Cloning and functional identification of anthocyanin synthesis-regulating transcription factor AaMYB4 in Aeonium arboreum ‘Halloween’

Rong Zhao
Suqian University
Su-Hua Li
Suqian University
Hao-Zhang Han
21011@squ.edu.cn

Suqian University
Li-Hua Zhang
Suqian University
Fang Wang
Suqian University
Nan Zhang
Suqian University

Research Article

Keywords: Aeonium arboreum ‘Halloween’, Succulents, Regulation of anthocyanin synthesis, MYB transcription factor, Negative regulation

Posted Date: January 30th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-3896321/v1

Additional Declarations: No competing interests reported.

Abstract

Succulents are widely appreciated for indoor decoration, primarily due to their vibrant leaf colors. However, the underlying mechanisms of leaf color development in succulents remain largely unexplored. In this study, we isolated AaMYB4, an MYB transcription factor that represses anthocyanin synthesis, from an ornamental succulent, *Aeonium arboreum* 'Halloween'. Our study employed 'Halloween' leaves for experimental analysis, incorporating gene cloning, bioinformatics, functional validation of transgenes, and yeast two-hybrid assays to investigate AaMYB4's role. The finding revealed that the full-length Open Reading Frame (ORF) of *AaMYB4* spans 825 base pairs, encoding 274 amino acids. Phylogenetic analyses suggest AaMYB4 as a transcription factor suppressing flavonoid biosynthesis. Comparative analysis of protein sequences revealed that *AaMYB4* functions as an R2R3-MYB transcription factor, characterized by a typical repressive EAR motif. *AaMYB4* was cloned into *Arabidopsis* by inflorescence infestation. The WT and stably overexpressing *AaMYB4* T_2-generation *Arabidopsis* were subsequently grown under stress conditions including nitrogen deficiency, high light exposure, 6% sucrose, and abscisic acid (ABA) treatment. The results indicated that the anthocyanin content was significantly reduced in *AaMYB4* overexpressing *Arabidopsis* compared with the WT under the four treatments described above, and the structural genes for anthocyanin synthesis were down-regulated in the *AaMYB4* transgenic *Arabidopsis*. Moreover, the expression levels of the positively regulated MYB and bHLH transcription factors involved in anthocyanin synthesis, specifically AtPAP1 and AtTT8, exhibited a significant downregulation in *Arabidopsis*. Yeast two-hybrid assays revealed no interaction between AaMYB4 and AaTT8, and the AaMYB4 protein itself can interact. This research confirms AaMYB4's role in inhibiting anthocyanin synthesis in 'Halloween' leaves, enriching our understanding of the molecular basis of leaf color formation in succulents. Additionally, it offers valuable genetic insights for developing new 'Halloween' colorful leaf varieties.

Introduction

Anthocyanins, water-soluble secondary flavonoid metabolites, are branched chain end products of flavonoid metabolism. Anthocyanins, typically stored in vesicles after synthesis, red, blue, or purple hues to the leaves, flowers, and fruits of plants, enhancing their aesthetic and commercial appeal, particularly in ornamental varieties (Mol et al., 1998; Zhao and Tao, 2015; Zhao et al., 2022). Anthocyanins have the ability to attract pollinating insects and help plants withstand Ultraviolet radiation, cold temperatures, and pathogenic attacks (Oliveira et al., 2020; Alappat and Alappat, 2020; Li et al., 2023). Moreover, the consumption of anthocyanins offers significant health advantages, including anti-inflammatory properties and potential contributions to weight management (Lee et al., 2017; Kelley et al., 2018).

Anthocyanin biosynthesis pathways are highly conserved across plant species, showing remarkable similarity (Holton and Cornish, 1995; Sunil and Shetty, 2022). The immediate precursor of anthocyanin biosynthesis is phenylalanine, which is catalyzed sequentially by phenylalanine deaminase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H), flavonoid-3'-hydroxylase (F3'H), dihydroflavonoid reductase (DFR), anthocyanidin synthase (ANS), and flavonoid

glycosidyltransferase (UFGT), and ultimately synthesizes coloured anthocyanins (Sunil and Shetty, 2022).

The regulation of anthocyanin synthesis-related enzyme genes is mainly attributed to transcription factors (TFs) such as MYB, bHLH, WD40, and their complexes (Xu et al., 2015; Liu et al., 2021). The influence of MYB transcription factors (TFs) on anthocyanin synthesis encompasses both facilitative and inhibitory roles (Yan et al., 2021; Li et al., 2022). Specifically, proteins within the S6 subgroup of the R2R3-MYB family, such as AtPAP1 (Chhon et al., 2020) and MdMYB10 (Espley et al., 2007) predominantly exert a positive regulatory effect (Naing and Kim, 2018). Conversely, the negative regulation of anthocyanin synthesis is principally mediated by MYB TFs within the S4 subgroup of the R2R3-MYB and the R3-MYB families (Chen et al., 2019), including AtMYB4 (Wang et al., 2020), BrMYB4 (Zhang et al., 2014), MdMYB16 (Xu et al., 2017) for R2R3-MYBs and AtMYBL2 (Matsui et al., 2008), AtCPC (Zhu et al., 2009), PtMYB182 (Yoshida et al., 2015) for R3-MYBs. Moreover, R3-MYB was shown to impede anthocyanin synthesis by inhibiting the formation of the complex responsible for anthocyanin synthesis via interacting with the bHLH protein in the MBW complex (Zhang et al., 2020; Zhao et al., 2022; Xie et al., 2022). In contrast, the mechanism of R2R3-type MYBs inhibition is not uniform; some are inhibited by blocking complex formation through interaction with bHLH proteins (Zhang et al., 2023; Hu et al., 2024), while others are inhibited by inhibiting their expression through direct binding to the promoter regions of anthocyanin synthesis-related enzyme genes (Xu et al., 2017). The MYB gene plays a central role in regulating anthocyanin synthesis, which is a finely tuned process governed by a precise regulatory mechanism. This regulation involves not only positively regulated transcription factors but also negatively regulated proteins in a timely manner. Most of the current research focused on the positive regulation of anthocyanins, the negative regulation of anthocyanin synthesis should receive more attention.

Succulents are widely appreciated for indoor decoration, primarily due to their vibrant leaf colors. However, the underlying mechanisms of leaf color development in succulents remain largely unexplored. *Aeonium arboreum* 'Halloween' Belongs to *Aeonium spp.* of the Sedum family, and is highly esteemed for its ornamental qualities. However, its leaf color is highly unstable and is affected by stress conditions such as light. Our previous research found that 'Halloween,' characterized by its green leaf color, transformed to red after a 14-day treatment of darkness followed by 3 days of light exposure. Furthermore, cornflavin-3-galactoside was identified as the main pigment causing the reddening of 'Halloween' leaves. Transcriptome sequencing indicated a significant downregulation of AaMYB4 expression following light exposure (Zhao et al., 2023), which shares the highest homology with *AtMYB4* in *Arabidopsis thaliana*. It was speculated that it might be a TF that negatively regulates the synthesis of anthocyanins in 'Halloween'. To verify this speculation, the current study cloned the *AaMYB4* gene, analyzed its protein sequence, and transferred it into *Arabidopsis thaliana* for functional investigation. Additionally, the study investigated its possible reciprocal proteins, aiming to provide theoretical support for revealing the mechanism of ‘Halloween’ leaf color formation.

Materials and Methods

Plant materials and growth conditions

One-year-old ‘Halloween’ cuttings were first dark-treated for 14 days and then transferred to light treatment (The photoperiod is specified as 16 h of light and 8 h of darkness, light intensity was set at 200–250 μmol/(m^2s)) for 3 days. Subsequently, the samples were immediately collected for gene cloning. The *Arabidopsis thaliana* used in this experiment was of the Columbia ecotype and was grown in an artificial climatological chamber at a temperature of 22°C with a photoperiod specified as 16 h of light (10,000 Lux) and 8 h of darkness.

Gene cloning

The encoding sequence information of AaMYB4 was obtained from the ‘Halloween’ leaf transcriptome database. Specific primers were designed to amplify the *AaMYB4* gene using high-fidelity polymerase with the 'Halloween' leaf cDNA as a template. The amplified sequence was subsequently linked to the T vector and sequenced. Primers used for *AaMYB4* gene cloning: AaMYB4F (5'-AACATGAGGAAGCCTTGCTG-3'), AaMYB4R (5'-CTTCATCCATCACATAAA GAGG-3').

Phylogenetic analysis alignment and multiple sequences

In MEGA7.0, the neighbor-joining (N-J) method was applied to construct the phylogenetic tree. The robustness of the constructed tree was assessed using the bootstrap method with 1000 replicates. Protein sequence alignments were obtained using DNAMAN V6.

Transcriptional activity analysis

The coding sequences of *AaMYB4* were successfully amplified by utilizing AaMYB4-T vector plasmids as a template and then ligated into the pGBKT7 plasmid using homologous recombination with EcoR I and BamH I as the digestion sites. The recombinant plasmid was subsequently transferred into AH109 using Frozen-EZ Yeast Transformation II Kit. The transcriptional activity of AaMYB4 was determined on single-deficient (SD/-Trp) media, triple-deficient (SD/-Trp-His-Ade) media, and triple-deficient media with the addition of X-α-Gal (SD/-Trp-His-Ade/X-α-gal). The primers used for transcriptional activity analysis were as follows: AaMYB4-BDF (5'-ATGGCCATGGAGGCCGAATTCAACATGAGGAAGCCTTGCTG-3'), AaMYB4-BDR (5'-CGCTGCAGGTCGACGGATCCCTTCATCCATCACATAAAGAGG-3').

Overexpression vector construction and model plant transformation

The complete open reading frame of *AaMYB4* was cloned into the expression vector pCAMBIA2300S through homologous recombination, utilizing KpnI and BamHI as digestion sites. The plasmid pCAMBIA2300S-*AaMYB4* was transferred into Agrobacterium tumefaciens GV3101 using the freeze-thaw method and used for the subsequent transformation of *Arabidopsis thaliana*. The *AaMYB4* genes were transferred into WT *Arabidopsis* using the Agrobacterium-mediated floral-dip method. Primers used for

AaMYB4 expression vector construction were as follows: AaMYB4-2300SF (5'-GAGCTTTCGCGAGCTCGGTACCAAC ATGAGGAAGCCTTGCTG-3'), AaMYB4-2300SR (5'-CAGGTCGACTCTAGAGAGGATCCC TTCATCCATCACATAAAGAGG-3'). Positive detection primers for AaMYB4 transgenic *Arabidopsis* were AaMYB4F and 2300SR (5'-GGAGAAACTCGAGCTTGC-3').

Treatment of AaMYB4 transgenic Arabidopsis thaliana

Nitrogen-deficient MS medium and 6% sucrose-mimetic MS medium were used to culture *Arabidopsis* seeds for 7 days under standard conditions. For the high light treatment, *Arabidopsis* was sown on MS medium and cultured in continuous light for 7 days. In the ABA treatment protocol, *Arabidopsis* was grown on MS culture medium for 7 days and the sterile seedlings were transferred to MS medium with the addition of 15 μmol ABA and further cultured for 14 days.

RNA extraction and fluorescence quantitative PCR

The leaves of the fourth to fifth whorl from the center of the 'Halloween' plant were used for RNA extraction and cDNA synthesis. The methodology of the above experiments is described in Zhao et al. (2023). Fluorescence quantitative PCR analysis was performed on 21-day-old *Arabidopsis* plants treated with ABA. RNA extraction and cDNA synthesis in *Arabidopsis* were conducted following the protocol described by Yang et al. (2018). The fluorescence quantitative PCR procedure was adopted by Zhao et al. (2023), using *AtACT2* as the internal reference gene. Primer sequences for Primer sequences for this PCR were obtained from Zhao et al. (2020).

Anthocyanin extraction and content determination

Seven-day-old and twenty-one-day-old *Arabidopsis* under treatments were used for anthocyanin extraction and content determination. The experiments were performed in accordance with the methodology outlined by Zhao et al. (2023).

Yeast two-hybrid

Homologous recombination was used to link AaMYB4 and AaTT8 into pGADT7 and pGBKT7 plasmids, respectively. The recombinant plasmids pGADT7-AaMYB4/AaTT8 and pGBKT7-AaMYB4/AaTT8 were transfected into a yeast strain, AH109 cells, following the protocols outlined in the transcriptional activity analysis section. Positive transformants were initially screened on double-deficient (SD/-Leu-Trp) medium and later transferred to four-deficient (SD/-Ade-His-Leu-Trp) medium and four-deficient medium supplemented with 25 mg/L X-α-Gal (SD/-Ade-His-Leu-Trp + X-α-gal). The primers used in the AaMYB4 and AaTT8 two-hybrid experiments were AaMYB4-ADF (5 '-GCCATGGAGGCCAGTGAATTCAACATGA GGAAGCCTTGCTG-3'), AaMYB4-ADR (5'- AGCTCGAGCTCGATGGATCCCTTCATCCATC ACATAAAGAGG-3'), AaMYB4-BDF, AaMYB4-BDR, AaTT8-ADF (5'- GCCATGGAGGCCAG TGAATTCAGTTTCTTGAATGGCTGCAG-3'), AaTT8-ADR (5'-AGCTCGAGCTCGATGGATC CTGATCGAAGGCTCATTGTTG-3'), AaTT8-BDF (5'-ATGGCCATGGAGGCCGAATTCAGTT TCTTGAATGGCTGCAG-3'), and AaTT8-BDR (5'- CGCTGCAGGTCGACGGGATCCTGATC GAAGGCTCATTGTTG-3').

Statistical analysis

Significance analysis of differences in anthocyanin content and gene expression levels between WT and *AaMYB4* transgenic plants was conducted using a two-tailed Student's *t*-test.

Results

Cloning and sequence analysis of AaMYB4

Our previous study revealed the downregulation of AaMYB4 expression in 'Halloween' leaves after light exposure, implying its involvement in the regulation of light-responsive anthocyanins synthesis. To verify their function, we cloned this gene.

The 'Halloween' leaf cDNA was used as a template to amplify the size fragment ranging from 750 bp ~ 1000 bp using a PCR instrument (Fig. 1B). Sequencing determined the fragment's length to be 837 bp. Analysis with ORF Finder software revealed that the *AaMYB4* gene's ORF spans 825 bp, encoding 274 amino acids (Fig. 1C). The genome database (Han et al., 2024) of 'Euro Purple' (a variety of the same series as 'Halloween') identified that AaMYB4's DNA structure includes three exons and two introns (Fig. 1A).

Phylogenetic analysis of the AaMYB4 amino acid sequence alongside MYBs regulating flavonoid synthesis suggests that AaMYB4 acts as a transcriptional repressor of flavonoid biosynthesis (Fig. 2). Comparative analysis further reveals that AaMYB4 belongs to the R2R3-MYB family and possesses the characteristic EAR motif indicative of a repressive function in the structural domain (Fig. 3).

Transcriptional properties assay of AaMYB4

To explore the protein transcriptional properties of *AaMYB4*, we incorporated the intact ORF of AaMYB4 into the pGBKT7 plasmid and transformed it into cells of yeast strain AH109, which was subsequently validated on three mediums (Fig. 4). The findings revealed that the yeast containing the empty vector, the pGBKT7-AaMYB4 vector, and the positive control exhibited normal growth on single-deficient medium (Fig. 4). The positive control grew normally on triple-deficient medium and triple-deficient media with the addition of X-α-Gal (Fig. 4), whereas the former two failed to grow on these two medium (Fig. 4), revealing that AaMYB4 cannot activate gene expression.

Construction of AaMYB4 plant expression vector and identification of transgenic Arabidopsis thaliana

The AaMYB4 overexpression vector was constructed using the homologous recombination method. To confirm its success, the vector underwent double-enzyme digestion with KpnI and BamHI, resulting in two fragments on electrophoresis. The sizes of these fragments matched the expected outcomes (Fig. 5A), indicating the successful construction of the AaMYB4 overexpression vector.

AaMYB4 was transformed into *Arabidopsis* by inflorescence infestation, and PCR was used for confirmation of transformation. The findings revealed that 9 out of 11 resistant plants were positive (Fig. 5B), and *AaMYB4* had been successfully transformed in *Arabidopsis*. Subsequently, a semi-quantitative analysis of 11 transgenic lines for AaMYB4 expression identified lines 8 and 11 as exhibiting the highest levels of overexpression and were designated as L1 and L2 lines.

Phenotypic analysis of AaMYB4 transgenic Arabidopsis thaliana

To ascertain the role of *AaMYB4* in regulating anthocyanin synthesis, the *Arabidopsis* was subjected to nitrogen deficiency, strong light, 6% sucrose, and ABA treatments. Subsequent analysis demonstrated that these treatments stimulated anthocyanin accumulation in WT seedlings. Conversely, anthocyanin levels were markedly diminished in *AaMYB4* transgenic Arabidopsis compared to WT. (Fig. 6A-D). Further quantification of anthocyanin content in treated seedlings revealed a significant reduction in Arabidopsis overexpressing AaMYB4 compared to WT across all treatment conditions. These findings suggest that AaMYB4 plays a role in inhibiting anthocyanin synthesis.

Expression analysis of anthocyanin synthesis-related genes in Arabidopsis overexpressing AaMYB4

Phenotypic analysis of AaMYB4-transgenic Arabidopsis thaliana revealed that AaMYB4 inhibits anthocyanin synthesis. This study aimed to elucidate the mechanism by which *AaMYB4* suppresses anthocyanin production in Arabidopsis. To this end, we conducted fluorescence quantitative PCR analysis on ABA-treated Arabidopsis, focusing primarily on the expression patterns of genes associated with anthocyanin synthesis. The findings demonstrated a downregulation in the expression of genes responsible for anthocyanin synthesis in AaMYB4-transgenic Arabidopsis (Fig. 7A). Furthermore, expression levels of the anthocyanin-synthesis-promoting MYB transcription factor AtPAP1 and the bHLH transcription factor AtTT8 were significantly reduced (Fig. 7B). Conversely, the expression of the MYB transcription factor AtMYBL2, which negatively correlates with anthocyanin synthesis, showed no significant difference between wild-type and AaMYB4-transgenic lines (Fig. 7B). The above results suggest that *AaMYB4* may inhibit anthocyanin synthesis by repressing *AtPAP1* and *AtTT8* which in turn affects the genes encoding the enzymes required for anthocyanin production, thereby inhibiting anthocyanin synthesis.

Exploration of AaMYB4 and AaTT8 interactions

Previous research has demonstrated that MYB proteins, which inhibit anthocyanin synthesis, frequently interact with bHLH transcription factors, such as TT8, to regulate the synthesis of anthocyanins. To investigate the interaction of AaMYB4 with AaTT8 in 'Halloween', relevant vectors were constructed and transferred into yeast strain AH109, which were subsequently validated on double-deficient medium, four-deficient medium, and four-deficient medium with the addition of X-α-Gal specialized for yeast. The findings indicated that across all test combinations, yeast strains exhibited normal growth on the double-deficient medium. However, on the four-deficient medium, only strains transformed with AaMYB4-AD and AaMYB4-BD combinations maintained normal growth. In contrast, strains harboring AaMYB4-AD/BD and

AaTT8-AD/BD combinations exhibited impaired growth. These findings indicated a lack of reciprocal interaction between AaMYB4 and AaTT8 proteins while demonstrating the self-interaction of AaMYB4 proteins. Only Yeast strains transformed with AaMYB4-AD and AaMYB4-BD exclusively exhibited blue coloration on X-α-Gal-supplemented, and four-deficient medium, indicating a direct interaction between AaMYB4 proteins.

Discussion

Anthocyanin production in plants is regulated through a series of transcription factors both in positive and negative senses (Yang Lin et al., 2014). FaMYB1, an R2R3-type MYB transcription factor from strawberry, was the first identified to inhibit anthocyanin synthesis. Its overexpression in tobacco results in a color shift of the petals from pink to white (Aharoni et al., 2001). R2R3-MYBs is another TF that inhibits anthocyanin synthesis in different plants such as turnip BrMYB4 (Zhang et al., 2014), apple MdMYB16 (Xu et al., 2017), and *Arabidopsis* AtMYB4 (Wang et al., 2020). All MYBs that inhibit anthocyanin synthesis belong to the S4 subgroup of R2R3-MYB and share a conserved repressive motif "pdLNLD/EL" (LaFountain and Yuan, 2021). Studies on R2R3-MYB genes suppressing anthocyanin synthesis primarily focused on model plants and have not been reported in succulents. Our study introduces 'Halloween' AaMYB4, isolated from succulents, as another member of the R2R3-MYB family harboring the aforementioned repressive motifs. Meanwhile, ectopic expression of *AaMYB4* in *Arabidopsis* causes a significant decrease in anthocyanin accumulation. AaMYB4 is the first transcription factor reported to negatively regulate anthocyanins in succulents.

R2R3-MYBs that inhibit anthocyanin synthesis can be further subdivided into 2 major classes, AtMYB4-like and FaMYB1-like (Yan et al., 2021). The former include AtMYB4, BrMYB4, MdMYB16, TgMYB4 (Hu al., 2015), and SlMYB7 (Zhang al., 2023), while the latter include FaMYB1, PhMYB27 (Albert et al., 2014), and VvMYBC2-L1 (Cavallini et al., 2015). FaMYB1-like proteins, including FaMYB1 (Aharoni et al., 2001) and PhMYB27 (Albert et al., 2014), interact with TT8-like proteins, a pivotal bHLH transcription factor that regulates anthocyanin synthesis. Notably, FaMYB1 and PhMYB27 interact with the petunia TT8-like protein AN1. Notably, AtMYB4 from the AtMYB4-like protein family shows no interaction with the TT8-like protein AtTT8 (Zimmermann et al., 2004). Similarly, there is an absence of supporting evidence for an interaction between MdMYB16 and the TT8-like protein MdbHLH3 (Xu et al., 2017). However, TgMYB4 (Hu al., 2015) and SlMYB7 (Zhang al., 2023) belonging to the AtMYB4-Like type were shown to interact with TT8-Like proteins. Therefore, this raises uncertainty about the interaction of AtMYB4-Like proteins with TT8-Like proteins. To investigate whether AaMYB4 interacts with TT8-like proteins, yeast two-hybrid experiments were carried out and findings revealed that AaMYB4 does not interact with AaTT8, but interestingly, AaMYB4 can self-interact. Similarly, apple MdMYB16 also exhibits self-interaction (Xu et al., 2017). The above results further suggest that MYB4-like proteins may not interact with TT8-like proteins but have the capability to self-interact.

It is generally believed that FaMYB1-like proteins inhibit anthocyanin synthesis by interacting with TT8-like proteins, thus preventing the formation of the MYB, bHLH, and WD40 complexes, which ultimately

represses anthocyanin synthesis (Yan et al., 2021). Conversely, AtMYB4-like proteins directly target the promoter regions of genes associated with anthocyanin production. For instance, Apple MdMYB16 directly binds to the promoter regions of *MdANS* and *MdUFGT* (Xu et al., 2017). We speculated that AaMYB4 might repress anthocyanin synthesis through direct binding to the promoter regions of anthocyanin synthesis-related enzyme genes and thus inhibit anthocyanin synthesis. This inference needs to be followed up with further studies.

Conclusions

In this study, we successfully cloned the full 825 bp coding sequence of the AaMYB4 transcription factor. Bioinformatics analyses revealed that AaMYB4 encodes a protein possessing an inhibitory domain. Moreover, overexpression of *AaMYB4* in *Arabidopsis thaliana* significantly reduced the anthocyanin contents and the expression level of genes associated with anthocyanin biosynthesis in transgenic plants. These findings confirm that AaMYB4 acts as a transcription factor that represses anthocyanin synthesis in 'Halloween". This study enriches our understanding on the molecular basis of leaf color formation in succulents. Additionally, it offers valuable genetic insights for developing new 'Halloween' colorful leaf varieties.

Declarations

Acknowledgements This work was funded by Suqian Sci & Tech Program (Grant No.K202207) and the basic science research (natural science) of colleges and universities in Jiangsu province (Grant No.23KJD210005)

Author Contribution The study was conceived by Rong Zhao, Su-Hua Li, Hao-Zhang Han, Li-Hua Zhang, Fang Wang, and Nan Zhang. Rong Zhao and Fang Wang cloned AaMYB4 and analysed its transcriptional activity; Su-Hua Li and Nan Zhang analyzed the expression of genes related to anthocyanin synthesis; Rong Zhao, Hao-Zhang Han, Li-Hua Zhang and performed the four treatment of AaMYB4 transgenic *Arabidopsis*; Rong Zhao wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Data Availability Not applicable.

Ethical Approval Not applicable.

Conflict of Interest The authors declare no competing interests.

References

1. Aharoni A, De Vos CH, Wein M et al (2001) The strawberry FaMYB1 transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. Plant J 28(3):319–332

2. Albert NW, Davies KM, Lewis DH et al (2014) A conserved network of transcriptional activators and repressors regulates anthocyanin pigmentation in eudicots. Plant Cell 26(3):962–980
3. Alappat B, Alappat J (2020) Anthocyanin Pigments: Beyond Aesthetics. Molecules 25(23):5500
4. Cavallini E, Matus JT, Finezzo L et al (2015) The phenylpropanoid pathway is controlled at different branches by a set of R2R3-MYB C2 repressors in grapevine. Plant Physiol 167(4):1448–1470
5. Chen L, Hu B, Qin Y et al (2019) Advance of the negative regulation of anthocyanin biosynthesis by MYB transcription factors. Plant Physiol Biochem 136:178–187
6. Chhon S, Jeon J, Kim J et al (2020) Accumulation of Anthocyanins through Overexpression of AtPAP1 in Solanum nigrum Lin. (Black Nightshade). Biomolecules 10(2). 10.3390/biom10020277
7. Espley RV, Hellens RP, Putterill J et al (2007) Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J 49(3):414–427
8. Holton TA, Cornish EC (1995) Genetics and Biochemistry of Anthocyanin Biosynthesis. Plant Cell 7(7):1071–1083
9. Hu X, Liang Z, Sun T et al (2024) The R2R3-MYB Transcriptional Repressor TgMYB4 Negatively Regulates Anthocyanin Biosynthesis in Tulips (Tulipa gesneriana L). Int J Mol Sci 25(1):563
10. Kelley DS, Adkins Y, Laugero KD (2018) A Review of the Health Benefits of Cherries. Nutrients 10(3):368
11. LaFountain AM, Yuan YW (2021) Repressors of anthocyanin biosynthesis. New Phytol 231(3):933–949
12. Lee YM, Yoon Y, Yoon H et al (2017) Dietary Anthocyanins against Obesity and Inflammation. Nutrients 9(10):1089
13. Li C, Yu W, Xu J et al (2022) Anthocyanin Biosynthesis Induced by MYB Transcription Factors in Plants. Int J Mol Sci 23(19):11701
14. Li Z, Ahammed GJ (2023) Hormonal regulation of anthocyanin biosynthesis for improved stress tolerance in plants. Plant Physiol Biochem 201:107835
15. Liu Y, Ma K, Qi Y et al (2021) Transcriptional Regulation of Anthocyanin Synthesis by MYB-bHLH-WDR Complexes in Kiwifruit (*Actinidia chinensis*). J Agric Food Chem 69(12):3677–3691
16. Matsui K, Umemura Y, Ohme-Takagi M (2008) AtMYBL2, a protein with a single MYB domain, acts as a negative regulator of anthocyanin biosynthesis in Arabidopsis. Plant J 55(6):954–967
17. Mol J, Grotewold E, Koes R (1998) How genes paint flowers and seeds. Trends Plant Sci 6(6):212–217
18. Naing AH, Kim CK (2018) Roles of R2R3-MYB transcription factors in transcriptional regulation of anthocyanin biosynthesis in horticultural plants. Plant Mol Biol 98(1–2):1–18
19. Oliveira H, Correia P, Pereira AR et al (2020) Exploring the Applications of the Photoprotective Properties of Anthocyanins in Biological Systems. Int J Mol Sci 21(20):7464
20. Sunil L, Shetty NP (2022) Biosynthesis and regulation of anthocyanin pathway genes. Appl Microbiol Biotechnol 106(5–6):1783–1798

21. Wang XC, Wu J, Guan ML et al (2020) Arabidopsis MYB4 plays dual roles in flavonoid biosynthesis. Plant J 101(3):637–652
22. Xie T, Zan X, Chen X et al (2022) An R3-MYB repressor, BnCPC forms a feedback regulation with MBW complex to modulate anthocyanin biosynthesis in Brassica napus. Biotechnol Biofuels Bioprod 15(1):133
23. Xu H, Wang N, Liu J et al (2017) The molecular mechanism underlying anthocyanin metabolism in apple using the MdMYB16 and MdbHLH33 genes. Plant Mol Biol 94(1–2):149–165
24. Xu W, Dubos C, Lepiniec L (2015) Transcriptional control of flavonoid biosynthesis by MYB-bHLH-WDR complexes. Trends Plant Sci 20(3):176–185
25. Yan H, Pei X, Zhang H et al (2021) MYB-Mediated Regulation of Anthocyanin Biosynthesis. Int J Mol Sci 22(6):3103
26. Yang N, Zhao K, Li X et al (2018) Comprehensive analysis of wintersweet flower reveals key structural genes involved in flavonoid biosynthetic pathway. Gene 676:279–289
27. Yoshida K, Ma D, Constabel CP (2015) The MYB182 protein down-regulates proanthocyanidin and anthocyanin biosynthesis in poplar by repressing both structural and regulatory flavonoid genes. Plant Physiol 167(3):693–710
28. Zhang H, Gong J, Chen K et al (2020) A novel R3 MYB transcriptional repressor, MaMYBx, finely regulates anthocyanin biosynthesis in grape hyacinth. Plant Sci 298:110588
29. Zhang L, Duan Z, Ma S et al (2024) SlMYB7, an AtMYB4-Like R2R3-MYB Transcription Factor, Inhibits Anthocyanin Accumulation in Solanum lycopersicum Fruits. J Agric Food Chem 71(48):18758–18768
30. Zhang L, Wang Y, Sun M et al (2014) BrMYB4, a suppressor of genes for phenylpropanoid and anthocyanin biosynthesis, is down-regulated by UV-B but not by pigment-inducing sunlight in turnip cv. Tsuda. Plant Cell Physiol 55(12):2092–2101
31. Zhao D, Tao J (2015) Recent advances on the development and regulation of flower color in ornamental plants. Front Plant Sci 6:261
32. Zhao X, Zhang Y, Long T et al (2022) Regulation Mechanism of Plant Pigments Biosynthesis: Anthocyanins, Carotenoids, and Betalains. Metabolites 12(9):871
33. Zhao J, Chen L, Ma A et al (2021) R3-MYB transcription factor LcMYBx from Litchi chinensis negatively regulates anthocyanin biosynthesis by ectopic expression in tobacco. Gene 812:146105
34. Zhao R, Li SH, Zhang LH et al (2023) Transcriptome Analysis Reveals the Effect of Light on Anthocyanin Synthesis in Leaves of Aeonium arboreum ‘Halloween’. Russ J Plant Physiol 70:64
35. Zhao R, Song X, Yang N et al (2020) Expression of the subgroup IIIf bHLH transcription factor CpbHLH1 from Chimonanthus praecox (L.) in transgenic model plants inhibits anthocyanin accumulation. Plant Cell Rep 39(7):891–907
36. Zhu HF, Fitzsimmons K, Khandelwal A et al (2009) CPC, a single-repeat R3 MYB, is a negative regulator of anthocyanin biosynthesis in Arabidopsis. Mol Plant 2(4):790–802

37. Zimmermann IM, Heim MA, Weisshaar B et al (2004) Comprehensive identification of Arabidopsis thaliana MYB transcription factors interacting with R/B-like BHLH proteins. Plant J 40(1):22–34

Figures

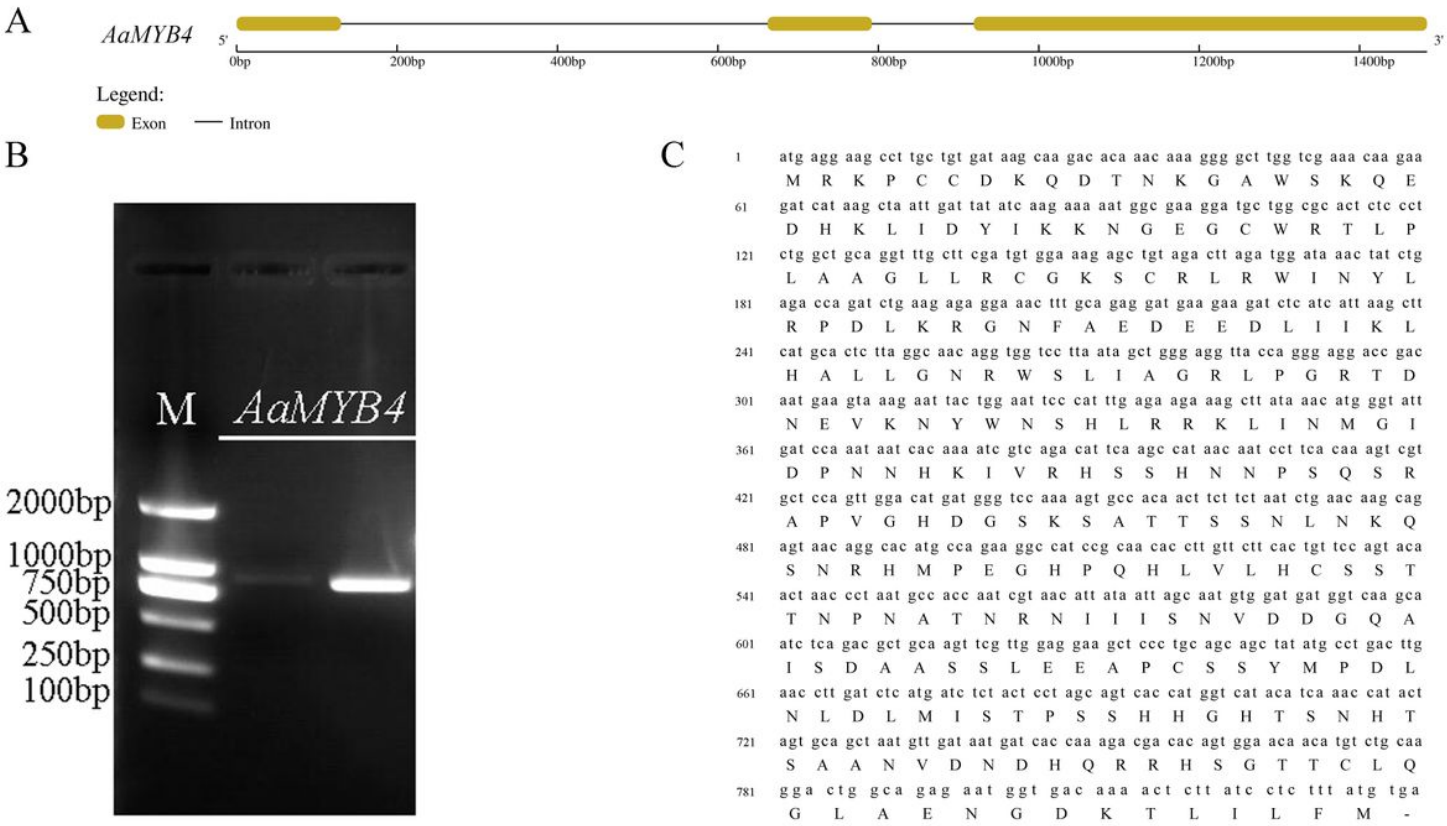

Figure 1

Cloning and gene structure analysis of *AaMYB4*

A: Gene structure of AaMYB4. B: PCR amplification of AaMYB4. C: Amino acid sequence of AaMYB4 protein

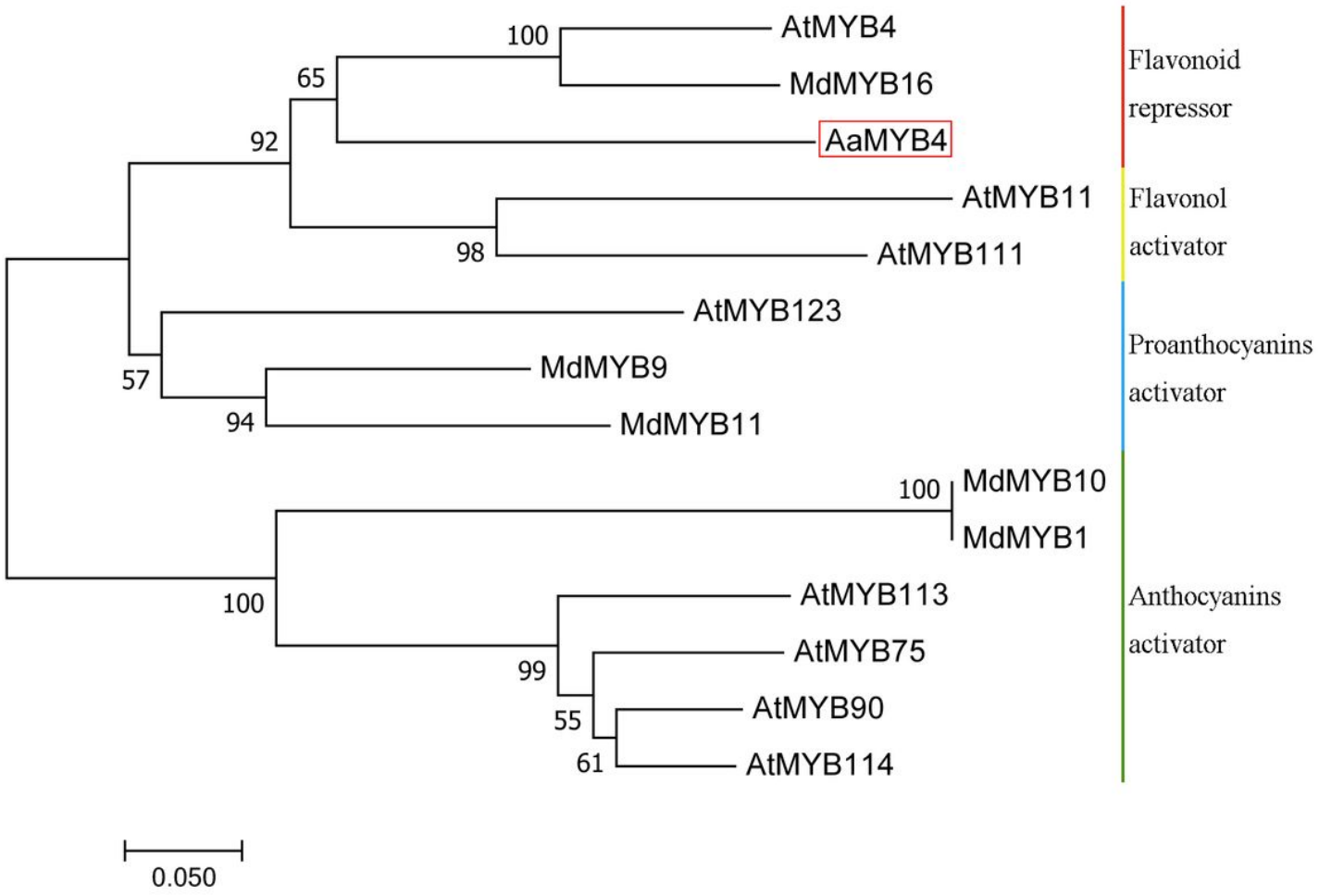

Figure 2

The phylogenetic tree analysis of AaMYB4 and flavonoid-regulated MYB proteins in other species

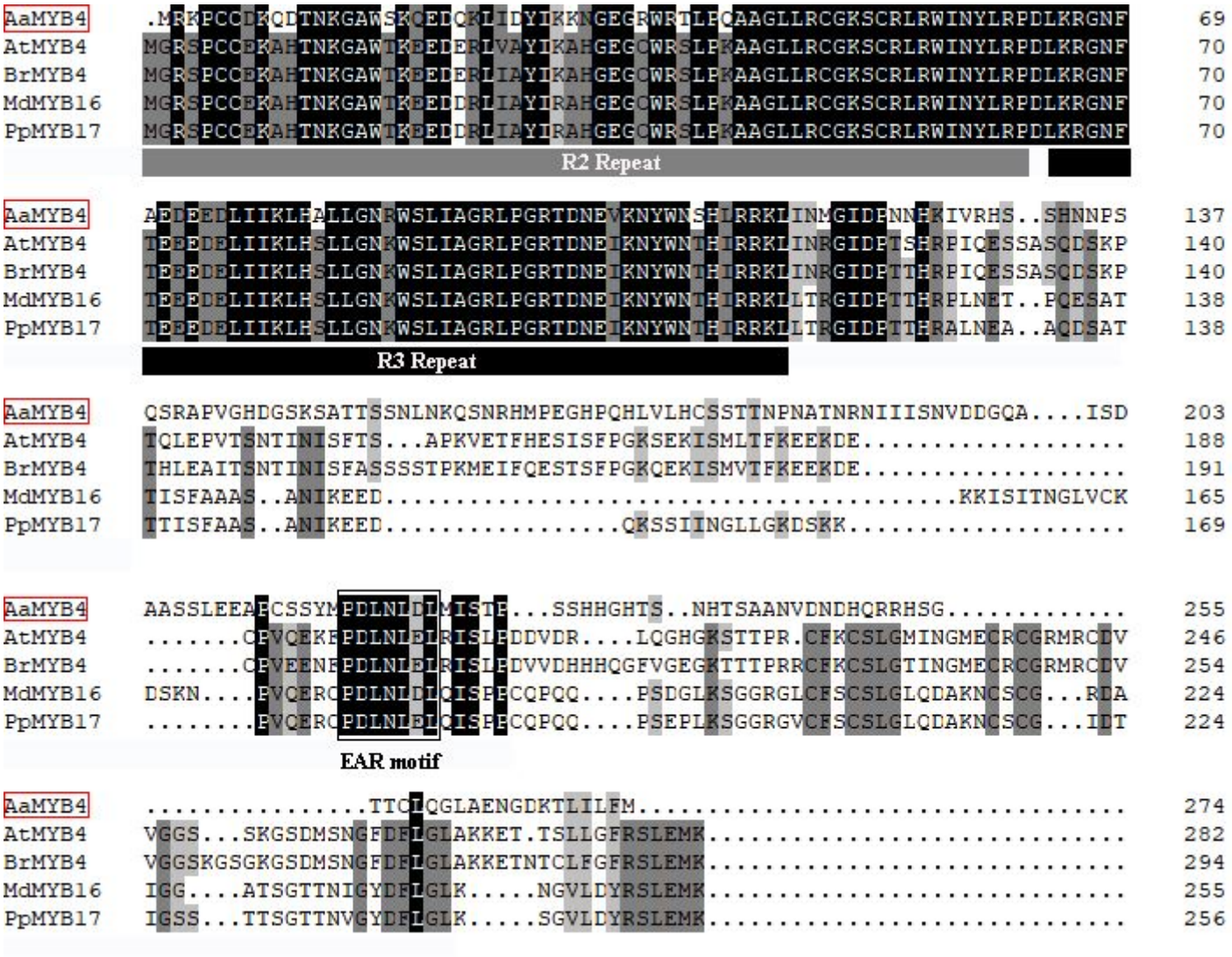

Figure 3

Sequence alignment of AaMYB4 protein

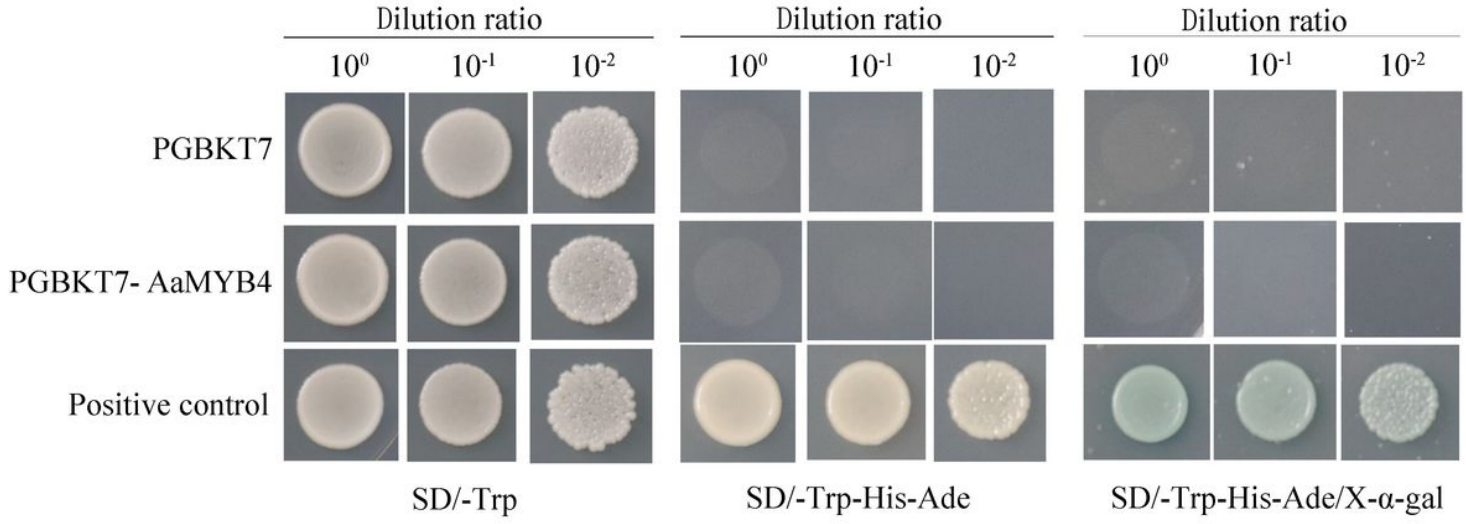

Figure 4

Analysis of the transcriptional activity of AaMYB4

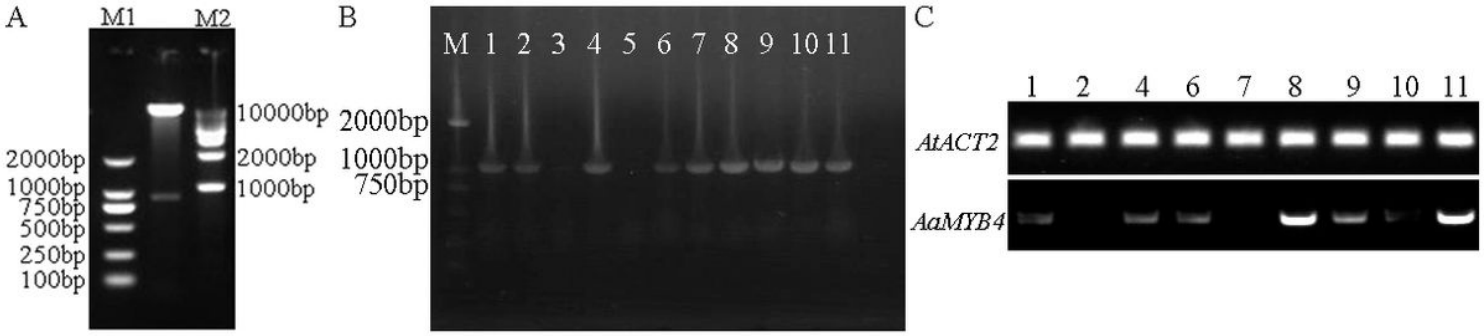

Figure 5

Double digestion of the *AaMYB4* expression vector and identification of its transgenic *Arabidopsis thaliana*

A : Double digestion of AaMYB4 expression vector. B : Positive identification of AaMYB4 transgenic *Arabidopsis.* C : Semi-quantitative analysis of *AaMYB4*in transgenic *Arabidopsis*

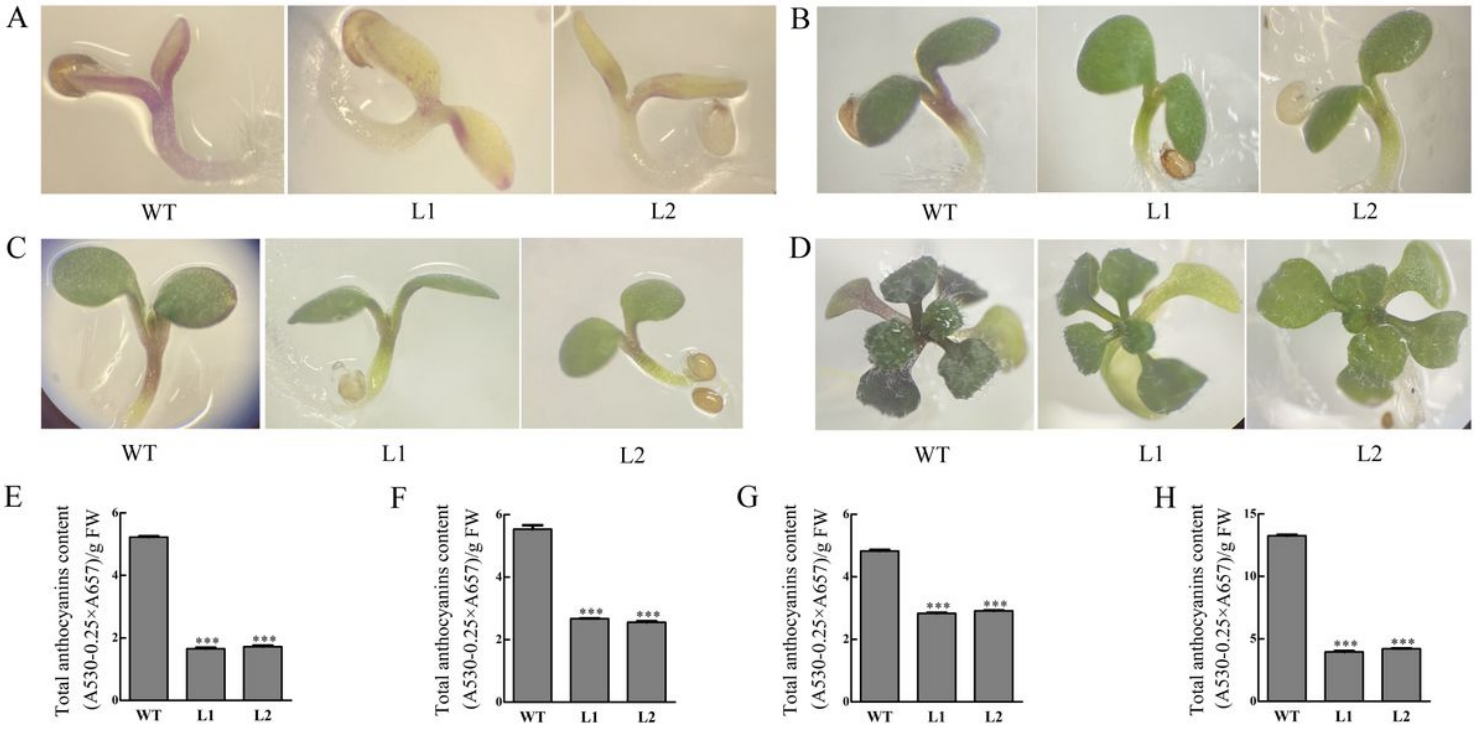

Figure 6

Phenotypic analysis of *AaMYB4* transgenic *Arabidopsis*

A : *Arabidopsis* seedlings under nitrogen deficiency treatment. B : *Arabidopsis* seedlings under high light treatment. C : *Arabidopsis* seedlings treated with 6% sucrose. D : *Arabidopsis* seedlings under ABA

treatment. E-H are the anthocyanin contents of *Arabidopsis* under nitrogen deficiency, high light, 6% sucrose and ABA treatments, respectively. ***P< 0.001.

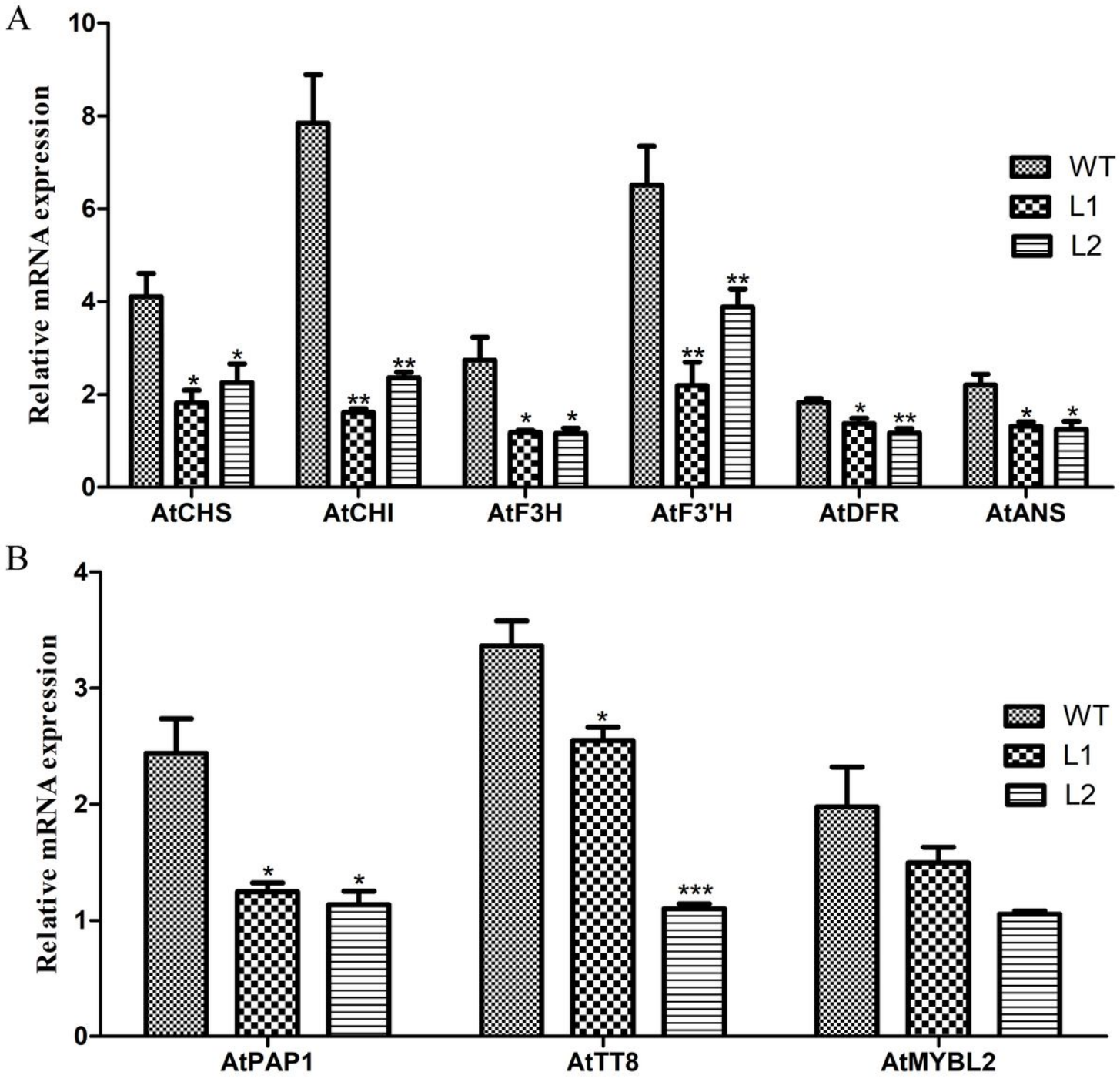

Figure 7

Expression analysis of anthocyanin synthesis-related genes in *Arabidopsis*overexpressing *AaMYB4*

A: Expression analysis of structural genes for anthocyanin synthesis in *Arabidopsis*. B: Expression analysis of transcription factors related to anthocyanin synthesis in *Arabidopsis*. *0.01<P< 0.05 **0.001<P< 0.01

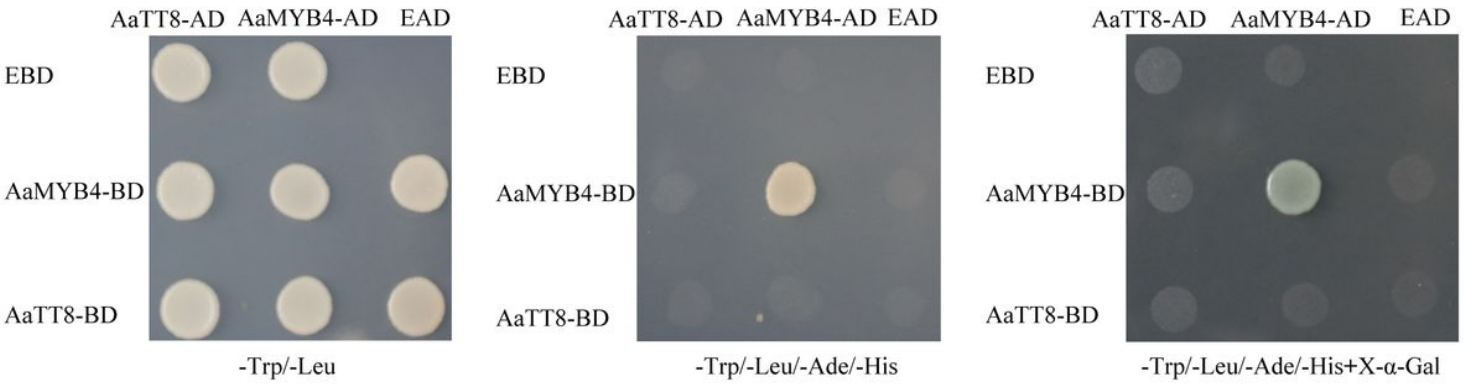

Figure 8

Exploring the interactions between AaMYB4 and AaTT8