amylase), EGL (endoglucanase), BG (beta-glucosidase), BFR (beta-fructofuranosidase), PGM (phosphoglucomutase), TPS (trehalose 6-phosphate synthase/phosphatase), and GN (glucan endo-1,3-beta-glucosidase) synthesis. Therefore, changes in enzyme expression in these glycolytic pathways are likely related to the potassium-influenced synthesis and accumulation of anthocyanins.

As shown in Supplementary Table 8, the KEGG pathway enrichment identified genes related to potassium transport. These genes were mainly divided into two categories: Potassium channel proteins (AKT, KAT, GORK, SKOR, KHA) and potassium transporter proteins (KUP). A total of 12 DEGs were detected, with only 2 up-regulated and 10 down-regulated in the potassium application treatment. This indicates that the expression of AKT and KUP genes in fruit is down-regulated by potassium.

Analysis of related genes in the biosynthesis pathway of blueberry anthocyanin

Further analysis of the DEGs identified through GO and KEGG functional classification and enrichment analysis revealed key enzyme genes encoding anthocyanin synthesis. The findings indicated that under potassium regulation, a total of 13 gene families were detected, all exhibiting differential expression (Supplementary Table 9). *PAL*, *CHS*, *CHI*, *F3'5'H*, *F3H*, *DFR*, *GST*, and *HCT* were primarily upregulated under potassium treatment, with relatively low expression levels in CK treatment. These genes are essential for the potassium-induced synthesis and accumulation of blueberry anthocyanins. Under KH-10 treatment alone, the highly expressed genes include *PAL*, *F3'5'H*, *DFR*, and *GST*, whereas *CHS*, *CHI*, *LAR*, *ANR*, *F3H*, and *HCT* are only highly expressed in YG-10.

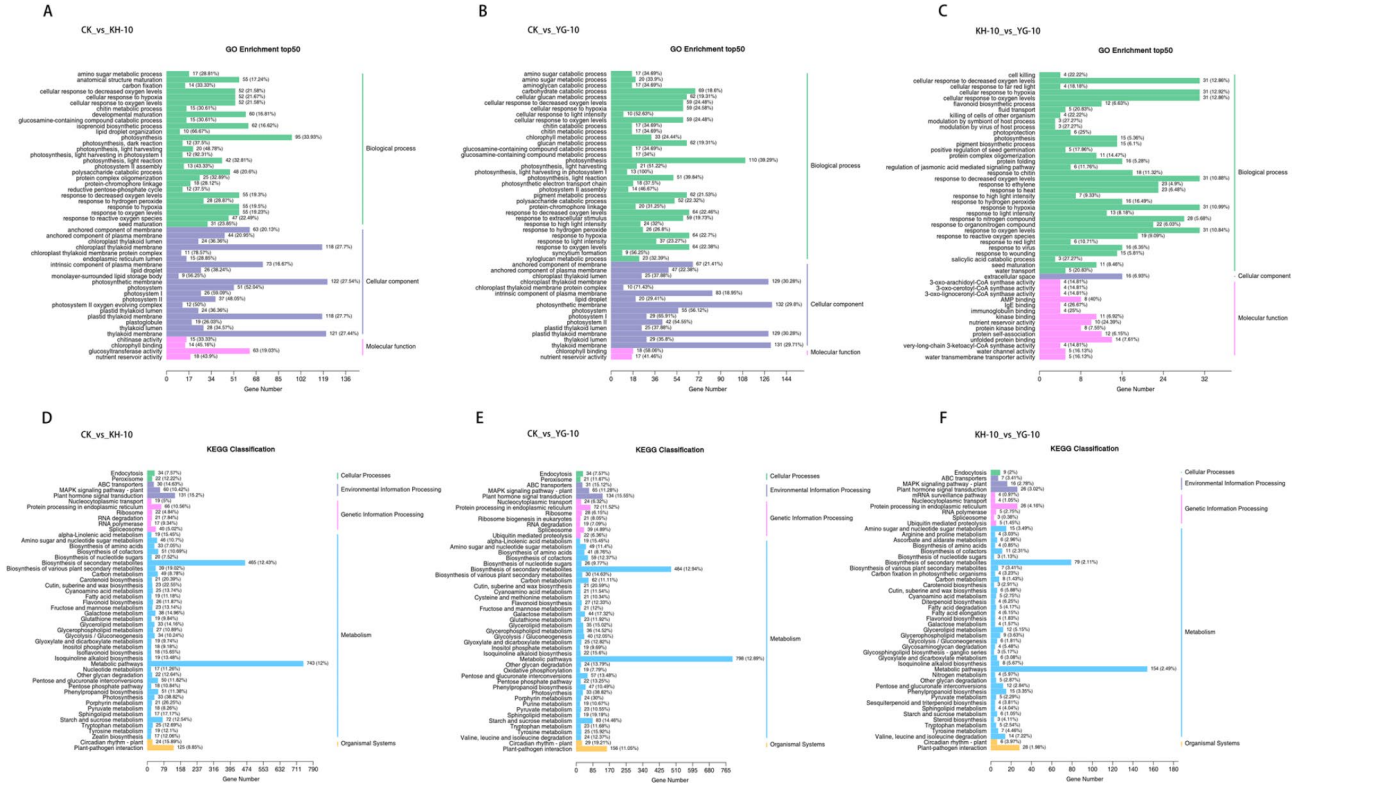

Fig. 4. GO and KEGG enrichment classification histograms of DEGs in fruits treated with potassium at different stages. The ordinate is the GO term category and KEGG pathway category of differential genes and is represented by different colors, and the abscissa is the number and percentage of enriched genes.

The expression heat map was created to analyse the key metabolite concentrations of structural genes in the anthocyanin biosynthesis pathway of blueberry fruit, based on the reported route for anthocyanin biosynthesis (Fig. 5). P-Coumaroyl-CoA produces Caffeoyl-CoA and Naringenin chalcone, respectively, through the actions of HCT and CHS, with CHS being the first rate-limiting enzyme in the pathway. Metabolomic and transcriptomic correlation analyses revealed that *CHS* regulation significantly increased the content of the downstream product Naringenin chalcone. This increase, in turn, promoted the synthesis of Naringenin via the action of *CHI*. Additionally, the up-regulation of *DFR* expression favored the synthesis of Pelargonidin. Eriodictyol generated various types of anthocyanins through the actions of *F3H*, *DFR*, and *F3'5'H*. Increased F3H activity promoted the accumulation of downstream Cyanidin. F3'5'H increases the content of dihydromyricetin, the latter under the action of DRF, and then produce Delphinidin, Peonidin, Cyanidin and Malvidin. By combining the metabolites of these pathways with gene expression, we can infer that potassium regulation promotes the synthesis of blueberry Cyanidin, Delphinidin, Malvidin, and Peonidin. The *F3'5'H*, *F3H*, and *DFR* genes catalyze the synthesis of flavonoids and anthocyanidins in the phenylpropanoid synthesis pathway. The differential expression of these genes is the primary reason for the differences in flavonoid content between fruits grown under different conditions: Potassium application and CK.

Integrated analysis of the transcriptome and metabolome

The substances enriched in the flavonoid and anthocyanin pathways, as identified by transcriptome and metabolome analyses, were examined. Analysis of the common differentially expressed genes and metabolites between fertilization treatment and CK revealed that *UFGT*, *F3H*, *CHI*, *HCT*, *C12RT1*, *DFR*, and *F3'5'H* were associated with metabolite production and catalyzed the synthesis of flavonoids and anthocyanins. Among the KH-10 and YG-10 treatments, *BZ1*, *ANR*, and shikimate O-hydroxycinnamoyltransferase were linked to the formation of metabolites and facilitated the synthesis of flavonoids and anthocyanins (Supplementary Table 10).

Analysis of gene expression through qRT-PCR

Based on the transcriptome and metabolome results, six structural genes involved in anthocyanin synthesis (*F3H*, *DFR*, *F3'5'H*, *C12RT1*, *HCT*, *UFGT*) were selected for qRT-PCR analysis, with blueberry GAPDH serving as the internal reference (Fig. 6A). The qRT-PCR results indicated that the expressions of the *F3H* and *F3'5'H* genes were significantly higher under potassium treatment compared to CK treatment, whereas the expressions of *UFGT* and *C12RT1* were inhibited. Specifically, the expressions of *F3'5'H* and *HCT* genes increased significantly at the flowering stage by 266.97% and 106.64%, and by 77.41% and 39.44% compared to CK and YG-10, respectively. Additionally, the expression of the *F3H* gene in young fruit increased significantly

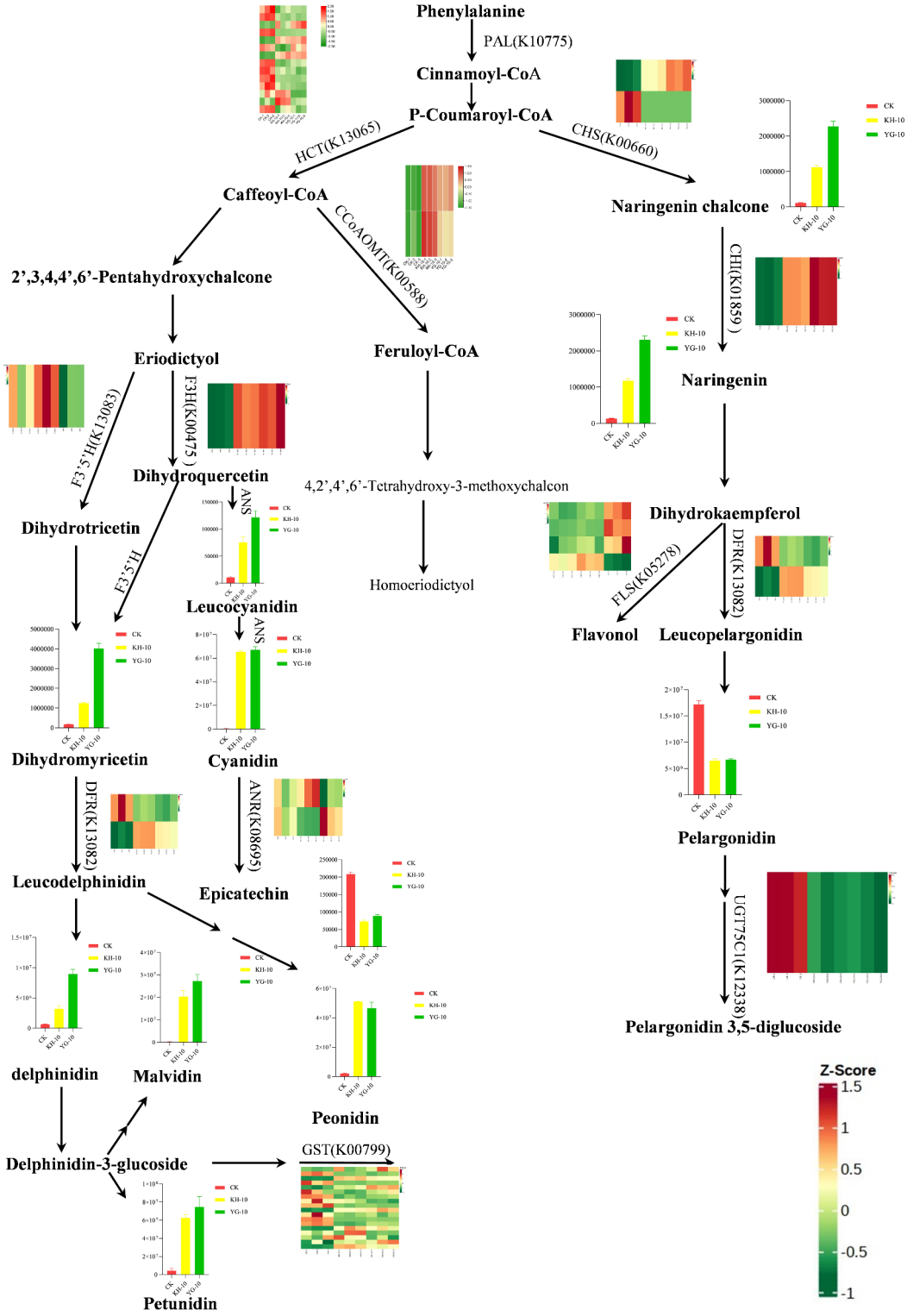

Fig. 5. Changes in the content of major metabolites and gene expression during anthocyanin synthesis in blueberry. (The bar chart represents the change in metabolite abundance, and the heat map represents the change in gene expression)

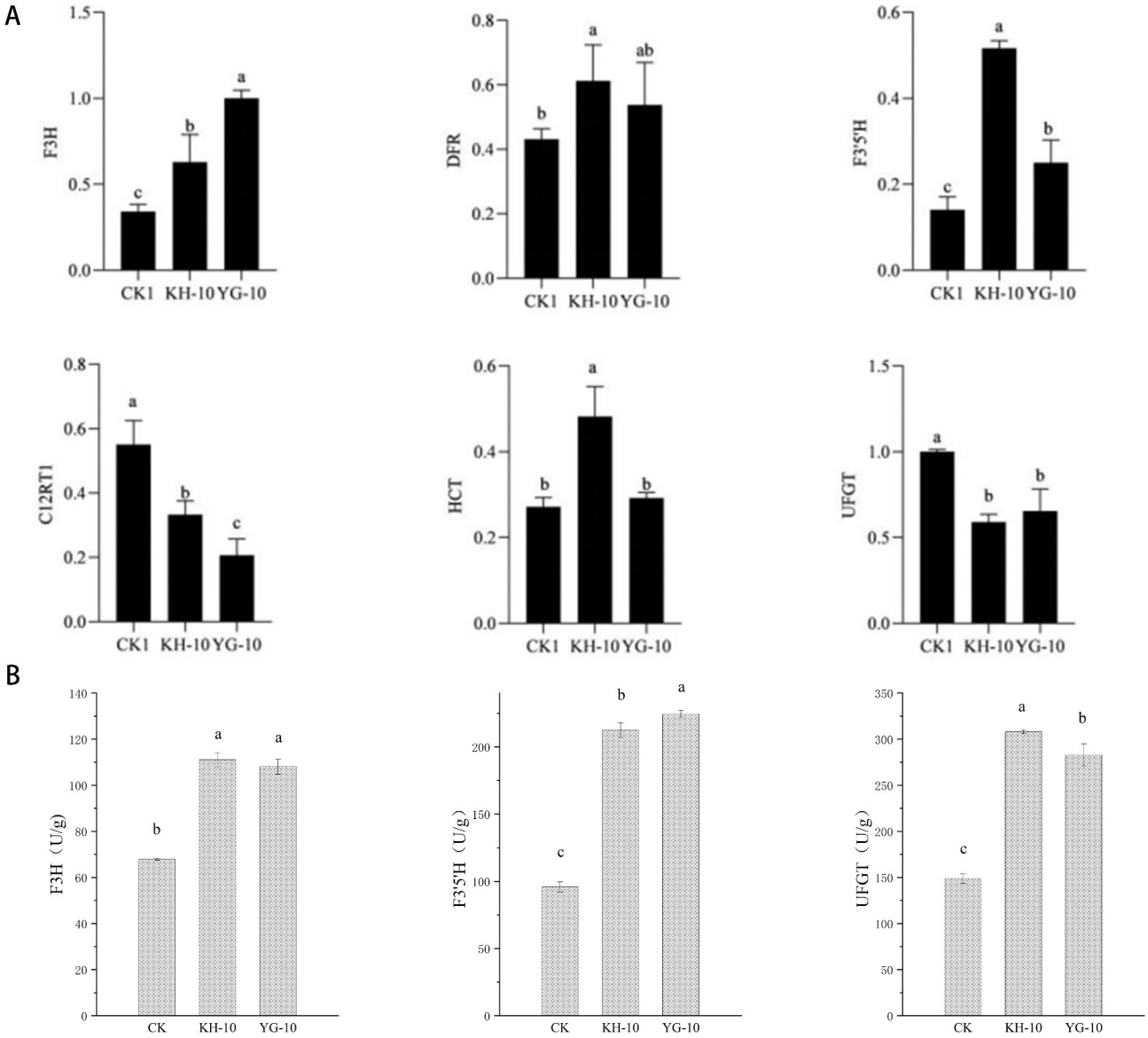

Fig. 6. Expression levels (A) of genes related to the flavonoid metabolism pathway and histograms (B) of related enzymes during anthocyanin synthesis in blueberry. Different lowercase letters indicate significant differences between treatments ($p < 0.05$).

by 192.28% and 59.03% compared to CK and KH-10, respectively. The expression patterns of genes differentially related to flavonoid biosynthesis in the transcriptome were in agreement with the qRT-PCR findings.

The activity of F3H, F3′5′H, and UFGT enzymes in the anthocyanin biosynthesis pathway

Anthocyanin biosynthesis depends on a series of enzymes. Based on the qRT-PCR results, the activities of F3H, F3'5'H, and UFGT enzymes were determined at different periods under potassium fertilizer treatment (Fig. 6B). All potassium-treated enzymes had significantly greater activity than CK. Specifically, F3H, F3'5'H, and UFGT in KH-10 were 63.65%, 121.99%, and 107.13% higher than in CK, respectively, while in YG-10, they were 59.14%, 134.49%, and 90.36% higher than in CK. Except for F3H, there were significant differences in F3'5'H and UFGT enzyme activities between KH-10 and YG-10. The results indicated that potassium significantly promotes the activity of key enzymes in the anthocyanin synthesis pathway, and its effect varies across different growth stages of blueberry.

Discussion

Flavonoids, a key group of plant-derived compounds, are present in blueberries in both aglycone and glycoside forms[40]. In this study, 338 flavonoids were identified, with potassium-treated samples showing the highest flavonoid content. Anthocyanin derivatives such as malvidin, petuniadin, and delphinidin are the primary pigments responsible for purple and black colors, while centaurin and pelargonidin contribute to bright red

fruits[41]. Pelargonidin anthocyanin is primarily found in red radish[42], and among all treated blueberry fruits, those treated with CK exhibited higher pelargonidin content, resulting in a slightly pink hue. This is consistent with the above conclusion. Malvidin, petunidin, and delphinidin are abundant in KH-10 and YG-10, which may explain why CK exhibits a different color compared to these treatments. In this research, anthocyanin derivatives were present in all samples, but significant differences were observed among the groups. Potassium treatment particularly enhanced the amounts of delphinidin, petunidin, cyanidin, and peonidin. This finding aligns with the outcomes of Ranganath K G's research[43]. Notably, the concentration of malvidin-3,5-di-O-glucoside (Malvin) was significantly higher in KH-10 compared to CK, YG-10 treatment also revealed higher levels of dark pigments, such as delphinidin-3-O-(6"-O-p-coumaroyl) glucoside. This indicates that potassium can regulate specific anthocyanin derivatives and affect the color of fruits[44,45].

Anthocyanins, which are polyphenolic compounds found abundantly in various plants, play a key role in providing color to fruits, vegetables, and flowers[46]. Besides anthocyanins, the types and levels of flavonoid metabolites upstream in the pathway are also associated with the coloration of blueberries. For instance, alfalfa, myricetin, and limonin were more abundant in potassium-treated fruits, being 107.35 to 204.25 times, 65.69 to 75.80 times, and 149.62 to 482.18 times higher than in CK, respectively. These changes in flavonoid composition are likely linked to the regulation of the phenylpropanoid pathway, which supplies precursors for flavonoid biosynthesis, thereby enhancing the fruit's coloration[41,47]These findings underscore the role of potassium in modifying both the types and levels of flavonoids, thus impacting the color of blueberry fruit.

The upregulation of genes associated with anthocyanin biosynthesis was much higher in the potassium-treated samples compared to the CK-treated samples, which is in line with earlier findings[48]. KEGG and GO enrichment analysis showed that metabolic pathways, hormones, and sugars in plants are closely related to flavonoid biosynthesis in blueberry fruits. It has been demonstrated that fruit flavonoid biosynthesis genes are influenced by potassium. For instance, in watermelon, the yield and quality of the fruit under potassium supply conditions were analyzed from a transcriptional perspective, revealing that the expression of the lycopene synthase gene was lower under low potassium conditions[49]. Flavonoids can be harmful in the cytoplasm, so to prevent toxicity, they are moved to the vacuole for storage or sequestration through the action of transporters. Specifically, ABC transporters have a special role in transporting plant flavonoids[27,50].

The synthesis of anthocyanin requires a phenylpropanoid pathway to provide its precursor. Flavonoid biosynthesis pathways and structural genes are highly conserved in higher plants, particularly in dicotyledonous plants[51], and have been extensively studied in *Arabidopsis thaliana*[52], grape[53], and petunia[54]. Flavonoids are initially derived from glucose produced by photosynthesis and then converted to phenylalanine via glycolysis, the pentose phosphate pathway, and the shikimic acid pathway. Anthocyanins are synthesized from phenylalanine as a substrate through the action of a series of key enzymes. According to Chen's research using omics technology, *PAL*, *4CL*, *HCT*, *CCoAOMT*, *CHS*, and *FLS* are the key genes regulating cucumber peel yellows. Thirty flavonoids were identified to be related to cucumber skin aging, including flavonoids, isoflavones, and two anthocyanins pelargonidin 3-O-beta-D-glucoside (Callistephin chloride) and Pelargonidin[55]. Potassium appears to act as a key modulator, enhancing enzyme gene expression and, in some cases, boosting enzyme activity. In this study, transcriptome sequencing and analysis of blueberry fruits treated with potassium identified eight structural genes in the anthocyanin synthesis pathway, all of which were highly expressed under potassium treatment: *PAL*, *CHS*, *CHI*, *F3'5'H*, *F3H*, *DFR*, *GST*, and *HCT*[56,57]. Therefore, increased potassium content promotes the up-regulation of enzyme gene expression in the anthocyanin synthesis pathway. The high expression of these genes likely explains why anthocyanin accumulation in fruits under potassium treatment is higher than in those under other treatments. During different treatment periods, *CHS*, *CHI*, *LAR*, *ANR*, *F3H*, and *HCT* were highly expressed under the YG-10 treatment. Proanthocyanidins share a biosynthetic pathway with anthocyanins up to the stage of leucoanthocyanidins, after which their pathways diverge. Highly expressed *CHS* and *CHI* promote the initiation process of flavonoid synthesis, while *LAR* and *ANR* are key enzymes in proanthocyanidin biosynthesis, converting colorless anthocyanidins to catechins and epicatechin, respectively. Potassium may promote the synthesis and accumulation of proanthocyanidins by up-regulating the expression of these genes.

The *AKT* gene plays a pivotal role in potassium transport and assimilation in leaves and fruits[58]. It has been reported that the expression of many potassium transporters in pear leaves and fruits is influenced by both low and high potassium conditions. The number of these genes under control treatment is greater than under high potassium treatment, including potassium channel proteins and potassium transporters[59]. These data are consistent with the results of this study. In this study, it was found that the gene expression levels of potassium transporters in fruits treated with potassium were down-regulated compared with CK. However, the expressions of *AKT1* and *KUP* in fruits were significantly up-regulated, consistent with the findings of Changwei Shen's study[59]. *AKT1* is the first potassium channel of the Shaker family to be cloned from Arabidopsis. Increasing AKT1 activity in watermelon roots has been reported to improve the uptake of K^+ when K^+ is deficient[60]. Song's study found that *KAT1*, an ABA-induced K^+ channel gene, increases expression with fruit maturation and is associated with the formation of red flesh[48,61].

K^+ is regarded as cofactors that play a crucial role in the action of several enzymes in plant cells[62]. The genes regulating K^+ participation in metabolic processes are crucial for the metabolic processes of many key enzymes, such as those responsible for pyruvate production and sugar metabolism[58,59,63,64]. This study identified an important upregulation of numerous genes related to metabolic processes in fruits treated with potassium. These genes include structural genes involved in starch and sucrose metabolism, glucose and fructose metabolism, and hexose metabolism. This indicates that anthocyanins need to form glucoside bonds with glucose, rhamnose-galactose, and other sugars after synthesis, eventually transforming into stable anthocyanins[65]. These results highlight the crucial role of the anthocyanin biosynthesis pathway in the blueberry's response to potassium treatment.

Conclusion

In this study, we identified 338 flavonoid compounds and 163 metabolites in the anthocyanin synthesis pathway using UPLC-MS/MS. Potassium application at the young fruit stage enhanced the accumulation of dark pigments, promoting early fruit color transformation. Transcriptome analysis revealed upregulation of key flavonoid and anthocyanin synthesis genes, including *UFGT*, *F3H*, *CHI*, *HCT*, *C12RT1*, *DFR*, and *F3'5'H*, under potassium treatment. Additionally, potassium transport genes, *AKT1* and *KUP*, were upregulated, indicating their potential involvement in anthocyanin synthesis. Overall, potassium regulates anthocyanin accumulation by modulating the expression of relevant genes, increasing both flavonoid diversity and enzymatic activity in the biosynthesis pathway. These findings provide valuable insights into the molecular mechanisms by which potassium influences anthocyanin synthesis in blueberry fruit.

Author contributions.

All authors contributed to the study conception. Material preparation was performed by Zejun Song. Data collection was accomplished by Boping Yu and Qian Li. Data analysis, experiment design and supervision were performed by Delu Wang. The draft of the manuscript was written by Ting Yan. All authors commented on previous versions of the manuscript and all authors read and approved the final manuscript.

Funding.

This work was financially supported by the National Natural Science Foundation of China. [Grant No. 31760205].

Data availability

The datasets presented in this study can be found the NCBI Gene Expression Omnibus (GEO) platform under accession numbers GSE277541.

Received: 31 August 2024; Accepted: 10 February 2025
Published online: 04 March 2025

References

1. Krishna, P., Pandey, G., Thomas, R. & Parks, S. Improving blueberry fruit nutritional quality through physiological and genetic interventions: A review of current research and future directions. *Antioxidants* **12**, 810 (2023).
2. Wang, H. et al. Identification of R2R3-MYB family in blueberry and its potential involvement of anthocyanin biosynthesis in fruits. *BMC Genom.* **24**, 505 (2023).
3. Kalt, W. et al. Recent Research on the Health benefits of blueberries and their anthocyanins. *Adv. Nutr. Bethesda Md.* **11**, 224–236 (2020).
4. Lin, Y. et al. Comparative transcriptome analysis of genes involved in anthocyanin synthesis in blueberry. *Plant. Physiol. Biochem.* **127**, 561–572 (2018).
5. Wang, J. et al. In vivo antioxidant activity of rabbiteye blueberry (*Vaccinium ashei* Cv. 'Brightwell') anthocyanin extracts. *J. Zhejiang Univ. -Sci B.* **24**, 602–616 (2023).
6. Curtis, P. J. et al. Blueberry anthocyanin intake attenuates the postprandial cardiometabolic effect of an energy-dense food challenge: results from a double blind, randomized controlled trial in metabolic syndrome participants. *Clin. Nutr.* **41**, 165–176 (2022).
7. Liu, Y. et al. Blueberry anthocyanins: Protection against ageing and light-induced damage in retinal pigment epithelial cells. *Br. J. Nutr.* **108**, 16–27 (2012).
8. Li, P. et al. Protective effects of anthocyanins on neurodegenerative diseases. *Trends Food Sci. Technol.* **117**, 205–217 (2021).
9. Wei, J. et al. Anthocyanins from Black Chokeberry (*Aroniamelanocarpa* Elliot) delayed aging-related degenerative changes of Brain. *J. Agric. Food Chem.* **65**, 5973–5984 (2017).
10. You, Q. et al. Comparison of anthocyanins and phenolics in organically and conventionally grown blueberries in selected cultivars. *Food Chem.* **125**, 201–208 (2011).
11. Chai, Z. et al. A comparative analysis on the anthocyanin composition of 74 blueberry cultivars from China. *J. Food Compos. Anal.* **102**, 104051 (2021).
12. Ogata, J., Kanno, Y., Itoh, Y., Tsugawa, H. & Suzuki, M. Anthocyanin biosynthesis in roses. *Nature* **435**, 757–758 (2005).
13. Nabavi, S. M. et al. Flavonoid biosynthetic pathways in plants: versatile targets for metabolic engineering. *Biotechnol. Adv.* **38**, 107316 (2020).
14. Hichri, I. et al. Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. *J. Exp. Bot.* **62**, 2465–2483 (2011).
15. Manzoor, M. A. et al. Flavonoids: A review on biosynthesis and transportation mechanism in plants. *Funct. Integr. Genomics.* **23**, 212 (2023).
16. Tohge, T., de Souza, L. P. & Fernie, A. R. Current understanding of the pathways of flavonoid biosynthesis in model and crop plants. *J. Exp. Bot.* **68**, 4013–4028 (2017).
17. Chalker-Scott, L. Environmental significance of anthocyanins in plant stress responses. *Photochem. Photobiol.* **70**, 1–9 (1999).
18. Jeong, S. T., Goto-Yamamoto, N., Kobayashi, S. & Esaka, M. Effects of plant hormones and shading on the accumulation of anthocyanins and the expression of anthocyanin biosynthetic genes in grape berry skins. *Plant. Sci.* **167**, 247–252 (2004).
19. Lin-Wang, K. et al. High temperature reduces apple fruit colour via modulation of the anthocyanin regulatory complex. *Plant. Cell. Environ.* **34**, 1176–1190 (2011).
20. Teng, S., Keurentjes, J., Bentsink, L., Koornneef, M. & Smeekens, S. Sucrose-specific induction of Anthocyanin Biosynthesis in Arabidopsis requires the MYB75/PAP1 gene. *Plant. Physiol.* **139**, 1840–1852 (2005).
21. Jia, Y. et al. Light and potassium improve the quality of *Dendrobium officinale* through optimizing transcriptomic and metabolomic alteration. *Molecules* **27**, 4866 (2022).
22. Solhjoo, S., Gharaghani, A. & Fallahi, E. Calcium and potassium foliar sprays affect fruit skin color, quality attributes, and mineral nutrient concentrations of 'Red Delicious' apples. *Int. J. Fruit Sci.* **17**, 358–373 (2017).
23. Hafsi, C., Debez, A. & Abdelly, C. Potassium deficiency in plants: effects and signaling cascades. *Acta Physiol. Plant.* **36**, 1055–1070 (2014).
24. Johnson, R. et al. Potassium in plants: Growth regulation, signaling, and environmental stress tolerance. *Plant. Physiol. Biochem.* **172**, 56–69 (2022).
25. Maity, A., Marathe, R. A., Sarkar, A. & Basak, B. B. Phosphorus and potassium supplementing bio-mineral fertilizer augments soil fertility and improves fruit yield and quality of pomegranate. *Sci. Hortic.* **303**, 111234 (2022).

26. Zareei, E., Javadi, T. & Aryal, R. Biochemical composition and antioxidant activity affected by spraying potassium sulfate in black grape (*Vitis vinifera* L. Cv. Rasha). *J. Sci. Food Agric.* **98**, 5632–5638 (2018).
27. Liu, Y. et al. Integrative analysis of metabolome and transcriptome reveals the mechanism of color formation in pepper fruit (*Capsicum annuum* L). *Food Chem.* **306**, 125629 (2020).
28. Schraer, S. M., Shaw, D. R., Boyette, M., Coupe, R. H. & Thurman, E. M. Comparison of enzyme-linked immunosorbent assay and gas chromatography procedures for the detection of cyanazine and metolachlor in surface water samples. *J. Agric. Food Chem.* **48**, 5881–5886 (2000).
29. Fraga, C. G., Clowers, B. H., Moore, R. J. & Zink, E. M. Signature-discovery approach for sample matching of a nerve-agent precursor using liquid chromatography – mass spectrometry, XCMS, and chemometrics. *Anal. Chem.* **82**, 4165–4173 (2010).
30. Kanehisa, M. & Goto, S. K. E. G. G. Kyoto Encyclopedia of genes and genomes. *Nucleic Acids Res.* **28**, 27–30 (2000).
31. Meng, L. & Feldman, L. A rapid TRIzol-based two-step method for DNA-free RNA extraction from *Arabidopsis* siliques and dry seeds. *Biotechnol. J.* **5**, 183–186 (2010).
32. Yu, J., Hulse-Kemp, A. M., Babiker, E. & Staton, M. High-quality reference genome and annotation aids understanding of berry development for evergreen blueberry (*Vaccinium darrowii*). *Hortic. Res.* **8**, 228 (2021).
33. Chen, S., Zhou, Y., Chen, Y. & Gu, J. Fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **34**, i884–i890 (2018).
34. Pertea, M. et al. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat. Biotechnol.* **33**, 290–295 (2015).
35. Liao, Y., Smyth, G. K. & Shi, W. featureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinforma Oxf. Engl.* **30**, (2014).
36. Love, M. I., Huber, W. & Anders, S. Moderated estimation of Fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, (2014).
37. Varet, H., Brillet-Guéguen, L., Coppée, J. Y., Dillies, M. A. SARTools a DESeq2- and EdgeR-based R pipeline for comprehensive differential analysis of RNA-Seq Data. *PLOS ONE.* **11**, e0157022 (2016).
38. Yu, G., Wang, L. G., Han, Y. & He, Q. Y. clusterProfiler: An R package for comparing biological themes among gene clusters. *Omics-J. Integr. Biol.* **16**, 284–287 (2012).
39. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2– ΔΔCT method. *Methods* **25**, 402–408 (2001).
40. Cook, N. C. & Samman, S. Flavonoids—Chemistry, metabolism, cardioprotective effects, and dietary sources. *J. Nutr. Biochem.* **7**, 66–76 (1996).
41. Jaakola, L. New insights into the regulation of anthocyanin biosynthesis in fruits. *Trends Plant. Sci.* **18**, 477–483 (2013).
42. Guo, X., Wang, D. & Shakeel, M. Transcriptome analysis reveals light-induced anthocyanin synthesis candidate genes in rabbiteye blueberry (*Vaccinium ashei*: Reade). *Biotechnol. Biotechnol. Equip.* **35**, 747–758 (2021).
43. Ranganath, K. G. et al. Profiling of anthocyanins and carotenoids in fruit peel of different colored mango cultivars. *J. Food Sci. Technol.* **55**, 4566–4577 (2018).
44. Lester, G. E., Jifon, J. L. & Rogers, G. Supplemental foliar potassium applications during muskmelon fruit development can improve fruit quality, ascorbic acid, and beta-carotene contents. *J. Am. Soc. Hortic. Sci.* **130**, 649–653 (2005).
45. Lester, G. E., Jifon, J. L. & Makus, D. J. Supplemental foliar potassium applications with or without a surfactant can enhance netted muskmelon quality. *HortScience* **41**, 741–744 (2006).
46. Norberto, S. et al. Blueberry anthocyanins in health promotion: A metabolic overview. *J. Funct. Foods.* **5**, 1518–1528 (2013).
47. Zhang, J., Qiu, X., Tan, Q., Xiao, Q. & Mei, S. A. Comparative metabolomics study of flavonoids in radish with different skin and flesh colors (*Raphanus sativus* L). *J. Agric. Food Chem.* **68**, 14463–14470 (2020).
48. Rogiers, S. Y., Coetzee, Z. A., Walker, R. R., Deloire, A. & Tyerman, S. D. Potassium in the grape (*Vitis vinifera* L.) Berry: transport and function. *Front. Plant. Sci.* **8**, (2017).
49. Zhong, Y. et al. Using rootstock to increase watermelon fruit yield and quality at low potassium supply: A comprehensive analysis from agronomic, physiological and transcriptional perspective. *Sci. Hortic.* **241**, 144–151 (2018).
50. Goodman, C. D., Casati, P. & Walbot, V. A. Multidrug resistance–associated protein involved in anthocyanin transport in *Zea mays*. *Plant. Cell.* **16**, 1812–1826 (2004).
51. Deng, Y. & Lu, S. Biosynthesis and regulation of phenylpropanoids in plants. *Crit. Rev. Plant. Sci.* **36**, 257–290 (2017).
52. Saito, K. et al. The flavonoid biosynthetic pathway in *Arabidopsis*: structural and genetic diversity. *Plant. Physiol. Biochem.* **72**, 21–34 (2013).
53. Czemmel, S., Heppel, S. C. & Bogs, J. R2R3 MYB transcription factors: Key regulators of the flavonoid biosynthetic pathway in grapevine. *Protoplasma* **249**, 109–118 (2012).
54. Beld, M., Martin, C., Huits, H., Stuitje, A. R. & Gerats, A. G. M. Flavonoid synthesis in *Petunia hybrida*: Partial characterization of dihydroflavonol-4-reductase genes. *Plant. Mol. Biol.* **13**, 491–502 (1989).
55. Chen, C. et al. Integrated Metabolome and transcriptome analysis unveils novel pathway involved in the formation of yellow peel in cucumber. *Int. J. Mol. Sci.* **22**, 1494 (2021).
56. Sun, T. et al. Transcriptional and metabolic responses of apple to different potassium environments. *Front. Plant. Sci.* **14**, 1131708 (2023).
57. Zhao, T., Xie, S. & Zhang, Z. Effects of foliar-sprayed potassium dihydrogen phosphate on accumulation of flavonoids in Cabernet Sauvignon (*Vitis vinifera* L). *J. Sci. Food Agric.* **103**, 4838–4849 (2023).
58. Armengaud, P., Breitling, R. & Amtmann, A. The potassium-dependent transcriptome of *Arabidopsis* reveals a prominent role of Jasmonic acid in nutrient signaling. *Plant. Physiol.* **136**, 2556–2576 (2004).
59. Shen, C. et al. Transcriptome analysis of differentially expressed genes induced by low and high potassium levels provides insight into fruit sugar metabolism of pear. *Front. Plant. Sci.* **8**, 938 (2017).
60. Sentenac, H. et al. Cloning and expression in yeast of a plant potassium Ion transport system. *Science* **256,** 663-665 (1992).
61. Fan, M. et al. Comparative transcriptome profiling of potassium starvation responsiveness in two contrasting watermelon genotypes. *Planta* **239**, 397–410 (2014).
62. Zörb, C., Senbayram, M. & Peiter, E. Potassium in agriculture – status and perspectives. *J. Plant. Physiol.* **171**, 656–669 (2014).
63. Ma, T. L., Wu, W. H. & Wang, Y. Transcriptome analysis of rice root responses to potassium deficiency. *BMC Plant. Biol.* **12**, 161 (2012).
64. Zeng, Q. et al. Transcriptome profiling of sugarcane roots in response to low potassium stress. *PLOS ONE.* **10**, e0126306 (2015).
65. Delgado, R., Martín, P., del Álamo, M. & González, M. R. Changes in the phenolic composition of grape berries during ripening in relation to vineyard nitrogen and potassium fertilisation rates. *J. Sci. Food Agric.* **84**, 623–630 (2004).

Author contributions

All authors contributed to the study conception. Material preparation was performed by Z. S. Data collection was accomplished by Q. Land B. Y. Data analysis, experiment design and supervision were performed by D. W. The draft of the manuscript was written by T. Y. All authors commented on previous versions of the manuscript and all authors read and approved the final manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-90060-w.

Correspondence and requests for materials should be addressed to D.W.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.