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Inducible overexpression of MYB118-like gene improves anthocyanin production in *Populus × canescens*

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Abstract

Anthocyanins are secondary metabolites which contribute different colors to the leaves. Anthocyanin biosynthesis is regulated by MYB transcription factors, which have been extensively studied and characterized in a diversity of plants. In this study, we identified a novel MYB transcription factor *MYB118L*, from *Populus* 'Zhonghua Hongye', which showed an elevated accumulation of anthocyanin than *Populus* clone ZL-2025. Subcellular localization analyses revealed that MYB118L-GFP fusion protein was specifically located in the nucleus. Transgenic plants overexpressing *MYB118L* driven by the stress-inducible Rd29A promoter showed a significant increase in anthocyanin production, resulting in a red coloration of the leaves under drought stress conditions. These plants also exhibited higher expression levels of genes involved in anthocyanin biosynthesis compared to the wild type, suggesting that *MYB118L* positively regulates the expression of these genes. Y1H and dual-luciferase assays confirmed that MYB118L can directly activate the promoters of *LAR1* gene. Our findings suggest that *MYB118L* is an essential transcription factor involved in the regulation of anthocyanin biosynthesis in poplar and could be utilized for genetic engineering of colorful tree species.

Keywords

Anthocyanin biosynthesis; *MYB118L*; *Populus canescens*; genetic engineering; Rd29A promoter

Introduction

Leaves are primary organs of woody plants and play a crucial role in photosynthesis. Leaf color diversity not only affects the growth and development of trees but also significantly impacts landscaping, beautification and ecological benefits (1, 2). During the 21st century, which has led to a growing demand for environmental enhancement, there has been an increased interest in using highly colorful tree species for garden landscape design (3). Endowing woody plants with high ornamental value has made the integration of colorful tree species increasingly popular for enhancing the aesthetic appeal of outdoor environment (2).

Higher plants possess three types of pigments in their leaves: flavonoids, carotenoids and chlorophyll (4). Among these, flavonoids include anthocyanins, flavones and flavonols, with anthocyanins being the most complex subgroup. Anthocyanins act as primary pigments, while

flavones and flavonols provide complementary colors (5). Anthocyanins, benzopyran derivatives present in plant vacuoles, determine the color of stems, leaves, sepals, flowers and fruits in hues such as red, pink, blue and purple (6). Anthocyanin biosynthesis, conserved across many plants, involves regulatory genes encoding transcription factors such as MYB, bHLH and WD40, controlling the expression of structural genes (7) and the enzymes controlled by these structural genes are crucial for anthocyanin biosynthesis (8). MYB transcription factors interact with bHLH and WD40 proteins to regulate the expression of structural genes, forming the MYB-bHLH-WD40 complex that plays a central role in the regulation of anthocyanin biosynthesis (9, 10). MYB transcription factors are critically involved in regulating the expression of structural genes (*CHS*, *CHI*, *F3H*, *F3'H*, *F3'5'H*, *DFR*, *ANS*, *ANR* and *LAR*) in the anthocyanin biosynthesis pathway, with most plant anthocyanins being positively regulated by these factors. Instances such as the overexpression of *AN1* in tomatoes, which enhances *CHS*, *CHI* and *DFR* genes and boosts anthocyanin level in fruit (11), illustrate the regulatory role of MYB transcription factors. In gentian, *GtMYB1* and *GtMYB2* separately control anthocyanins and flavonols synthesis, potentially under the coordination of *GtbHLH1* (12). Similarly, *IbMYB1* in sweet potato manages genes *CHS*, *CHI*, *F3H*, *DFR*, *ANS* and *UFGT*, increasing anthocyanin content (13). Genome sequencing has uncovered MYB genes across plants with a focus on model plants like *Arabidopsis*, rice and soybean. These factors are integral to plant development and secondary metabolite synthesis, including anthocyanins (14-17). Despite these findings, the function of MYB factors in poplar trees requires further study.

In this study, we focused on isolating the MYB gene from *Populus* 'Zhonghua Hongye', anticipated to regulate anthocyanin biosynthesis. Gene expression analysis revealed an up-regulation homolog of MYB118 named MYB118L in 'Zhonghua Hongye' versus the ZL-2025 clone. Subcellular localization confirmed MYB118L-GFP fusion's nucleus specificity. Overexpression of MYB118L in *Populus canescens*, a natural hybrid of *P. alba* and *P. tremula*, yielded transgenic plants with enhanced production of anthocyanins, manifesting as red coloration under stress. This was accompanied by an elevated transcription of essential anthocyanin biosynthesis genes. Our study also demonstrated MYB118L's direct binding to the MBS cis-element, indicating a positive regulatory role. Dual-luciferase assays substantiated MYB118L's capacity to activate the *LAR1* promoter. Overall, our study highlights MYB118L's significance as a transcription factor in anthocyanin regulation in poplar, with broader applications in the genetic enhancement of tree color.

Materials and Methods

Plant material and treatments

Populus clone ZL-2025 and its red leaf mutant *Populus* 'Zhonghua Hongye' were originally obtained from Siyang County, Suqian City, Jiangsu Province. They were acclimated in soil and grown under controlled environmental conditions with a growth chamber. The chamber was set to a 16-h light cycle, with a photosynthetic photon flux density of 200 μmol m^{-2} s^{-1} and a constant temperature of 26 °C.

Both the non-transformed and transgenic plants *P. canescens* were used in this study. The plants were micro propagated *in vitro* on solid MS medium in a growth chamber (15). For inducing drought stress, 2-month-old potted seedlings of transgenic and non-transformed *P. canescens* were transferred to an environmental simulation greenhouse. Uniform seedlings were subjected to moderate drought stress by withholding watering for a period of 7 days, while maintaining all other growth conditions optimal.

Amino acid structural analysis of MYB118L

To investigate the evolutionary relationship of *MYB118L* with other MYB transcription factors, a phylogenetic tree was constructed using homologous MYB transcription factors. The neighbour joining method with partial deletion gap treatment was employed and the tree's reliability was assessed using a bootstrap analysis with 1000 replicates via MEGA7 software. Amino acid sequences for alignment were retrieved from NCBI and the alignment was performed using DNAMAN7.0.

Subcellular localization of MYB118L fusion protein

The subcellular localization of the *MYB118L* protein was determined by fusing the full-length coding sequences of MYB118L to the N-terminal of green fluorescent protein (GFP), creating a MYB118L-GFP fusion construct in the pCAMBIA1302 vector. The plasmid was introduced into *Nicotiana benthamiana* leaves via Agrobacterium-mediated transformation for transient expression. The resulting fluorescence was observed and recorded using a confocal laser scanning microscope to ascertain the protein's nuclear localization.

Vector construction and production of transgenic poplars

The full-length coding sequence (CDS) of *MYB118L* was amplified from *Populus* 'Zhonghua Hongye' cDNA and cloned into the modified pBI121 vector under the control of the stress-inducible Rd29A promoter. The construct was verified by DNA sequencing and subsequently introduced into *Agrobacterium tumefaciens* strain EHA105 for plant transformation. *P. canescens* was transformed using the leaf disk method, with co-cultivation of leaf discs with *A. tumefaciens* for 10 min, followed by selection on MS medium containing 50 mg/L kanamycin. Regenerated shoots were rooted on the MS basal medium and then transferred to sterile soil for acclimatization.

RT-PCR for gene expression analysis

Total RNA was extracted from leaf samples using the RNAprep Pure Reagent Kit (Tiangen, China). First-strand cDNA synthesis was performed using approximately 1 μg of total RNA and oligo dT primers. Semi-quantitative PT-PCR (RT-PCR) was conducted as described (18), followed by agarose gel electrophoresis to analyse the PCR

products. Quantitative real-time PCR (qRT-PCR) was performed using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and SYBR Premix ExTaq reagent (TaKaRa) to determine the relative expression level of target genes, normalized against actin expression.

Anthocyanin measurement

To quantify anthocyanin accumulation in response to stress, leaves from transgenic and wild-type *P. canescens* were sampled. Anthocyanins were extracted using 0.1 % HCl/methanol and the extracts were centrifuged. The supernatants were diluted treated with chloroform to remove chlorophylls. The absorbance of the aqueous phase was measured at 530 nm and anthocyanin content was calculated based on the A530 value per gram fresh weight (FW). All experiments were repeated at least 3 times to ensure reproducibility and statistical significance.

Yeast one-hybrid assay

The *PtrLAR1* promoter containing the MYB-biding site MBS, was used to assess the DNA-binding activity of MYB118L. The promoter region was inserted into the pLacZi vector to create a reporter construct. *MYB118L* cDNA was cloned into pB42AD vector to generate an activation domain fusion expression vector. Yeast one-hybrid assays were conducted using EGY48 yeast cells cotranformed with the reporter and effector vectors. Positive interactions were identified by the growth of yeast clones on the selective media containing X-gal.

Transient transcription dual-luciferase assays

The *PtrLAR1* promoter was cloned into the pGreenII0800-LUC vector to create a reporter vector. The *MYB18L* CDS was inserted into the pGreenII 62-SK vector to generate an effector vector. These vectors were transformed into *A. tumefaciens* strain EHA105. Tobacco leaves were infiltrated with the transformed Agrobacterium, and the leaves were analysed using a plant live imaging system 2 days post-infiltration to measure LUC/REN using a Dual Luciferase Reporter Gene Assay Kit (Yeasen).

Results and Discussion

MYB118L was a transcription factor relating to the red leaf of *Populus* 'Zhonghua Hongye'

In the previous study, we identified a MYB transcription factor (TF) gene that showed increased expression in the red leaf mutant of *Populus* ‘Zhonghua Hongye’ compared to its wild type counterpart, ZL-2025. This MYB TF gene is (predicted to be) involved in the regulation of anthocyanin biosynthesis. Based on this observation, we speculated that the up regulation of this MYB TF gene contributed to the enhanced production of anthocyanins in the mutant. This MYB TF gene, *MYB118L*, from *Populus* ‘Zhonghua Hongye’ was cloned and compared with MYB factors involved in anthocyanin and proanthocyanidin biosynthesis in poplar, and named *PzhMYB118L*. Through amino acid sequence alignment, we discovered that PzhMYB118L exhibited high homology with PdMYB118, a known MYB factor involved in anthocyanin and pro-anthocyanidin biosynthesis in poplar (Fig. 1B, C). In *Populus,* the MYB118 subfamily includes 3 members, PtrMYB118, PtrMYB119 and PtrMYB120, all of which contain an R2R3 repeat domain at their N-terminus, which is a typical domain of MYB transcription factors involved in anthocyanin and pro-anthocyanidin biosynthesis.

Next, we examined the expression levels of *MYB118L in Populus* ‘Zhonghua Hongye’ and ZL-2025 and observed a significant up-regulation of *MYB118L* in *Populus* ‘Zhonghua Hongye’ (Fig. 1A). This indicates that *MYB118L* may play an important role in anthocyanin biosynthesis in *Populus* ‘Zhonghua Hongye’, similar to the previously reported PtrMYB119. These findings provide valuable insights for further investigating the molecular mechanism underlying anthocyanin biosynthesis in poplar.

For subcellular localization of *MYB118L*, we utilized a transient expression method in tobacco leaves. In contrast to the ubiquitous distribution of free GFP, the fluorescence signal of the *MYB118L*-GFP fusion protein was specifically localized to the nucleus (Fig. 2). This result clearly indicates that *MYB118L* functions in the nucleus and may be related to transcriptional regulation.

Overexpression of *MYB118L* leads to increased anthocyanin in transgenic poplar

A plasmid containing the Rd29A promoter driving *MYB118L* overexpression was constructed and transformed into *P. canescens* (Fig. 3A). PCR analysis confirmed the integration of *MYB118L* in the regenerated plant lines (Fig. 3B). The transgenic plantlets were transferred to soil and grown in a greenhouse (Fig. 3C). Under drought stress conditions, the leaves of transgenic plants overexpressing *MYB118L* gradually turned red and accumulated higher levels of anthocyanins compared to wild-type plants (Fig. 3D).

Poplars overexpressing *MYB118L* show induced expression of genes in the anthocyanin biosynthesis pathway

Anthocyanin accumulation has been shown to have a positive correlation with the expression of anthocyanin biosynthetic genes(19-21). Overexpression of MYB118L in genetically modified *P. canescens* resulted in a significant increase in anthocyanin production (Fig. 4B), probably achieved through the direct or indirect regulation of genes involved in anthocyanin biosynthesis. To investigate the transcriptional activation activity of *MYB118L* on anthocyanin biosynthesis genes, we manipulated the expression of the MYB18L-inducible promoter in transgenic *Populus canescens* under drought stress. Under these conditions, *MYB118L* expression was induced in *Populus canescens* (Fig. 4A) and semi-quantitative PCR revealed a significant increase in the expression levels of anthocyanin biosynthesis pathway-related genes, including *F3'H, ANR1* and *LAR1*, in the leaves of *MYB118L*-overexpressing plants (Fig. 4C). These findings suggest that *MYB118L* may directly or indirectly regulate the expression of structural genes involved in anthocyanin biosynthesis. These findings shed light on the regulatory mechanisms of anthocyanin biosynthesis in *Populus* species and offer valuable insights for future research.

Fig. 1. *MYB118L* belongs to the MYB family of TFs involved in anthocyanin biosynthesis. (A) The expression level of *MYB118L* in *populus* 'Zhonghua Hongye' and ZL-2025; (B) Phylogenetic tree constructed from the NJ method using the *MYB118L* in *Populus* 'Zhonghua Hongye' and homologous proteins of other species. GenBank accession numbers or locus IDs are as follows: AtMYB75 (*Arabidopsis thaliana*, AT1G56650.1), AtMYB113 (*A. thaliana*, AT1G66370.1), PaMYB1 (*Populus alba*, XP_034902279.1), PeMYB113 (*P. euphratica*, XP_011021395.1), PtMYB1 (*P. trichocarpa*, XP_024444002.1), PdMYB118 (*P. deltoids*, Potri.017G125800). The phylogenetic tree constructed using MEGA7 software. The bootstrap values setting 1000 repetitions; (C) Multiple sequence alignment of *MYB118L* and other homologous proteins. GenBank accession numbers as above.

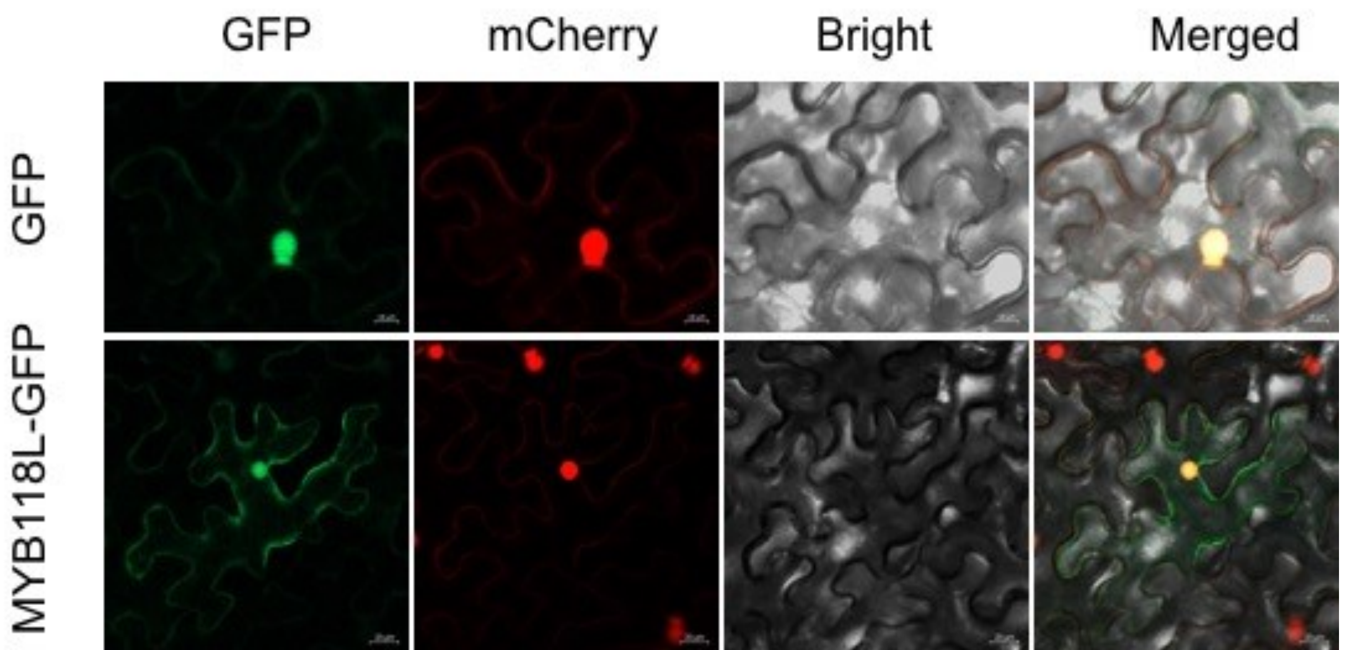

Fig. 2. Subcellular localization of *MYB118L*. Nucleus-localized marker protein (H2B-mCherry) was co-expressed with *MYB118L*-GFP fusion protein in tobacco

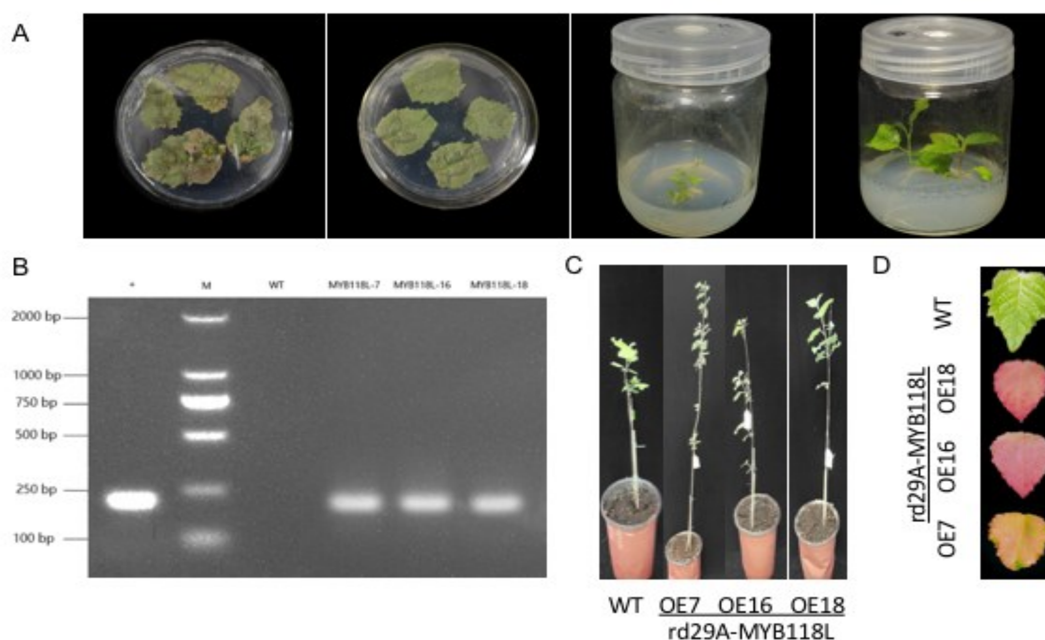

Fig. 3. Overexpression of *MYB118L* in *P. canescens* leads to red leaf phenotype of transgenic plants. (A) Agrobacterium-mediated *P. canescens* transformation and plant regeneration; (B) Detection of *MYB118L* by PCR. Lane +: positive control (plasmid pBI121-rd29A-MYB118L), Lane M: 2-kb DNA ladder, lane WT: negative control (wild-type poplar), lanes MYB118L-7, MYB118L-16, MYB118L-18, putative transgenic poplar lines; (C) The phenotype of transgenic poplars grown in soil under normal condition; (D) Red leaf phenotype photographed on the adaxial side.

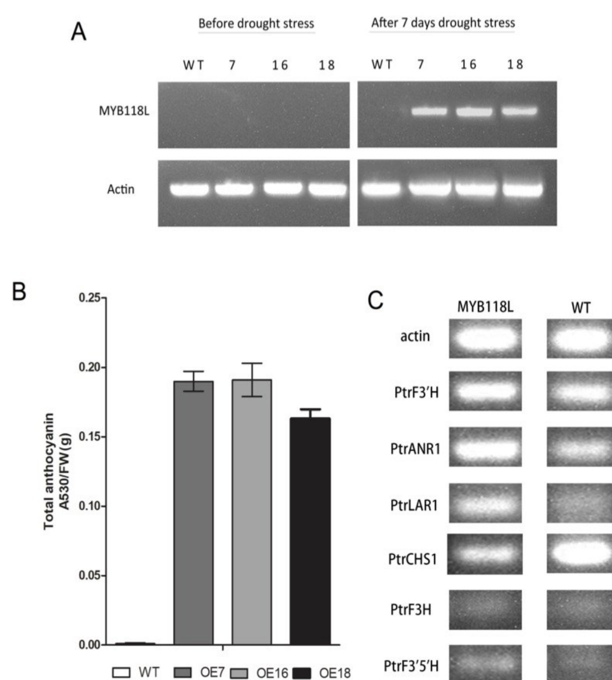

Fig. 4. Expression of anthocyanin biosynthesis genes and anthocyanin content in MYB118L transgenic poplars. (A) *MYB118L* expression in the transgenic lines by RT-PCR before and after drought stress; (B) Anthocyanin content in leaves of transgenic plants after 7 days of drought stress. Error bars indicate standard errors of three biological replicates; (C) RT-PCR analysis of anthocyanin biosynthetic genes. *PtrF3'H*: flavanone 3'-hydroxylase gene; *PtrANR1*: anthocyanidin reductase gene; *PtrLAR1*: leucoanthocyanidin reductase gene; *PtrCHS1*: chalcone synthase gene; *PtrF3H*: flavanone 3-hydroxylase; *PtrF3'5'H*: flavonoid 3'5'-

MYB118L could directly bind to the promoter of *PtrLAR1* gene and activate its expression

To further verify the up-regulation of anthocyanin biosynthesis related genes in *MYB118L* transgenic poplars, we analysed the ability of *MYB118L* to activate the promoters of these genes using yeast one-hybrid and transient transcriptional activation assays. In the yeast one-hybrid experiment, we introduced 2 expression vectors into the experimental group: placZi-4xMBS and pB42AD-MYB118L. placZi-4xMBS contains the MBS regulatory element, while pB42AD-MYB118L contains the transcription factor MYB118L. Simultaneous introduction of these 2 vectors into yeast cells enabled the detection of any interaction between *MYB118L* and the MBS regulatory element. Analysis of the experimental results revealed an interaction between the MBS regulatory element and the transcription factor MYB118L (Fig. 5A). This indicated that MYB118L is the transcription factor of the MBS regulatory element and can regulate the expression of its downstream gene. The findings of this experiment are significant for studying transcriptional regulation networks and exploring novel gene regulation mechanisms.

To further confirm whether *MYB118L* directly targets the LAR1 promoter, we performed a dual luciferase assay. The experimental group consisted of three leaves co-injected with pGreenII 62-SK-MYB118L and pGreenII 0800-Luc-ProLAR1, while the control group comprised 3 sets of leaves injected with pGreenII 62-SK-MYB118L and pGreenII 0800-Luc, pGreenII 62-SK and pGreenII 0800-Luc-ProLAR1 and pGreenII 62-SK and pGreenII 0800-Luc respectively. The results of the assay showed that *MYB118L* directly activated the LAR1 promoter, displaying a higher LUC/REN ratio compared to the control group (Fig. 5B, C). Our findings indicate that *MYB118L* functions as a transcription factor that regulates anthocyanin-related genes in P. canescens and can directly regulate the LAR1 promoter.

Discussion

The poplar tree (*Populus* spp.),traditionally recognized for its green foliage, occasionally presence of red leaves, indicating a sophisticated regulatory mechanism for anthocyanin-mediated coloration. Within the diverse MYB transcription factor family, key regulators of anthocyanin biosynthesis have been identified across a spectrum of plant species. *AtPAP1* is instrumental in anthocyanin production in seedings, While *AtMYB113/114* and *AtPAP2* exhibit partial redundancy in this way (22). Similarly, *SlAN2* in tomatoes (*Solanum lycopersicum*) (23) and a variety of MYB transcription factors in petunia (24), apple (25), peach (26) and Freesia hybrid (27) have been shown to regulate anthocyanin biosynthesis. This regulatory role is further emphasized by the direct binding of MYB-TFs to the promoters of anthocyanin biosynthetic genes, as demonstrated by *PpMYB108* in peach (26) and *AaMYB2* in *Anthurium andraeanum* (28), where specific promoter interactions dictate gene expression patterns. In this study, we successfully cloned the *MYB118L* transcription factor from *Populus*

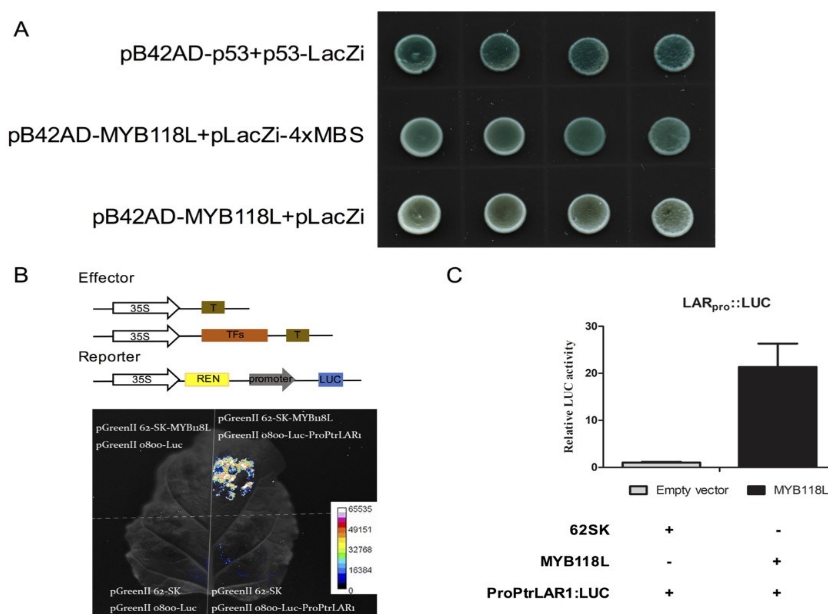

Fig. 5. MYB118L activates the expression of genes involved in anthocyanin biosynthesis. Yeast one-hybrid assay showed that MYB118L could bind to MBS element. Positive control: P53; negative controls: pLaczi-MBS and pB42AD; The experimental samples: pLaczi-4×MYBS and pB42AD-MYB118L; (B) Dual-luciferase assays showing that MYB118L activates the transcriptional activation of *PtrLAR1*; (C) The LUC/REN ratios represent relative activities of MYB118L on the promoters of *PtrLAR1*. Error bars indicate standard errors of three biological replicates.

'Zhonghua Hongye', a red-leaf poplar mutant. Our findings indicate that *MYB118L* positively regulates the expression of anthocyanin-related genes by activating their promoters, thereby modulating the red coloration display. The integration of *MYB118L* with mutant plants and anthocyanin synthesis genes underscored its pivotal role in red color formation, a discovery that parallels the regulatory mechanisms observed in other species.

The isolation and functional characterization of *MYB118L* from *Populus* 'Zhonghua Hongye' have laid the groundwork for its application in the genetic engineering of ornamental tree species. Our research revealed that plants overexpressing *MYB118L* accumulate elevated levels of anthocyanins and exhibit enhanced expression of anthocyanin synthesis genes compared to wild-type controls. Sequence analysis and subcellular localization studies confirmed the nuclear localization of *MYB118L* and its capacity to regulate the downstream *LAR1* gene, solidifying its classification as a member of the MYB transcription factor family with typical characteristics. This study expands upon the work of *AcMYBF110* in golden kiwifruit (*Actinidia chinensis*) (29), where promoter specificity was shown to be crucial for anthocyanin regulation.

Our study, while elucidating the role of MYB118L in anthocyanin biosynthesis under drought stress in *P. canescens*, has certain limitations. The primary focus on drought stress, although ecologically pertinent, does not encompass the full spectrum of abiotic stresses that can influence anthocyanin synthesis. Other stressors such as temperature fluctuations, salinity and nutrient availability also play significant roles in regulating anthocyanin production, as evidenced in various plant species (30). These observations highlight the multifaceted nature of plant stress responses and the need for a broader examination of stress factors in future research. Moreover, our findings are specific to *P. canescens* and the applicability of these results to other poplar species or woody plants requires further investigation. Comparative analyses across different plant species and stress conditions could provide a more comprehensive understanding of MYB transcription factors in anthocyanin regulation.

Looking forward, we aim to further elucidate the regulatory mechanisms of *MYB118L* within the anthocyanin synthesis pathway and assess its potential for functional application across diverse plant species. This research direction is in line with contemporary efforts to understand and harness the complexity of transcriptional regulation in plants.

To summarize, the transgenic *MYB118L P. canescens* developed in this study holds considerable promise as a novel tree species with enhanced visual and ecological attributes. The identification of *MYB118L* as a critical transcription factor in anthocyanin biosynthesis not only enriches our understanding of coloration mechanisms in plants but also provides a foundation for developing ornamental trees with improved adaptability to arid or cold climates. Our findings present a promising starting point for future research endeavors focused on the genetic enhancement of tree species for aesthetic and environmental benefits.

Conclusion

In this study, we isolated an MYB gene regulating anthocyanin biosynthesis from *Populus* 'Zhonghua Hongye'. Gene expression analyses demonstrated that *a MYB118* homologous gene, *MYB118L*, was up-regulated in *Populus* 'Zhonghua Hongye' compared with ZL-2025. Subcellular localization analyses showed that MYB118L-GFP fusion protein was specially located in nucleus. When overexpressed in *P. canescens,* transgenic plants overexpressing *MYB118L* produced more anthocyanins and turned their color into redness under stress conditions. Consistently, transcripts of some important anthocyanidin biosynthesis genes were significantly increased in transgenic plants. Furthermore, the results also revealed that *MYB118L* could bind to MBS cis-element directly for positive regulation. Dual-luciferase assays revealed that *MYB118L* can directly activate the promoter of *LAR1* gene. Overall, our data indicated that *MYB118L* functions as an essential transcription factor regulating anthocyanin biosynthesis in poplar and could be used for the genetic engineering of colorful tree species.

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Authors' contributions

WZ and ZH contributed to data analysis and manuscript preparation. WQ and RZ planned and designed the research. WZ, WQ, YL, YY performed the experiments, WZ, WQ and XH wrote the manuscript and coordinated its revision. GQ and JX helped in sample preparation and data collection. All authors read and provided helpful discussions and approved the final version.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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