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Comparison of Transcriptional Profiles of Flavonoid Genes and Anthocyanin Content During Fruit Development in Chinese Bayberry (*Myrica Rubra* Sieb. & Zucc.)

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Anthocyanins in the fruit of the Chinese bayberry (Myrica rubra Sieb. & Zucc.) are a major focus of research. In the current study, we investigated anthocyanin, total flavonoid, and phenolic acid profiles, as well as the expression of genes involved in anthocyanin biosynthesis, during fruit development in eight cultivars of Chinese bayberry. Total flavonoid and phenolic acid levels were relatively higher in green stage fruits, decreased in turning stage fruit, and increased in ripe fruit. Anthocyanin profiling revealed a contrasting pattern; anthocyanin levels were lower during fruit ripening and increased sharply in mature fruit. Expression profiling of anthocyanins showed coordinated expression of multiple anthocyanin biosynthesis genes during the development process. However, different genes played a predominant role at different stages. Flavanone-3-hydroxylase and flavonoid-3′-hydroxylase played predominant roles in the accumulation of total flavonoids and phenolic acids during the early stage of development, whereas chalcone synthase played a predominant role in the accumulation of anthocyanins during the ripening stage, implying regulation of anthocyanins varies with development stage.

1. Introduction

Myrica rubra Sieb. & Zucc. (Chinese bayberry), one of the six Myrica species native to China, has become the most economically important fruit crops in southern China because of its high anthocyanin content. It has been reported that anthocyanins from Chinese bayberry can protect β-cells from oxidative stress-mediated injury (Zhang et al., 2013) and play important roles in promoting good health and reducing the risk of chronic diseases (Philpott et al., 2004). In addition to their health benefits, anthocyanins in Chinese bayberry contribute to fruit quality and ultimately influence consumer acceptance (Liu et al., 2013; zhang et al., 2008). Whereas increasing anthocyanin content is essential for improving fruit appearance and nutritional quality, while understanding the genetic changes underlying variations in pigment levels is the firest crucial for solving this problem.

Anthocyanins are synthesized by a series of structural genes encoding chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H), flavonoid-3′-hydroxylase (F3′H), flavonoid-3′,5′-hydroxylase (F3′5′H) dihydroflavonol-4-reductase (DFR), anthocyanidin synthase (ANS), and UDP glucose:flavonoid-3-O-glucosyltransferase (UFGT) (Holton and Cornish, 1995). These structural genes are always regulated by a heterotrimeric complex composed of a MYB transcription factor (TF), a basic helix-loop-helix (bHLH) TF, and a WD40 protein (Spelt et al., 2000; Schwinn et al., 2006). It has been reported that the TF MrMYB1 is involved in anthocyanin accumulation in Chinese bayberry fruits, and a nonsense mutation in the MYB1 protein is responsible for no or low expression of MYB1 in white and red fruits (Niu et al., 2010). However, it is currently not known how TFs of MYB1 regulate the structural genes or which genes play the primary role during different stages of development.

In this study, we conducted differential gene expression profiling of eight *Myrica* species through four different stages of fruit development. In parallel, the accumulation of anthocyanins, total flavonoids, and total phenolic acids was quantified, and the relationships between the transcriptional profiles and flavonoids present in the

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eight cultivars were examined, the aim of this work was to determine the expression pattern of anthocyanin genes during fruit development.

2. Material and methods

2.1 Plant material and treatments

Myrica rubra Sieb. & Zucc. belongs to the genus Myrica in the family Myricaceae. Eight cultivars of Myrica ((C1: Toukuiao, a subspecies of Biqi; C2: Wanqi, a subspecies of Biqi; C3: Dongkui, a subspecies of Dongkui; C4: Zaoqi, a subspecies of Biqi; C5: Donglingwumei, a subspecies of Tanmei; C6: Biqi, a subspecies of Biqi; C7: Yongjiabaiyangmei, a subspecies of white Myrica; C8: Yuanqiaoshuimei, a species of Tanmei)) were collected at four developmental stages (S1: young fruit period; S2: stone hardening period; S3: turning color period; and S4: mature period; 28, 44, 60, and 76 days after full bloom, respectively) from the Zhejiang Citrus Research Institute in Taizhou City (Zhejiang Province, China).

Fruit flesh was cut into small pieces, immediately frozen in liquid nitrogen, and stored at -80 °C for RNA extraction and other analyses. For physical and chemical analysis, 1 g of frozen fruit sample was ground and extracted in 10 mL of methanol acidified with 1.0% HCl at 24 °C for 24 h. The extract was centrifuged at 5,000× g for 10 min and the supernatant was used for analysis of phenolic acids, flavonoids, and anthocyanins. Analyses were conducted in triplicate.

2.2 Determination of total anthocyanins

Total anthocyanin concentration was determined using a modified pH differential method, as described previously (Wolfe et al., 2003). A general spectrophotometer (T6 New Century, Purkinje General Instrument Co., Beijing, China) was used to measure the absorbance of the extraction solution in buffers of pH 1.0 and pH 4.5 at 520 nm and 700 nm, respectively. Absorbance readings were converted to total mg of cyanidin-3-glucoside per g dry weight of bayberry using a molar extinction coefficient of 26,900 and a molecular weight of 449.2. Total anthocyanins = $A \times MW \times B \times 100 \times V/\xi$, where $A = [(A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5}]$, MW indicates molecular weight, B indicates dilution ratio, V indicates final volume, and ξ indicates molar extinction coefficient. Three replicates were performed for each analysis.

2.3 Determination of total flavonoids

Total flavonoids were determined using the aluminum chloride colorimetric assay (Jia et al., 1999). A known volume of extract was placed in a 10 mL volumetric flask and diluted to 5 mL with distilled water. Then, 0.3 mL $NaNO_2$ (1:20) was added, followed by 3 mL of $AlCl_3$ (1:10) after 5 min, 2 mL of 1 M NaOH was added after a further 6 min, and distilled water was added for a total volume of 10 mL. The solution was mixed again, and a general spectrophotometer (T6 New Century, Purkinje General Instrument Co.) was used to measure absorbance at 510 nm. The total flavonoid content was expressed as mg rutin equivalents per g fresh mass. Samples were analyzed in three replicates.

2.4 Determination of total soluble phenolic acid content

Total phenolic acid content was estimated using the Folin-Ciocalteu colorimetric method (Zhang et al., 2008). The extract of ripe fruit was diluted ten times, and the extract from fruit of other maturities and cultivars was diluted five times. The diluted extract (0.5 mL) was oxidized with 0.5 mL of 0.5 M Folin-Ciocalteu reagent and methanol was used as the extracting solvent. The reaction was neutralized with 1 mL saturated sodium carbonate (75 g/L). After 2 h at room temperature, absorbance was read at 760 nm using a spectrophotometer (T6 New Century, Purkinje General Instrument Co.). Gallic acid was used as a standard, and results were expressed as mg gallic acid equivalent (GAE)/100 g of fresh weight.

2.5 Gene expression analysis

Total RNA was extracted from fruit using an RNAiso Plus kit (TaKaRa, China), and cDNA synthesis was performed using the PrimeScript™ reagent kit with the gDNA Eraser (Perfect Real Time) reverse transcription system (TaKaRa), using gene-specific primers reported by Niu et al. (2010), all according to the respective manufacturer's protocols.

All reactions were performed using SYBR Green Master Mix (SYBR® Premix Ex Taq™, TaKaRa) according to the manufacturer's protocol, using an Illumina Eco qPCR system. Reactions were performed in triplicate using 12.5 μL of 10× buffer, 0.4 M of each primer, 2 μL of diluted cDNA, and nuclease-free water to a total volume of 20 μL. The real-time quantitative PCR (qPCR) conditions were as follows: preincubation at 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s. Fluorescence was measured at the end of each annealing step.

qRT-PCR reactions were normalized using the Ct value corresponding to the reference gene (actin) to minimize variation in cDNA template levels. Cultivar C7 at S1 was selected as the calibration sample. Relative

expression levels were quantified using the $2^{-\triangle\triangle}$ method (Pfaffl, 2001). qPCR data are technical replicates with error bars, representing mean ± SE (n = 3).

2.6 Statistical analysis

All data are shown as mean ± SD. Data were subjected to analysis of variance (ANOVA) using SPSS software (SPSS Statistics 17.0). The statistical significance of differences was calculated using Student's t-test for single comparisons or using an ANOVA for multiple comparisons. The criterion employed for statistical significance was $p < 0.05$.

3. Results

3.1 Concentration of anthocyanins, total flavonoids, and phenolic acids

To study the patterns of anthocyanin, flavonoid, and phenolic acid accumulation during ripening of Chinese bayberry, total content of anthocyanins, flavonoids, and phenolic acids was quantified in eight cultivars(C1 to C8) during four developmental stages (S1 to S4). Anthocyanin accumulation was found to be low in the first three stages of development then increased sharply during S4, reaching approximately 85 mg 100 g^{-1} FW in the cultivar that showed the highest levels, C1. However, no anthocyanins were detected in the C7 cultivar at any stage of development (Figure 1A).

Total flavonoids showed a different pattern; levels were relatively high during S1 and S2, decreased in S3, and then increased during S4. Flavonoid content reached the highest levels of approximately 150 mg 100 g^{-1} FW during S4 in the C1 cultivar, which also showed the highest anthocyanin content among the eight cultivars (Figure 1B). Total phenolic acid accumulation showed an almost identical pattern to flavonoids, declining during the first three stages, and sharply increasing during S4 (Figure 1C).

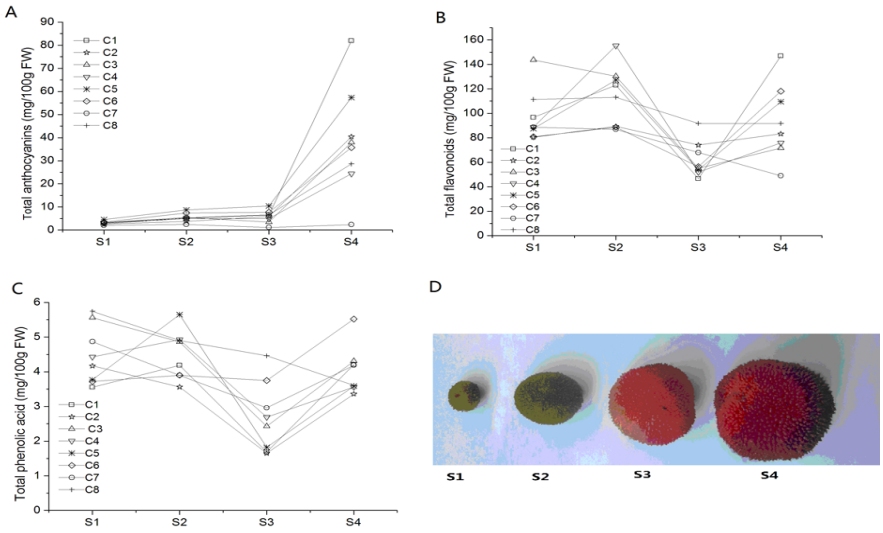

Figure 1: Total accumulation patterns of anthocyanin, flavonoids, and phenolic acid during various developmental stages. C1: Toukuiao, a subspecies of Biqi; C2: Wanqi, a subspecies of Biqi; C3: Dongkui, a subspecies of Dongkui; C4: Zaoqi, a subspecies of Biqi; C5: Donglingwumei, a subspecies of Tanmei; C6: Biqi, a subspecies of Biqi; C7: Yongjiabaiyangmei, a subspecies of white Myrica; C8: Yuanqiaoshuimei, a species of Tanmei; S1: young fruit period; S2: stone hardening period; S3: turning color period; and S4: mature period; 28, 44, 60, and 76 days after full bloom, respectively.

3.2 Expression of anthocyanin biosynthetic genes in different cultivars of Chinese bayberry

Expression of anthocyanin biosynthesis genes was studied in the eight cultivars during the fruit development process. The first step of the anthocyanin biosynthesis pathway is mediated by *CHS*. During the initial developmental stages (S1 and S2), expression of *CHS* was low, and during S3 and S4 transcription increased in all cultivars, especially in C5. Similar expression patterns were observed for *F3H, F3'H, DFR*, and *UFGT*, in almost all of the eight cultivars, although in the case of *ANS*, no significant increase was observed during fruit development—a unique pattern among the transcription profiles studied (Figure 2). The TF *MYB* showed a distinct transcription profile; a decrease in *MYB* expression was observed during S2, followed by a slight increase during S3 and S4 in all cultivars.

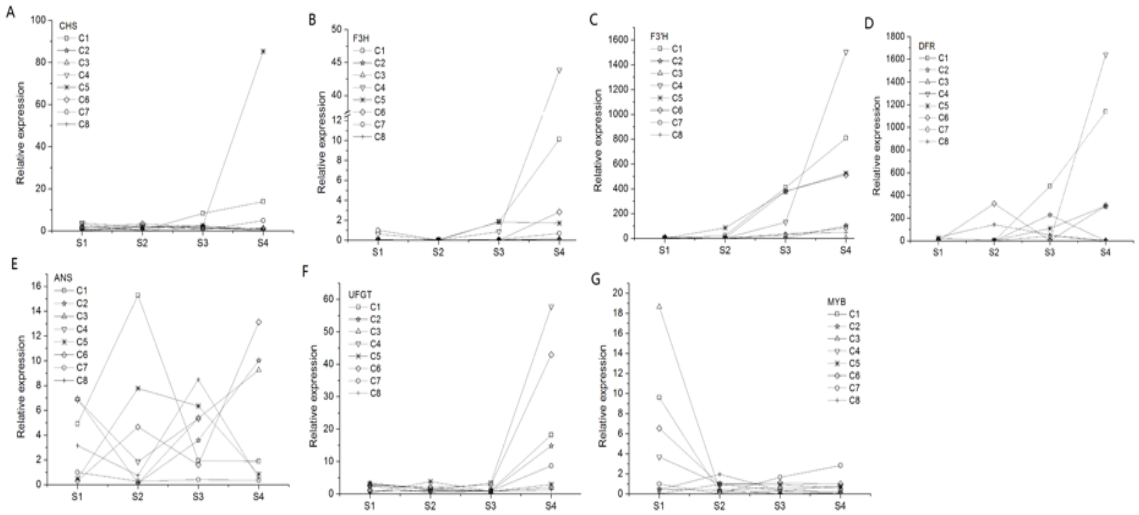

Figure 2: Transcriptional analysis of genes involved in anthocyanin biosynthesis during fruit development of Chinese bayberry. The expression profile of CHS(A); F3H(B);F3'H(C); DFR(D); ANS(E); UFGT(F); MYB(G)

Overall, almost all structural genes in the anthocyanin pathway in Chinese bayberry were up-regulated during development (Figure 2), in accordance with the increase in anthocyanin content from S1 to S4 (Fig. 1A). These results suggest a causal relationship between structural gene expression and anthocyanin accumulation.

The relationship between gene expression and flavonoid content was calculated among the eight tested cultivars at all four development stages. During S1, the expression level of *F3'H* was positively correlated with total flavonoid content (r = 0.8792; Fig. 3A) and total phenolic acid content (r = 0.59; Figure 3B). During S2, anthocyanin content was positively correlated with the expression of *F3H* (r = 0.8084), *UFGT* (r = 0.8814), and *MYB* (r = 0.448), whereas total phenolic acid content was positively correlated with the expression of *F3H* (r = 0.5459) and *MYB* (r = 0.4977). In contrast, total flavonoid content was negatively correlated with the expression of *DFR*. During S3, anthocyanin content was positively correlated with the expression of *CHS* (r = 0.315) and *F3H* (r = 0.533), and during S4, only expression of *CHS* was found to be positively correlated with anthocyanin content.

4. Discussion

In this study, we determined anthocyanin, flavonoid, and phenolic acid accumulation and the expression profiles of genes in the anthocyanin pathway in eight cultivars of Chinese bayberry during four developmental stages. Our parallel investigations at the gene and metabolite levels in eight different cultivars enabled us to assess the impact of genetic and developmental variation on flavonoid metabolism in Chinese bayberry, and the combined information from molecular and biochemical analyses provides an overall picture of flavonoid metabolism in Chinese bayberry.

Phenolic acid was present in the fruit throughout all of the developmental stages, and its concentration steadily declined from S1 to S3 and then slightly increased during S4, corresponding to a high level of flavonoids in the initial stage. This result implies that phenolic acid synthesis occurs primarily in the early stages of fruit development when sufficient pools of flavan-3-ols are produced for phenolic acid synthesis. It is generally assumed that high levels of phenolic acid in young fruit hinders consumption by seed-dispersers and functions as an antimicrobial to protect against pathogens and other fungi (Treutter, 2006; Petkov and Sotirov, 2013;) and the difference of flavonoids among those species may be caused by the difference of ecosystem in China (Liang, 2014). During S1, expression of *F3'H* was positively correlated with accumulation of total flavonoids and total phenolic acid, which indicates that high levels of F3′H transcripts account for the synthesis and accumulation of flavonols during the early stage of fruit development. F3′Hs catalyze the hydroxylation of the flavonoid B-ring at the 3′ positions, resulting in the formation of anthocyanin or flavonols (Liu et al., 2014). Thus, during the initial stages of development when anthocyanin content is very low, phenolic acid may be largely dependent on the regulation of flavonoid hydroxylase genes, which is consistent with our results in Chinese bayberry. In addition, similar phenomena have been observed to occur in the fruit

of apple, where the phenolic acid hydroxylation pattern is the result of high *F3'H* gene expression (Han et al., 2010).

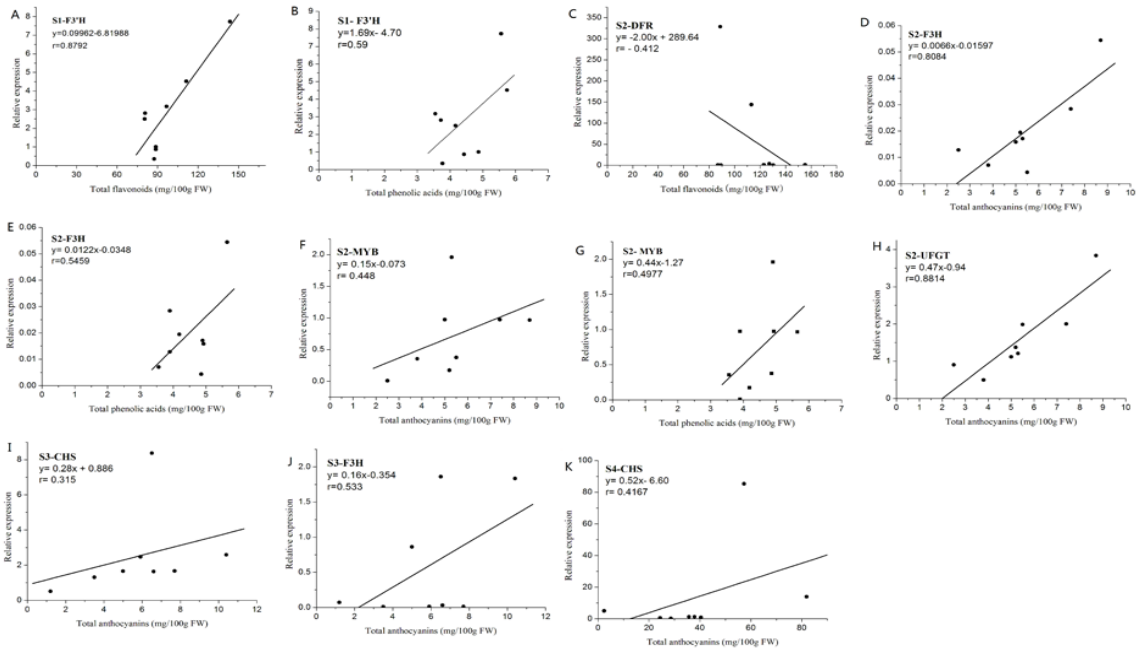

Figure 3. Correlation between concentration of anthocyanin, flavonoid, and phenolic acid, and the expression profiles of anthocyanin biosynthesis genes in Chinese bayberry at different stages ($p < 0.01$).

While phenolic acid content was greater during the initial stages of development in the current study, anthocyanin synthesis began during S3 and peaked during S4, and a similar pattern has been reported from strawberries (Carbone et al., 2009). Expression profiles of *CHS*, *F3H*, *F3'H*, *DFR*, and *UFGT* were parallel with the accumulation of anthocyanin, suggesting that the corresponding genes are involved in anthocyanin biosynthesis and likewise reflected in a concerted activation of anthocyanin gene expression. It should be noted that the expression of *F3'H* and *DFR* were the fastest growing genes during fruit development, possibly because the major anthocyanin present in Chinese bayberry fruits is cyanidin (more than 95% of total pigment) (Bao et al., 2005), which requires a high ratio of *F3'H/F3'5'H* (Streisfeld and Rausher, 2010). Expression of *F3'H* appears to determinate the component of anthocyanins. Although *F3'H* and *DFR* were the fastest growing genes during fruit development, accumulation of anthocyanin was only positively correlated with *CHS* expression (r = 0.4167, Figure 3K) during S4, thus, it is likely that *CHS* plays an important role in accumulation of anthocyanin.

The synthesis of anthocyanins, flavonoids, and phenolic acids is known to be regulated by TFs of MYB1 in Chinese bayberry (Niu et al., 2010). During S2 to S4, anthocyanin accumulation was parallel to increasing expression of *MYB1*, suggesting that anthocyanin is regulated by the TFs of MYB1, which is in agreement with previous results (Niu et al., 2010). However, the highest expression of *MYB1* was observed during the initial stage of fruit development, corresponding with high levels of phenolic acid. It is tempting to hypothesize that TFs of MYB1 not only activate anthocyanin synthesis at a later stage during fruit ripening, but that they also contribute to phenolic acid synthesis in the early stages of development by regulating expression of some phenolic acid-specific transcripts, such as anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR) (Zifkin et al., 2011). It is likely that this pathway shows temporal and spatial specificity of gene regulation; however, in order to elucidate this, further investigation is necessary to test the functionality and specificity of *MYB1* in the context of Chinese bayberry fruit development.

5. Conclusion

In conclusion, the current study found that accumulation of anthocyanins showing coordinated expression of multiple anthocyanin biosynthesis genes during the development process, but different genes played a predominant role at different stages, it showed that flavanone-3-hydroxylase and flavonoid-3′-hydroxylase played predominant roles in the accumulation of total flavonoids and phenolic acids during the early stage of

development, whereas chalcone synthase played a predominant role in the accumulation of anthocyanins during the ripening stage, implying that regulation of anthocyanins varies with development stage, it is likely that this pathway shows temporal and spatial specificity of gene regulation.

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