Tree Genetics and Molecular Breeding 2025, Vol.15, No.3, 98-107
http://genbreedpublisher.com/index.php/tgmb

Feature Review Open Access

Cloning and Functional Analysis of Key Genes Involved in Anthocyanin Biosynthesis in *Morella rubra*

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Tree Genetics and Molecular Breeding, 2025, Vol.15, No.3 doi: 10.5376/tgmb.2025.15.0012
Received: 17 Apr., 2025
Accepted: 19 May, 2025
Published: 30 May, 2025

Preferred citation for this article:
Liu Z., Huang D.D., and Wang W.F., 2025, Cloning and functional analysis of key genes involved in anthocyanin biosynthesis in *Morella rubra*, Tree Genetics and Molecular Breeding, 15(3): 98-107 (doi: 10.5376/tgmb.2025.15.0012)

Abstract This study reviews the research achievements made in recent years regarding the synthesis of anthocyanins in *Morella rubra*. Key genes such as *CHS*, *CHI* and *F3H* play an important role in the synthesis process of anthocyanins. In addition, some factors that regulate these genes, such as R2R3-MYB, bHLH and WD40, also play a role in the regulatory process. There is also a mutually cooperative relationship among them. To figure out how these genes work, researchers employed methods such as cloning genes, analyzing expression patterns, and also studied the epigenetic regulation of genes. This study also introduces the application prospects of molecular breeding, genetic engineering and CRISPR technology in improving the color and nutritional value of *Morella rubra*. It is hoped that these achievements can provide a scientific basis for improving the quality of *Morella rubra* and also bring practical methods for planting and breeding.
Keywords *Morella rubra*; Anthocyanin biosynthesis; MYB transcription factor; Molecular breeding; Epigenetic regulation

1 Introduction

Morella rubra is a common fruit tree, and its fruits come in a variety of colors, ranging from white, red to deep purplish red. These color differences are mainly due to the varying content of anthocyanins in the fruits. Anthocyanins not only make the fruit look good and improve its appearance, but also are beneficial to human health and can help prevent some diseases. They are very important nutrients in *Morella rubra* (Niu et al., 2010; Shi et al., 2021). Therefore, the amount of anthocyanins directly affects the quality and nutritional value of *Morella rubra* (Duan et al., 2015; Li et al., 2023).

Anthocyanins are synthesized through a process called the “biosynthetic pathway”, which requires some structural genes, such as *CHS*, *CHI*, *F3H*, *F3’H*, *DFR*, *ANS* and *UFGT*. The expression of these genes is regulated by some transcription factors, especially the proteins of the MYB, bHLH and WD40 families (Liu et al., 2013b; Yan et al., 2021). In *Morella rubra*, some R2R3-MYB transcription factors such as MrMYB1 and MrMYB9 can promote anthocyanin synthesis, while MrMYB6 has the opposite effect and inhibits anthocyanin accumulation (Shi et al., 2021; Li et al., 2023). In addition, MrbHLH1 and MrWD40-1 can also cooperate with MYB protein to better promote the formation of anthocyanins (Liu et al., 2013a). The expression of these genes varies in different varieties and fruit development stages, which determines the depth of fruit color and the amount of anthocyanins (Niu et al., 2010; Duan et al., 2015).

This study mainly aims to identify these key genes and investigate how they affect the color change of *Morella rubra*. Through the cloning research of some key genes, it explores their expression and role during the fruit development process. This study hopes that these results can provide assistance in improving the fruit color and nutritional value of *Morella rubra*, and also offer tools and theoretical support for future molecular breeding.

2 Biology and Distribution of *Morella rubra*

2.1 Botanical characteristics and genetic background

Morella rubra is a common evergreen fruit tree in southern China. It is dioecious, that is to say, the male and female plants are separate, and only the female plants can bear fruit. Research has found that female plants have a slightly higher number of alleles and heterozygosity, but the difference is not significant compared to male plants.

Although the overall differences are not significant, female and male strains can be clearly distinguished by some specific gender markers (such as ZJU062 and ZJU130). Zhejiang is the region with the richest genetic diversity of *Morella rubra*. Varieties such as 'Biqi', 'Dongkui' and 'Pink' are derived from different genetic resources (Jia et al., 2015). Now, the genome, transcriptome and germplasm resources of bayberry have been sorted out and a database has been established, laying a good foundation for breeding research (Ren et al., 2021).

2.2 Geographic distribution and ecological adaptability

Morella rubra are mainly distributed in the tropical and subtropical regions of our country. Due to its suitable climate, Guangdong Province is a place where a large number of *Morella rubra* are grown. Different varieties are distributed in 41 counties. There are also many wild *Morella rubra* in Guangxi, and these wild populations have a high degree of genetic diversity. Their distribution is closely related to the local climate and environment. According to the color and luster of the peel, *Morella rubra* can roughly be divided into three types: black, red and white. Varieties of different colors also have their own characteristics and are suitable for promotion and cultivation. From the perspective of population genetics, the reason why *Morella rubra* can be widely cultivated in China is their strong ability to adapt to the environment. Meanwhile, gene drift and limited gene flow also have an impact on its genetic structure.

2.3 Fruit development and pigmentation traits

The flesh development process of *Morella rubra* is a rather complex one, which is regulated by many hormones and genes. Plant hormones such as auxin (IAA), jasmonic acid (JA), abscisic acid (ABA), and gibberellin change levels at different stages of fruit development. Especially the interaction among IAA, JA and ABA plays a particularly significant role during critical periods. The study also found that some genes, such as *LAX2*, *LAX3* (responsible for auxin transport), *JAZ6* (regulating JA signaling), *KAN1* and *KAN4* (involved in multiple hormone signaling), are closely related to pulp development. Immunofluorescence experiments also revealed that auxin was mainly concentrated in the vascular bundles and outer cells in the middle of the pulp. This uneven distribution might affect the shape of the pulp (Figure 1) (Fu et al., 2025). In addition, the fruit color of *Morella rubra* is classified into three types: black, red and white. The differences in color and luster are due to the variations in the accumulation of pigments such as anthocyanins.

3 Anthocyanin Biosynthesis Pathway

3.1 Phenylpropanoid and flavonoid pathways

The synthesis process of anthocyanins is part of two pathways: phenylpropane metabolism and flavonoid synthesis. This process starts with phenylalanine and, through the action of some enzymes, will generate the precursors of flavonoids. Then, plants convert these precursor substances into various flavonoids through the flavonoid synthesis pathway, among which anthocyanins are included (Pratyusha and Sarada, 2022; Dutt et al., 2023). This path not only determines the color of the plants but is also related to their stress resistance, growth and development, and other functions.

3.2 Key enzymes: CHS, CHI, F3H, DFR, ANS, UFGT

During the synthesis of anthocyanins, some structural genes play significant roles, such as *CHS*, *CHI*, *F3H*, *DFR*, *ANS*, and *UFGT*. These enzymes will transform substances one by one and finally synthesize different types of anthocyanins (Raziq et al., 2024; Zhu et al., 2025). Research has found that the expression levels of these genes can affect the content of anthocyanins, and their expressions also vary at different developmental stages or in different organs. For instance, when the fruit begins to change color, the expression of these genes will significantly increase, which is conducive to the synthesis and accumulation of anthocyanins (Dutt et al., 2023; Sun et al., 2025).

3.3 Regulatory genes: MYB, bHLH, and WD40 transcription factors

In addition to structural genes, the synthesis of anthocyanins is also controlled by some regulatory proteins, mainly three types of transcription factors: MYB, bHLH and WD40. These proteins can together form a regulatory group called the MBW complex, which can directly control the expression of structural genes and is the core of the entire regulatory network (Chen et al., 2019; Jiang et al., 2023). Among them, the factors of the

R2R3-MYB family play a leading role, responsible for initiating or inhibiting the expression of anthocyanidin-related genes; While bHLH and WD40 help MYB function better (Zhao et al., 2019; Peng et al., 2020; Karppinen et al., 2021). In addition, some MYB transcription factors may also be related to hormone signals or stress responses, further refining the regulation of anthocyanin synthesis (Pratyusha and Sarada, 2022).

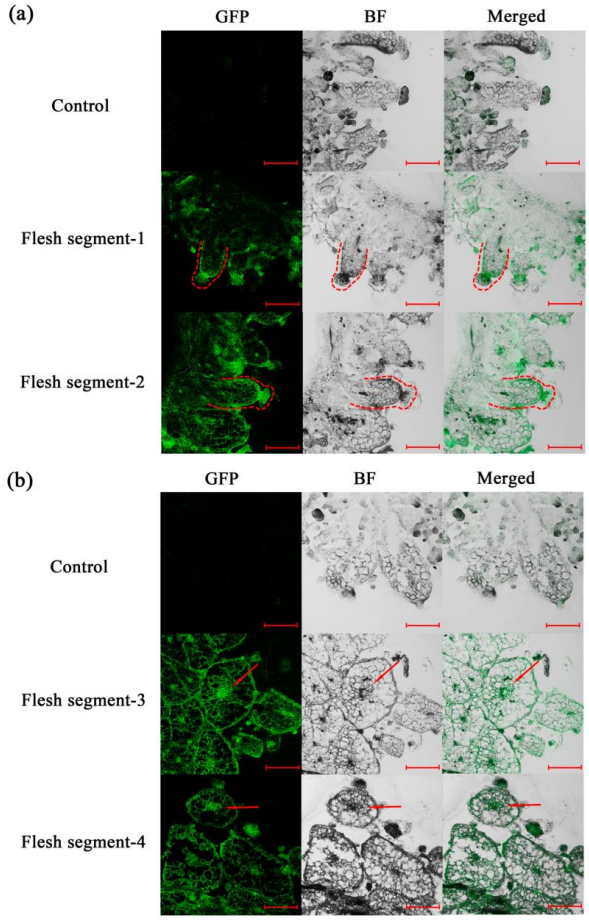

Figure 1 Immunofluorescence localization of auxin in the (a) longitudinal and (b) transverse sections of flesh segments (Adopted from Fu et al., 2025)

Image caption: The bayberry variety used for the immunofluorescence localization analysis was 'Biqi', and all scale bars were set to 200 μm. In (a), the dashed line indicates a longitudinal section of a single flesh segment, where the auxin fluorescence signals are enriched at the top and side walls of the flesh segment, forming a continuous linear distribution along the contour marked by the dashed line. In (b), the arrow indicates the central vascular bundle within the cross-section of the flesh segment, where the enrichment of auxin fluorescence signals is observed (Adopted from Fu et al., 2025)

4 Advances in Cloning of Anthocyanin Biosynthetic Genes in *Morella rubra*

4.1 Gene discovery strategies and transcriptome profiling

In China, researchers mainly identified the genes related to anthocyanin synthesis in *Morella rubra* through transcriptome sequencing and comparative analysis. They conducted transcriptome sequencing on fruits of different colors (such as white, red, and deep purple-red) and at different developmental stages, and screened out some structural genes and regulatory genes that are closely related to anthocyanin accumulation. For instance, a study identified 60 genes of the WD40 family using the RNA-Seq database and, by comparing their expression levels with anthocyanin contents, identified some of them as key regulatory factors (Liu et al., 2013a; Cao et al., 2021). In addition, by comparing the gene expression of red and white fruits, a MYB transcription factor called MrMYB9 was also discovered, which promotes anthocyanin synthesis (Li et al., 2023).

4.2 Isolation and characterization of structural genes

At present, the structural genes that have been identified in *Morella rubra* mainly include: *CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, *ANS* and *UFGT* (Liu et al., 2013b). The expression levels of these genes vary in different fruit colors and

developmental stages, and have a strong correlation with the amount of anthocyanins (Niu et al., 2010). The study also found that *CHS* plays a major role at fruit ripening, while *F3H* and *F3'H* are relatively important for the synthesis of flavonoids and phenolic acids during the early fruit growth stage (Duan et al., 2015).

4.3 Identification of regulatory genes in *Morella rubra*

The synthesis of anthocyanins is also controlled by some regulatory factors, mainly three types of proteins: MYB, bHLH and WD40, which together form the MYB-BHLH-WD40 complex. In *Morella rubra*, MrMYB1 is a very crucial MYB protein, and its expression is consistent with the accumulation of anthocyanins (Niu et al., 2010). MrMYB9 is also a regulatory factor that can promote anthocyanin synthesis (Li et al., 2023). In the bHLH family, the expression of MrbHLH1 and MrMYB1 is synchronous, and the two can combine to form a complex, activating the expression of structural genes. The regulatory ability of MrbHLH2 is relatively weak (Liu et al., 2013b). Among the WD40 family, MrWD40-1 can form a complex together with MrMYB1 and MrbHLH1 to enhance anthocyanin synthesis, but MrWD40-2 does not have this function (Liu et al., 2013a). In addition, *MrMYB6* has been found to be a negative regulatory factor. It can bind to other proteins and inhibit the synthesis of anthocyanins and proanthocyanidins (Figure 2) (Shi et al., 2021).

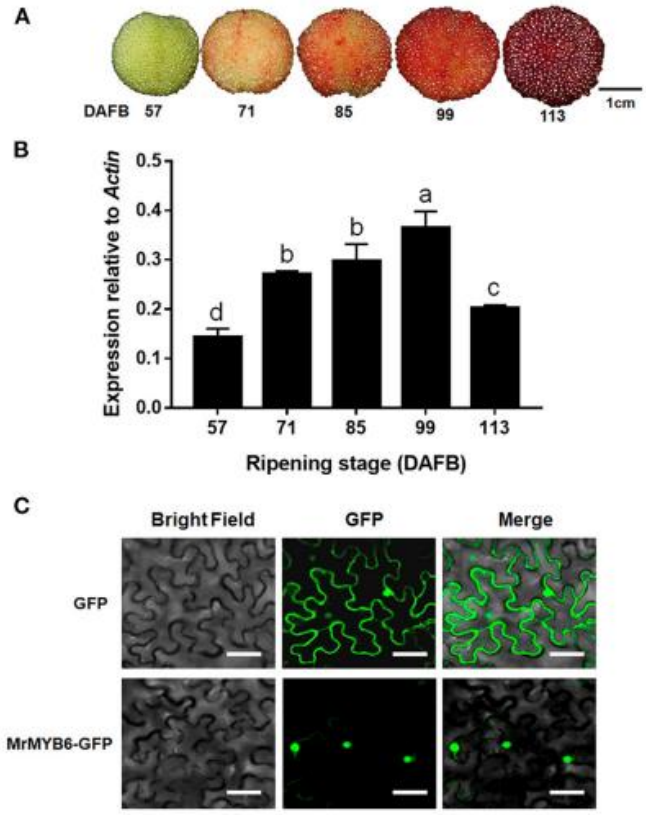

Figure 2 *MrMYB6* expression pattern during bayberry fruit development (Adopted from Shi et al., 2021)

Image caption: (A) The bayberry fruits at 57, 71, 85, 99, and 113 days after full bloom (DAFB) used in this study. Scale bar represents 1 cm. (B) Expression levels of *MrMYB6* at different stages. The data were normalized to the *MrACT* expression level. Error bars indicate the mean ± *SE* of three replicate reactions. Letters (a, b, c, and d) represent significant difference between wild-type and transgenic plants according to the Duncan's multiple range test ($P < 0.05$). (C) Subcellular localization of MrMYB6 in tobacco leaf epidermal cells. Scale bar represents 50 μm (Adopted from Shi et al., 2021)

5 Gene Expression Patterns and Regulation

5.1 Temporal and spatial expression during fruit development

Genes related to anthocyanin synthesis show significant temporal and spatial expression differences during fruit development. Research has found that when fruits are close to ripening, the content of anthocyanins increases significantly. The distribution of anthocyanins is also different between the peel and the pulp. Take sweet cherries and red pears as examples. Structural genes such as *PacANS*, *ANS* and *UFGT* have the highest expression levels

when the fruits turn red, and they directly determine how much anthocyanin can accumulate (Yang et al., 2015; Yang et al., 2021). In addition, the expression of some regulatory genes, such as *MYB* and *bHLH*, is also closely related to these structural genes. Some regulatory factors are expressed only in specific sites, such as the endopeel of kiwifruit, which can control the synthesis of local anthocyanins (Wang et al., 2019).

5.2 Hormonal and environmental modulation

The synthesis of anthocyanins is not only determined by genes, but also influenced by plant hormones and the external environment. Hormones such as abscisic acid (ABA) can enhance the expression of regulatory genes and thereby promote the accumulation of anthocyanins (Karppinen et al., 2021). In the environment, light is one of the most important factors. It can induce the expression of MYB transcription factors, thereby activating structural genes and increasing anthocyanin synthesis. For instance, after apples are exposed to light, the expression of MdMYB1 increases, and anthocyanins also rise accordingly. In addition, temperature changes and some adverse conditions, such as drought or cold damage, can also indirectly affect anthocyanin production by influencing these regulatory factors (Ma et al., 2021; Yan et al., 2021).

5.3 Co-expression networks and transcriptional hierarchies

The process of regulating anthocyanin synthesis is very complex and requires the cooperation of many transcription factors. The three proteins, MYB, bHLH and WD40, can form a complex, and MYB plays a major role in this process. They jointly control the expression of structural genes (Wang et al., 2019; Sharma et al., 2024). Studies using co-expression network analysis have found that transcription factors such as PacbHLH13, PacbHLH74, and PacMYB308 are expressed together with structural genes and can directly activate the promoters of key enzymes (Yang et al., 2021). Moreover, because genes have the processes of replication and differentiation, members of the MYB family have gradually formed different functional branches, such as controlling the synthesis of cyanidin or delphinidin (Karppinen et al., 2021). In addition, the expression of anthocyanin genes is also influenced by epigenetics. For example, the interaction between *H2A.Z* and *H3K4me3* also regulates the process of anthocyanin synthesis (Cai et al., 2018).

6 Functional Analysis and Verification Approaches

6.1 Heterologous expression in model plants

Heterologous expression is a common method used to verify whether a certain gene is useful. Researchers often transfer regulatory genes from *Morella rubra*, such as *MrMYB1*, *MrbHLH1* and *MrWD40-1*, into model plants like tobacco to see if they increase anthocyanins. The experiment found that when *MrMYB1* and *MrbHLH1* were overexpressed in tobacco, anthocyanins significantly increased, and the expression of related structural genes also enhanced (Niu et al., 2010; Liu et al., 2013b). In addition, if *MrMYB1*, *MrbHLH1* and *MrWD40-1* are expressed together, the effect will be stronger. This also indicates that these three genes are very crucial in regulating anthocyanins (Liu et al., 2013a).

6.2 Virus-induced gene silencing (VIGS) and overexpression studies

Virus-induced gene silencing (VIGS) and overexpression experiments are two commonly used methods in molecular biology. By using VIGS to “turn off” a certain gene, one can observe whether anthocyanins have decreased, thereby determining whether this gene is involved in anthocyanin synthesis. Conversely, if certain genes (such as *MrMYB1* and *MrMYB6*) are “turned on”, changes in anthocyanins or other related substances can also be observed. For example, after *MrMYB6* is overexpressed in tobacco, the contents of anthocyanins and proanthocyanidins decrease instead, indicating that it plays an inhibitory role (Shi et al., 2021). These methods can directly indicate which genes are promoting and which are inhibiting anthocyanin synthesis (Niu et al., 2010; Liu et al., 2013b).

6.3 Subcellular localization and protein-protein interactions

Scientists also conducted some cell localization experiments to see exactly where the proteins work within the cells. Whether proteins will bind to each other can also be verified through experiments such as yeast two-hybrid and BiFC. For instance, the yeast two-hybrid experiment confirmed that MrMYB1 can interact with MrbHLH1 and MrWD40-1. These three proteins can assemble into a complex specifically for regulating anthocyanin

synthesis (Shi et al., 2021). These findings also indicate that the regulatory process of anthocyanins is actually quite complex. It is not a single gene acting alone, but rather multiple proteins working together.

7 Genetic and Epigenetic Regulation of Anthocyanin

7.1 Promoter elements and cis-regulatory motifs

There are many cis-regulatory elements in the promoters of genes related to anthocyanin synthesis. These small fragments can sense light, hormones and developmental signals, thereby regulating whether genes are functioning or not. Transcription factors such as R2R3-MYB recognize these elements. Some can activate genes, and some can inhibit them, thus affecting the amount of anthocyanins (Zhao et al., 2023). Moreover, the natural differences in the promoter regions of different varieties can also cause variations in anthocyanin content (Yang et al., 2022).

7.2 DNA methylation and histone modification

DNA methylation and histone modification are another set of regulatory mechanisms, which belong to epigenetic regulation. Studies have found that if the methylation level in the promoter region is high, the related genes are prone to suppression and anthocyanins are less (Sun et al., 2023). For instance, in chrysanthemums, if the methylation of the promoter of the *CmMYB6* gene is high, the flower color will be light. But if methylation is removed, anthocyanins will come back (Tang et al., 2022). In addition, some modifications on histones can also regulate expression. H3K4me3 can promote gene expression, while H2A.Z has the opposite effect, and the two balance each other (Cai et al., 2018). Histone demethylases (such as JMJ25, IBM1) also regulate the activity of anthocyanin genes by eliminating certain modifications (such as H3K9me2) (Fan et al., 2018; Zheng et al., 2019; Fan et al., 2024).

7.3 Small RNAs and post-transcriptional regulation

Small RNAs (such as miRNA and siRNA) are also involved in regulation. These small molecules can directly bind to the mRNA of anthocyanin synthesis-related genes, causing them to degrade or be untranslated (Zhao et al., 2023). Some can also affect transcription factors, thereby indirectly regulating the entire anthocyanin synthesis pathway (Khan and Abbas, 2023). These mechanisms work together to ensure that plants can flexibly control the synthesis of anthocyanins according to different developmental stages or environmental changes.

8 Case Study: Functional Analysis of *MrMYB1* in Regulating Anthocyanin Accumulation

8.1 Background and research hypothesis

The fruit color of *Morella rubra* is very rich, ranging from white, red to deep purple-red. This color difference is mainly caused by the varying content of anthocyanins. The synthesis process of anthocyanins is controlled by multiple genes, among which MYB transcription factors play a very important role. Studies have found that *MrMYB1* is a key R2R3-MYB transcription factor in bayberry, and its expression level is highly correlated with the amount of anthocyanins in the fruit (Niu et al., 2010). So it is speculated that *MrMYB1* might affect the color of *Morella rubra* by regulating the expression of genes related to anthocyanin synthesis.

8.2 Experimental design and key findings

Researchers first examined the expression of *MrMYB1* in different colored varieties of *Morella rubra* and found that the higher the expression of this gene, the more anthocyanins there were in the fruits. When they wrapped the fruits in fruit bags, the expression of *MrMYB1* and other anthocyanin synthesis genes decreased, and the anthocyanin content also decreased accordingly. Then, they overexpressed *MrMYB1* in tobacco, resulting in a significant increase in anthocyanins. They also found that the promoters of some key enzymes were activated. In addition, meaningless mutant versions of *MrMYB1* were also found in the white and red varieties. This mutation may render this gene “ineffective”, resulting in a lighter fruit color (Niu et al., 2010). Subsequent experiments also indicated that *MrMYB1* could interact with *MrbHLH1* and *MrWD40-1* to form a complex, and this combination could significantly promote the synthesis of anthocyanins (Liu et al., 2013a; Liu et al., 2013b).

8.3 Implications for fruit color improvement in bayberry breeding

MrMYB1 is an important gene that regulates the synthesis of anthocyanins in *Morella rubra*. The level of its expression will directly affect the color of the fruit. In the future, if *MrMYB1* and its partner genes can be regulated through molecular breeding, it will be possible to cultivate new varieties of *Morella rubra* with brighter

colors and better nutrition. This is also of great help in improving the fruit color of *Morella rubra* and enhancing their quality.

9 Biotechnological Applications and Crop Improvement

9.1 Molecular breeding for enhanced anthocyanin

The color of the fruit of *Morella rubra* is mainly determined by the amount of anthocyanins. Anthocyanins not only make fruits look good in color, but also have high nutritional and health care value. Studies have shown that R2R3-MYB transcription factors, such as *MrMYB1* and *MrMYB9*, are very crucial in the anthocyanin synthesis process, and their expression levels have a strong relationship with anthocyanin accumulation (Niu et al., 2010; Li et al., 2023). By using molecular breeding methods and selecting varieties with high expression levels of MrMYB1, it is possible to breed new varieties with brighter colors and higher nutritional value (Yan et al., 2021). In addition, crops with high anthocyanin content are more tolerant to environmental stresses (such as low temperature and drought), which is very helpful for improving the adaptability of crops (Sun et al., 2021).

9.2 Marker-assisted selection (MAS) and genetic engineering

Now, scientists have identified multiple genes related to anthocyanin synthesis, such as *MrCHI*, *MrF3'H*, *MrANS*, *MrUFGT*, as well as regulatory factors like *MrMYB1*, *MrbHLH1*, and *MrWD40-1*. These genes have all been cloned and functionally verified (Liu et al., 2013b; Li et al., 2023). These genes can be used as molecular markers to help select bayberry varieties with better color more quickly. If genetic engineering, such as transgenic or gene editing techniques, is employed, the expression of these genes can be directly regulated, thereby controlling the amount of anthocyanins. For example, overexpression of *MrMYB1* can increase anthocyanins (Niu et al., 2010; Liu et al., 2013a), while inhibiting negative regulatory factors such as *MrMYB6* can reduce the decomposition of anthocyanins (Shi et al., 2021). These methods can all be applied to the improvement of fruit color and nutrition (Chaves-Silva et al., 2018; Yan et al., 2021).

9.3 CRISPR/Cas-based genome editing potential

New gene editing tools like CRISPR/Cas enable us to precisely modify key genes related to anthocyanins (Han, 2024). By adjusting regulatory factors such as MYB, bHLH, and WD40, or modifying certain structural genes, the entire pathway of anthocyanin synthesis can be optimized, thereby breeding bayberry varieties with better color and higher nutrition (Chaves-Silva et al., 2018; Yan et al., 2021). This technology can also be used to remove negative regulatory factors such as MrMYB6, thereby allowing more anthocyanins to accumulate in the fruit (Shi et al., 2021). The development of these genetic technologies has opened up new ideas for fruit tree breeding and quality improvement in the future.

10 Concluding Remarks

In recent years, scientists have made significant progress in the research of genes related to anthocyanin synthesis in *Morella rubra*. Research has found that there are many transcription factors belonging to the MYB family in *Morella rubra*. Among them, *MrMYB1* and *MrMYB9* are *R2R3-MYB* genes. They are particularly important in regulating anthocyanin synthesis and have a strong relationship with the amount of anthocyanin in the fruit. There are also two important proteins, one called MrbHLH1 and the other MrWD40-1. Together with MYB protein, they form a complex, which can significantly increase the accumulation of anthocyanins. However, there are also some MYB factors, such as MrMYB6, which can have the opposite effect and instead inhibit the synthesis of anthocyanins and proanthocyanidins. These achievements help us better understand how the color of *Morella rubra* is formed and also lay a foundation for future breeding and nutritional improvement.

However, the current understanding of these regulatory genes is still not comprehensive enough. How transcription factors like MYB, bHLH and WD40 interact with each other, what different functions their members have, and how they specifically regulate downstream genes - these questions still require further research. In addition, it is still not very clear how environmental factors such as light and temperature affect anthocyanin synthesis, as well as the relationship between genetic differences and appearance colors among different varieties. Moreover, many functional verifications are currently conducted on other plants. *Morella rubra* themselves do not yet have a mature transformation system, which also limits further research.

In the future, different technical means such as genomics, transcriptomics and metabolomics can be used together for research to systematically clarify the regulatory network of anthocyanin synthesis in *Morella rubra*. If single-cell omics or spatial transcriptomics techniques can be used, it is also possible to observe how genes change in different tissues, at different developmental stages, or in different environments. At the same time, a genetic transformation system of its own should also be established for *Morella rubra*. This will enable more accurate verification of gene functions and can also be used for molecular breeding. In-depth research on the functional differences of gene families such as MYB, bHLH, and WD40 and their cooperative relationships can help breed new varieties of *Morella rubra* with better color and higher nutrition, and also provide theoretical support and technical methods for improving the quality of *Morella rubra*.

Acknowledgments

GenBreed Publisher appreciates the comments from two anonymous peer reviewers on the manuscript of this study.

Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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