

Metabolome and Transcriptome Analyses of Red-colored Leaves From *Eucommia Ulmoides* 'Huazhong No. 12'

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Abstract

Background: *Eucommia ulmoides* 'Oliver' is an economically important tree species with highly medicinal and ecological values that is naturally distributed in China. *E. ulmoides* Huazhong No. 12' (H12) is the only red-leaf genotype of this species. In this study, the pigment contents of H12 and *E. ulmoides* 'Huazhong No. 11' (H11, green leaves) were determined. The differential metabolites in H12 and H11 were detected by UPLC-MS/MS, and the differentially expressed genes were screened by transcriptome. Then the key metabolites and corresponding gene regulation in anthocyanin related metabolic pathway were analyzed.

Results: The chlorophyll a, chlorophyll b and carotenoid contents in H12 leaves were lower than H11, while the total anthocyanin content in H12 was 4.06 times higher than in H11. There were 96 up-regulated metabolites in H12, including anthocyanin, proanthocyanidins, flavonoid and flavonol. Among them, four differentially expressed anthocyanins were identified. A total of 8,368 differentially expressed genes were selected from transcriptome between H12 and H11. The flavone and flavonol biosynthesis pathway, anthocyanin pathway and photosynthetic pathway were analyzed. Finally, *EuCHI*, *EuF3'H*, *EuF3'5'H*, *EuDFR* and *Eu3MaT1* were recommended as the key genes. The cyanidin, cyanidin 3-malonyl-glucoside and cyanidin 3, 5-glucoside were responsible for the H12 red leaves.

Conclusion: This study revealed the metabolites and gene regulation of anthocyanin synthesis, and the potential function between the anthocyanin and photosynthetic gene expression in *E. ulmoides* red leaves. Notably, the results also provided a reference for the study of other plant leaf-coloring mechanisms.

Background

Anthocyanins are natural pigments in plant tissues. The plant leaves are red-purple because they are rich in pelargonidin and cyanidin, the basic unit of anthocyanin[1]. And anthocyanins are thought to provide photoprotection under stressful conditions[2]. On the one hand, red leaves had higher anthocyanin content, filtering out a portion of visible light. In addition, anthocyanin absorbed blue-green waveband to prevent excessive light damage to leaves. On the other hand, anthocyanin was thought to be able to eliminate the accumulation of reactive oxygen species (ROS) as strong antioxidant[3]. Therefore, the study of anthocyanin synthesis in leaves is of great significance for the analysis of plant ecological adaptability.

The anthocyanin metabolism and molecular regulation of the anthocyanin biosynthetic pathway have been well described in the fruit and flowers of several plants, which share the same upstream enzymatic steps with proanthocyanidin and flavonoid biosynthetic pathways[4–6]. In the pink flowers of anthocyanin-rich tea (*Camellia sinensis*), cyanidin 3-O-glucoside and petunidin 3-O-glucoside were found to have direct impacts on anthocyanin biosynthesis via metabolome[7]. Peonidin, cyanidin and their

glycoside derivatives were confirmed to be the main anthocyanins in the peels of purple asparagus[8]. Anthocyanin synthesis is involved in flavonoid-related pathways.

First, phenylalanine, as a substrate, is catalyzed by phenylalanine ammonia-lyase (PAL). Then, cinnamic acid 4-hydroxylase (C4H), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3',5'-hydroxylase (F3'5'H), dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase are activated successively. Then anthocyanidins can be modified into other forms of anthocyanins owing to the actions secondary glycosidation, acetylation, methoxylation[9]. The genes that control the synthesis of these enzymes are enzymatic genes. In addition to enzymatic genes, transcription factors (TFs) also play important roles in anthocyanin synthesis [10]. The key TFs, including MYB TFs, basic helix-loop-helix (bHLH) and WD40 proteins, can work alone or form the MYB–bHLH–WD40 ternary complex that regulates the expression of anthocyanin synthesis genes[11, 12]. And glutathione S-transferase (GST), as an important transport protein, transported the anthocyanins synthesized in the endoplasmic reticulum to the vacuole for storage[13].

Non-photochemical quenching (NPQ) is an important photoprotective mechanism which is activated in few seconds upon exposure to excess light. The PsbS protein of photosystem II could dissipate excessive energy and maintain the efficient operation of photosynthesis[14]. However, this process produced harmful ROS in the chloroplast, including O_2^- , H_2O_2 . Then, O_2^- was rapidly converted H_2O_2 to by superoxide dismutase (SOD), and H_2O_2 could be eliminated by catalase (CAT) in chloroplast. The reduction reaction depended on the presence of glutathione[2]. So, what was the relationship between anthocyanin and photoprotective function? Xu proposed a model that anthocyanin could eliminate excessive ROS and maintain photosynthetic capacity[3]. Moreover, the leaves with high content of anthocyanin and flavonoids showed strong antioxidant capacity in *Arabidopsis thaliana*[15]. Hughes believed that anthocyanin in winter leaves act as light filters and may act as antioxidant pools[16]. However, Zhang hold an opinion that anthocyanin was mainly used as light filter rather than antioxidant in the leaves of *Begonia semperflorens*[2].

Eucommia ulmoides is native to China and has been extensively cultivated across 27 provinces (24°50'N–41°50'N, 76°00'E–126°00'E) because of its high economic value and adaptability [17]. *E. ulmoides* leaves is a potential bioactive material, which contains chlorogenic acid, phenols, terpenes, flavonoids[18]. Notably, *Eucommia ulmoides* 'Huazhong No. 12' is a special variety with red leaves, which expressed red color in summer and autumn. In addition, only palisade tissue was red in leaves. Previously, the study on 6-year-old red-leaves of *E. ulmoides* showed that anthocyanin was the main reason for the formation of leaf color, and the main factors affecting the content of anthocyanins were PAL enzyme, activity, temperature, pH value and light, while soluble sugar content has no significant effect on anthocyanin[19]. And the total flavonoids content of red leaves *E. ulmoides* was significantly higher than that of green leaves[20]. Here, we combined the metabolome with the transcriptome to analyze the significantly different gene expression levels and substances present in H12 and *E. ulmoides* 'Huazhong No. 11' (H11, green leaves). In addition to identifying specific anthocyanins in H12, we also revealed the accumulation of a set of flavonoids, anthocyanins, procyanidin A and procyanidin B,

accompanied by the regulation of structural genes in the phenylpropanoid and flavonoid biosynthetic pathways.

Results

Photosynthetic Pigment and anthocyanin Content of H11 and H12 Leaves

The detection revealed that chlorophyll a, b, a + b and carotenoid contents of the H12 leaves were lower than those of H11 leaves. And the chlorophyll a, b, a + b and carotenoid contents of the H12 were 0.54-, 0.71-, 0.58-, 0.54- fold compared with H11. However, the total anthocyanin content in H12 leaves was 4.06 times higher than that of H11. The results suggested that anthocyanin was responsible for the redness of H12 leaves (Fig. 2).

Secondary Metabolite Assay and Differential Metabolite Assay Filter

In this experiment, 556 metabolites were detected, including 14 anthocyanins, 40 flavonoids, 38 flavonols, 19 flavonoid glycosides, 17 flavanones, 19 saccharides, 28 hydroxycinnamoyl derivatives, 17 quinic acid derivatives, 10 catechin derivatives, 5 proanthocyanidins, 55 amino acids, 54 organic acid derivatives, 46 amino acid derivatives, 46 nucleotide derivatives, 15 benzoic acid derivatives, 13 kinds of cinnamon derivatives and other substances.

Based on the criteria of $VIP \geq 1$ and fold change ≤ 0.5 or fold change ≥ 2 , 165 differential metabolites were screened. Among them, 96 and 69 metabolites were up-regulated and down-regulated, respectively, in H12 compared with H11. The 96 differentially up-regulated metabolites mainly contained 4 anthocyanins, 2 proanthocyanidins, 20 flavones, 19 flavonols, isoflavones. The contents of peonidin O-hexoside, cyanidin 3-O-malonylhexoside, cyanidin O-syringic acid, cyanidin 3,5-O-diglucoside and cyanidin in H12 were 137.38-, 2.30-, 57.39-, 45.70- and 10.50-fold higher than in H11, respectively. Type A and B proanthocyanidins are dimeric flavonoids, and the contents of proanthocyanidin A1 and A2 in H12 were 4.15 and 89.46 times higher than in H11. The 69 differentially down-regulated metabolites mainly included 6 amino acids, 9 amino acid derivatives, 15 organic acid derivatives lipid-fatty acids and so on. 18 selected differential metabolites are listed in Table 1. In addition, several important mass spectrograms are shown in Figure S1.

KEGG Enrichment Analyses of Differential Metabolites in H11 and H12

The KEGG pathway enrichment analysis revealed that the biosynthesis of flavone and flavonol, flavonoid, anthocyanin, as well as that of amino acids, changed significantly in H12 compared with H11. First, in the flavone and flavonol biosynthesis pathway (ko00941), 17 flavone substances were enriched in this pathway and 10 metabolites were highly expressed in H12. Notably, dihydroquercetin was up-regulated, with its content being 7.99 times higher in H12 than in H11. Most importantly, in the anthocyanin biosynthesis pathways (ko00942), the contents of cyanidins and cyanidin 3,5-glucoside in H12 were 10.51 and 45.71 times higher than in H11, respectively. Thus, cyanidins and cyanidin 3,5-glucoside

appear to be substances involved in the purple-red appearance of H12 leaves. Moreover, in biosynthesis of amino acids pathway (ko01230), the contents of 7 metabolites including tryptophan and glutamine decreased significantly. But in the glutathione metabolism pathway (ko00480), the contents of glutathione disulfide and glutathione were up-regulated in H12. The pathway maps mentioned above were shown in Figure S2.

Transcriptome Analysis

For the transcriptome sequencing, the average H12 sample produced 594,236,10 clean reads, the base error distribution rate was 0.02%, and the GC content was 46.57%. The average H11 sample produced 60,047,905 clean reads, the base error distribution rate was 0.02%, and the GC content was 46.76%. And the clean data have been submitted to SRA database of NCBI (Accession ID: SRR12569328 and SRR12569327). The sequenced reads were compared with the reference genome of *E. ulmoides*. The average rates of total mapping for H12 and H11 were 95.53% and 95.36%, respectively. There were 17,503 genes expressed in H12, 17,765 genes in H11, and 16,687 genes were expressed in both leaf types. DEGs met the criteria of \log_2 (fold change) ≥ 1 and corrected $P \leq 0.005$. In total, 8,368 DEGs in H12 vs H11 were identified.

The GO analysis annotated 12,893 genes that mapped to the biological process, cell component and molecular functional classes. The COG annotation placed 24,851 genes into 25 COG categories. The KEGG database annotated 11,104 genes, the Swiss-Prot database annotated 20,382 genes, the Pfam database annotated 20,000 genes, and the Nr database annotated 25,233 genes. The number of genes annotated in the Nr database was the highest, followed by the COG database. The DEGs were subjected to a GO enrichment analysis (Figure S3). The KEGG enrichment of DEGs was shown in Figure S4, which reflected the differential metabolic processes of H11 and H12 during the same period in leaf tissues.

Photosynthesis Biosynthetic Pathways

To figure out the relationship between anthocyanin and photoprotection, the expression of photosynthetic genes in *E. ulmoides* red leaves were analyzed. Photosynthesis is related to photosynthesis pathways (ko00195) and photosynthesis antenna proteins pathway (ko00196), which had 5 parts, including photosystem II, photosystem I, cytochrome b6/f complex, photosynthetic electron transport and F-type ATPase. They were shown in Figure S5. Thus, the 13 DEGs related to photosynthesis in H11 was significantly up-regulated than that in H12. And the 5 genes in H11 is down-regulated than that in H12 in Table 2. This result showed that most of photosynthesis DEGs in red leaves of H12 were down-regulated at that time.

Combined analysis of key metabolites and corresponding genes related to Flavonoid and Anthocyanidin Biosynthetic Pathways

In order to speculate the regulation of metabolites and genes in the red leaves of *E. ulmoides*, metabolome and transcriptome were used to analyze the anthocyanins and flavonoids biosynthetic

pathways of H12. Firstly, *CHI* gene (EUC08195) was up-regulated 1.06-fold in H12 compared with H11. Next, the *F3'5'H* (EUC01284) was down-regulated - 3.34-fold while *F3'H* (EUC03526) was up-regulated 1.22-fold, which lift the content of dihydroquercetin 10.51-fold up. Then, *DFR* (EUC00272, MSTRG.13706, MSTRG.13719, MSTRG.15870, MSTRG.15871) were up-regulated 2.57-, 1.72-, 1.69-, 1.86-, 1.34-fold, respectively. The corresponding result was dihydroquercetin was more transformed to promote the synthesis of cyanidin, and cyanidin was up-regulated 7.99-fold. Finally, the structural gene *3MaT1* (EUC11625) was up-regulated 1.22-fold. Cyanidin 3-malonyl-glucoside and cyanidin 3, 5-glucoside were up-regulated 1.58- and 5.51-fold, respectively. The results of the combined analysis are shown in Fig. 3. Therefore, cyanidin, cyanidin 3-malonyl-glucoside and cyanidin 3, 5-glucoside were the key substances for the red color of H12 leaves. And *EuCHI*, *EuF3'H*, *EuF3'5'H*, *EuDFR* and *Eu3MaT1* were the key regulatory genes. Notably, the content of procyanidinA1 and procyanidinA2 of H12 were up-regulated 4.15-fold and 89.46-fold, respectively. This is the first time to detect proanthocyanidins in H12 leaves, and the antioxidant activity of them provided a basis for the development and utilization of *E. ulmoides*.

Candidate Transcription Factors

Among all of the transcripts, a total of 1931 transcripts were annotated as transcription factors (TFs) which were divided into 44 TF families. Among these TF families, 123 MYB transcripts was the largest, followed by 118 MYB-related and 100 bHLHs. And differential expressed transcripts were screen from MYBs, MYB-related family, bHLHs and WD40 TFs. Then, the phylogenetic analysis and alignment were performed to find the candidate MYBs and bHLHs involved in anthocyanin biosynthesis. Moreover, the matched sequences were picked from published paper and NCBI.

The phylogenetic tree and alignment analysis results in Fig. 4 indicated EUC13670 (up-regulated, 2.05-fold) was probably involved in the synthesis of anthocyanin and proanthocyanin[21, 22]. Like other anthocyanin MYBs, EUC13670 contained the highly-conserved R2R3 domain and bHLH binding motifs at the N-terminus. The motif [D/E]Lx₂[R/K] x₃Lx₆Lx₃R is important for interaction with bHLH TFs[23]. In addition, the conservative [K/R]Pxxx[K/T][F/Y] sequence in the C-terminal was part of the signature for MYBs that positively regulated anthocyanin biosynthesis[24]. Intriguingly, EUC13670 was also likely to regulate procyanidins synthesis in *E. ulmoides*.

The deduced amino acid sequence of EUC06643, EUC01933, EUC18920 and EUC04483 were align with other bHLHs regulating anthocyanin biosynthesis. However, EUC04483 (down-regulated, -1.47-fold) was proved to be bHLH-MYC TF with helix-loop-helix DNA-binding domain and R2R3-MYB transcription factors in N-terminal. As shown in Fig. 5, the box 11, 18 and 13 domain at the N-terminus were key to the interaction of bHLH and MYB[25].

Previous studies showed that GST transported anthocyanins from endoplasmic reticulum to vacuolar membrane as a carrier. *AcGST1* promoter was directly bound with *AcMYB110* to regulate anthocyanins in kiwifruit (*Actinidia chinensis*)[26]. The transcriptome analysis indicated *GST* (EUC05510, EUC04745,

EUC12154, EUC0882, EUC12155) were up-regulated 2.47-, 1.29-, 1.11-, 1.17-, 2.02-fold, respectively. The expression of *GST* genes was significantly increased in the red leaves of *E. ulmoides*.

RT-qPCR Verification

To verify the RNA-Seq results, we selected 10 genes (2 flavonoids, 4 anthocyanin and 3 photosynthetic metabolism pathway genes). The RT-qPCR results showed the up-regulated and down-regulated gene expression levels in the leaves of H11 and H12 were consistent with RNA-Seq (Fig. 6).

Discussion

The metabolite and gene regulation in leaf anthocyanin synthesis

Most of the previous studies analyzed the physiological, cytological and molecular causes of leaf color formation. Then metabolome and transcriptome metabolism analysis were popular in the era of multi omics, showing the large-scale metabolites and gene regulation of anthocyanin synthesis in leaves. In this study, 4 anthocyanins, 2 proanthocyanidins, 20 flavones, 19 flavonols, isoflavones of *E. ulmoides* leaves were qualitatively and quantitatively analyzed based on UPLC-MS/MS. And the *EuCHI*, *EuF3'H*, *EuF3'5'H*, *EuDFR* and *Eu3MaT1* were the key genes responsible for the red *E. ulmoides* leaves via the combined analysis of transcriptome and metabolome. Finally, cyanidin, cyanidin 3-malonyl-glucoside and cyanidin 3, 5-glucoside were important to red leaves of H12. The pattern of flavonoid-related metabolites and gene expression was first constructed in H12. Another similar study showed that the content of 7 anthocyanins in red leaf were higher than that in green leaf, and 19 key MYB regulators were co-expressed with the flavonoid–anthocyanin biosynthetic genes in leaf color formation of *Lagerstroemia indica* cv. Ebony Embers[27]. The changes of flavonoid related metabolites, enzyme activity and gene expression in the leaves color development of *Cymbidium sinense* 'Red Sun' were detected via metabolome. The above results indicate that transcriptome and metabolome have potential in finding the relation of flavonoid-related metabolites and gene regulation in leaf color formation.

Structural genes and transcription factors involved in anthocyanin synthesis

The types and content of anthocyanins that were commonly colored could directly affect the color of plant tissue[28]. The combined analysis showed that the *F3'5'H* was down-regulated while *F3'H* was up-regulated, which resulted in the activation of cyanidin pathway and the blocking of delphinidin pathway. Therefore, the reason for the red leaves of H12 may be the substrate competition between *F3'5'H* and *F3'H*. *F3'5'H* encodes a flavonoid 3',5'-hydroxylase belonging to the *cyp75a* subfamily, while *F3'H* encodes a flavanone 3-hydroxylase belonging to the *cyp75b* subfamily[29]. *F3'5'H* catalyzed naringenin and dihydrokaempferol into dihydromyricetin while *F3'H* catalyzed them into dihydroquercetin. Besides, there were reports that *FLS* could affect anthocyanin synthesis through substrate competition. *FLS* changed the ratio of flavonol and anthocyanin, and finally changed the flower color of *Petunia hybrida*[30]. *FLS* competed with *DFR*, causing the white flower mutant of *Muscari botryoides* Mill to loss cyanidin compound compared with the blue-flowered [31].

The MYB TF (EUC13670) and bHLH (EUC04483) were supposed to regulated anthocyanin synthesis in H12 leaves. Wang reported R2R3-MYB TF MYB6 of *Populus tomentosa* was mainly expressed in young leaves and the overexpression of MYB6 in transgenic poplar resulted in the significant up-regulation of anthocyanin and procyanidin accumulation[32]. In addition, *PpGST* together with *PpMYB10.1* might regulate the anthocyanin accumulation[33]. The key TFs could regulate the expression of anthocyanin synthesis genes. For example, R2R3-MYB proteins MYB11, MYB12, and MYB111 participate in transcriptional activation of the early biosynthetic genes *CHS*, *CHI* and *F3H*[34], whereas R2R3-MYB TFs PAP1, PAP2, MYB113, MYB114 and TT2 activate the late biosynthesis enzymes in Arabidopsis seedling[35]. Furthermore, AcMYB123/AcbHLH42 complex regulated promoters of *AcANS* and *AcF3GT1* that encode the dedicated enzymes for anthocyanin biosynthesis in the inner pericarp of the red-centered kiwifruit *Actinidia chinensis* cv. 'Hongyang'[4]. Thus, further experiments are needed to prove the function of *EuMYB*, *EubHLH* and *EuGST* in the process of leaf color formation, and predict their downstream target genes.

Potential photoprotective function of anthocyanins in *E. ulmoides*

Transcriptome showed that most of photosynthesis DEGs in H12 were down-regulated. In addition, the previous research reported the photosynthetic capacity differences between H11 and H12 leaves. The light compensation point of H12 was $24.00 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ while that of H11 was $26.00 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. And the light saturation point of H12 was $496.00 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ while that of H11 was $521.00 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. the maximum net photosynthetic rate of H12 was $16.91 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ while that of H11 was $18.50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Consequently, the capacity of red leaves to utilize strong light was weaker than green leaves in *E. ulmoides*. However, the maximum quantum yield of PSII (Fv/Fm) of H12 was significantly higher than that of H11[20]. Gould and Vogelmann found that the red leaves had less photosynthesis than green leaves in *Quintinia serrata*[36]. The study about red leaves of *Begonia semperflorens* showed the Fv/Fm in red leaves was significantly higher than that in green leaves during high-light stress. And red leaves were less sensitive to green light than green leaves because anthocyanin acted as light filters[2].

Additionally, anthocyanins were putative to be antioxidant and mediators of ROS-induced signaling cascades[37]. Manetas hold an opinion that anthocyanins could protect tissues from ROS produced by chloroplasts[38]. However, the other study confirmed that under high light stress, the O_2^- production rate and H_2O_2 content of green leaf were 71.34% and 6.85% higher than those of red leaf in *Begonia semperflorens*. Simultaneously, the non-enzymatic antioxidant activities of the reaction system determined by DPPH were higher in red leaves while the enzyme antioxidant activities in red leaves were lower than those in green leaves[2]. The Arabidopsis leaves with anthocyanins experiment suggested that the main function of anthocyanins was light attenuation rather than antioxidant in photoprotection. Thus, anthocyanin in the palisade tissue of *E. ulmoides* might play a role of light curtain. And more experiments are needed to supplement, such as the determination of antioxidant capacity and ROS content in H12 and H11 leaves under high light stress, as well as SOD, peroxidase, CAT enzyme activity.

Conclusions

This is the first report on metabolome and transcriptome analyses of red-colored leaves from *E. ulmoides* 'Huazhong No. 12'. The anthocyanins, proanthocyanidins, flavones, flavonols, isoflavones were up-regulated in the H12 than H11. Meanwhile, *EuCHI*, *EuF3'H*, *EuDFR* and *Eu3MaT1* genes were up-regulated, which enriched in the 'flavone and flavonol biosynthesis' and 'anthocyanin biosynthesis' pathway. Association analysis indicated that *EuCHI*, *EuF3'H*, *EuF3'5'H*, *EuDFR* and *Eu3MaT1* were key genes and cyanidin, cyanidin 3-malonyl-glucoside and cyanidin 3, 5-glucoside were responsible for the H12 red leaves. Furthermore, that most of photosynthesis DEGs in red leaves of H12 were down-regulated. The results provide a reference for other plants to study the mechanism of leaf coloration at transcriptional and metabolic levels, and analyze the potential relationship between photosynthetic physiological function and gene regulation in red leaf plants.

Methods

Plant Materials and Treatments

The grafted seedlings of H12 (red leaves) and H11 (green leaves) were selected, and typical leaves were collected as materials on July 2018 (Fig. 1B). The materials were developed from the Paulownia Research & Development Center, State Forestry and Grassland Administration, Henan, China. And they were planted at the affiliated Yuanyang experimental site (Northern latitude 34°78'; East longitude 113°71'). Notably, *Eucommia ulmoides* 'Oliver' 'Huazhong No. 12' (H12) was identified as superior tree species by Henan Provincial Forest Variety Examination Committee in 2013. The certificate was shown in Additional File 7. Experimental trees were grown under the same environment conditions along with the controls. The leaves for the metabolome study, transcriptome sequencing and RT-qPCR validation were quickly frozen in liquid nitrogen and stored at -80 °C. Three biological replicates were collected per sample.

Leaf Pigment Content Determination

The chlorophyll and carotenoid contents of H12 and H11 leaves were determined using the direct extraction method[39]. The leaves were weighed to 0.1 g (accurate to 0.01 g) after removing the veins and 80% acetone was added to 10 ml. Then, the supernatant was extracted after 72 h in darkness. The absorbance values at 445, 644 and 662 nm were determined using an ultraviolet spectrophotometer (JINGHUA Instruments 752, Shanghai Jinghua Technology Instruments Co., Ltd.). The anthocyanin content was determined. Samples of 0.1 g (accurate to 0.01 g) were ground in 10 ml 1% HCL methanol solution for 4 h and centrifuged for 15 min at 3,500 × g. The supernatant contents were determined at 530 nm and 600 nm. The calculated formula was described in the previous article[40].

Metabolite

The extraction and separation of freeze-dried leaf samples, metabolite identification and quantification were done at MetWare Biotechnology Co., Ltd (Wuhan, China) and described in detail by Fu Wang[41]. The Ultra Performance Liquid Chromatography Mass Spectrometer (UPLC-MS) was connected to an electrospray ionization (ESI)-triple quadrupole-linear ion trap–mass spectrometer /MS system (Q TRAP-MS/MS, Applied Biosystems 6500 Q TRAP) in this experiment. The characteristic ions of each metabolite were screened through the triple quadrupole (QQQ) mass spectrometer. Then the metabolites were qualitatively analyzed based on the Metware database and public database of metabolite information. The relative contents of corresponding metabolite were represented by the integrals of chromatographic peak area, which were calculated and corrected by MultiQuant (version 3.0.2)[42]. In addition, partial least squares discriminant statistical analysis was used to maximize the metabolite differences between two groups of samples. Variable importance in projection (VIP) ≥ 1 and fold change ≥ 2 or ≤ 0.5 were used as screening criteria[43].

RNA-Seq

Total RNA of each sample was extracted using a SuperPure plant polyRNA rapid extraction kit (GeneAnswer). RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, U.S.A.) and the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA), respectively. RNA integrity was validated using 1% agarose gel electrophoresis. Then, RNA-Seq libraries were prepared for sequencing at Majorbio Technology Co., Ltd (Shanghai, China). The Illumina Novaseq 6000 platform was used for the transcriptome sequencing. Raw reads were filtered to obtain high-quality reads. The clean reads were aligned with the reference genome using the software Hisat2 (version 2.1.0)[44]. After each sample was spliced using StringTie software (version 1.3.3b), the expression levels of genes and transcripts were quantified using the software RSEM (version 1.3.1), and quantitative index was expressed in Fragments Per Kilobase of exon model per Million mapped fragments (FPKM)[45]. All of the genes were then searched against protein databases, Nr, COG, KEGG and Swiss-Prot.

Differentially expressed genes (**DEGs**) was calculated using DESeq2 (version 1.24.0) by edgeR (version 3.24.3). DEGs met the criteria of $|\log_2(\text{fold change})| \geq 1$ and corrected $P \leq 0.05$. All the DEGs were analyzed for gene ontology (GO) enrichment using goatools (Version 0.6.5)[46] and KEGG enrichment using KOBAS software (version 2.1.1).

The TFs were annotated into the PlantTFDB database. The amino acid sequences of the MYB and bHLH TFs used for construct phylogenetic tree and sequence alignment were acquired from the NCBI database (<http://www.ncbi.nlm.nih.gov>). The phylogenetic tree image was generated using MEGA 7.0 (the neighbor-joining method with 1000 bootstrap replicates was used). The sequences alignment was conducted by DNAMAN version 9.

Real-Time Quantitative PCR (RT-qPCR) Validation

In total, 12 related genes were selected for RT-qPCR, and the primer list is shown in Table S1. The SYBR Premix Ex Taq™ kit (TaKaRa, Japan) was used, and the RT-qPCR expression assays were performed on a CFX96 platform (Bio-Rad, USA). The *GADPH* gene (*EUC05716*) was used as the relative quantitative reference gene. Three technical replicates were performed for each sample. The expression levels of the genes in the list were calculated using the $2^{-\Delta\Delta C_t}$ method[47]. The mean values \pm standard deviations were determined, and an analysis of variance followed by Duncan's new multiple range test were performed for the significance analysis.

Abbreviations

3MaT1

Anthocyanin 3-O-glucoside-6"-O-malonyltransferase; 4CL:antisense 4-coumaric acid coenzyme a ligase; ANS:anthocyanidin synthase; bHLH:basic helix-loop-helix; C4H:cinnamic acid 4-hydroxylase; CAT catalase; CHS:chalcone synthase; CHI:chalcone isomerase; DEGs:Differentially expressed genes; DFR:dihydroflavonol 4-reductase; F3H:flavanone 3-hydroxylase; F3'5'H:flavonoid 3',5'-hydroxylase; FLS:flavonol synthase; FPKM:Fragments Per Kilobase of exon model per Million mapped fragments; GT1:anthocyanidin 5,3-O-glucosyltransferase; GST:glutathione S-transferase; LAR:leucoanthocyanidin reductase; NPQ:Non-photochemical quenching; PAL:phenylalanine ammonia-Lyase; ROS:reactive oxygen species; RT-qPCR:Real-Time Quantitative PCR; SOD:superoxide dismutase; UPLC-MS:Ultra Performance Liquid Chromatography Mass Spectrometer; ESI-Q TRAP-MS/MS:electrospray ionization-triple quadrupole-linear ion trap–mass spectrometer /MS system; VIP:variable importance in projection.

Declarations

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Authors' contributions

Zhu Jingle and Du Hongyan designed the experimental scheme and provided the plant materials; YangYun and Chen Meng Jiao wrote the main manuscript text; Yang Yun and Ding Huanhuan prepared prepared Figures and Tables; all the authors reviewed the manuscript.

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Availability of data and materials

The transcriptome clean data have been submitted to SRA database of NCBI (Accession ID: SRR12569328 and SRR12569327).

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agreed to publish.

Competing interests

The authors declare no competing interests.

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Figures

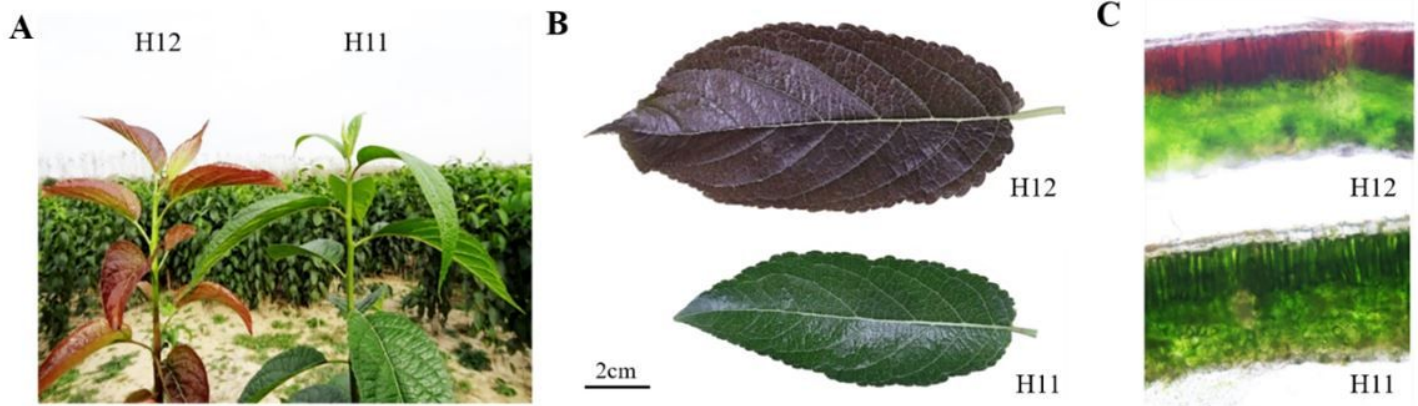


Figure 1

Leaves of *E. ulmoides* 'Huazhong No. 12' (H12) and *E. ulmoides* 'Huazhong No. 11' (H11, CK). (A) Leaves from H12 and H11 seedlings. (B) Mature leaves of H12 and H11 used as experimental materials. (C) Bare-hand sectioning of H12 and H11 leaves. The palisade tissue of H12 was observed to be red under the light microscope.

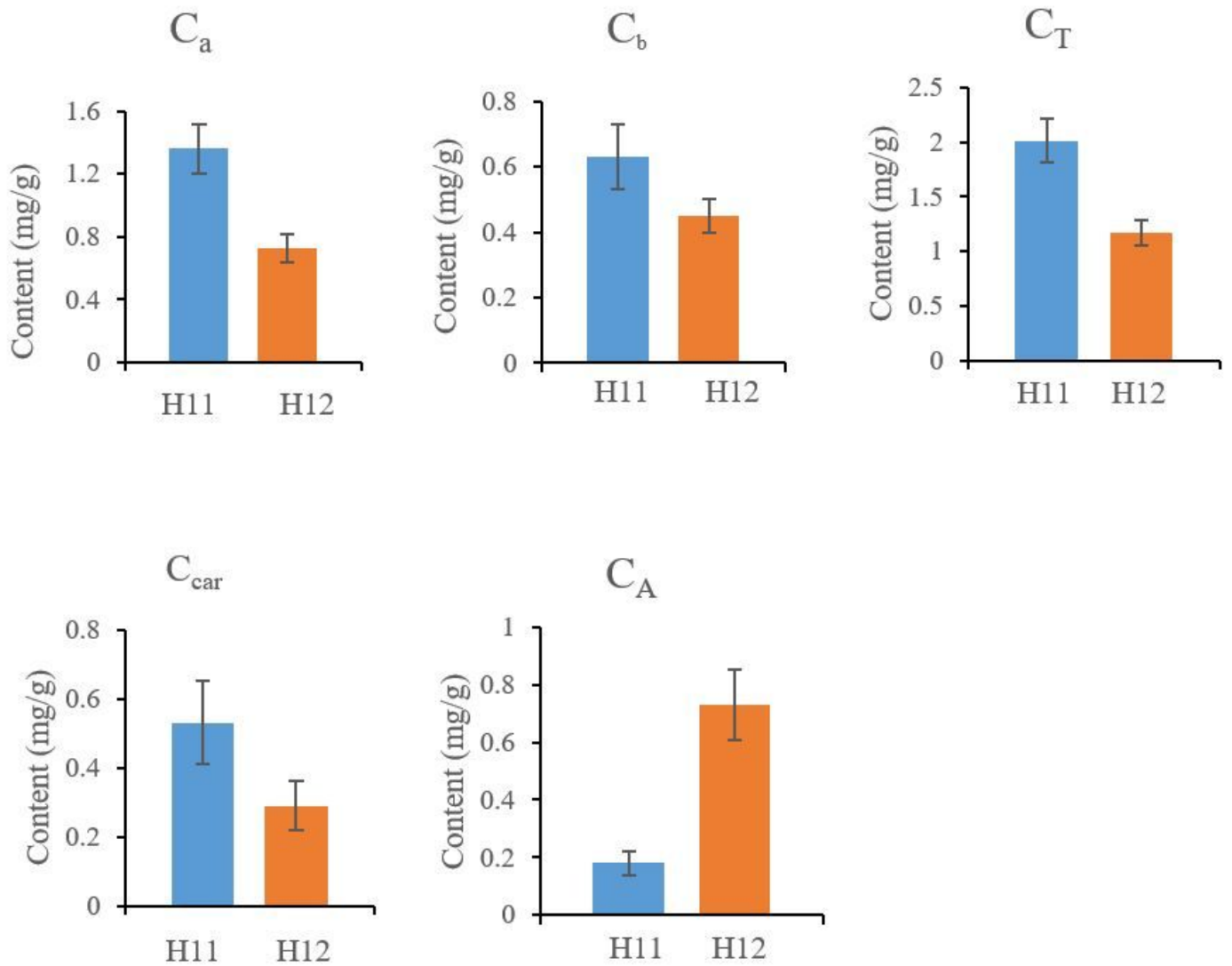


Figure 2

Photosynthetic pigments and anthocyanins content in leaf color between H11 and H12, the significance level of data difference was $P \leq 0.05$. Here Chlorophyll a content (C_a); Chlorophyll b content (C_b); total chlorophyll content (C_T) = $C_a + C_b$; Carotenoid content (C_{car}); anthocyanins content (C_A).

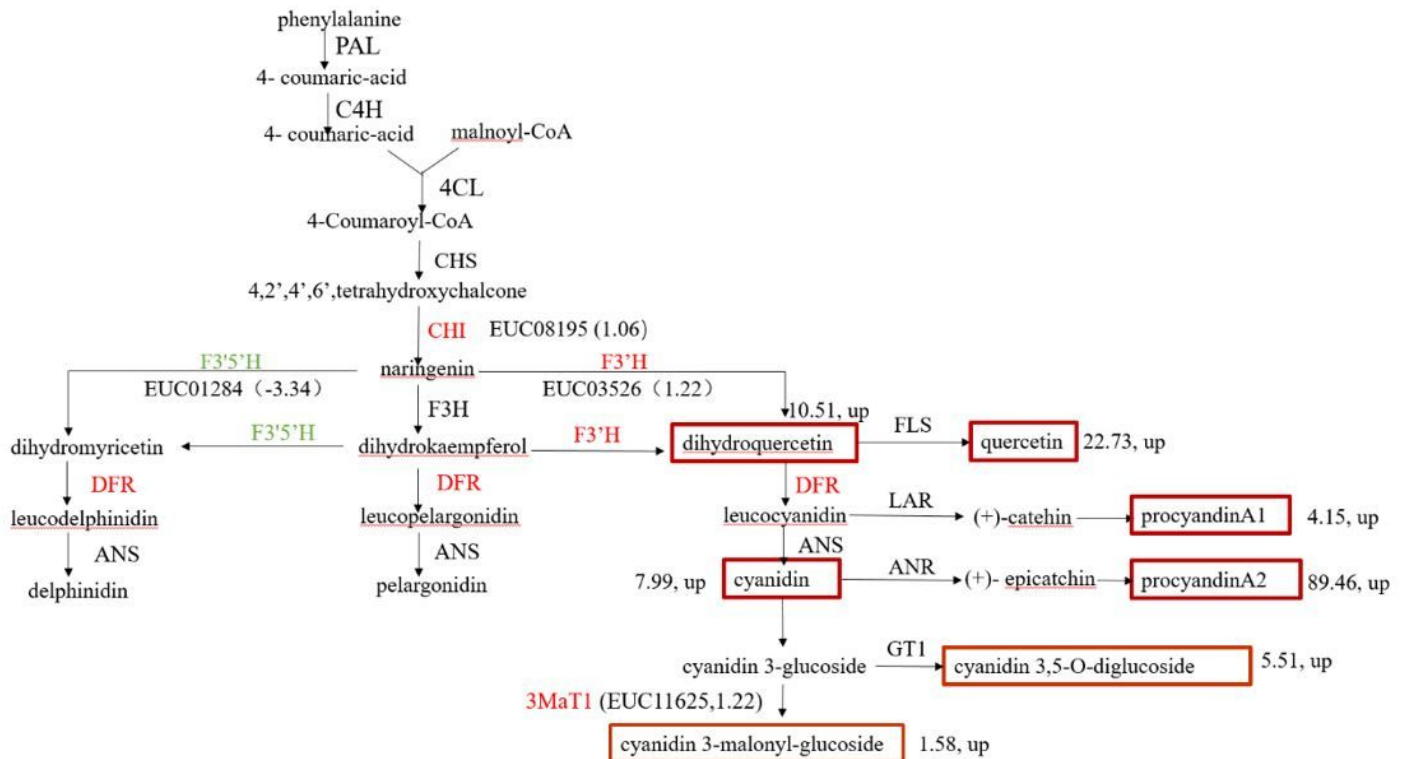


Figure 3

The results of transcriptome and metabolomic analysis on Flavonoid, and anthocyanidin biosynthetic pathways. Genes showed that red was up-regulated, while green was down regulated. Metabolites showed that red was up regulated. Phenylalanine ammonia-lyase (PAL). Then, cinnamic acid 4-hydroxylase (C4H), antisense 4-coumaric acid coenzyme a ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavanoid 3'-monooxygenase (F3'H), flavonoid 3',5'-hydroxylase (F3'5'H), dihydroflavonol 4-reductase (DFR), by anthocyanidin synthase (ANS), flavonol synthase (FLS), leucoanthocyanidin reductase (LAR), anthocyanidin 5,3-O-glucosyltransferase (GT1), anthocyanin 3-O-glucoside-6"-O-malonyltransferase (3MaT1).

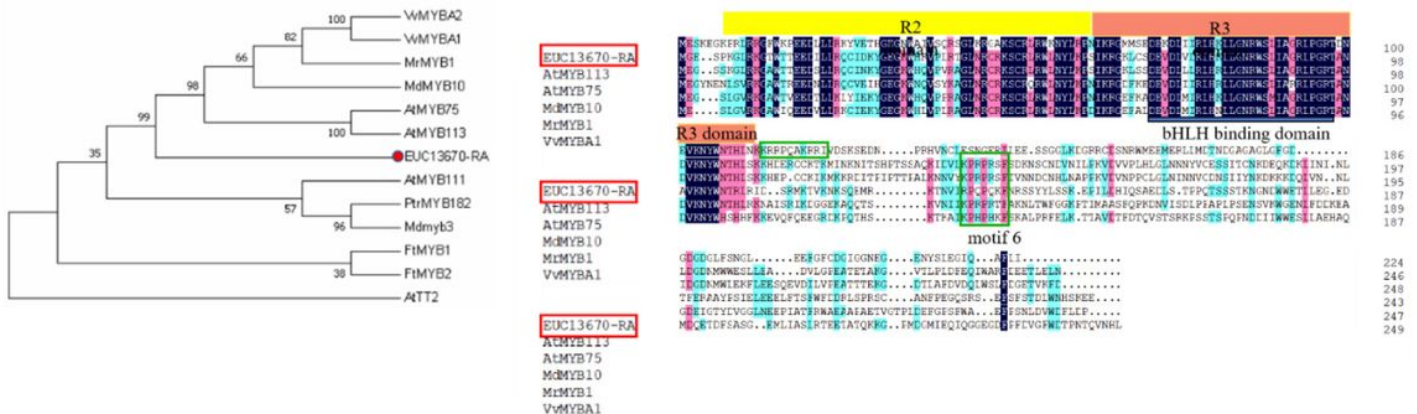


Figure 4

Phylogenetic relationships and alignment in anthocyanin or proanthocyanin MYB regulators in other species. GenBank accession numbers were as follows: (1) anthocyanin promoting MYB: VvMYBA1 (BAD18977.1), VvMYBA2 (ABL14065.1), MrMYB1 (ADG21957.1), MdMYB10 (ACQ45201.1), AtMYB75 (NP_176057.1), AtMYB113 (NP_176811.1); (2) proanthocyanins regulating MYB: AtMYB111 (NP_199744.1), MdMYB3 (AEX08668.1), FtMYB1 (AEC3297.1), FtMYB2 (AEC32976.1), AtTT2 (OA091653.1); (3) MYB regulating anthocyanin and proanthocyanins: PtrMYB182 (AJI76863.1). The EUC13670 was the putative anthocyanin and proanthocyanins MYB regulator in *E. ulmoides*.

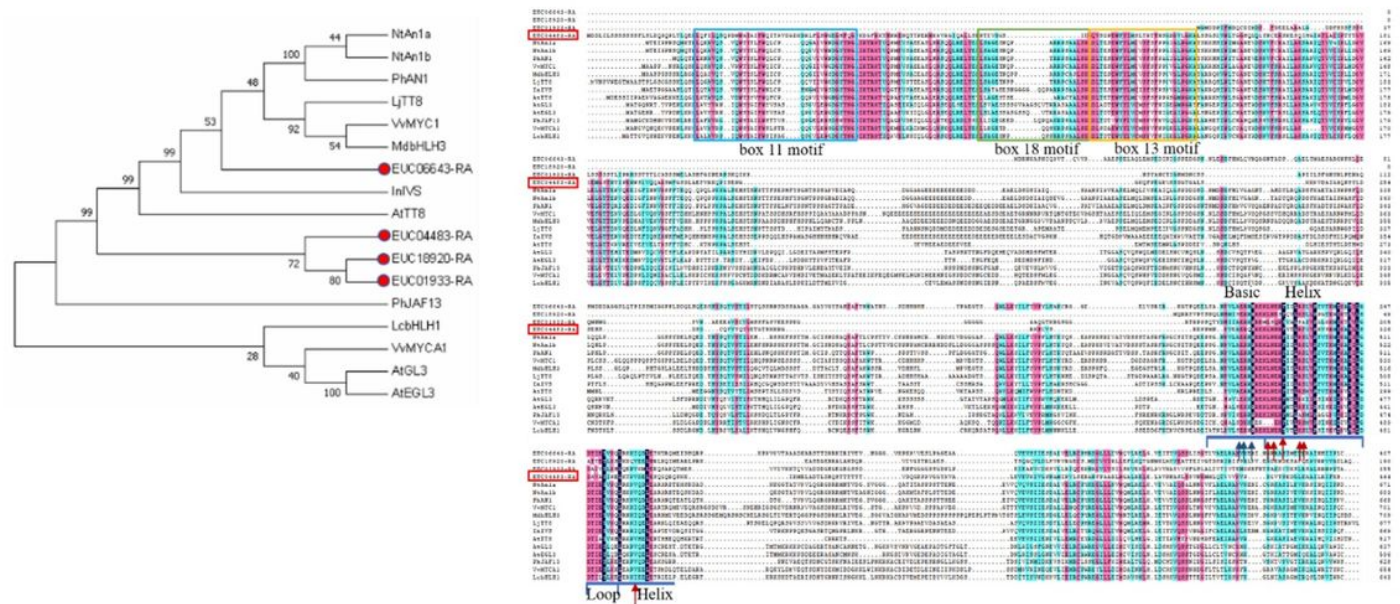


Figure 5

Phylogenetic relationships and alignment among putative bHLHs in *E. ulmoides* and anthocyanin bHLH regulators in other species. GenBank accession numbers were as follows: NtAn1a (AEE99257), NtAn1b (AEE99258), PhAN1 (AAG25927), VvMYC1 (ACC68685), MdbHLH3 (ADL36597), LjTT8 (BAH28881), InIVS (BAE94394), AtTT8 (CAC14865), PhJAF13 (AAC39455), LcbHLH1 (APP94122.1), VvMYCA1 (ABM92332), AtGL3 (NP 680372), and AtEGL3 (NP 176552). Then, the EUC06643, EUC01933, EUC18920 and EUC04483 were selected. The results indicated EUC04483 was likely candidate for bHLH TF regulating anthocyanin in *E. ulmoides*.

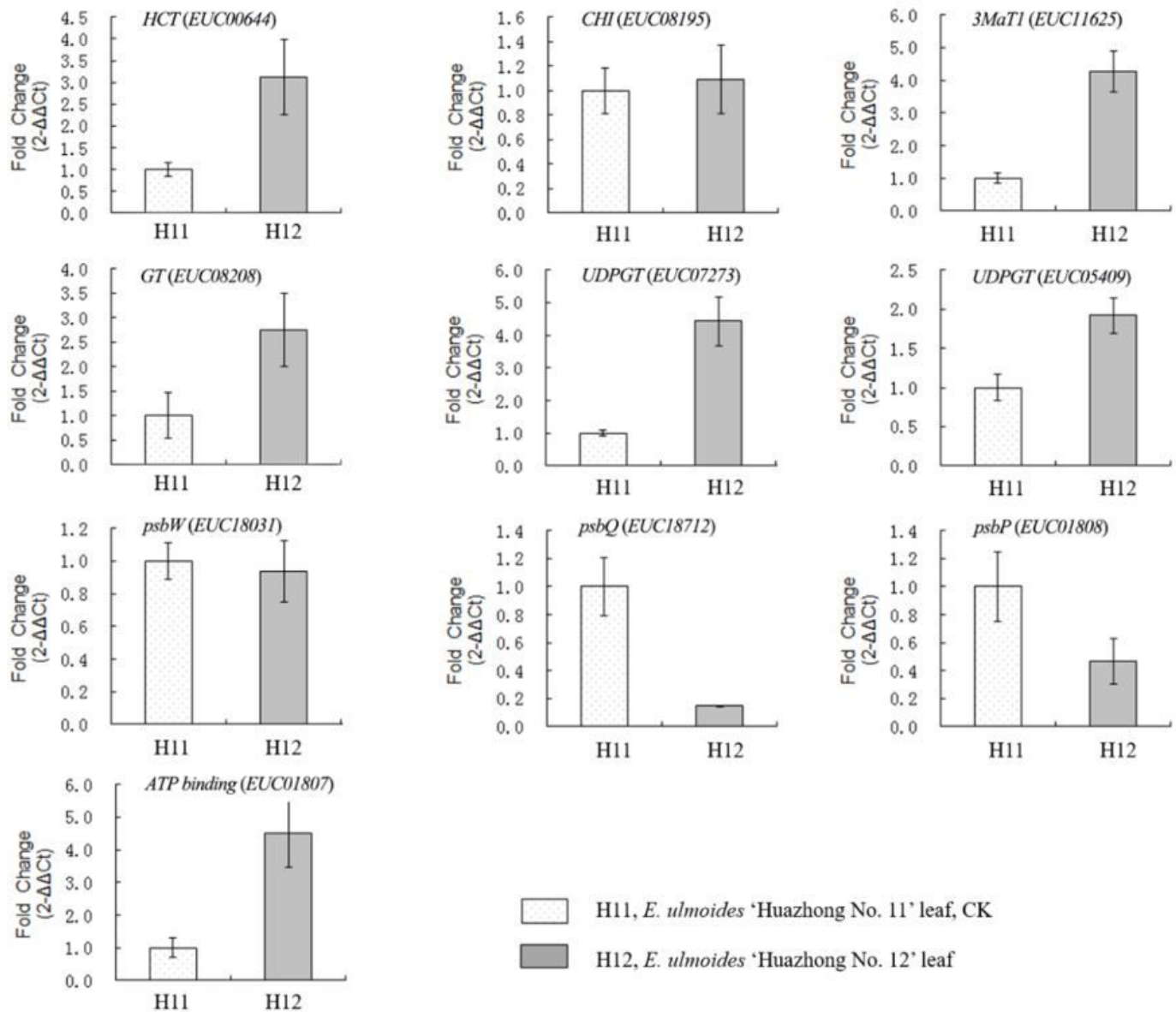


Figure 6

The RT-qPCR results of gene expression levels in the leaves of H11 and H12 were consistent with RNA-Seq.

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