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The 3AT gene determines fruit color of *Myrica rubra* (Myricaceae)

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ABSTRACT. *Myrica rubra* has been cultivated for more than 2000 years it is one of most popular fruits in south China. We compared three *M. rubra* cultivars, "*Muye*" (MY), "*DongKui*" (DK), and "*Zao Jia*" (ZJ) to determine the causes of the differences in fruit color. We found changes in the anthocyanin and carotenoid contents during the fruit coloring and maturity phases. The anthocyanin contents of the three cultivars increased from the initial fruit coloring phase to the maturity phase. During this period, the total contents of anthocyanin and cyanidin in MY were higher than those in ZJ by 8.57 and 26.45% and higher than those in DK by 80.16 and 129.37%, respectively. RNA-Seq based transcriptome analysis of veraison and mature berries of the three *M. rubra* cultivars was conducted. The total numbers of genes and N50 lengths were 33,033 and 1,426 for MY, 35,166 and 1,145 for DK, and 31,374 and 476 for ZJ, respectively From the initial color-turning phase to the maturity phase, there were three up-regulated and three down-regulated genes in MY, while there were 3,058 up-regulated and 65 down-regulated genes in DK and 1,484 up-regulated and 1,169 down-regulated genes in ZJ. Based on functional insight of the differentially expressed genes, 3-Amino-1,2,4-triazole (3AT) would be the key gene responsible for color variation in these three cultivars. We concluded that the 3AT gene controls *M. rubra* berry color. Based on qRT-PCR, we found that expression of 3AT was much higher in MY and ZJ. This result was consistent with our RNA-Seq analysis. In conclusion, cy-3glu and

peonidin are pigments that have a significant effect on the color of *M. rubra* fruit, and 3AT is a functional gene that regulates these pigments. These results could have practical significance for *M. rubra* selection strategies.

Key words: *Myrica rubra*; Chinese bayberry; Fruit color; 3AT gene

INTRODUCTION

Bayberry (*Myrica rubra*) has a cultivated history of more than 2000 years; it is one of six *Myrica* species native to China, and the wild-growing history for this plant goes back about 7000 years (Fang et al., 2006a). The fruit has a unique sweet-sour taste and exquisite flavor and ripens in the hot and rainy season from May to July (Fang et al., 2006b). Chinese bayberry is a rich source of anthocyanins (Zhang et al., 2015). C3G is the most abundant of these (Sun et al., 2012). The colors of Chinese bayberry are produced by anthocyanins that accumulate with the increasing ripening stages (Shi et al., 2014). Anthocyanin synthesis is highly dependent on light conditions, and ultraviolet (UV) light from 280 to 320 nm (UV-B) reportedly has a strong effect on anthocyanin synthesis (Arakawa et al., 2019). Anthocyanins provide colors ranging from salmon-pink through red and from violet to nearly black in a variety of plant sources (Bao, 2005). All anthocyanins expressed red-pink hues (330°-13.2°) in acidic pH and blues (230°-262°) in alkaline pH (Sigurdson et al., 2019).

Anthocyanins have proven to be good for human health (Sulc et al., 2017; Vio Michaelis et al., 2019); they are secondary plant metabolites of the flavonoid family. As a potential radical scavenger (Rajan et al., 2018), antioxidant activity is highly correlated with phenolic contents (Chen et al., 2016), demonstrated DNA-protective, antiproliferative (Diaconeasa et al., 2015), anti-inflammatory (Mackert et al., 2016; Abdel-Aal et al., 2018), cardio-protective (Rupasinghe et al.,2018), anti-thrombotic chemo-preventive (Olivas-Aguirre et al., 2016), and antioxidant effects (Bakuradze et al., 2019) and prevents insulin resistance in diabetic patients (Li et al., 2015) both in vitro and in vivo. Anthocyanins from Chinese bayberry extracts could decrease graft apoptosis after transplantation (Zhang et al., 2013) and protect β cells from H_2O_2-induced cell injury (Zhang et al., 2011). C3G isolated from mulberry fruit has potential for the prevention of diabetes by preventing oxidative stress-induced β-cell apoptosis (Lee et al., 2015). C3G has potential as an anti-cancer agent (Cho et al., 2017) against gastric adenocarcinoma cells (Sun et al., 2012). Anthocyanins (ACNs) are a group of polyphenolic pigments The balance between biosynthesis and degradation determines its accumulation, which results in increased resistance to chilling and pathogens (Sivankalyani et al., 2016; Liu et al., 2018).

Conventional solvent extraction (CE), ultrasound-assisted extraction (UE) (Wang et al., 2016) and high-speed counter-current chromatography (HSCCC) (Sun et al., 2012) were used in the extraction of anthocyanins. Analytical methods for anthocyanin content include NIR hyperspectral imaging (Chen et al., 2015), ultra-performance liquid chromatography with triple quadrupol mass spectrometric (UPLC-QqQ-MS/MS) (Lukic et al., 2019), UPLC-DAD-MSE (Strauch et al., 2019), UHPLC-PDA-fluorescence (Diaz-Garcia et al., 2013), LC-ESI-Q-TOF-MS (X. Zhang et al., 2015), LC-(+ESI)MS/MS (Sulc et al., 2017) and NMR analysis (Oliveira et al., 2019).

Anthocyanins are light-dependent and are not produced in the absence of light; both anthocyanin biosynthetic and regulatory genes are down-regulated greatly in the dark (Dong et al., 2019). The biological metabolic mechanisms of anthocyanin synthesis and color formation depend on the up-regulation of key genes encoding enzymes in the anthocyanin synthesis and sugar metabolic pathways. The stability of anthocyanins is closely correlated to the concentration and structure of the sugars, and the fructose is negatively correlated with anthocyanins preservation (Leng et al., 2013). The composition of sugars in mature bayberry was 69% sucrose, 13-17% glucose, and 10-14% fructose (Xie et al., 2005). When fructose was at a concentration of <5%, sucrose, glucose, and fructose all functioned to protect coloration. However, when the fructose concentration reached above 20%, the anthocyanin concentration dropped to 53.49% compared with the peak. To improve the antioxidative capacity when processing fruits and vegetables, the method of extracting co-pigments, such as VC, from the juice can improve the stability of pigments (Griesser et al., 2008).

In this study, we found that the "*Muye*" (MY) fruit was significantly different from that of the other cultivars, with reddish black color and strong antioxidant capacity. Accordingly, two related cultivars, "*DongKui*" (DK) and "*ZaoJia*" (ZJ), which mature earlier than the "*BiQi* "(BQ) by approximately 10 days, were selected to detect the relevant physical and chemical indicators of the important developmental stages. RNA-Seq detection technology was applied to explore the antioxidant activity of the fruits of the cultivars and to analyze the underlying mechanism of color changes.

MATERIAL AND METHODS

Three bayberry cultivars, *DongKui* (DK), *Muye* (MY), and *ZaoJia* (ZJ), were collected during the color-turning and maturity phases. *Dongkui* (DK) is a late-maturing variety; it originated in Huangyan, Zhejiang Province. The fruit is nearly round with purple-red flesh, the weight of single fruit was 25-40g. *Muye* (MY) was the midseason variety, which originated in Lanxi, Zhejiang Province. The fruit is round and dark in color and of the short round carneous column; the weight of single fruit was 15-18g. *ZaoJia* (ZJ) was the early maturing variety and a budding variety of *BiQi*, which originated in Lanxi, Zhejiang Province. The fruit is round and dark color of the round and blunt flesh column; the weight of a single fruit is 18-25g. For each cultivar, 3 kg of fruits were randomly collected from production trees over 10 years old and stored in a -20℃ freezer until required.

The cy-3glu (CC), delphinidin (DC), pelargonidin (PelC), peonidin (PeoC), and carotenoid standards were purchased from Sigma. Chromatography grade methanol was purchased from Tedia. All other reagents were produced in China and were analytically pure. Kit information: Trans Script One-Step gDNA Removal and cDNA Synthesis Super Mix Kit (Trans Bionovo Co., Ltd., Beijing, China).

Detection of Anthocyanins and Carotenoids

Extractions were performed in triplicate with a mixture of 0.1% concentrated hydrochloric acid and 80% methanol using three biological replicates. The specifics of the procedure were as follows: pipette 10 mL of the mixture containing 0.1% of concentrated hydrochloric acid and 80% of methanol to a 50 mL test tube. Weigh 1 g of fully ground,

fresh sample, and transfer to the test tube. Ensure all fruit extracted are fully soaked in the extraction solution. The sample-containing extraction solutions were kept in an incubator at 37ºC overnight (without light exposure). Each extraction was contained in quartz cuvettes and performed in triplicate. A mixture containing 0.1% concentrated hydrochloric acid and 80% methanol was used as a blank. Sample solutions were scanned at 400-800 nm using dilutions where necessary and the scans recorded. The scan results showed that anthocyanin had a maximum absorption at 530 nm. The absorbance of samples at the maximum absorption wavelength was measured and the anthocyanin content was calculated according toa previous report (Wang et al., 2006).

Analysis of Active Ingredients in Fresh Fruits

Agilent high-performance liquid chromatography (HPLC) was used to analyze Vitamin C (VC) and fructose according to the method described by (Wang et al., 2015). TSS was measured using an RA-620 saccharometer and pH was measured using an MP512-01 pH meter.

RNA-SEQ Transcriptome Sequencing

The research group and the sequencing company obtained the materials according to the requirements specified by Chen et al. (2016). The fruit collection and treatment before RNA-SEQ were performed independently by the research group. Sequencing was performed by Bio-Marker. The resulting data were analyzed and organized by the research group.

Quantitative PCR Amplification

A plant RNA extraction kit (DP432) from Tien Gen Biotech (Beijing) was used to extract RNA from bayberry fresh fruits. The cDNA reverse transcription kit with gDNA removed was used for cDNA transcription. For each cultivar, three biological replicates were extracted. Quantitative PCR primers for 3AT were designed and the primer sequences were as follows. F primer: TTCTACCTCTTGCATGCCCA, and R primer: GAAGCTGACATGAATGCCGT. The bayberry fruits cDNAs were first amplified using the primers. The PCR products were then sequenced to ensure that the primers only amplified 3AT genes. When performing quantitative PCR, a Light Cycler 96 (Roche) quantitative PCR system was used with FastStart Essential DNA Green Master Mix (Roche). The reference gene was the bayberry actin gene using the F primer: AATGGAACTGGAATGGTCAAGG, and the R primer:
GCCAGATCTTCTCCATGTCATCCCA.

Assembly of Sequencing Results

Repeated measures were performed for the fruits of the three cultivars. The ≥Q30 of the processes were all greater than 92.31%. Assembly analysis was then performed by Trinity as follows: the sequence reads were broken into shorter segments, called K-mers. The K-mers were then extended to long segments, known as contigs. The overlaps between

the contigs were exploited to obtain the components. Finally, the De Bruijn method and sequencing read message were used to recognize the transcript sequence from the components.

Unigene Function Annotation

The Unigene sequences were compared with databases such as NR using BLAST software. After predicting the Unigene amino acid sequence, data were compared to the Pfam database using HMMER to obtain the Unigene annotation information.

SSR and SNP Analysis

Unigene screens greater than 1 kB were subjected to SSR analysis using MISA software. The comparison software STAR designed for RNA-Seq was used to compare the reads and the Unigene sequences of each sample. The single nucleotide polymorphism (SNP) SNP Calling process for RNA-Seq was accomplished using GATK.

Gene Expression Analysis

BLAT was used to compare the reads and Unigenes measured from each sample. The expression level was estimated according to the comparison results. The expression abundances of the corresponding Unigenes were expressed using FPKM values.

Function Annotation and Enrichment Analysis for Differentially Expressed Genes

An analysis of differentially expressed genes across samples was performed using DE-Seq, and the gene set that was differentially expressed across two conditions was obtained. The screening standards for the process were as follows: FDR < 0.01 and fold change ≥ 2. Based on the expression of genes in different samples, functional annotation was performed for the differentially expressed genes that were identified. The differentially expressed genes across all samples that were annotated in the GO database were subjected to enrichment analysis using the topGO software.2.

RESULTS

Differences Color of three Bayberry Cultivars by Morphological Observation

To study the differences in color, mature bayberry fruits from three *Myrica rubra* cultivars, MY, DK and ZJ, were selected in this study for morphological observation and analysis of physical and chemical parameters. The results in Figure 1 showed that the fruits of the three *Myrica Rubra* cultivars differed significantly in color when they ripened. ZJ (Figure 1, left) is dark red, and maturation occurred approximately 10-15 days earlier than that of the *BiQi*. MY (Figure 1, middle) is red-black and matured two to five days later than the fruit of the *BiQi*. DK (Figure 1, right) is currently the world's largest fruit, with an average fruit weight of 25 g or more and a maximum fruit weight of 50 g or more, which is

three times that of other large-fruited varieties. Its color is red and the fruit mature approximately 15 days later than those of the *BiQi*.

Figure 1. Three bayberry cultivars (left→right ZJ, MY and DK) and the comparison of fruit fading between soaking in liquor for four years and 30d fruit.

Differences in Related Physical and Chemical Parameters.

Using a visible light-ultraviolet spectrophotometer, the content of anthocyanin in the fresh fruits was found to be 30.1498±2.7267 mg/100g in MY, 27.7658±1.4095 mg/100g in ZJ, and 16.7356±0.3753 mg/100g in DK. This result is consistent with the color of the mature flesh. The components of anthocyanin were detected by HPLC. For the Cy-3glu assay, the fruit content was 2046.04 μg/g for MY, 1618.53 μg/g for ZJ, and 892.15 μg/g for DK. The content in MY was 26.45% higher than that of ZJ by and 129.37% higher than that of DK and these differences were highly significant, as shown in Figure 2 (A, B). Measurements of carotenoid and chlorophyll showed the contents were increased by 2.05 and 174%, respectively, in ZJ, while the contents in DK were decreased by 47.95 and 13.05%, respectively, and the contents in MY were decreased by 40.04 and 37.78%, respectively, and these differences were also highly significant. In comparison with the same period, the measurements showed the quercetin content in MY was significantly higher than that in DK and ZJ from the fruit color-turning phase compared to the mature phase. During the color-turning phase, the contents were

13.8860 mg/g in MY, 0.6551 mg/g in DK, and 0.1724 mg/g in ZJ. Similarly, in the mature phase, although the content in MY was decreased to 8.7802 mg/g and the contents in DK and ZJ rose to 0.8948 mg/g and 0.7573 mg/g, respectively, there was still a difference of 9-10-fold. The results of the myricetin test were consistent with the above findings, with the content in MY being highly significantly different at both the color-turning phase and the mature phase compared to DK and ZJ. During the color-turning phase, the myricetin content in MY was as high as 0.2622 mg/g, while that in DK and ZJ was only 0.0730 mg/g and 0.1040 mg/g, respectively; at the mature phase, the myricetin content in MY was 1.2700 mg/g, while that in DK and ZJ was only 0.0641 mg/g and 0.8759 mg/g, respectively (Figure 3).

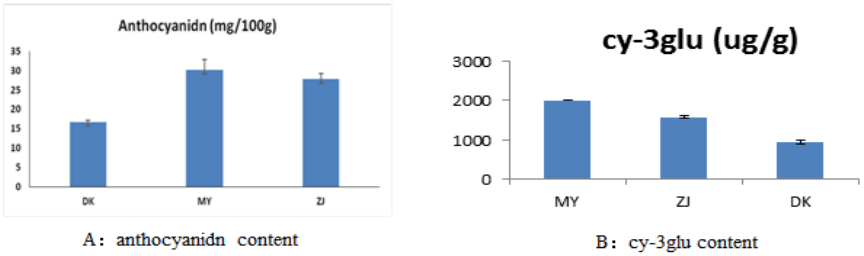

Figure 2. Determination of pigment content in ripe fruit of *Myrica rubra* by spectrophotometry.

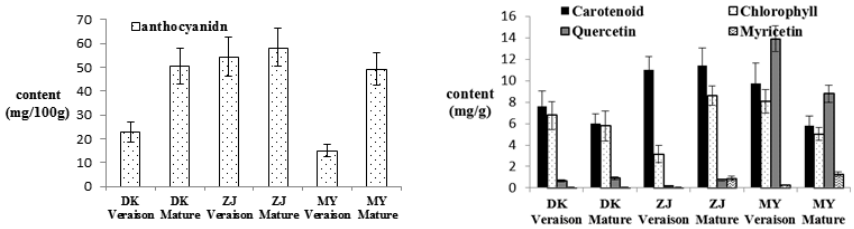

Figure 3. Detection of related active components in different developmental stages of bayberry fruit.

Besides, we also measured the three auxiliary pigments, alpha carotenoids, beta carotenoids, and lycopene (Table 1), and no alpha carotenoids or lycopene were detected. In the beta carotenoid assay, a concentration of 0.069 μg/mL and content of 0.3422 μg/g could be detected only in MY; none was detected in DK, and the content was only 0.0954 μg/g; no beta carotenoid could be detected in ZJ. Also, antioxidants such as VC, sugar and Ph were also measured. The VC content was 2.12 mg/100 g in the MY fruits, 4.20 mg/100 g in DK, and 1.79 mg/100 g in ZJ. Of all the sugars, sucrose accounted for 74.36% in MY, 76.20% in DK, and 75.68% in ZJ; glucose accounted for 12.62, 15.35, and 14.84%, respectively, in MY, DK, and ZJ; and fructose accounted for 9.47, 11.16, and 10.25%, respectively, in MY, DK, and ZJ. The results of the Ph test showed that the acid content in MY fruit was higher than that in the other two cultivars, suggesting that naturally occurring moderately acidic fruits contribute to the improvement of antioxidant activity. The pH of each cultivars was 2.63 for MY, 2.92 for DK, and 2.85 for ZJ.

Table 1. Changes in carotenoids content of *Myrica rubra* fruit flesh.

Material	α- Carotenoid		β- Carotenoid		Lycopene	
	Detection concentration	Content	Detection concentration	Content	Detection concentration	Content
DK	not detected	not detected	not detected	0.095421103	not detected	not detected
ZJ	not detected	not detected	not detected	not detected	not detected	not detected
MY	not detected	not detected	0.069	0.342261905	not detected	not detected

Note: *Dongkui* (DK), *Zaojia* (ZJ), *Muye* (MY), the same as below. The sample dilution volume was 10ml, and the sample quality was 2.03 + 0.02. Detection concentration: μg/ml, sample content: μg/g.

RNA-Seq Sequencing of the Mature Fruits of the Three Varieties.

The fruits of the three varieties at the color-turning and mature phases were collected and RNA-Seq technology was used to analyze the differentially expressed genes and identify the differences in expression of anthocyanin synthesis and regulatory pathway genes during fruit development in the different varieties of bayberry. In this experiment, two biological replicates for each sample were used. After sequencing, a total of 55.49 Gb clean data were obtained. The average data volume of each sample was 4.12 Gb, and the percentage of Q30 bases was 92.31% or higher. After quality control of these raw data, 99573 unigenes were obtained by splicing without relying on a reference genomes, including 37,621 1 Kb unigenes (Table 2). As the length range increased, the difference was highly significant. For example, when the length range was 200-300 bases, the percentage of unigenes was 39.58% in DK, 43.15% in MY, and 31.84% in ZJ, while the clustered results (all unigenes) of the three unigene libraries were 40.07%. When the length range was >2000, the percentage of unigenes was 6.64% in DK, 8.46% in MY, and 4.35% in ZJ, while the percentage of all unigenes was 8.93%.

Table 2. Statistical results of gene assembly for three *Myrica rubra* fruit transcriptomes.

Length Range	DK Unigenes	MY Unigenes	ZJ Unigenes	All Unigenes
200-300	13918(39.58%)	14253(43.15%)	9989(31.84%)	39898(40.07%)
300-500	8517(24.22%)	7557(22.88%)	5888(18.77%)	22742(22.84%)
500-1000	6291(17.89%)	5116(15.49%)	5336(17.01%)	16827(16.90%)
1000-2000	4103(11.67%)	3313(10.03%)	4430(14.12%)	11211(11.26%)
2000+	2335(6.64%)	2794(8.46%)	1364(4.35%)	8891(8.93%)
Total Number	35166	33033	31374	99573
Total Length	87,920,155	58,588,866	33,469,082	13,984,0189
N50 Length	1,145	1,426	476	1,445
Mean Length	678.64	717.70	48.534	75,020,35

Analysis of SSRs and SNPs Using RNA-Seq.

Gene structure analysis based on Unigene was performed, and the SSR analysis resulted in 18,872 SSR markers (Table 3) and following the screening, seven pairs of SSR primers were designed (Table 4). Through gene testing (using N50 length), the total number of genes in the color-turning phase was 35166and 1,145 and 33033, and 1,426 in the mature phase (Table 2). During the color-turning phase, the number of differentially expressed

genes and the Unigene ratios with the corresponding length interval were all significantly higher than those in the mature phase. The Unigene screens that were greater than 1 kB were subjected to SSR analysis using MISA software (Table 3). The SSR markers found were treated with Primer 3 for primer design (Table 4). The gene homozygosity of the three tested materials was significantly different, with 75.40% for MY, 67.39% for DK, and 68.52% for ZJ during the maturity phase (Table 5). Hence, the gene homozygosity of MY was 8.01% higher than that of DK and 6.88% higher than that of ZJ.

Table 3. Statistical results of SSR analysis for three *Myrica rubra* fruit transcriptomes.

Search Item	Number
Total number of sequences examined	37,621
Total size of examined sequences (bp)	83,952,494
Total number of identified SSRs	18,872
Number of SSR containing sequences	13,162
Number of sequences containing more than 1 SSR	4,060
Number of SSRs present in compound formation	1,130
Mono nucleotide	10,282
Di nucleotide	5,853
Tri nucleotide	2,554
Tetra nucleotide	124
Penta nucleotide	29
Hexa nucleotide	30

Table 4. SSR primer information for three *Myrica rubra* fruit transcriptomes.

Gene_ID	FPr1(5'-3')	Tm	Size	RPr1(5'-3')	Tm	Size	PSize	Start	End
CL1Contig3	TCTTCAAAGCTTCCTTCCCA	59.93	20	CTGCCGCAAGGTCTGAGTAT	60.42	20	230	220	449
CL1Contig9	TTTCCTCAACAGCCTTGTGTT	59.77	21	AATGGAATTGGGCAATGAAA	60.13	20	212	610	821
CL1Contig9	TTTCATTGCCCAATTCCATT	60.13	20	CCTTCTAGCTTGGAAGCCCT	59.98	20	247	802	1048
CL1Contig12	CAGAACCGGGCTACGTGTAT	60.02	20	CGTTGTCTCCTTATTCCCCA	59.93	20	228	2618	2845
CL1Contig22	ACCTCGATGACCGTGAGTTC	60.12	20	ATCCATCCTGACTGGCTGAC	60.08	20	232	6164	6395
CL1Contig22	CCACAACTTGCAACGAAGAA	59.88	20	CAACTTTATGCGCTCCCTTT	59.36	20	233	6500	6732
CL1Contig24	CTAACGGTCAGCTTTGGAGG	59.87	20	GGCACATCTAAACCAACCCA	60.76	20	279	761	1039
CL1Contig35	TTGAAGTGTGCCAAAATTGC	59.71	20	ACCCAATTATTTTCACCGCA	60.19	20	106	100	205

Table 5. SNP statistical table for *Myrica rubra.*

Samples	HomoSNP	HeteSNP	AllSNP
Dk Veraison	138244	62477	200723
Dk Mature	146405	70828	217233
MY Veraison	116720	35807	152527
MY Mature	138305	45106	183411
ZJ Veraison	94334	41076	135410
ZJ Mature	97102	44593	141695

Differentially expressed genes in the fruit maturity phase based on RNA-Seq data.

The 37,621 unigenes obtained by splicing were used as the reference genes to analyze the differentially expressed genes from the fruit color-turning phase to the mature phase in each cultivar. First, 37,621 unigenes were obtained for each sample's clean reads map, and the ratio of clean reads to mapped unigenes was different for the three samples, ranging from 79.25% (the lowest) to 86% (the highest) (Table 6). The FPKM values of each unigene at different phases were compared in pairs, and a total of nine comparisons were obtained (Table 7). The differences between them were highly significant for all varieties from the veraison to the mature phase. There were six AllDEGs in MY, including three up-regulated and three down-regulated genes; 3,123 AllDEGs in DK, including 3,058 up-regulated and 65 down-regulated genes; and 2,519 AllDEGs in ZJ, including 1,484 up-regulated and 1,169 down-regulated genes. The differences in the different varieties in the same phase showed the same trend. According to the analysis of the genetic differences between MY and ZJ during veraison, there were 110 AllDEGs, 75 of which were up-regulated and 35 of which were down-regulated. Similarly, during maturity, there were 1,069 AllDEGs, 577 of which were up-regulated and 492 were down-regulated. The genetic differences between MY and DK during veraison were analyzed. There were 1,516 AllDEGs, 902 of which were up-regulated and 614 of which were down-regulated. Similarly, at maturity, there were 4,913 AllDEGs, 1,648 of which were up-regulated and 3,265 of which were down-regulated. The analysis of the differences between DK and ZJ showed that during veraison, there were 2,683 AllDEGs, 1,085 of which were up-regulated and 1,598 of which were down-regulated. Similarly, during the maturity phase, there were 8,687 AllDEGs, 1,300 of which were up-regulated and 7,567 of which were down-regulated.

Table 6. Comparison between sequencing data and assembly results for *Myrica rubra.*

ID	Clean Reads	Mapped Reads	Mapped Ratio
Dk Veraison	20797309	17461762	83.93%
Dk Mature	18113934	15125138	83.50%
MY Veraison	17254397	13643431	79.25%
MY Mature	187005745	15641651	83.63%
ZJ Veraison	17611081	15146851	86.00%
ZJ Mature	17662147	14788813	83.64%

Table 7. Comparison of the number of differentially expressed genes in *Myrica rubra.*

DEG Set	All DEG	up-regulated	down-regulated
Dk Veraison to Mature	3123	3058	65
Dk Veraison Vs MY Veraison	1516	902	614
Dk Veraison Vs ZJ Veraison	2683	1085	1598
Dk Mature Vs MY Mature	4913	1648	3265
Dk Mature Vs ZJ Mature	8867	1300	7567
MY Veraison to Mature	6	3	3
ZJ Veraison to Mature	2519	1484	1169
MY Veraison Vs ZJ Veraison	110	75	35
MY Mature Vs ZJ Mature	1069	577	492

Eight coding genes (Table 8) were identified for a representative analysis of the sequencing results. Taking CL10003Contig1 as an example, the length was 1,021, depth was

59.4466, coverage was 0.99834, FPKM was 16.3756, and TotalReads and UniqReads were both 281. The expression effects and the complexity of this gene were the highest compared to the other six genes, while the second and third were CL10000Contig1 and CL10002Contig1 respectively. The detection results of CL10001Contig1 and CL10005Contig1 for depth, coverage, FPKM, TotalReads, UniqReads and MultiReads were all shown as 0. The TotalReads of CL10004Contig1 was only one. It should be noted that the function of CL10007Contig1 may be special. The MultiReads value for CL1007Contig1 was 1, while that of all other encoding genes was 0. Moreover, the TotalReads and UniqReads values of CL10007Contig1 were 8 and 7, while the other seven coding genes had the same values for these two reads.

Table 8. Comparison of gene expression results in *Myrica rubra.*

Gene_ID	Length	Depth	Coverage	FPKM	TotalReads	UniqReads	MultiReads
CL10000Contig1	2187	24.6955	0.9758	6.8287	251	251	0
CL10001Contig1	601	0.0000	0.0000	0.0000	0	0	0
CL10002Contig1	1045	3.4890	0.8987	1.0249	18	18	0
CL10003Contig1	1021	59.4466	0.9834	16.3756	281	281	0
CL10004Contig1	377	0.5385	0.5370	0.1578	1	1	0
CL10005Contig1	1313	0.0000	0.0000	0.0000	0	0	0
CL10006Contig1	654	2.6361	0.7588	0.8188	9	9	0
CL10007Contig1	1104	1.7826	0.6968	0.4312	8	7	1

To perform functional enrichment analysis of the differentially expressed genes in each phase shown in Table 7, annotation and molecular enrichment were performed using seven databases (Table 9): COG, GO, KEGG, KOG, Pfam, Swiss-Prot, and NR. The NR database showed the best annotation, followed by Pfam. Of the 114,651 unigenes in the total annotations, NR had 106,830 unigenes, accounting for 93.18%, of which the unigenes of ≥300 nt accounted for 95.36% and the unigenes of ≥1000 nt accounted for 98.96%. Based on the results of the functional comparisons of the above seven databases, topGO was used to perform the enrichment analysis (Table 10). Among the eight nodes, the KS node 2-alkenal reductase [NAD(P)] activity was 5.4e-20, which was the most significant, followed by the node non-membrane spanning protein tyrosine kinase activity, with a KS of 1.2e-12 (Table 9). Next, the results of the KEGG enrichment of the differentially expressed genes were analyzed. Of the eight pathway names, the metabolic pathway values (Q value) enriched in the ko00970 and ko00966 pathways were 1.2362e-01 and 2.6076e-01, respectively. Although the enrichment factors of the other six pathways were close to or exceeded the former two, their KS values were all 1.0000e+00, with a significant difference of 10 and 20 times (Table 11).

Table 9. Comparison of the number of differentially expressed gene annotations in *Myrica rubra.*

DEG Set	Annotated	COG	GO	KEGG	KOG	Pfam	Swiss-Prot	nr
Dongkui Veraison to Mature	3,123	1,501	1,829	1,292	2,370	2,616	1,970	3,106
Dongkui Veraison Vs MuYe Veraison	1,516	567	876	296	844	1,205	1,147	1,509
Dongkui Veraison Vs ZaoJia Veraison	2,682	1,192	1,705	795	1,632	2,168	2,067	2,675
Dongkui Mature Vs MuYe Mature	6	4	4	2	3	6	6	6
Dongkui Mature Vs ZaoJia Mature	4,913	2,181	2,829	1,583	3,380	4,023	3,461	4,864
MuYeVeraison to Mature	8,966	4,255	4,749	3,395	6,685	7,585	6,206	8,804
ZaoJia Veraison to Mature	2,519	999	1,541	480	1,239	1,960	1,936	2,516

Table 10. Differential gene expression topGO enrichment results (molecular function) in *Myrica rubra.*

GO.ID	Term	Annotated	Significant	Expected	KS
GO:0032440	2-alkenal reductase [NAD(P)] activity	323	21	25.49	5.4e-20
GO:0004715	non-membrane spanning protein tyrosine kinase activity	88	3	6.95	1.2e-12
GO:0004842	ubiquitin-protein transferase activity	343	36	27.07	5.2e-09
GO:0004713	protein tyrosine kinase activity	220	14	17.36	6.9e-08
GO:0004683	calmodulin-dependent protein kinase activity	108	6	8.52	1.8e-06
GO:0042973	glucan endo-1,3-beta-D-glucosidase activity	40	11	3.16	2.1e-06
GO:0016740	transferase activity	10891	837	859.56	2.5e-06
GO:0004672	protein kinase activity	2422	135	191.15	2.6e-06

Table 11. Summary of 8 pathway enrichment in *Myrica rubra.*

Pathway	KO	Enrichment_Factor	Q_value
Aminoacyl-tRNA biosynthesis	ko00970	0.73	1.2362e-01
Glucosinolate biosynthesis	ko00966	0.22	2.6076e-01
Valine, leucine and isoleucine biosynthesis	ko00290	0.76	1.0000e+00
RNA transport	ko03013	0.87	1.0000e+00
Phagosome	ko04145	0.81	1.0000e+00
Synthesis and degradation of ketone bodies	ko00072	0.57	1.0000e+00
Ribosome biogenesis in eukaryotes	ko03008	0.87	1.0000e+00
Nicotinate and nicotinamide metabolism	ko00760	0.67	1.0000e+00

Differential expression of the 3AT gene in Bayberry cultivars with different fruit colors

Based on the in-depth analysis of differentially expressed genes obtained from the RNA-Seq analysis of the mature fruits of the three bayberry cultivars, these differential genes were compared with the conserved genes in *Arabidopsis thaliana* and known anthocyanin synthesis pathway genes. It was found that a gene annotated with 3AT was differentially expressed in the three bayberry cultivars during the maturation phase. To verify the correctness of the transcriptome results, QRT-PCR was performed to verify the expression of the 3AT gene. The 3AT gene expression (FC) as detected by PCR was 1.0653±0.3969 in DK, 20.1219±2.7522 in MY, and 15.7536±1.5840 in ZJ. The content in MY was 18.9 times that of DK and 1.28 times that of ZJ, and these differences were highly significant (Figure 4). Since 3AT is a key gene in the anthocyanin synthesis pathway, by comparing anthocyanidin content in Figure 3 with 3AT expression in Figure 4, it is speculated that the differential expression of the 3AT gene may lead to differences in the color of the three bayberry fruits.

Changes in anthocyanin and carotenoid contents before and after soaking. *Myrica rubra* soaked in liquor not only has many functions, such as digestion, dehumidification, heat relief, Shengjin, cough relief, digestion and cold resistance, but also has the function of anti-cancer and softening blood vessels. It has a history of processing for more than 1000 years. Three varieties of DongKui (DK), Muye (MY) and ZaoJia (ZJ) were randomly picked at the turning and maturing stages for more than 10 years, and their fruits were 3 kg each, soaked in 52-degree rice liquor 2500 ml.

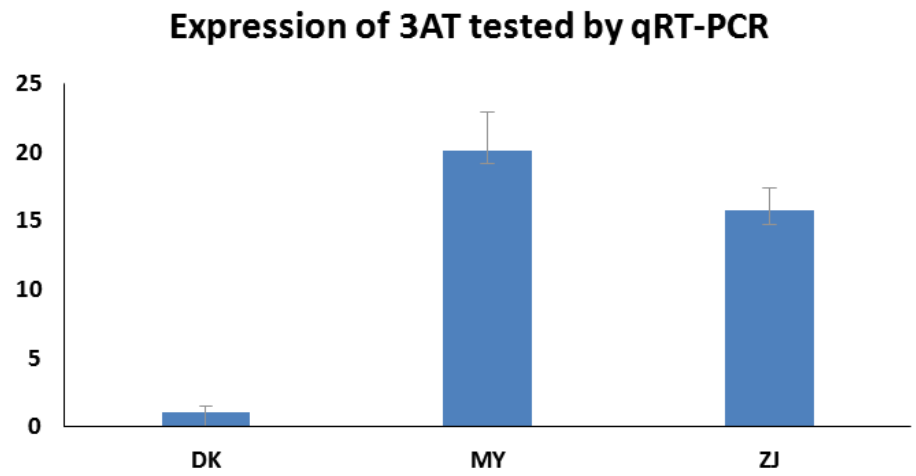

Figure 4. Expression of 3AT tested by qRT-PCR in the fruits of the bayberry cultivars.

Detection of anthocyanin, carotenoid, quercetin and Myricetin before and after soaking fresh fruit in the wine. The results showed that the highest content of anthocyanin (Figure 5A) in fresh fruit was MY with 78.74 mg/100g, meanwhile, the lowest degradation rate in alcohol was 24.12%. The contents of DK and ZJ were 64.74 mg/100g and 61.24 mg/100g, and degradation rates of 32.84 and 44.82% respectively. Carotenoid (Figure 5B) was the most abundant component in the fruit before and after soaking. The content of carotenoid in MY after soaking was 18.79% higher than that of DK and 28.67% higher than that of ZJ, respectively. The concentration and content of cyanidin were more than twice that of DK and ZJ before soaking, the content of cyanidin decreased significantly, only slightly higher than that of DK and less than that of ZJ after soaking. The concentration and content of delphinidin before immersion were more than twice that of the control. After immersion, the concentration and content of delphinidin were almost the same as that of ZJ, and the difference between delphinidin and DK was significant. Concentrations and contents of α-carotenoids and lycopene were not detected, β-carotenoids were detected only before MY immersion, with a concentration of 0.069 ug/mL (Table 12). As can be seen from Figure 6, vitamin C content of MY and ZJ fruits before soaking was less than twice that of DK, and was not detected after soaking. There is no significant difference in sucrose, glucose and fructose, and no varieties were detected after soaking. There were some differences in pH values between different varieties and MY was lower than the other two varieties before and after soaking. Overall, MY was more abundant in anthocyanins and functional components than the other two varieties. The morphological comparison also confirmed that MY bayberry was not easy to fade, and the wound healing rate of fresh fruits was high and the decay rate was low. The comparative experiment of soaking wine showed that the alcohol resistance of MY bayberry with a long duration was more remarkable than that of MY bayberry (Figure 1).

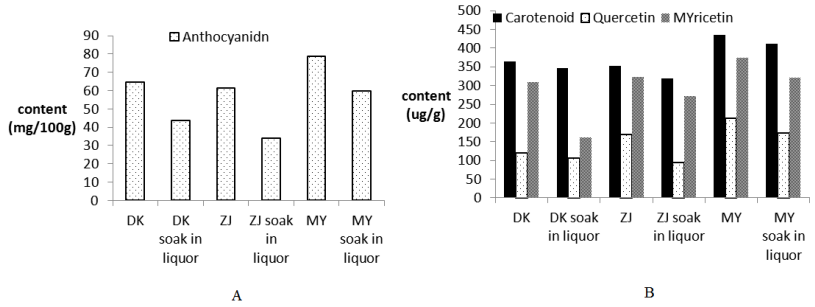

Figure 5. Different active ingredients content in bayberry fruit before and after liquor soaking.

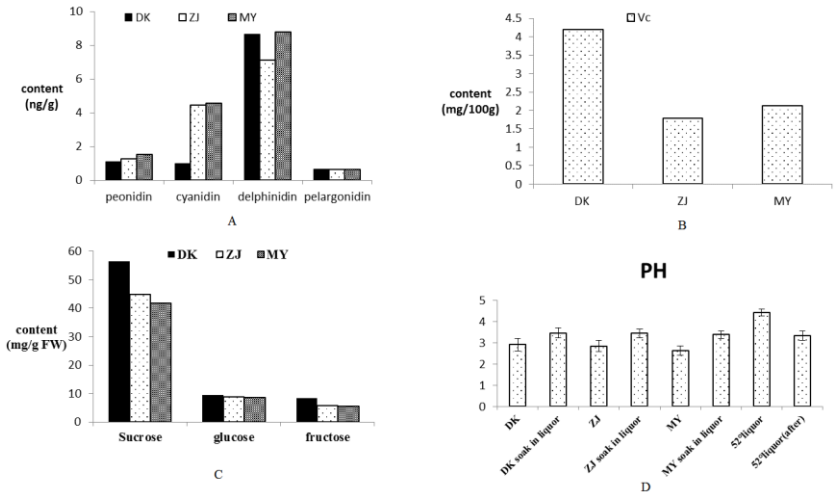

Figure 6. Comparison of VC, sugar and pH pigments content in bayberry fruit before and after liquor soaking.

Table 12. Carotenoid content changes before and after liquor soaking of the bayberry fruits.

Material	α- Carotenoid		β- Carotenoid		Lycopene	
	Detection concentration	Content	Detection concentration	Content	Detection concentration	Content
DK	not detected	not detected	not detected	0.095421103	not detected	not detected
DK soak in liquor	not detected	not detected	0.013	0.064676617	not detected	not detected
ZJ	not detected	not detected	not detected	not detected	not detected	not detected
ZJ soak in liquor	not detected	not detected	0.017	0.084241824	not detected	not detected
MY	not detected	not detected	0.069	0.342261905	not detected	not detected
MY soak in liquor	not detected	not detected	<0	<0	not detected	not detected

DISCUSSION

The synthesis of anthocyanin involves complex biological, biochemical, and molecular biological processes. The study by Takeshi et al. (2015) indicated that the structural base of anthocyanin in *Clitoria ternatea* was 3-O-glucosyltransferase. Sun studied

the biological and molecular biological characteristics of 3-O-glucosyltransferase during the anthocyanin synthesis process in a Freesia hybrid (Sun et al., 2016). It was found that Fh3GT1 is a type of flavonoid 3-O-glucosyltransferase that uses UDP-glucose as the sugar donor. In the maturing process of strawberry fruits, the biosynthesis of flavonoids is achieved by downregulating the expression of anthocyanin glucosyltransferase. As the flavonoid compounds that control the oxidation of bayberry juice, anthocyanin, quercetin, and myricetin are the key factors in pigment stability. Our study showed that the color of MY fruits was dark and that the flavonoid content was high. Hence, the antioxidative activity of MY was significantly higher than the other two cultivars. From the color-turning phase to the mature phase, flavonoid increased to 226% in MY, 122% in DK, and only 6.78% in ZJ. Therefore, the color stability in MY was significantly higher than in the other two cultivars. Among the constituent pigments, the functions of Cy-3-glu, peonidin, and carotenoid were the most apparent. The result was consistent with the results presented by Chen, Wang and Huang (Wang et al., 2008; Huang et al., 2014). These researchers suggested that the anthocyanin in bayberry was positively correlated with maturity. The total phenol was closely correlated to the total anthocyanin and the antioxidative activity (R^2=0.85).

The main component of anthocyanin was Cy-3-glu, which made up 92% of the anthocyanin in bayberry fruits, 27% of total phenol, and 58% of flavonoid, and the contribution toward antioxidative activity was more than 85%. Zhang et al. (2015) studied 17 different types of bayberry and suggested that phenolic compounds are critical to antioxidative activity. Additionally, soluble sugar was positively correlated with antioxidative activity. A high CIRG value indicates high antioxidative activity. In this study, it is believed that the level of sugar has a certain influence on the stability of the pigment. High sugar content and a high pH content are not conducive to pigment stabilization. Relatively, the sucrose contents in the bayberry varieties were 41.75 mg/g FW for MY, 44.83 mg/g FW for ZJ, and 56.43 mg/g FW for DK. Therefore, lower sugar content was conducive to the suppression of fading. The pH test showed that the pH value of MY was significantly lower than that of the other two varieties. This result is consistent with the results of Zhang et al. (2015), which showed “good stability of anthocyanins with pH 3.0-3.5 at the ripening stage of fresh bayberry fruit”.

During this analysis, we screened nine genes and found that the absolute content and stability of Cy-3-glue and peonidin in MY were significantly better than for the other two cultivars. In MY, there were three up-regulated, three down-regulated, and three non-differentially expressed genes from the color-turning phase to the maturity phase. In DK, there were two, three, and four genes that were up-regulated, down-regulated, and not differentially expressed, respectively. In ZJ, there were four and five genes that were up-regulated and not differentially expressed. These results suggest that the genes provided a significant balancing function in the process of bayberry fruit development and are advantageous in stabilizing pigments. The results of the study showed that Cy-3-glu has an important impact on the color of bayberry fresh fruit during different developmental periods. MY is darker in color than DK and ZJ because the 3AT gene regulates Cy-3-glu. Our results were similar to the results found in the study by Niu (Niu et al., 2010), who studied the accumulation of anthocyanin at the mature stage of three different bayberry cultivars, white (*Shuijing*, SJ), red (*Dongkui*, DK), and dark red-purple (*Biqi*, BQ). Their results showed that the differential expression of anthocyanin accumulation in bayberry

fruits was the result of the cooperation between multiple genes. Of these genes, UFGT and F3'H were critical, and MrMYB1 regulated the differentially expressed genes across the three cultivars. Therefore, it is reasonable to believe that 3AT is the key gene in regulating bayberry color stability. Cloning of this gene is an important objective in the next stage of creating cultivars for further processing. The results would have practical significance for *M. rubra* industrial restructuring.

Long-term immersion test of *M. rubra* with similar color confirmed that the main pigments that are not easy to fade are Cy-3-glu and peonidin. The reason why the decomposition rate of these pigments in alcohol is low and the absolute value content is high is that they are regulated by 3AT gene. Therefore, it is reasonable to think that 3AT is the key gene to regulate the color stability of *M. rubra*. Cloning of this gene will be an important goal for the next stage of processing germplasm creation.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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