

Integrated Transcriptome and Proteome Analysis Provides Insights into Anthocyanin Accumulation in the Leaves of Red-Leaved Poplars

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Integrated transcriptome and proteome analysis provides insights into anthocyanin accumulation in the leaves of red-leaved poplars

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Abstract

The red-leaved poplar cultivars ‘Quanhong’ and ‘Xuanhong’ are bud mutations of *Populus deltoides* cv. ‘Zhonglin 2025’. These cultivars are valued for their beautiful shape, lack of flying catkins, and ornamental leaf colors. However, the understanding of the molecular mechanism of anthocyanin accumulation in the leaves of red-leaved poplars is still unclear. Here, we profiled the changes of pigment content, transcriptome and proteome expression in the leaves of three poplar cultivars and the results showed that the ratios of anthocyanin to total chlorophyll in both red-leaved poplars were higher than that in ‘Zhonglin 2025’, indicating that the anthocyanin was highly accumulated in the leaves of red-leaved poplars. Based on the results of integrated transcriptome and proteome analysis, 15 and 11 differentially expressed genes/proteins involved in anthocyanin synthesis were screened in ‘Quanhong’ and ‘Xuanhong’, respectively, including the *CHS*, *F3H*, and *DFR* genes. Among the 120 transcription factors, 3 (HY5, HYH, and TTG2), may be directly involved in the regulation of anthocyanin synthesis in both red-leaved poplars. This study screens the candidate genes involved in anthocyanin accumulation in the leaves of red-leaved poplars and lays a foundation for further exploring the molecular mechanism of leaf red coloration in red-leaved poplars.

Keywords: Red-leaved poplar, Transcriptome, Proteome, Anthocyanin, Transcription factor

Introduction

With increasing demand for landscape ecological construction, plants with colorful leaves are becoming more popular due to their brilliant leaf colors and high ornamental value. For example, *Ulmus pumila* cv. Jinye, *Sophora japonica* cv. Golden Stem, *Melaleuca bracteata* F. Muell and *Acer rubrum* L. are widely used in landscape ecological construction due to their stable leaf color throughout the growing season. At present, germplasm resources for tree species with colorful leaves remain scarce; therefore, their breeding is of great significance for improving landscape ecological construction. The *Populus deltoides* ‘Quanhong’ and *Populus deltoides* ‘Xuanhong’ are red-leaved cultivars produced by bud mutation from *Populus deltoides* cv. ‘Zhonglin 2025’; they have excellent ornamental characteristics, such as brilliant leaf color, beautiful tree shape, and a lack of flying catkins. ‘Quanhong’ poplar leaves gradually change from purple-red to purplish-green to orange as the seasons change, whereas ‘Xuanhong’ poplar has brighter leaves that change gradually from bright red at the budding stage to orange at the deciduous stage (Guo, 2013; Wang et al., 2019a). Therefore, for the breeding and promotion of new colorful-leaf tree species, it is of great significance to explore the molecular mechanism of the leaf color difference between red-leaved poplars and ‘Zhonglin 2025’.

In addition to environmental factors (e.g., light (Lu et al., 2009; Albert et al., 2009), temperature (Lu et al., 2009; Miller et al., 2011), and soil (Roe et al., 2014)), plant leaf color is determined by the content and distribution of pigments such as chlorophyll, carotenoids (Car), flavonoids, and betaine (Tanaka et al., 2010). Among these, anthocyanins, which belong to the flavonoid family, determine the color of fruits, leaves, and flowers in most plants (Miller et al., 2011); the redness of the red-leaved poplar leaves is also caused by anthocyanin accumulation (Guo, 2013). Anthocyanins have many biological functions, including pollinator attraction, fruit dehiscence, reduction of the damage caused by ultraviolet (UV) light, and resistance against biological and abiotic stresses such as diseases and

insect pests (Miller et al., 2011; Winkel-Shirley, 2001; Wang et al., 2019b). The anthocyanin biosynthetic pathway is a branch of the flavonoid pathway in plants (Huang et al., 2016). Following phenylpropane synthesis, anthocyanins are synthesized in the cytoplasm by 4-coumarate-CoA and malonyl-CoA, which is catalyzed by enzymes encoded by a series of structural genes, including *CHS*, *CHI*, *F3H*, *F3'H*, *F3'5'H*, *DFR*, *ANS*, and *3GT*, followed by modifications of glycosylation, methylation, and acylation to form stable anthocyanins and allow entry into the vacuole, in a process mediated by glutathione S-transferase (GST) and MATE-type transporters (Vogt, 2010; Zhang et al., 2019; Luo et al., 2018). Anthocyanin synthesis is mainly regulated by MBW complexes including MYB, basic helix-loop-helix (bHLH), and WD40-repeat proteins, as well as other transcription factors such as bZIP and WRKY (Xu et al., 2014; Gelderen et al., 2018; Gonzalez et al., 2016). These transcription factors bind to the cis-acting elements of the structural genes involved in anthocyanin synthesis to regulate their expression.

Anthocyanin synthesis and transport are controlled by a series of structural genes and regulated by a variety of transcription factors, and the mutation of any one of these genes can cause changes in plant coloration. Many genes that regulate or are involved in anthocyanin synthesis have been isolated and characterized. For example, overexpression of *PtrMYB119*, a R2R3-MYB transcription from *Populus trichocarpa*, promotes anthocyanin production in hybrid poplar (Cho et al., 2016). The poplar MYB transcription factor *PtrMYB182* can inhibit the expression of *F3H* gene, thereby inhibiting the synthesis of proanthocyanins and anthocyanins (Yoshida et al., 2015). The overexpression of structural genes such as *PyDFR*, *PyANS*, and *PyUGT* in pear can promote anthocyanin biosynthesis and increase anthocyanin accumulation (Zhu et al., 2018). Many molecular mechanisms of plant color variation due to mutations in anthocyanin synthesis-related genes have also been unveiled. In purple cauliflower, a Harbinger DNA transposon inserted into the upstream regulatory region of the *BoMYB2* gene was found to upregulate the expression of the *BoMYB2* gene and the anthocyanin metabolism pathway (Chiu et al., 2010). In red apples, multiple repeats of a promoter segment caused *MdMYB10* gene autoregulation, which resulted in anthocyanin accumulation in the fruit and redness of the pulp (Espley et al., 2009). Grape peel whitening is caused by simultaneous mutation of the anthocyanin-specific transcription factors *VvMYBA1* and *VvMYBA2*, resulting in abnormal expression of the *3GT* gene, which is involved in anthocyanin synthesis (Walker et al., 2007).

Anthocyanin synthesis is affected by a variety of genes and complex environmental factors, so the correct research ideas and methods should be adopted to analyze the molecular mechanisms of color variation. Genes are the main directors of life activities, and proteins are the main executor of life activities (Song and Yu, 2019). With the continuous development and improvement of sequencing technology, transcriptome and proteomics have been widely used in biological research, but a single transcriptome or proteomics analysis only provides unilateral information at the transcriptional level or translation level (Yuan et al., 2019; Wang et al., 2019d; Dong et al., 2018). Therefore, the integrated analysis between transcriptome and proteomics is beneficial to the study of multi-level regulation of gene expression process, which is an effective way to study the molecular mechanisms underlying many biological traits (Vogel and Marcotte, 2012). In this study, we analyzed differences in mRNA and protein expression using transcriptome and proteome sequencing between red-leaved poplar cultivars

(‘Quanhong’ and ‘Xuanhong’) and ‘Zhonglin 2025’, and identified the key genes and proteins related to red leaf coloration.

Materials and methods

Plant materials

Three poplar cultivars (‘Zhonglin 2025’, ‘Quanhong’ and ‘Xuanhong’) were obtained from the Baoding Zhongsen Agriculture and Forestry Technology Co., Ltd. in Baoding city, Hebei province, China. This experiment was conducted in the experimental fields of Hebei Agricultural University (38.83°N, 115.45°E). At the end of March 2018, annual branches of ‘Zhonglin 2025’, ‘Quanhong’, and ‘Xuanhong’ poplar cultivars were cut into 20-cm cuttings. From each poplar species, 30 cuttings with uniform thickness and three to four full buds were selected and cut in the experimental field under identical conditions. Tilling of the soil and weeding were performed regularly, and water was applied when there was little to no rainfall to ensure normal seedling growth. Leaf sample collection for the three poplar species were performed on sunny and cloudless days in July 2018. The measurement of pigment content, as well as RNA-seq and proteomics analyses of the three poplar species were all performed three biological replicates.

Measurement of pigment content

The 3rd to 5th fresh leaves from the top of each poplar were washed and dried, and the midrib was removed. The leaves were then shredded and weighed; we added 10 mL 95% alcohol to 2-g samples of the shredded leaves, and extraction was performed for 24 h in the dark at room temperature. The chlorophyll a (Chla), chlorophyll b (Chlb), and Car content in the extract solution were determined using a spectrophotometer (722S; Shanghai Precise Science Instrument Co., Shanghai, China) at wavelengths of 440, 645, and 663 nm. We then added 10 mL 2% hydrochloric acid methanol solution to a 50-mL beaker containing 1 g of shredded leaves. Extraction was performed for 2 h in the dark at room temperature. After visually confirming that the leaf tissue had turned completely white, the extract solution was made up to 50 mL with 2% hydrochloric acid methanol solution. Anthocyanin content in the extract solution was measured at a wavelength of 530 nm.

RNA extraction and sequencing

The 3rd to 5th healthy and mature fresh leaves from the top of the tree were sampled from the three poplar species, and total RNA was then extracted from the leaves using a plant RNA extraction kit (SENO Biological Technology Co., Ltd., Zhangjiakou, Hebei, China) according to the manufacturer's instructions. The concentration and quality of RNA samples were determined using a NanoDrop 2000 spectrophotometer (Thermo, Waltham, MA, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. After RNA quality testing, 1 µg RNA per sample was used as input material for RNA sample preparation. Sequencing libraries were generated using the NEBNext Ultra RNA Library Prep Kit for Illumina New England Biolabs [NEB], Ipswich, MA, USA) following the manufacturer's recommendations. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was conducted using divalent cations at elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5×). First-strand cDNA was synthesized using random hexamer primer and Moloney murine leukemia virus (M-MuLV) reverse

transcriptase (RNase H⁻). Second-strand cDNA synthesis was then performed using DNA polymerase I and RNase H. The remaining overhangs were converted into blunt ends via exonuclease/polymerase activity. After adenylation of the 3' ends of the DNA fragments, NEBNext Adaptor with a unique hairpin loop structure was used for ligation prior to hybridization. To preferentially select cDNA fragments 250–300 bp in length, the library fragments were purified using the AMPure XP system (Beckman Coulter, Brea, CA, USA). Then, 3 µL USER enzyme mix (NEB) was incubated with size-selected, adaptor-ligated cDNA at 37°C for 15 min, followed by 5 min at 95°C prior to polymerase chain reaction (PCR). PCR was performed using Phusion High-Fidelity DNA polymerase, universal PCR primers and Index (X) Primer (NEB). Finally, PCR products were purified using the AMPure XP system and library quality was assessed using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Clustering of the index-coded samples was performed using a cBot Cluster Generation System with the TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, library preparations were sequenced on the Illumina NovaSeq platform and 150-bp paired-end reads were generated.

To ensure the quality and reliability of the data analyses, the raw data were pre-processed. In this step, clean reads were obtained by removing reads containing adapter, reads containing poly-N, and low-quality reads from the raw data. Hisat2 software (ver. 2.0.5; <http://ccb.jhu.edu/software/hisat2/index.shtml>) was used to compare the obtained clean reads to the reference genome of *Populus trichocarpa* in the National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/genome/98>). Raw sequences have been deposited at the NCBI SRA database (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA692065>). Gene expression levels were expressed as fragments per kilobase million (FPKM), with absolute fold change ($|FC|$) > 2 and adjusted p (p_{adj}) < 0.05 as thresholds for screening differentially expressed genes (DEGs). We used the R package *clusterProfiler* for Gene Ontology (GO) enrichment analysis of DEGs, and to further test for statistical enrichment of DEGs in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. We considered p_{adj} < 0.05 to indicate significant enrichment.

Protein extraction, tandem mass tag (TMT) labeling, and mass spectrometry analysis

The same plant materials were used for proteome and transcriptome analyses. Samples were ground individually in liquid nitrogen and lysed with lysis buffer containing 100 mM NH₄HCO₃ (pH 8), 6 M urea and 0.2% sodium dodecyl sulfate (SDS), followed by 5 min of ultrasonication on ice. The lysate was centrifuged at 12,000 × g for 15 min at 4°C and the supernatant was transferred to a clean tube. Extracts from each sample were reduced with 10 mM DTT for 1 h at 56°C, and then alkylated with sufficient iodoacetamide for 1 h at room temperature in the dark. Then, samples were completely mixed with four parts precooled acetone by vortexing, and incubated at –20°C for at least 2 h. Samples were centrifuged and the precipitate was collected. The pellet was washed twice with cold acetone and dissolved in dissolution buffer, which contained 0.1 M triethylammonium bicarbonate (TEAB, pH 8.5) and 6 M urea. Protein quality was analyzed using the Bradford protein quantification kit and 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

Protein samples were then digested with trypsin and TEAB buffer, and the peptides were labeled with acetonitrile-dissolved TMT labeling reagent. All labeling samples were mixed with an equal

volume of reagent, desalted, and lyophilized. The lyophilized powder was dissolved in mobile phase A (2% acetonitrile; pH adjusted to 10.0 using ammonium hydroxide). The peptides were fractionated on a L3000 high-performance liquid chromatography (HPLC) system (Rigol, Beijing, China) with a C18 column (Waters BEH C18, 4.6 × 250 mm, 5 μm). A 1-μg sample was taken from the supernatant of each fraction and separated using an EASY-nLCTM 1200 ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher, Waltham, MA, USA). The separated peptides were analyzed using a Q Exactive HF mass spectrometer (Thermo Fisher).

The resulting spectra from each fraction were searched separately against the *Populus trichocarpa* database using the Proteome Discoverer 2.2 search engine (Thermo). The protein quantitation results were statistically analyzed using the Mann–Whitney U test. Differentially expressed proteins (DEPs) were defined using $|FC| > 1.2$ and $p_{adj} < 0.05$ as thresholds. GO analysis was conducted using InterProScan 5 to search the non-redundant protein database, and the Clusters of Orthologous Groups (COG) and KEGG databases were used to analyze the protein family and pathway (Wei et al., 2008). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the iProX partner repository with the dataset identifier PXD023640 (<http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PX023640>).

Construction and analysis of protein-protein interaction network related to anthocyanin synthesis

The protein–protein interaction network was constructed using the protein sequences of differentially expressed TFs and differentially expressed genes/proteins involved in the anthocyanin synthesis pathway in the online software STRING (<http://stringdb.org>) using the TAIR accessions. The confidence level of minimum required interaction score parameters is set at 0.3 and other parameters are default.

Quantitative real-time PCR validation

The twelve genes that related to anthocyanin synthesis were used for validating gene expression by quantitative real-time PCR (qRT-PCR). All primers were designed using the Premier Primer 5.0 software (Supplementary Table S1) and synthesized by Shanghai ShengGong Biological Engineering Technology Service Co., Ltd. (Shanghai, China). A total of 1.0 μg RNA was used to synthesize cDNA with the First Strand cDNA Synthesis Kit (SENO Biological Technology Co., Ltd., Zhangjiakou, Hebei, China). qRT-PCR was performed using a Mx3005P system (Agilent Technologies, USA) with SYBR Green chemistry and *18S ribosomal RNA (rRNA)* as the housekeeping gene. The reaction was conducted as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 5 s, 56°C for 30 s, and 72°C for 34 s. Each reaction was performed with three biological replicates and gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Results

Analysis of pigment content in the leaves of red-leaved poplars

As we all know that the immediate reason for the color change of plant leaves is due to the change of the content and proportion of various pigments in the leaves. In order to understand the changes of pigment content in the leaves of red-leaved poplars, we first measured the contents of chlorophyll,

carotenoid and anthocyanin in the leaves of the three poplar species. The results showed that the chlorophyll a content in ‘Quanhong’ was slightly higher than that in ‘Zhonglin 2025’, while the chlorophyll a content in ‘Xuanhong’ was significantly lower than that in ‘Zhonglin 2025’ (Fig. 1B). The change tendency of chlorophyll b content in both red-leaved poplars was the same as that of chlorophyll a, and the difference was significant (Fig. 1C). The carotenoid content of both red-leaved poplars was lower than that of ‘Zhonglin 2025’, and the carotenoid content of ‘Xuanhong’ was significantly lower than that of ‘Zhonglin 2025’ (Fig. 1D). The anthocyanin content in both red-leaved poplars was significantly higher than that in ‘Zhonglin 2025’ (Fig. 1E). It is important that the ratios of anthocyanin to chlorophyll in the ‘Quanhong’ and ‘Xuanhong’ were 5.00 and 15.79, which were much higher than that of ‘Zhonglin 2025’ (0.38). It can be seen that the significant increase in the proportion of anthocyanins in the leaves of red-leaved poplars led to the redness of red-leaved poplars leaves. Although the contents of chlorophyll and carotenoids in both red-leaved poplars have changed and their change tendencies are different, no matter whether the chlorophyll content increases (QH) or decreases (XH), the red-leaved poplar leaves will turn red (Fig. 1A), which showed that the anthocyanin accumulation plays a direct role in the leaf coloration of red-leaved poplars.

General characterization of the transcriptome data

To identify the key genes related to red leaf coloration, we performed RNA sequencing (RNA-Seq) on the leaves of ‘Quanhong’, ‘Xuanhong’, and ‘Zhonglin 2025’ poplars. We obtained 58.6 Gb of clean bases, with an average output for each sample of about 6.5 Gb. The error rate, Q20, Q30, and GC contents of all samples were within acceptable ranges, and the efficiency of comparing the sequencing data of all samples with the reference genome exceeded 80%, indicating high-quality transcriptome data (Supplementary Table S2).

In this study, we identified a total of 37,846 genes, including 37,271 annotated genes and 575 predicted new genes. We screened a total of 6,792 DEGs in ‘Quanhong’, of which 3,084 were significantly upregulated and 3,708 were significantly downregulated; a total of 4,398 DEGs were screened in ‘Xuanhong’, including 2,103 significantly upregulated genes and 2,295 significantly downregulated genes (Fig. 2A). Venn diagram analysis showed a total of 2,779 genes that were differentially expressed in both red-leaved poplars (Fig. 2B). Altogether, many DEGs were detected in the red-leaved poplars, with slightly more genes downregulated than upregulated.

To investigate the functional features of these DEGs, we performed GO enrichment analysis; DEGs were classified into three categories: biological processes (BP), cellular components (CC), and molecular functions (MF). DEGs in ‘Quanhong’ and ‘Xuanhong’, showed significantly greater enrichment in structural constituent of ribosome and ADP binding, respectively, among the MF category genes. No DEGs in ‘Xuanhong’ were significantly enriched in the BP or CC categories, whereas in ‘Quanhong’, the most significantly enriched GO terms in the BP and CC categories were translation and ribosomes, respectively (Supplementary Table S3). DEGs in ‘Quanhong’ and ‘Xuanhong’ were significantly enriched in 21 and 18 of the KEGG pathways, respectively ($p_{\text{adj}} < 0.05$), including carbon fixation in photosynthetic organisms, biosynthesis of amino acids, biosynthesis of secondary metabolites, metabolic pathways, carbon metabolism, glycolysis/gluconeogenesis, tyrosine metabolism, cysteine and methionine metabolism, isoquinoline alkaloid biosynthesis, flavonoid

281 biosynthesis and alpha-linolenic acid metabolism (Fig. 2C and 2D). DEGs in ‘Quanhong’ were
282 significantly enriched in more GO terms and metabolic pathways than those in ‘Xuanhong’. These
283 results indicate that gene mutation changed the expression levels of many genes in both red-leaved
284 poplars, many of which are involved in the synthesis and metabolism of organics, amino acids and
285 secondary metabolites, which may be due to the secondary effect caused by anthocyanin accumulation.

286 **General characterization of the proteome data**

287 TMT labeling was performed to analyze the protein expression profiles of both red-leaved poplars and
288 ‘Zhonglin 2025’. The results showed that a total of 323,559 spectra were generated from the nine
289 samples, as well as 27,521 peptides and 6,145 proteins (Supplementary Table S4). Using the $|FC| > 1.2$
290 and $p < 0.05$ thresholds for screening DEPs resulted in the screening of 2,786 and 2,333 DEPs from
291 ‘Quanhong’ and ‘Xuanhong’, respectively.

292 To study the biological functions of DEPs, and the biochemical metabolic pathways and signal
293 transduction pathways involved, we conducted GO and KEGG enrichment analyses. GO enrichment
294 analysis showed that DEPs in ‘Quanhong’ and ‘Xuanhong’ poplars were most significantly enriched in
295 small molecule metabolic process and organonitrogen compound metabolic process, respectively, in the
296 BP category; catalytic activity and unfolded protein binding, respectively, in the MF category; and
297 cytoplasm and cell, respectively, in the CC category (Supplementary Table S5). DEPs in ‘Quanhong’
298 and ‘Xuanhong’ were significantly enriched in 48 and 26 metabolic pathways, respectively, mainly
299 including carbon metabolism, metabolism pathways, biosynthesis of secondary metabolites, carbon
300 fixation in photosynthetic organisms and biosynthesis of amino acids (Fig. 3).

301 **Integrated transcriptome and proteome analysis**

302 To explore changes in the metabolic pathways of red-leaved poplars in more detail, and to screen out
303 key genes related to red leaf coloration, we conducted an integrated analysis of the transcriptome and
304 proteome. We screened 769 DEGs and DEPs in ‘Quanhong’; 676 showed the same trend, while 93 of
305 which showed the opposite trend. Only 399 DEGs and DEPs were screened in ‘Xuanhong’, of which
306 344 showed the same trend and 55 showed the opposite trend (Fig. 4). KEGG enrichment analysis of
307 DEGs and DEPs obtained by integrated analysis showed that the genes/proteins in ‘Quanhong’ were
308 significantly enriched in 23 KEGG pathways, whereas those in ‘Xuanhong’ were significantly enriched
309 in only 8 KEGG pathways, perhaps due to the fewer DEGs and DEPs screened from ‘Xuanhong’.
310 DEGs and DEPs in both ‘Quanhong’ and ‘Xuanhong’ were significantly enriched in important
311 metabolic pathways including photosynthesis, phenylpropanoid biosynthesis, and flavonoid
312 biosynthesis (Supplementary figure S1 and S2). We identified 15 and 11 genes/proteins from
313 ‘Quanhong’ and ‘Xuanhong’, respectively, that were involved in anthocyanin synthesis; these included
314 the phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI),
315 flavanone-3 β -hydroxylase (F3H), and flavanone-3'-hydroxylase (F3'H) genes. The expression of most
316 of these genes was significantly upregulated (Fig. 5 and Supplementary Table S6). It can be seen that
317 the synergistic expression of these structural genes related to anthocyanin synthesis together promoted
318 the accumulation of anthocyanins in the leaves of red-leaved poplars.

319 **qRT-PCR confirmation of selected genes**

320 To verify the expression level of the transcriptome in this study, we selected nine genes that are

differentially expressed in the anthocyanin synthesis pathway for qRT-PCR analysis. The results showed that most of these genes were upregulated, consistent with the transcriptome analysis. This result verified the expression levels of structural genes involved in anthocyanin synthesis, and the reliability of the RNA-Seq gene expression data (Fig. 6). In addition, the expression levels of these genes were very different between the 'Quanhong' and 'Xuanhong', and most of them had higher expression levels in 'Quanhong', such as CHS gene, PAL gene and DFR gene. This different expression pattern may be the reason for the different accumulation of anthocyanins in both red-leaved poplars (Fig. 1E), resulting in the difference between the leaf colors of the both red-leaved poplars (Fig. 1A).

Identification of transcription factors involved in anthocyanin synthesis

To screen out transcription factors involved in anthocyanin synthesis in the red-leaved poplars, we analyzed all identified genes and proteins. A total of 120 transcription factors were screened, including 6 bHLH, 19 bZIP, 10 C2H2, 12 C3H, 12 MYB, 6 Trihelix, and 55 other transcription factors (Supplementary Figure S3). There were 22 and 17 transcription factors in 'Quanhong' and 'Xuanhong', respectively, the expression levels of which changed significantly in both the transcriptome and proteome (Supplementary Table S7).

Differentially expressed structural genes involved in anthocyanin synthesis, and the differentially expressed transcription factors identified above, were used to form a protein-protein interaction network (Fig. 7). In both 'Quanhong' and 'Xuanhong', there were close interactions among the structural genes that control anthocyanin synthesis. The key genes controlling anthocyanin synthesis were directly regulated by three transcription factors: TTG2, HYH, and HY5. TTG2 is a transcription factor that encodes the WRKY family and HYH and HY5 are both transcription factors of the bZIP family. These results show that while TTG2 interacts with more structural genes that control anthocyanin synthesis, HYH and HY5 interact with each other, as well as with other transcription factors. The mRNA and protein abundance of these three TFs were significantly increased in both red-leaved poplars, but the magnitude of the increase was somewhat different, which may also be the reason for the different expression patterns of structural genes related to anthocyanin synthesis in both red-leaved poplars. Through qRT-PCR analysis, we found that the expression trends of these three TFs among the three poplar cultivars are the same as the transcriptome data, indicating that the regulation modes of the three TFs in both red-leaved poplars are slightly different (Fig. 8). To sum up, TTG2, HYH, and HY5 may play important regulatory roles in anthocyanin synthesis.

Discussion

At present, the pathway of anthocyanin synthesis in plants has been relatively clear. Many studies have shown that the change of anthocyanin content or proportion will directly affect the color of leaves. For example, the change in color of herbaceous peony leaves from purple to green is caused by a reduction in anthocyanins and accumulation of chlorophyll (Tang et al., 2020). The leaf colors of coleus vary according to the relative proportions of chlorophyll and anthocyanin (Nguyen and Cin, 2009). The high expression of *BrMYB2* gene in Chinese cabbage activates the anthocyanin biosynthesis pathway, promotes the expression of anthocyanin biosynthesis related genes, and finally leads to the accumulation of anthocyanins to form purple head Chinese cabbage (He et al., 2020). Similarly, the

content and proportion of anthocyanin in the leaves of red-leaved poplars also changed significantly. The anthocyanin content in ‘Quanhong’ and ‘Xuanhong’ was 14.7 and 11.5 times that in ‘Zhonglin 2025’, respectively. Although the change trend of chlorophyll content in the leaves of both red-leaved poplars was opposite, no matter whether the chlorophyll content increased (QH) or decreased (XH), the ratios of anthocyanin to chlorophyll in the leaves of both red-leaved poplars were much higher than of ‘Zhonglin 2025’, which shows that the accumulation of anthocyanins is the direct cause of the redness of red-leaved poplar leaves.

The biosynthesis of anthocyanins is under the control of multiple structural genes, some of which have been isolated and characterized in plants such as pear (Yang et al., 2013), apple (Kim et al., 2003), lettuce (Zhang et al., 2018), rose (Sui et al., 2018), and sweet potato (Wang et al., 2018). Through integrated analysis of the transcriptome and proteome, we screened 15 and 11 DEGs and DEPs related to anthocyanin synthesis in ‘Quanhong’ and ‘Xuanhong’, respectively. The *CHS* gene is the first key gene in anthocyanin synthesis (Tian et al., 2011). We identified two *CHS* genes (LOC7460519 and LOC7466313) in both ‘Quanhong’ and ‘Xuanhong’, of which the LOC7460519 gene shows opposite expression patterns in the transcriptome and proteome from both red-leaved poplars, whereas the mRNA and protein levels of the LOC7466313 gene were significantly upregulated in both red-leaved poplars; this indicated that the two *CHS* genes have different expression patterns in the process of controlling anthocyanin synthesis. The expression levels of the *CHI* (LOC7459237), *F3H* (LOC18099191, LOC18099194), and *DFR* (LOC7454367) genes were significantly upregulated, and all played roles in anthocyanin accumulation in the leaves of both red-leaved poplars. However, some structural genes related to anthocyanin synthesis were differentially expressed only in one type of red-leaved poplar. For example, the *UFGT* gene (LOC7465981) was differentially expressed only in ‘Quanhong’, while the *4CL* (LOC7494541) and *ANS* (LOC7490928) genes were differentially expressed only in ‘Xuanhong’; this indicated that the leaf coloration mechanisms of the red-leaved poplars were not identical. There were no significant differences in the expression levels of anthocyanin modification-related genes or *GST* genes, which are responsible for anthocyanin transport, between the red-leaved poplars, indicating that the higher anthocyanin content in red-leaved poplars was due to upregulated expression of structural genes upstream of anthocyanin biosynthesis. The above results indicate that the synergistic expression of genes such as *CHS*, *CHI*, *F3H* and *DFR* together promoted the accumulation of anthocyanins in the leaves of red-leaved poplars.

In addition to structural genes related to anthocyanin synthesis, transcriptional regulators also play an important role in anthocyanin biosynthesis. Increasing numbers of transcription factors related to anthocyanin biosynthesis have been isolated and characterized, most of which are MYB family transcription factors (which regulate anthocyanin synthesis by interacting with bHLH and WD40 in a MYB-bHLH-WD40 [MBW] complex). This regulation pattern has been found in most plant species, including *Arabidopsis thaliana* (Xu et al., 2014), barley (Strygina et al., 2017), and strawberry (Schaart et al., 2013). In this study, we used differentially expressed transcription factors and structural genes related to anthocyanin synthesis to construct a protein–protein interaction network, and found that the transcription factors that may directly involved in the regulation of anthocyanin biosynthesis-related structural genes in both red-leaved poplars were HY5 (Long hypocotyl 5), HYH (HY5-homolog) and

TTG2 (TRANSPARENT TESTA GLABRA2). HY5, a key positive regulator of photomorphogenesis, regulates the expression of related functional genes downstream of light signal transduction, whereas HYH, a homologous protein of HY5, has a function similar to that of HY5; the protein content of HYH is also regulated by HY5. Studies have shown that HY5 and HYH are two important transcription factors in the bZIP family involved in anthocyanin synthesis. For example, HY5/HYH, which bind to the promoters of genes such as *CHS*, *F3H* and *DFR*, can play a key role in the anthocyanin accumulation of Arabidopsis seedlings induced by low temperature (Zhang et al., 2011). The purple tomato cultivar ‘Indigo Rose’ has been used to specifically induce an *Slhy5* mutant through CRISPR/Cas9 technology, leading to the discovery that the bZIP transcription factor HY5 plays a key role in controlling anthocyanin accumulation in response to light (Qiu et al., 2018). TTG2 is also regulated by MYB and the bHLH complex, and acts as a ligand to regulate the expression of structural genes controlling anthocyanin synthesis (Ishida et al., 2007). Therefore, in addition to MYB, bHLH, and WD40, proteins encoded by some bZIP and WRKY family genes are also important regulatory factors in the process of anthocyanin biosynthesis, and may be directly involved in anthocyanin accumulation in the leaves of red-leaved poplars.

MYB transcription factors are one of the largest families in plants, in which R2R3-MYB transcription factors play an important role in the regulation of anthocyanin synthesis (Naing and Kim, 2018). In poplars, *PtrMYB119* and *PtrMYB6* can positively regulate the synthesis of anthocyanin (Cho et al., 2016; Wang et al., 2019c), while *PtrMYB182* played a negative regulatory role (Yoshida et al., 2015). However, we did not find R2R3-MYB transcription factors in the results of protein-protein interaction analysis, which may be due to R2R3-MYB transcription factors did not play a leading and critical role in anthocyanin accumulation in red-leaved poplar leaves. The red-leaved poplars are bud mutations of ‘Zhonglin 2025’, and its leaf redness is caused by gene mutation. Therefore, the screening of mutant genes in red-leaved poplars and how the three transcription factors (HY5, HYH and TTG2) screened above regulate the anthocyanin accumulation in red-leaved poplar leaves need for further in-depth research.

Conclusion

In this study, we first measured pigment content in the leaves of both red-leaved poplars and ‘Zhonglin 2025’ and found that the proportion of anthocyanin increased significantly in the leaves of both red-leaved poplars. We screened 6,792 DEGs and 2,786 DEPs in ‘Quanhong’ and 4,398 DEGs and 2,333 DEPs in ‘Xuanhong’, respectively. Based on the results of integrated analysis, 15 and 11 differentially expressed genes/proteins involved in anthocyanin synthesis were further screened in ‘Quanhong’ and ‘Xuanhong’, respectively. In the protein-protein interaction network analysis, three transcription factors (HY5, HYH, and TTG2) interacted with several structural genes involved in anthocyanin biosynthesis, and may directly participate in and regulate anthocyanin synthesis in the leaves of red-leaved poplars. The results of this study lay a foundation for further exploring the molecular mechanism of leaf red coloration in red-leaved poplars.

Declarations

Funding

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Competing interests

The authors declare that they have no conflict of interest.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Code availability

Not applicable.

Availability of data and material

All relevant data are within this article and its additional files.

Author contributions

JZ and MY designed the experiments. XC and HL carried out the experiments and wrote the manuscript. SW and CZ revised the manuscript. All authors have read and approved the manuscript.

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Figure legends

Figure 1. Comparative analysis of the pigment content in the leaves of ‘Zhonglin 2025’ (ZL2025), ‘Quanhong’ (QH) and ‘Xuanhong’ (XH). (A) comparison of the three poplar leaves; (B) ChI a content; (C) ChI b content; (D) carotenoid content; (E) anthocyanin content.

Figure 2. Analysis of differentially expressed genes (DEGs). (A) the statistics of DEGs of QH/ZL2025 and XH/ZL2025; (B) the venn diagram of the number between QH/ZL2025 and XH/ZL2025; (C) KEGG Pathway enrichment analysis of DEGs in QH/ZL2025; (D) KEGG Pathway enrichment analysis of DEGs in XH/ZL2025.

Figure 3. Analysis of differentially expressed proteins (DEGs). (A) volcano plot of differential expressed proteins in QH/ZL2025; (B) volcano plot of differential expressed proteins in XH/ZL2025; (C) KEGG Pathway enrichment analysis of DEPs in QH/ZL2025; (D) KEGG Pathway enrichment analysis of DEPs in XH/ZL2025.

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Figure 5. Anthocyanin metabolism pathway and structural gene expression in the QH/ZL2025 and XH/ZL2025. PAL: Phenylalanine ammonia-lyase; C4H: Trans-cinnamate 4-monooxygenase; 4CL: 4-coumarate:CoA ligase; CHS: Chalcone synthase; CHI: Chalcone isomerase; F3H: Flavanone-3 β -hydroxylase; F3'H: Flavonoid-3'-hydroxylase; DFR: Dihydroflavonol reductase; ANS: Anthocyanin synthase; UFGT: UDP-glucose flavonoid 3-glucosyltransferase; GT/MT/AT: Glucosyltransferase/Methyltransferase/Acyltransferase; GST: Glutathione S-transferase.

Figure 6. qRT-PCR validation of differential gene expression. All data are presented as the mean $\pm$ SEM. (*, $P < 0.05$; **, $P < 0.01$).

Figure 7. Protein–protein interaction network constituted by protein sequences of differentially expressed transcription factors and structural genes controlling anthocyanin synthesis in ‘Quanhong’ (A) and ‘Xuanhong’ (B), respectively. The line color indicates the type of interaction evidence including known interactions, predicted interactions and other. The line thickness indicates the strength of data support and line shape indicates the predicted mode of action. The set organism: *Arabidopsis thaliana*.

634 The confidence level of minimum required interaction score parameters is set at 0.3 and other
635 parameters are default.

636

637 **Figure 8.** qRT-PCR validation of the expression of three transcription factors (HY5, HYH and TTG2).

638 All data are presented as the mean $\pm$ SEM. (*, $P < 0.05$; **, $P < 0.01$).

Figures

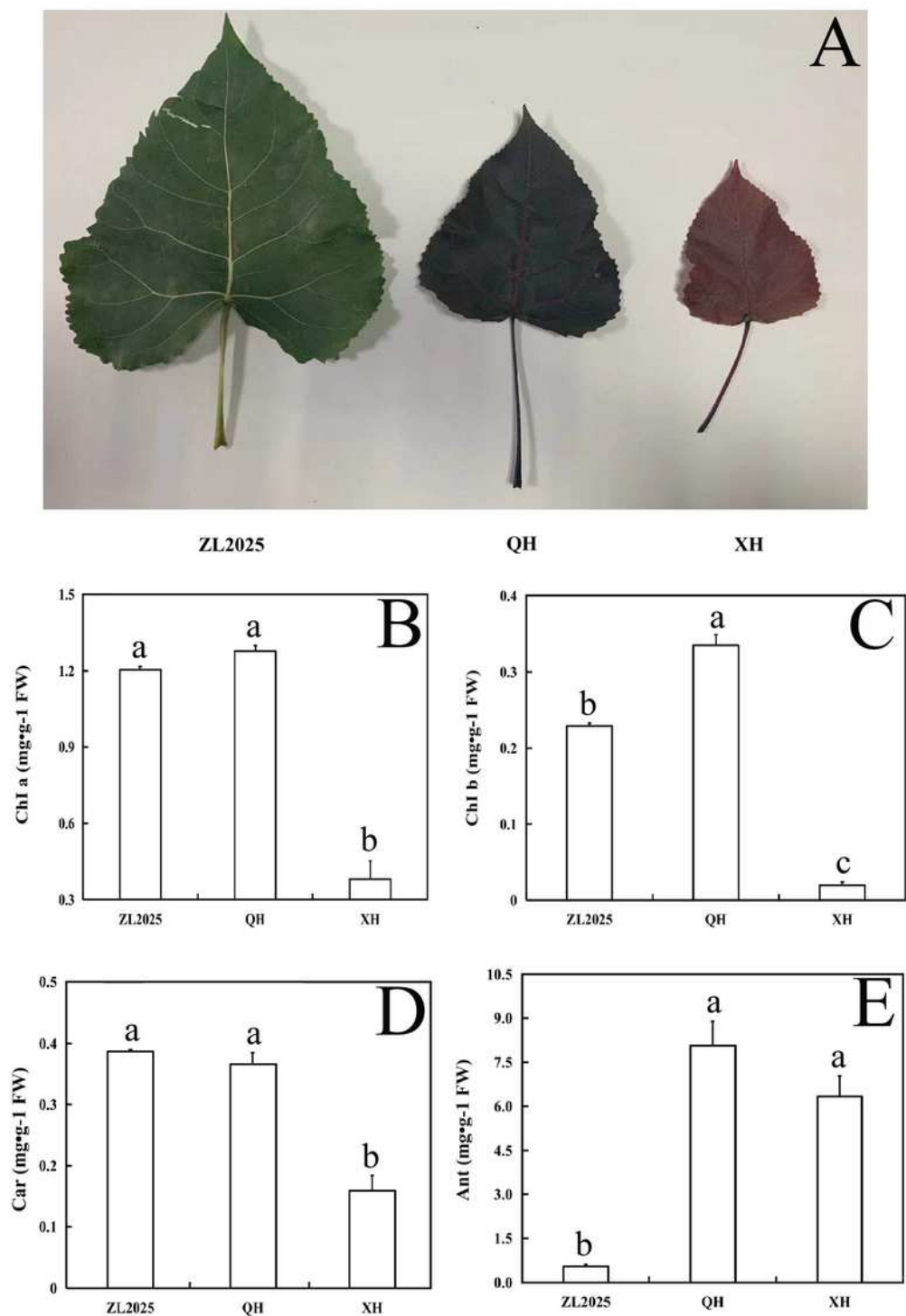


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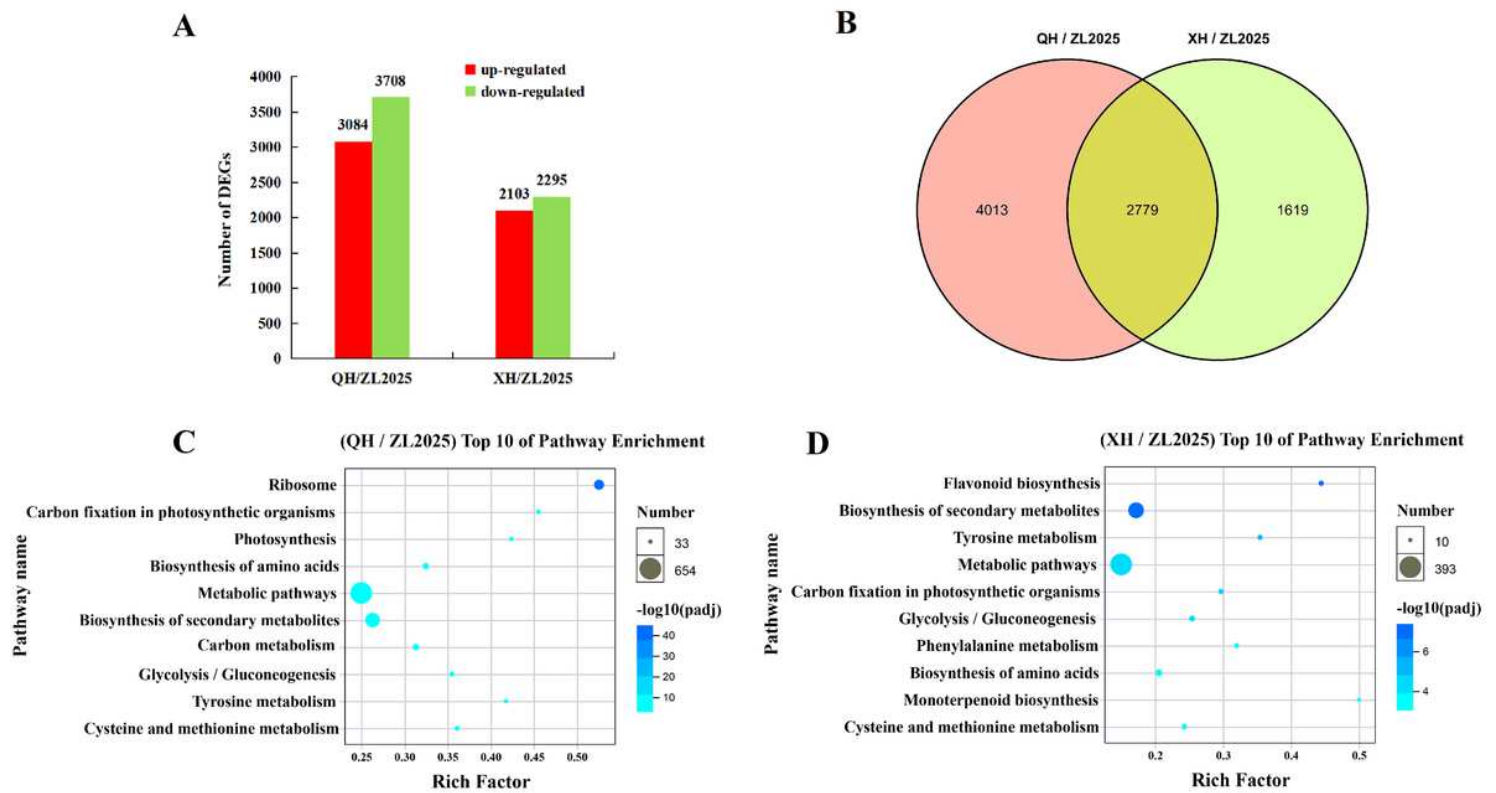


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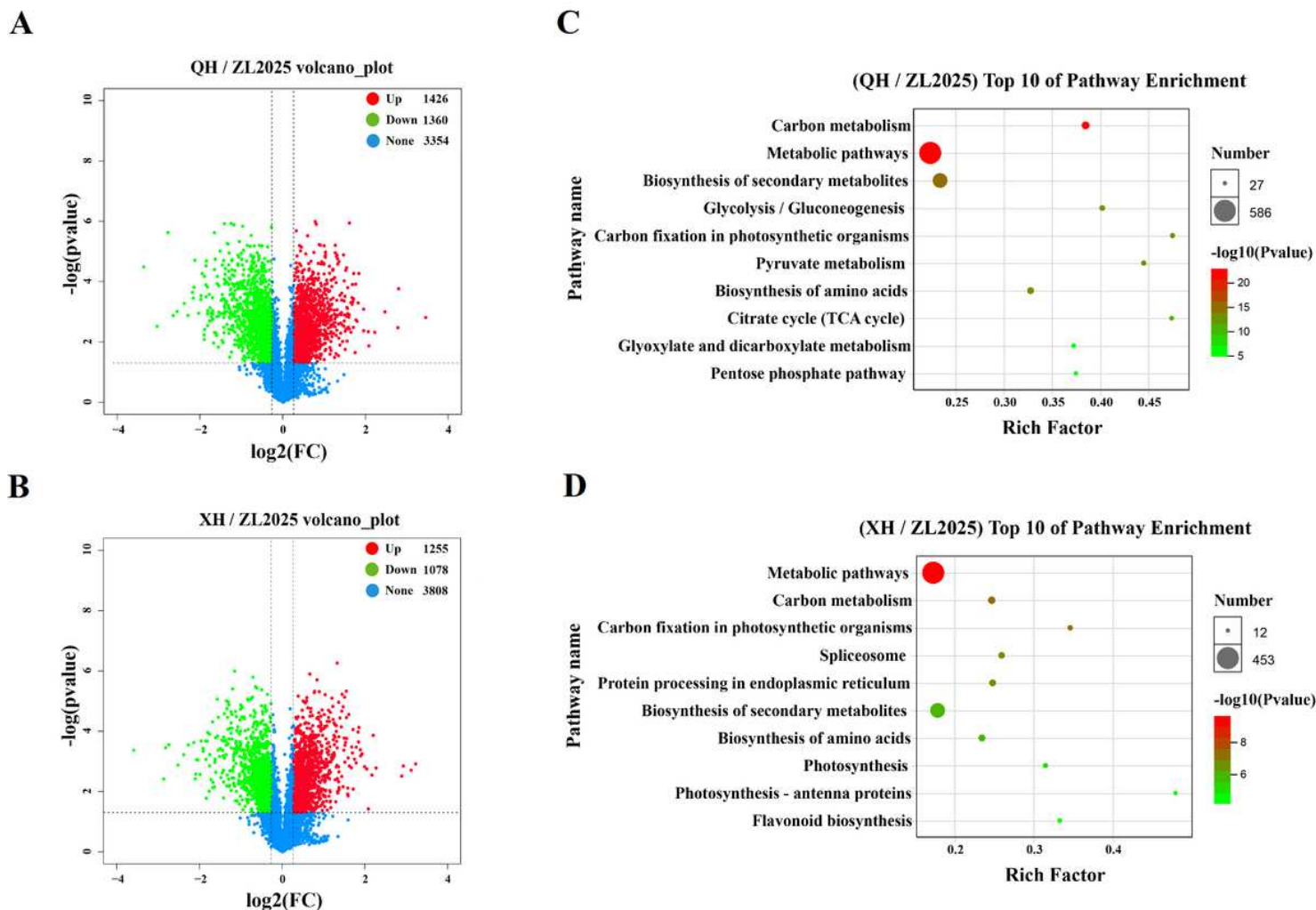


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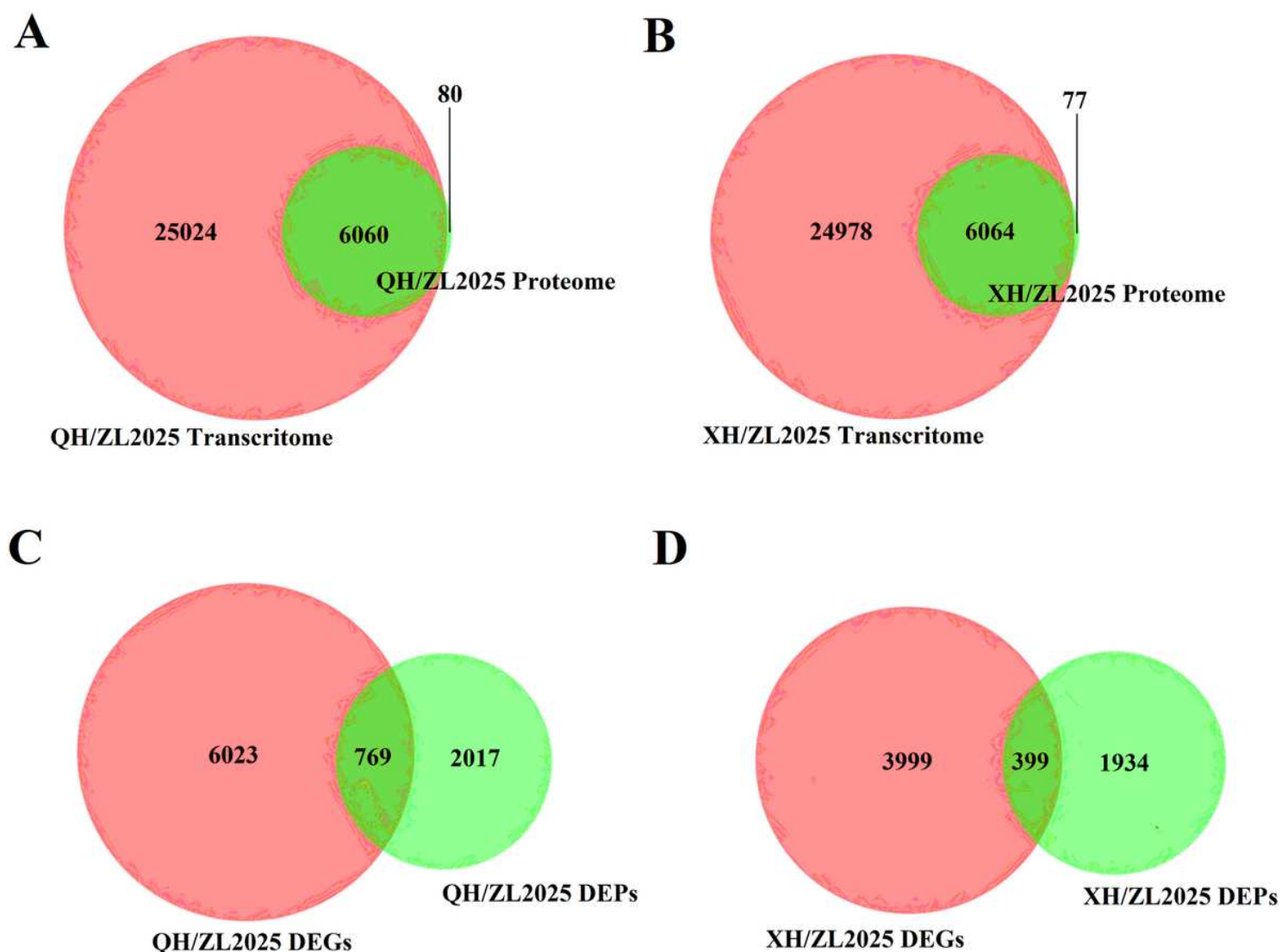


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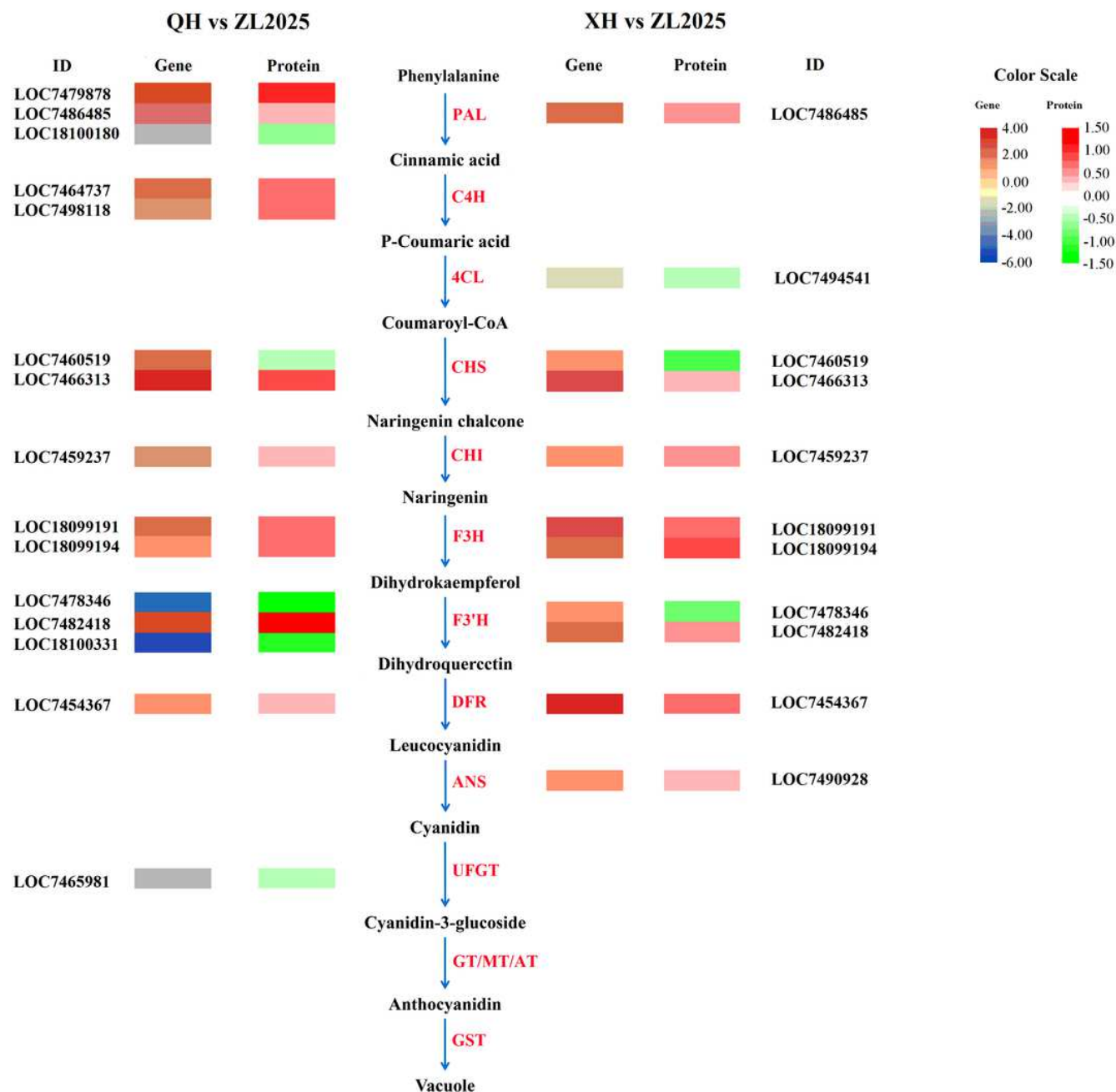


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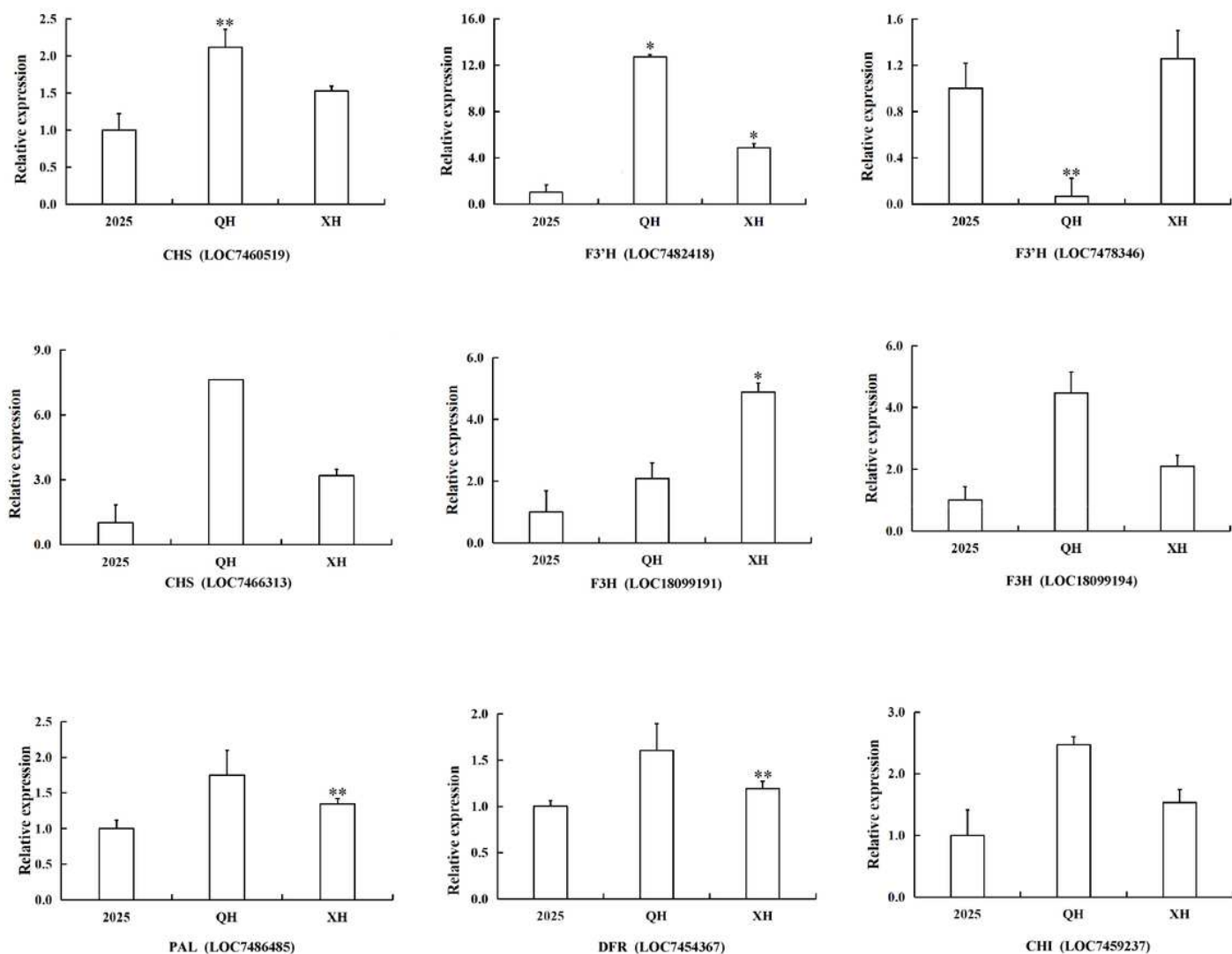


Figure 6

qRT-PCR validation of differential gene expression. All data are presented as the mean $\pm$ SEM. (*, $P < 0.05$; **, $P < 0.01$).

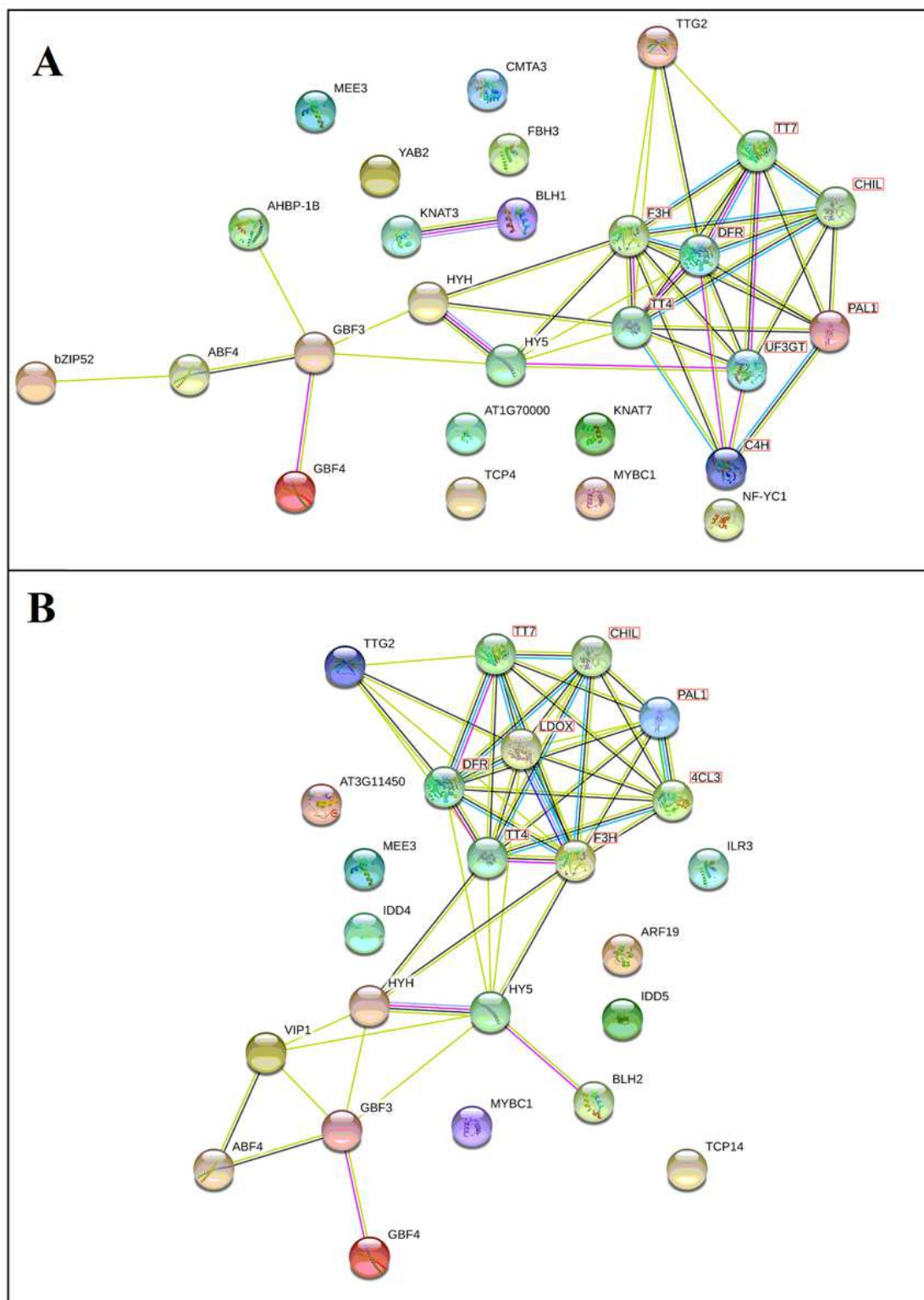


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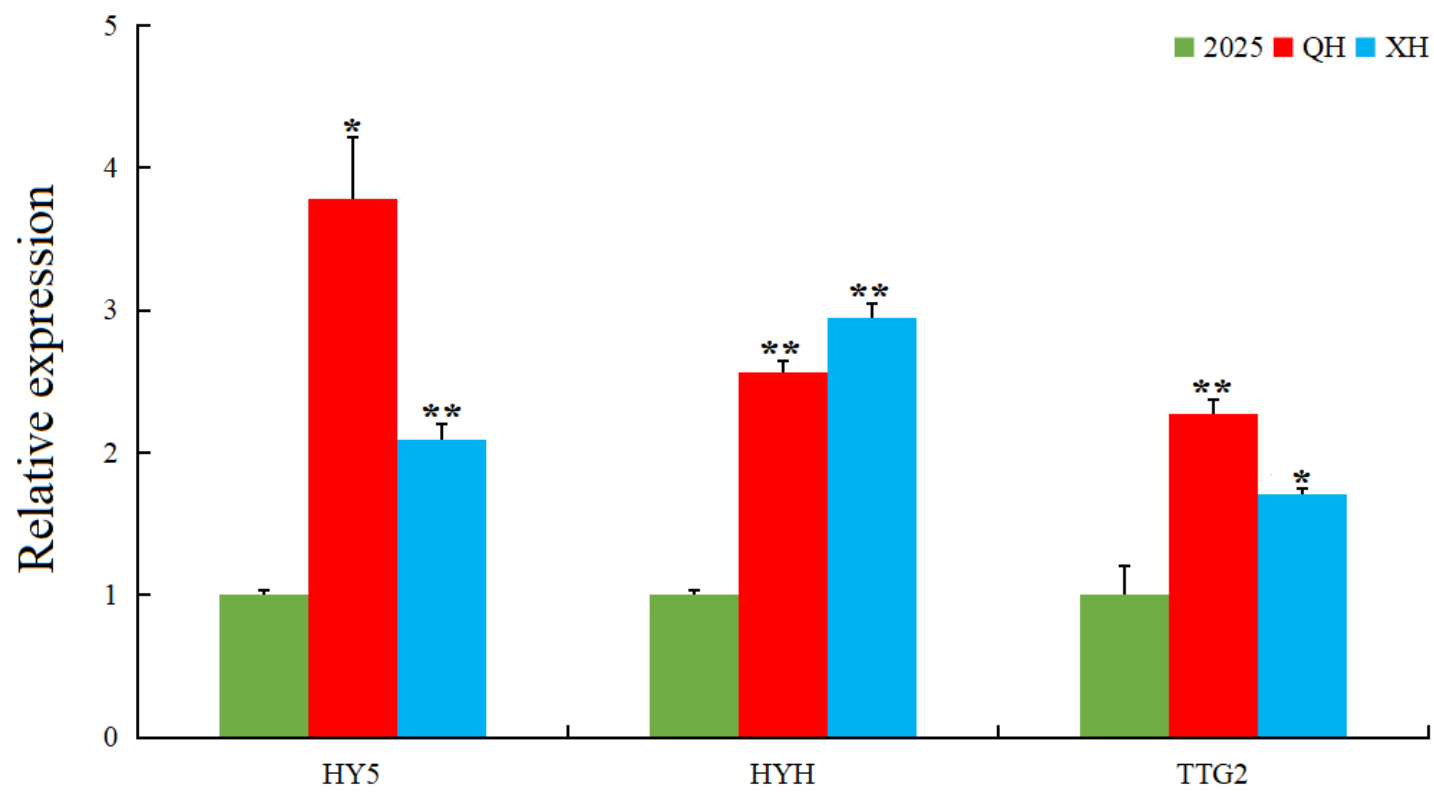


Figure 8

qRT-PCR validation of the expression of three transcription factors (HY5, HYH and TTG2). All data are presented as the mean $\pm$ SEM. (*, $P < 0.05$; **, $P < 0.01$).

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