



AcMYB1 Interacts With AcbHLH1 to Regulate Anthocyanin Biosynthesis in *Aglaonema commutatum*

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Aglaonema commutatum is one of the most popular foliage plants with abundant leaf phenotypes; therefore, anthocyanin coloration is a vital economic trait in *A. commutatum*. However, the molecular mechanisms underlying anthocyanin biosynthesis and its regulation remain unclear. In this study, *AcMYB1* and *AcbHLH1*, transcription factor genes related to an R2R3-myeloblast (MYB) and a basic helix–loop–helix (bHLH), respectively, were isolated from *A. commutatum* “Red Valentine” and functionally characterized. *AcMYB1* and *AcbHLH1* were found to interact by Y2H and BiFC assay. *AcMYB1* was grouped into the AN2 subgroup and shared high homology with the known regulators of anthocyanin biosynthesis. Gene expression analysis showed that both *AcMYB1* and *AcbHLH1* have similar expression patterns to anthocyanin structural genes and correlate with anthocyanin distribution in different tissues of *A. commutatum*. Light strongly promoted anthocyanin accumulation by upregulating the expression of anthocyanin-related genes in *A. commutatum* leaves. Ectopic expression of *AcMYB1* in tobacco remarkably increased anthocyanin accumulation in both vegetative and reproductive tissues at various developmental stages. These results provide insights into the regulation of anthocyanin biosynthesis in *A. commutatum* and are useful for breeding new *A. commutatum* cultivars with enhanced ornamental value.

Keywords: *Aglaonema commutatum* “Red Valentine”, anthocyanin pathway, R2R3-MYB, transcriptional regulation, transgenic tobacco

INTRODUCTION

Anthocyanins are broadly located in plant species and are responsible for a wide range of coloration, such as purple, blue, and pink, in plant flowers, fruits, and leaves (Winkel-Shirley, 2001). Anthocyanins are vital secondary metabolites that attract insect pollinators and help defend plants against biotic and abiotic stresses (Schaefer et al., 2004). In vegetative organs, anthocyanins act as a barrier to protect photosynthetic cells from intense light (Hughes et al., 2005). More importantly, there is growing evidence that anthocyanins are beneficial to human health by reducing the rates of cardiovascular disease, obesity, diabetes, lung disease, and cancer (Hou, 2003; Martin et al., 2011; Ha et al., 2015).

The biochemistry and enzymology of the anthocyanin pathway are among the most widely studied pathways in plant secondary metabolites, and almost all encoding enzymes have been isolated and characterized (Mol et al., 1998; Koes et al., 2005). Chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), flavonoid 3'-hydroxylase (*F3'H*),

and flavonol synthase are the early biosynthetic genes, which result in the production of flavonols and different flavonoid compounds. The late biosynthetic genes include dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*), and UDP-glucose-flavonoid-3-*O*-glucosyltransferase (*UFGT*), which lead to the production of anthocyanins (Lepiniec et al., 2006). After glycosylation, methylation, and acylation, water-soluble anthocyanin compounds are transported to vacuoles for stable storage (Zhao and Dixon, 2010). At the transcriptional level, anthocyanin structural genes are usually conservatively regulated by the MBW protein complex containing R2R3-myeloblast (MYB), basic helix-loop-helix (bHLH), and WD40 proteins (Feller et al., 2011; Hichri et al., 2011; Xu et al., 2015). In particular, MYBs play vital roles in anthocyanin biosynthesis, and their expression levels influence anthocyanin accumulation (Chen et al., 2017; Feng et al., 2020). MYBs often interact with bHLHs to co-regulate anthocyanin biosynthesis (Patra et al., 2013; Xu et al., 2015). In the model plant *Arabidopsis thaliana*, the R2R3-MYB genes *PAP1*, *PAP2*, *MYB113*, and *MYB114*, and the bHLH genes *TT8*, *GL3*, and *EGL3* were identified as critical genes that participate in the regulation of anthocyanin biosynthesis (Nesi et al., 2000; Payne et al., 2000; Gonzalez et al., 2008). In *Nicotiana tabacum*, the MYB transcription factor (TF), NtAn2, can regulate anthocyanin accumulation in tobacco flowers by interacting with NtAn1a and NtAn1b to activate the promoter of *DFR* and *CHS* (Pattanaik et al., 2010; Bai et al., 2011).

Anthocyanin accumulation in leaves, usually showing a red leaf color, can greatly increase horticultural value and stress resistance. Additionally, plant materials rich in anthocyanin are important germplasm resources for genetic breeding and pigment bioengineering (Tian et al., 2015). *PdMYB118*, identified in a red leaf mutant of *Populus deltoids*, acts as a key transcriptional regulator of leaf anthocyanin accumulation (Wang et al., 2019). In peaches, a novel branch of the MYB genes, *PpMYB10.4*, can activate leaf anthocyanin accumulation and form red leaf coloration (Zhou et al., 2014). In purple kale, *BoPAP1* responds to low temperatures to induce anthocyanin biosynthesis in the leaves (Zhang et al., 2012). Similar studies have also been reported on crabapples (Tian et al., 2015), potatoes (D'Amelia et al., 2014), and *Gynura bicolor* (Shimizu et al., 2011).

Aglaonema commutatum is a well-known foliage plant native to tropical Asian countries such as India, Thailand, and Vietnam (Du et al., 2013). *A. commutatum* have leaves with abundant colors and mosaic points, are shade tolerant and moisture resistant, and have few pests and diseases, which makes them ideal indoor ornamental and leaf-cutting plants (Gao, 2018). Leaf color is a considerable economic trait of *A. commutatum* sought by breeders, but little is known about the molecular mechanisms of anthocyanin biosynthesis regulation in *A. commutatum*.

In this study, an R2R3-MYB and a bHLH TF were identified as potential anthocyanin biosynthesis regulators from the *A. commutatum* "Red Valentine" leaf RNA-seq database, and further analyzed. Expression trend analysis showed that the mRNA expression levels of anthocyanin structural genes correlated with those of *AcMYB1* and *AcbHLH1*. Overexpression analyses of *AcMYB1* in tobacco further demonstrated its vital role in anthocyanin regulation. Our study may lay the foundation for further genetic studies on the diversity of leaf color and can

be applied to breeding plants with desirable color traits in *A. commutatum*.

MATERIALS AND METHODS

Plant Materials and Treatment

Seedlings of *A. commutatum* "Red Valentine" that were 2-year-old were potted in the greenhouse of the South China Botanical Garden, Chinese Academy of Sciences (Guangzhou, China) and received natural light with a 60% shade cloth. The temperature and relative humidity of the greenhouse range from 15 to 34°C and 75–99%, respectively. Three stages for leaf development were defined as follows: stage 1, curly and white (S1, 7 days), stage 2, unfolded and light pink (S2, 28 days), and stage 3, mature and dark red (S3, 35 days). The leaves of three developmental stages, roots, stems, and flowers samples were frozen in liquid nitrogen immediately after collection before being stored at –80°C in November 2019 for analysis of anthocyanin content and gene expression levels.

To study the effect of light on anthocyanin biosynthesis and related gene expression, the *A. commutatum* seedlings at stage S2 were grown under 12 h light/dark cycle or dark conditions for 5 days in a phytotron (DGXM-508, Jiangnan Instrument Factory, Ningbo, China) at 28°C with 8,000 Lux light intensity. Subsequently, leaf samples were collected for analysis of gene expression and anthocyanin content. Following growth for 5 days, samples were immediately stored at –80°C for further study. Each sample contained three biological replicates.

Anthocyanins Content Analysis

The pH difference method was used to measure the total anthocyanin content in *A. commutatum* and transgenic *Nicotiana tabacum* cv. NC89 (tobacco) tissues (Wrolstad et al., 1982). Briefly, fresh samples (0.1 g) were extracted with 2 ml methanol (with 0.05% hydrochloric acid) overnight at 0°C. The absorbance at 510 and 700 nm was measured using a microplate reader (Tecan Infinite, Männedorf, Switzerland). The following equation was used to calculate the total anthocyanin content: $\Delta_A \times DF \times M \times 1,000 / (\epsilon \times W)$. where $\Delta_A = (A_{510} - A_{700})_{pH 1.0} - (A_{510} - A_{700})_{pH 4.5}$, DF is the dilution factor, M is the molecular weight of cyanidin-3-glucoside (449.2 g mol⁻¹), ϵ is the molar absorptivity (26,900, molar extinction coefficient (L mol⁻¹ · cm⁻¹) for cyanidin-3-glucoside), and W is the sample weight (g).

Transcript Isolation and Sequence Analysis

The leaf transcriptome data of *A. commutatum* (Accession number: PRJNA793608) were used to screen for R2R3-MYB-like and bHLH-like genes by BLASTx searching from Nr and Swiss-Prot protein databases based on (i) similarity scores with the known anthocyanin-related TF genes in other species and (ii) correlations with anthocyanin structural gene expression. The transcripts were obtained from transcriptome assembly data, and the coding sequences were cloned using the SMARTer RACE cDNA Amplification Kit (Takara Biomedical Technology Co., Ltd.; Beijing, China), and amplified *via* PCR with 2× Super Pfx DNA Polymerase (Covin Biotech Co., Ltd.; Taizhou, China) and the pClone007 Versatile Simple Vector Kit (TSINGKE

Biotech Co., Ltd.; Beijing, China) for Sanger sequencing and subsequent analyses. DNAMAN (v8.0.8.789) was used to analyze the amino acid sequence alignment and MEGA (v7.0.26) was used to construct phylogenetic trees using the neighbor-joining method with 1,000 bootstrap replicates. The GenBank accession numbers of the MYB and bHLH proteins are listed in **Supplementary Table S1**.

RNA Extraction, cDNA Synthesis, and Gene Expression Analysis

Total RNA was isolated from tobacco as well as the roots, stems, flowers, and leaves of *A. commutatum* using a plant RNA kit (Polysaccharides and Polyphenolics-rich, Hua Yueyang, Beijing, China) with RNase-free DNase I (Takara Biomedical Technology Co., Ltd.; Beijing, China) to remove genomic DNA contamination. RNA (1 μ g) was used for cDNA synthesis using the TransScript II One-Step gDNA Removal and cDNA Synthesis kit (TransGen Biotech Co., Ltd.; Beijing, China).

Key genes related to anthocyanin biosynthesis were screened from the *A. commutatum* “Red Valentine” leaf RNA-Seq database (BioProject ID: PRJNA793608). We identified 10 anthocyanin biosynthetic genes: *AcCHS*, *AcCHS2*, *AcCHI*, *AcF3H*, *AcF3'H*, *AcDFR1*, *AcDFR3*, *AcANS*, *AcUFGT1*, and *AcUFGT2*. The expression levels of *AcMYB1* and *AcbHLH1* as well as these 10 genes were analyzed in the root, stem, flower, and three developmental leaf stages of *A. commutatum*. The expression levels of *AcMYB1* in transgenic tobacco leaves and flowers were also determined. The expression levels of nine structural genes and two bHLH TF genes (*NtPAL*, *NtCHS*, *NtCHI*, *NtF3H*, *NtF3'H*, *NtF3'5'H*, *NtIDFR*, *NtANS*, *NtUFGT*, *NtAn1a*, and *NtAn1b*, respectively) involved in anthocyanin biosynthesis were compared in the transgenic and control tobacco leaves and corollas. Finally, the mRNA abundance of six anthocyanin structural genes (*NbCHS*, *NbCHI*, *NbF3H*, *NbDFR*, *NbANS*, *NbUFGT*) in *Nicotiana benthamiana* leaves were examined 8 days after infiltration. Gene expression levels were determined using real-time quantitative PCR (qRT-PCR) with PerfectStart Green qPCR SuperMix (TransGen Biotech Co., Ltd., Beijing, China) with a LightCycler 480 II (Roche, Mannheim, Germany) according to the following conditions: 94°C for 30 s, 45 cycles at 94°C for 5 s, 60°C for 30 s. Elongation factor 1- α (*AcEF-1 α* , Accession number: OM688333), *NtActin* (Accession number: X69885), and *NbActin* (Accession number: JQ256516) were used as reference genes. The Ct value of each sample was calculated via LightCycler 480 software (Roche, version: 1.5.1.62) and the relative expression was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), where $\Delta Ct = Ct$ (target gene) – Ct (reference gene) and $\Delta\Delta CT = \Delta Ct$ (experimental group) – ΔCt (control group). Gene-specific primer pairs are listed in **Supplementary Table S2**. Three biological replicates and three technical replicates were performed.

Subcellular Localization

The *AcMYB1* and *AcbHLH1* open reading frames, without the stop codons, were inserted into the BglII and KpnI sites of the pSAT6-EYFP-N1 vector which is a yellow fluorescent protein (YFP) driven by the 35S Cauliflower mosaic virus

(35S) promoter. The final plasmids, 35S::AcMYB1-YFP, 35S::AcbHLH1-YFP, control 35S::YFP, and nuclear marker 35S::mCherry, were introduced into *A. thaliana* mesophyll protoplasts by polyethylene glycol (PEG)-mediated transient transformation (Yoo et al., 2007). Co-transformation with the control 35S::YFP and nuclear marker 35S::mCherry were used as a negative control. After incubation at 20°C for 20 h, the protoplasts were detected using a Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany).

Yeast Two-Hybrid Assay

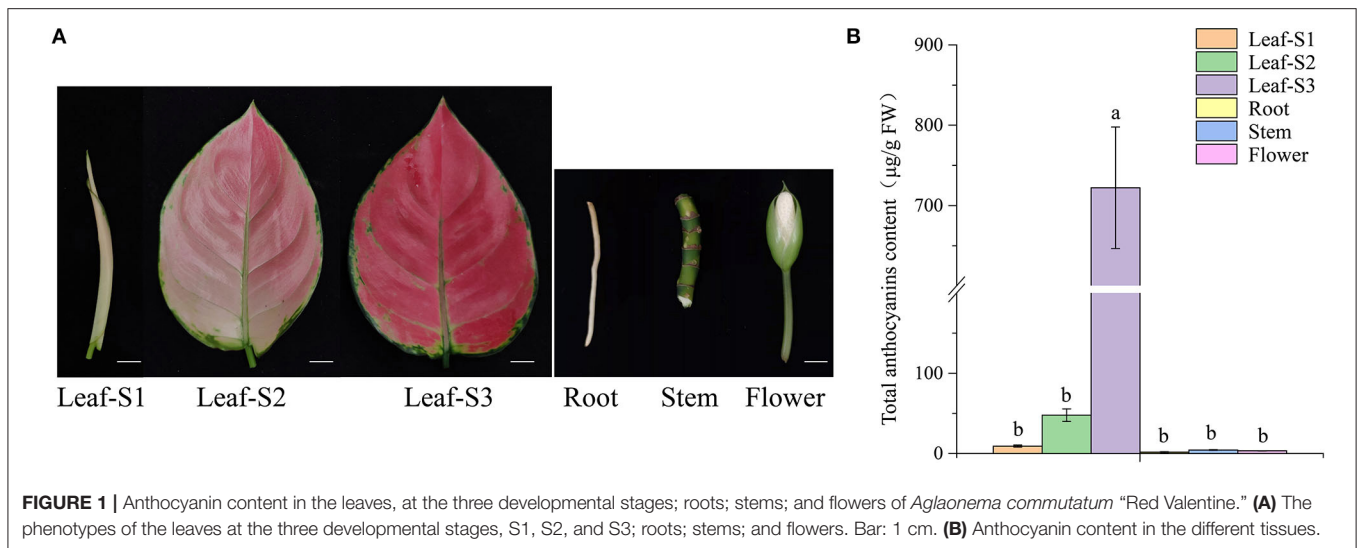
Yeast two-hybrid (Y2H) analysis was performed using the Matchmaker Two-Hybrid System 3 (Clontech; Takara Bio USA, Inc.; San Jose, CA, United States). The coding region of *AcbHLH1* was inserted into the bait vector pGBK-T7 (GAL4 DNA-binding domain), and *AcMYB1* was fused to the prey vector pGAD-T7 (GAL4 activation domain). Vector pGADT7-T and pGBKT7-53 were used as the positive controls. All constructs were transformed into the yeast strain AH109 (Clontech, Takara Bio USA, Inc.; San Jose, CA, United States) using the PEG/LiAc method, according to the protocol handbook. All transformed yeast cells were selected on a synthetic drop-out medium without leucine and tryptophan (SD–Leu–Trp) at 30°C for 3 days. Colonies that survived from double selection plates were then screened for growth on a quadruple selection SD medium lacking adenine, histidine, leucine, and tryptophan (SD–Ade–His–Leu–Trp) containing 30 mM 3-amino-1,2,4-triazole (3-AT) solution and 25 mg/L X- α -Gal.

Bimolecular Fluorescence Complementation

For the biomolecular fluorescence complementation (BiFC) assay, the *AcMYB1* and *AcbHLH1* ORFs, without stop codons, were inserted into the pSPYNE and pSPYCE vectors containing the N- and C-terminal halves of YFP (Walter et al., 2004). Recombinant plasmids were then introduced into *A. thaliana* mesophyll protoplasts by PEG-mediated transient transformation (Waadt et al., 2008). After incubation at 20°C for 20 h, the protoplasts were observed using a Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany).

Overexpression of *AcMYB1* in Tobacco

To overexpress *AcMYB1* in tobacco, the coding sequence of *AcMYB1* was inserted into the pGreen-C17 vector, which was triggered under the control of the CaMV 35S promoter. The resulting vector, pGreen-C17-AcMYB1, was transferred into *Agrobacterium tumefaciens* strain EHA105 and then transformed into tobacco via the leaf disc method (Zhang et al., 2007). Empty vector-infected tobacco plants were used as controls. The T1-generation leaves and corollas from the three transgenic lines and control plants were collected for the anthocyanin concentration (Section Anthocyanins Content Analysis) and gene expression (Section RNA Extraction, cDNA Synthesis, and Gene Expression Analysis) analyses.



Transient Expression Assay

The transient over-expression experiments in *N. benthamiana* leaves were performed according to the method described by Palapol et al. (2009). Briefly, 35S-promoter-driven *AcMYB1* and *AcbHLH1* constructs were inserted into *Agrobacterium tumefaciens* strain GV3101 via electroporation. *N. benthamiana* plants with 6–8 leaves were solely or simultaneously infiltrated with a needleless 1-ml syringe into the abaxial sides of the fourth or fifth leaves, and *Agrobacterium* with an empty vector was used as a control. Anthocyanin content was measured and digital photographs were taken 8 days after infiltration. Anthocyanin content was measured with the pH difference method as described in Section Anthocyanins Content Analysis.

Statistical Analysis

All experiments were performed on at least three independently grown biological replicates. All values represent the mean \pm SE. Differences between the treatment groups were examined using SPSS software (one-way ANOVA, Duncan test), and the significant differences ($p < 0.05$) are indicated with different letters, respectively.

RESULTS

Determination of Anthocyanin Concentration in *A. commutatum*

Leaves of *A. commutatum* “Red Valentine” has white color and stay curly at leaf-S1. At leaf-S2, leaf color changes from white to light pink and appears deep red at leaf-S3 (Figure 1A). Anthocyanin content in the leaves of *A. commutatum* significantly increased with developmental age. Additionally, trace amounts or no anthocyanins were detected in the roots, stems, and flowers (Figure 1B).

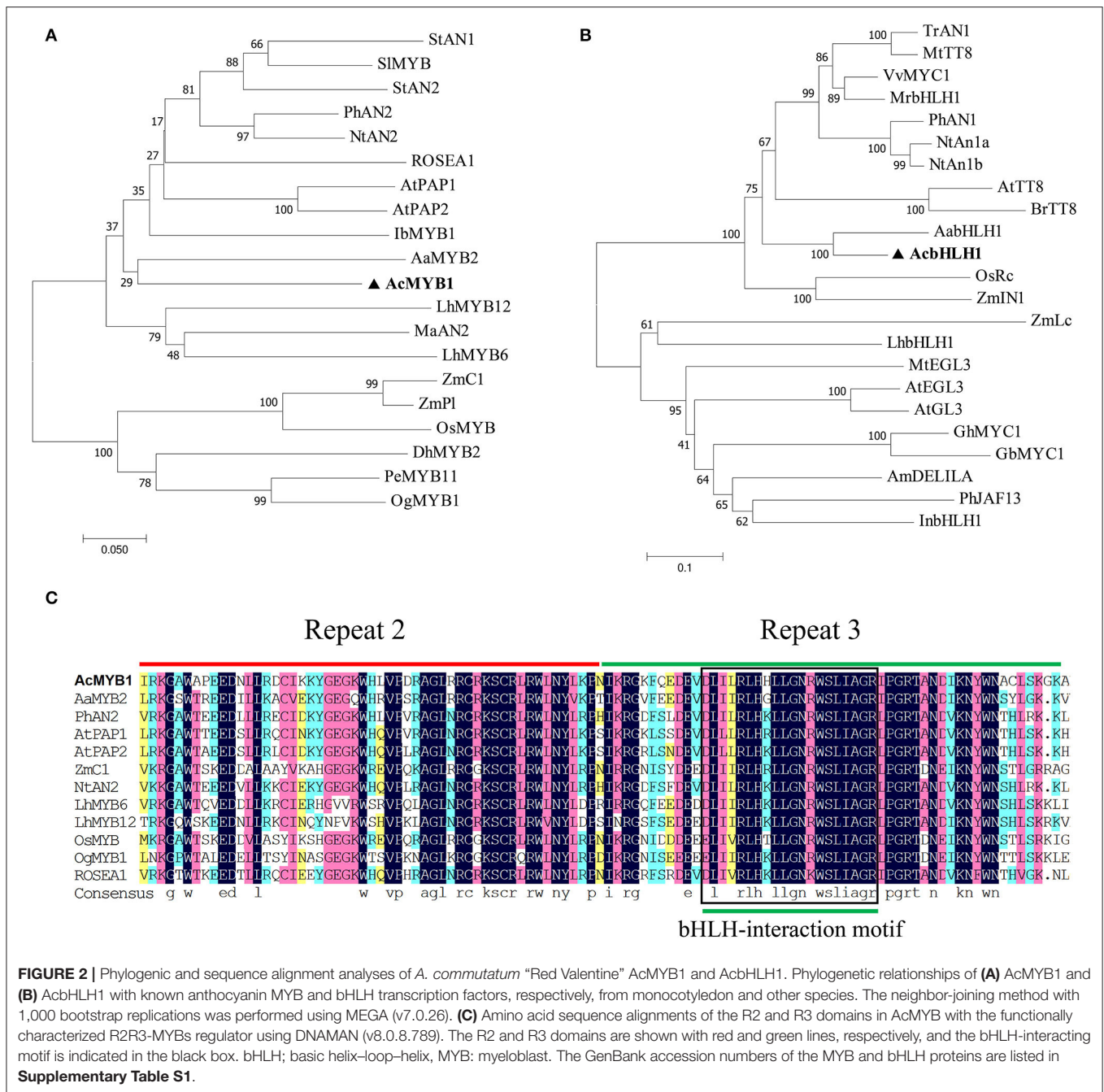
Isolation and Sequence Analysis of *AcMYB1* and *AcbHLH1*

In this study, R2R3-MYB and bHLH TFs were isolated from the leaf transcriptome database of *A. commutatum*

“Red Valentine.” The candidate genes were cloned from the cDNA of *A. commutatum* leaves by RACE PCR, sequenced, and named *AcMYB1* and *AcbHLH1*. The results showed that *AcMYB1* and *AcbHLH1* contained 969 and 2,115 bp ORFs, encoding proteins of 322 and 712 amino acids, respectively. The neighbor-joining phylogenetic tree showed that *AcMYB1* clustered with monocot-positive anthocyanin regulators, including *AaMYB2*, *LhMYB6*, *LhMYB12*, and *MaAN2* (Figure 2A). Similarly, *AcbHLH1* formed a cluster with *AabHLH1* and other bHLH TFs associated with anthocyanin biosynthesis in several plant species (Figure 2B). Alignment analysis showed that a highly conserved R2R3 domain is contained in the *AcMYB1* protein for DNA binding at the N-terminus, which contains a crucial bHLH-interacting motif in the R3 domain for interactions with bHLH TFs (Figure 2C) (Zimmermann et al., 2004). We also found an N-terminal MYB-interacting region and conserved bHLH domain in the *AcbHLH1* protein (Supplementary Figure S1) (Pattanaik et al., 2008). These results suggested that *AcMYB1* and *AcbHLH1* may interact and play roles in anthocyanin biosynthesis.

Expression Levels of *AcMYB1*, *AcbHLH1*, and Anthocyanin Structural Genes

The gene expression analyses showed that all the candidate structural genes, including *AcMYB1* and *AcbHLH1*, had significantly higher expression levels in the three developmental leaf stages compared to those in the other tissues and reached a peak at leaf stage two except *AcF3H* (Figure 3). Furthermore, the correlation analysis revealed that the gene expression correlation coefficient between *AcMYB1* or *AcbHLH1* and structural genes was in the range of 0.73–0.99 and 0.75–0.98. This result implies that transcript abundance of *AcMYB1* and *AcbHLH1* are correlated with those of the most anthocyanin structural genes and they are likely involved in the regulation of anthocyanin biosynthesis in *A. commutatum*.



Localization and Protein–Protein Interaction of AcMYB1 and AcbHLH1

Subcellular localization analysis in *A. thaliana* leaf protoplasts revealed that both AcMYB1 and AcbHLH1 were specifically localized in the cell nucleus (Figure 4). In *A. commutatum*, AcMYB1 and AcbHLH1 showed conserved interacting motifs, similar mRNA expression patterns in different tissues, and co-localization in the nucleus, suggesting a possible interaction. To test this hypothesis, we used Y2H and BiFC assays. For the Y2H assay, all the transformed colonies grew well on

SD/-Leu/-Trp, indicating their successful transformation. The co-transformed colonies of pGADT7-AcMYB1 + pGBKT7-AcbHLH1 displayed distinct blue coloration on SD/-Leu/-Trp/-His/-Ade, indicating that AcMYB1 and AcbHLH1 physically interacted (Figure 5A). In the BiFC assays, YFP fluorescence signals were observed only when pSPYNE/AcMYB1 and pSPYCE/AcbHLH1 were co-expressed and no fluorescence was detected in cells that contained control vectors (Figure 5B). These results further confirm that AcbHLH1 and AcbMYB1 may interact in *A. commutatum* cells.

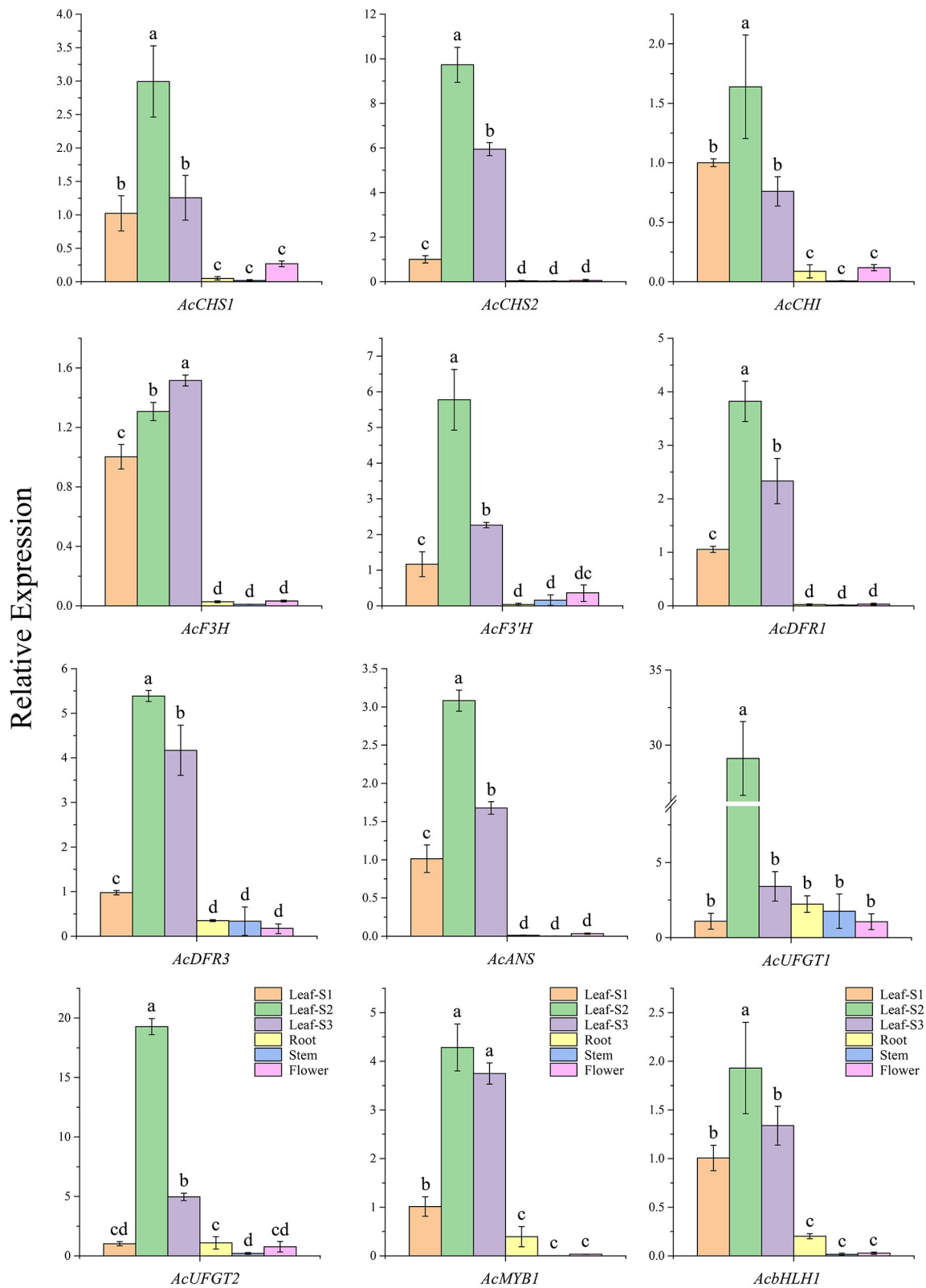
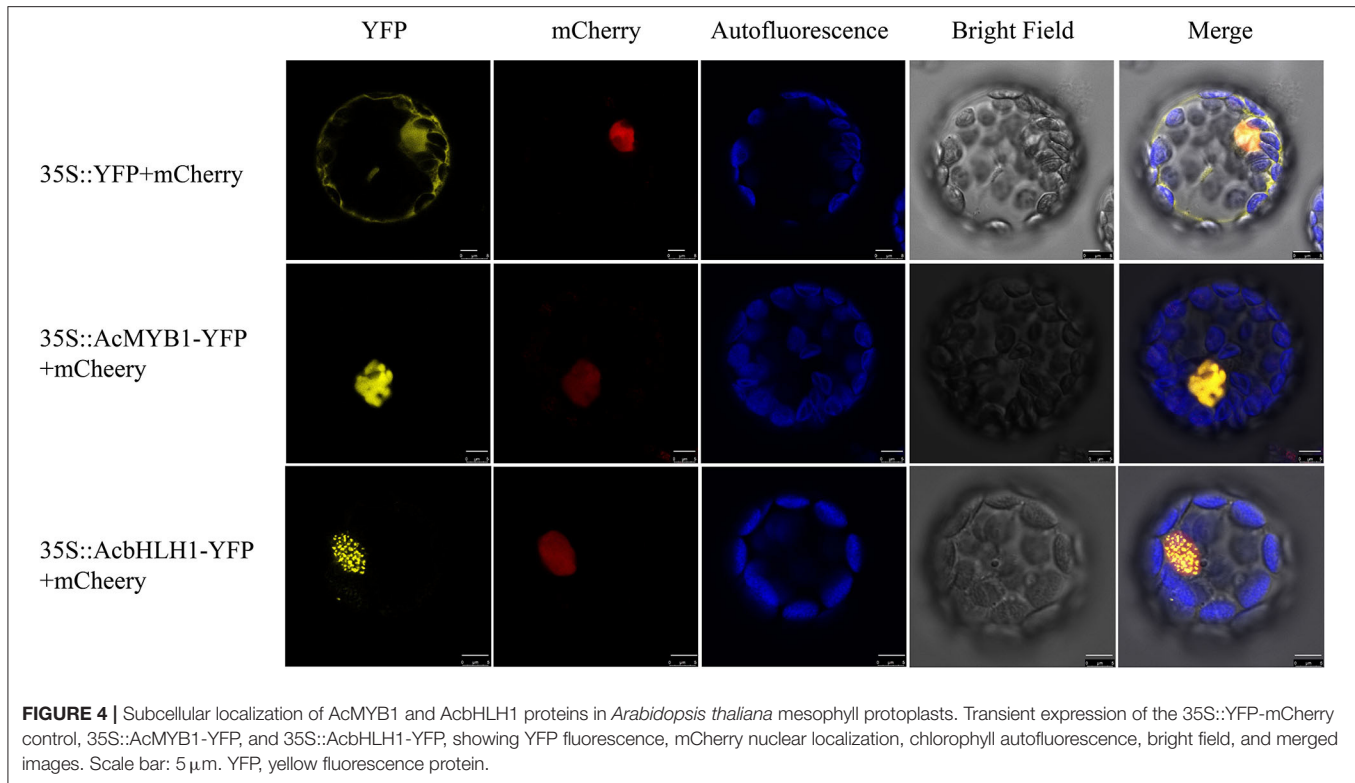


FIGURE 3 | The relative expression levels of 10 anthocyanin structural genes, *AcMYB1*, and *AcbHLH1* in various tissues of *A. commutatum* “Red Valentine” by qRT-PCR. The relative expression was calculated based on the $2^{-\Delta\Delta CT}$ method using *AcEF-1 α* gene as the reference gene.



Functional Analysis of AcMYB1 in Tobacco

To investigate the role of *AcMYB1* in regulating the anthocyanin biosynthetic pathway, overexpression (OE) under the control of the CaMV-35S promoter was carried out in tobacco. More than 20 independent transgenic lines were generated using genomic PCR. The results of phenotypic changes showed that pigment levels were significantly increased in both vegetative and reproductive tissues relative to that in the control plants. In particular, the OE-*AcMYB1* line showed a remarkable change in leaf color, and the mature leaves of the transgenic plants displayed an observably darker red color than that of the control plants. In addition, the corolla color of OE-*AcMYB1* tobacco changed from light pink to deep red, and increased anthocyanin accumulation was markedly visible in the anthers, filaments, calyxes, ovary walls, and seed coats (Figures 6A–L). Anthocyanin content determination confirmed that the anthocyanin extracted from the three lines of OE-*AcMYB1* tobacco leaves and corollas were markedly higher than those in the controls (Figures 6M,N).

To reveal the potential target genes in OE-*AcMYB1* transgenic plants, the mRNA expression level of structural genes and two bHLH TF genes involved in anthocyanin biosynthesis were verified by RT-PCR assay in tobacco leaves and corollas. A high expression level of *AcMYB1* was first confirmed in three lines by qRT-PCR (Figures 7A,B). Expression analysis showed that the 11 anthocyanin regulatory genes were significantly upregulated in the leaves of all three transgenic lines compared to those of the control plants (Figure 7C). However, in the corollas of the three transgenic tobacco plants, only *NtCHI*, *NtANS*, *NtUFGT*, and *NtAn1b* showed significantly higher expression levels than that of the control (Figure 7D). These results indicated

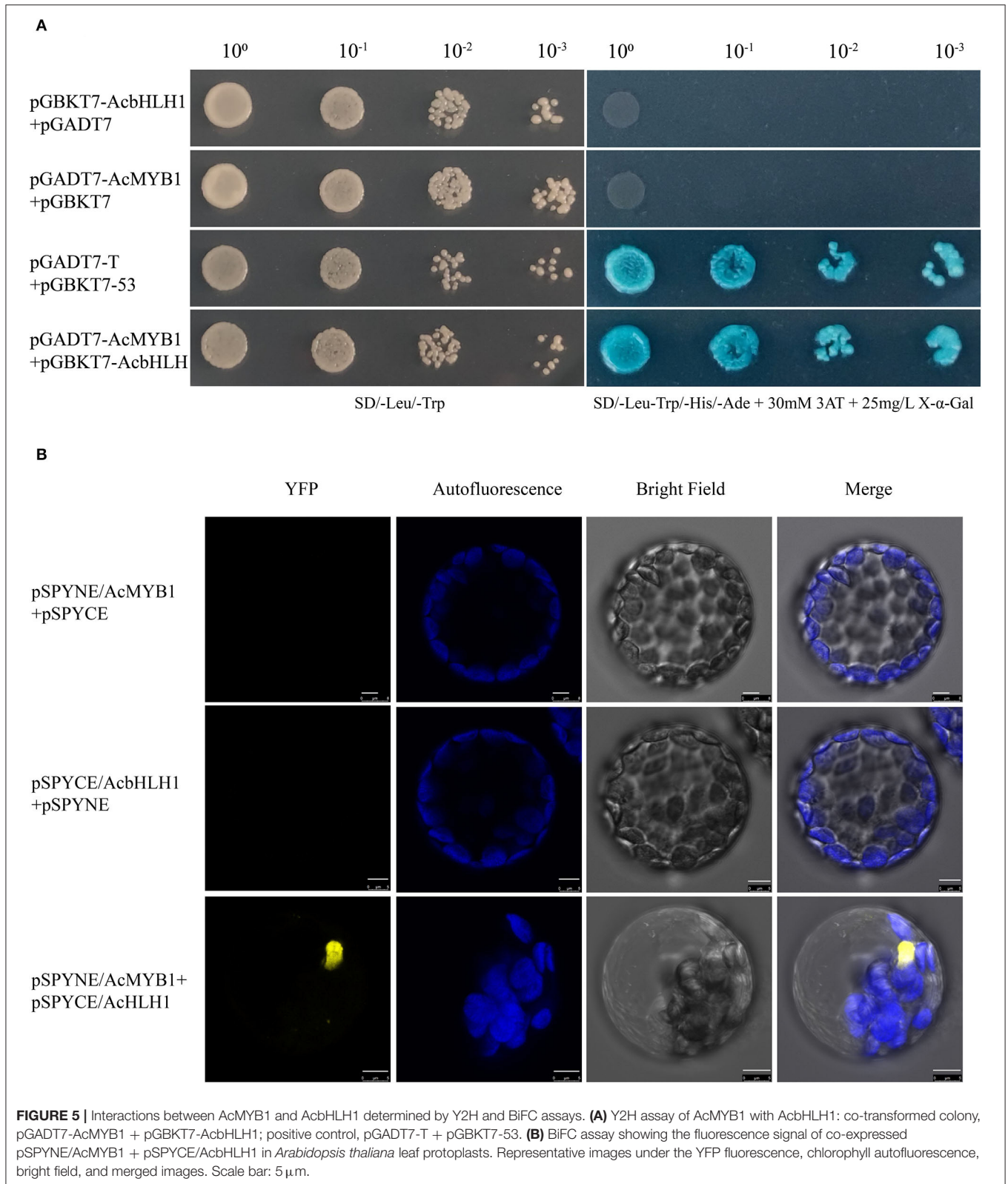
that *AcMYB1* could upregulate or activate the expression of key anthocyanin structural genes and bHLH TFs, ultimately promoting anthocyanin accumulation in transgenic tobacco.

Transient Expression of AcMYB1 and AcbHLH1 in *N. benthamiana* Leaves

An *Agrobacterium*-mediated transient assay was further performed to investigate the regulation of *AcMYB1* and *AcbHLH1* on anthocyanin biosynthesis. The results showed that a slight accumulation of anthocyanin was detected in the leaves inoculated with the control or *AcbHLH1* construct, while patches of anthocyanin were observed in *AcMYB1* and *AcMYB1* + *AcbHLH1* inoculated leaves (Figures 8A–E). The anthocyanin content in *AcMYB1* + *AcbHLH1* leaves is 1.36 times that of the *AcMYB1* leaves (Figure 8E). Moreover, the expression of anthocyanin biosynthetic genes were strongly up-regulated in the leaves infiltrated with *AcMYB1* and *AcMYB1* + *AcbHLH1* constructs (Figure 8F). Interestingly, *NbCHI*, *NbANS*, *NbDFR*, and *NbUFGT* displayed significantly higher expression levels in simultaneous inoculation of 35S::*AcMYB1* and 35S::*AcbHLH1* constructs than solely infiltrating with 35S::*AcMYB1*. Taken together, we predicted that the ability of *AcMYB1* in anthocyanin regulation could enhance by the interaction with *AcbHLH1*.

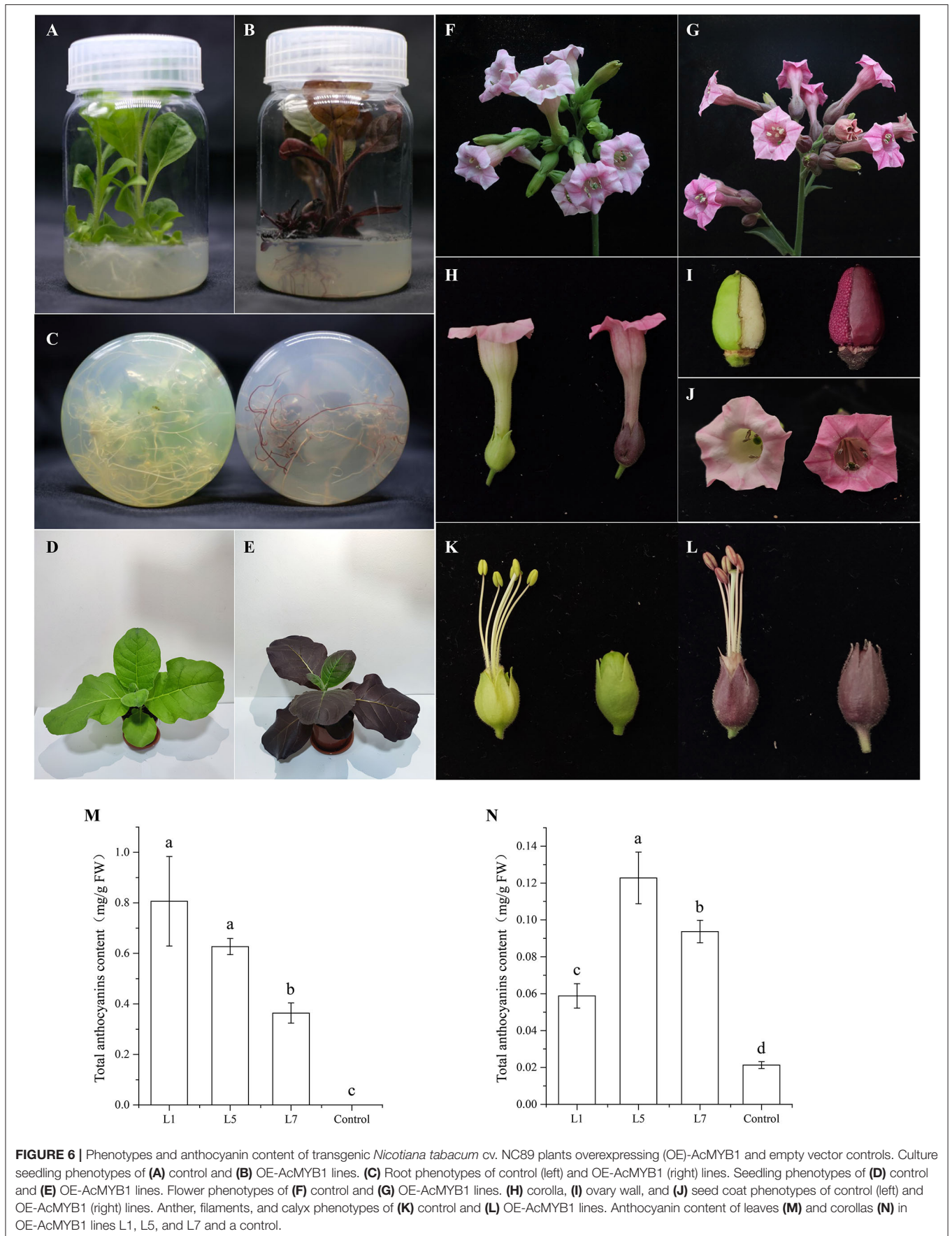
Influence of Light on Gene Expression and Anthocyanin Accumulation in *A. commutatum*

Comparative analysis was conducted between 2-year-old *A. commutatum* “Red Valentine” seedlings after growing in



the dark or light for 5 days. As shown in **Figure 9A**, the leaf color was bright red in light treatment plants and pale pink in dark-grown plants. The leaf anthocyanin content

in light treatment seedlings is 11.2-fold higher than that of the seedlings growing in dark (**Figure 9B**). Furthermore, the transcriptional level of AcMYB1, AcbHLH1, and 10 anthocyanin



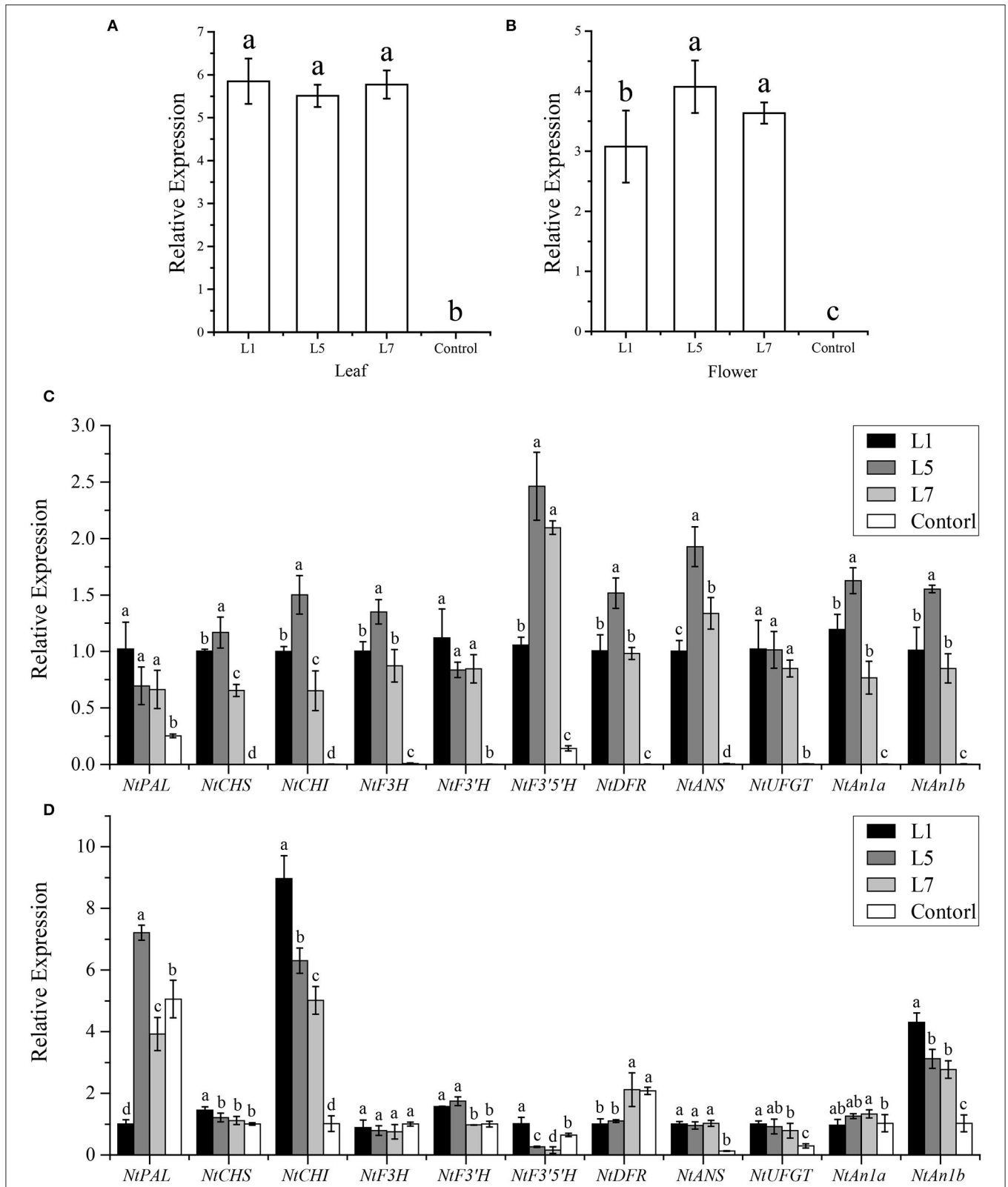
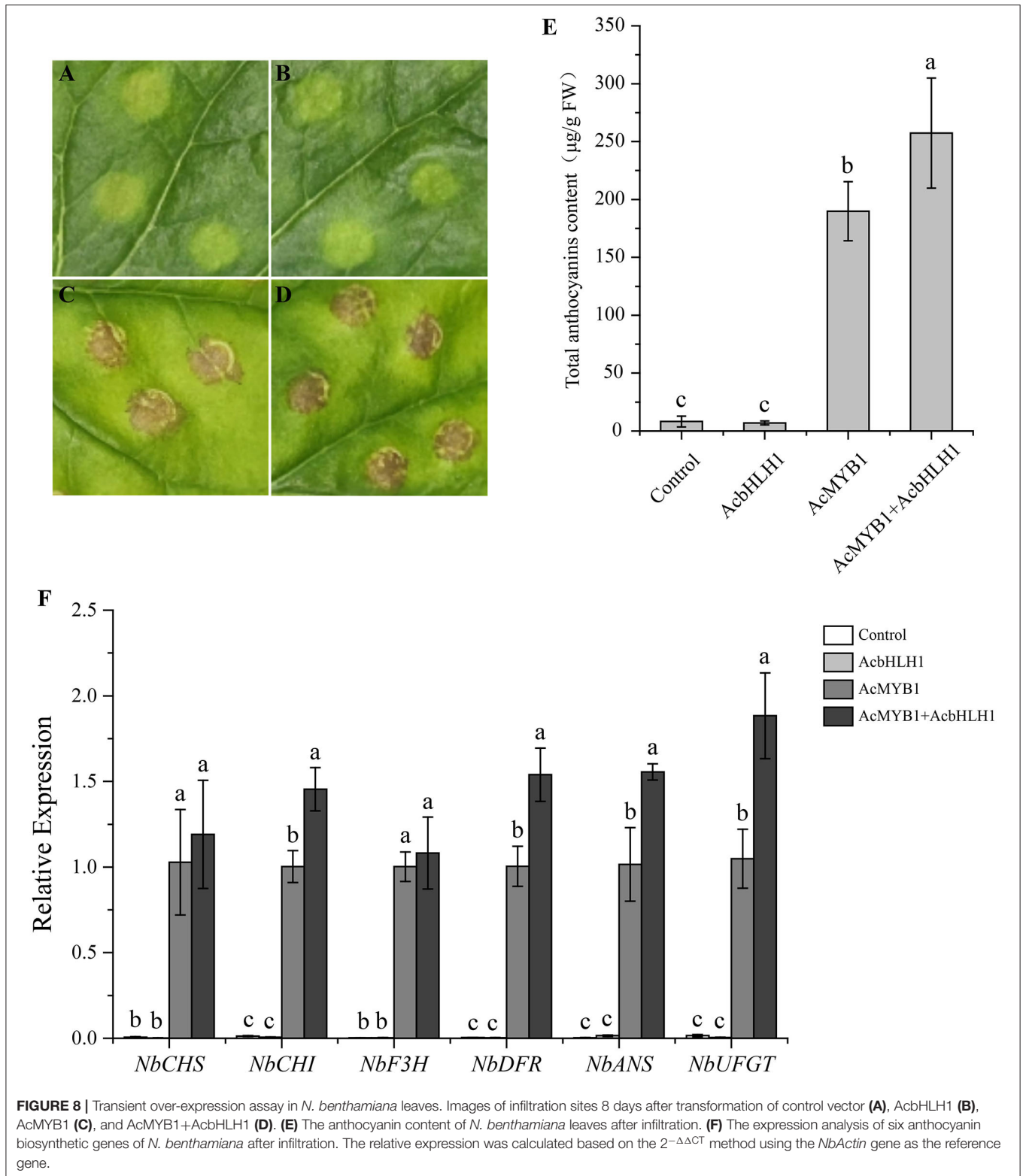
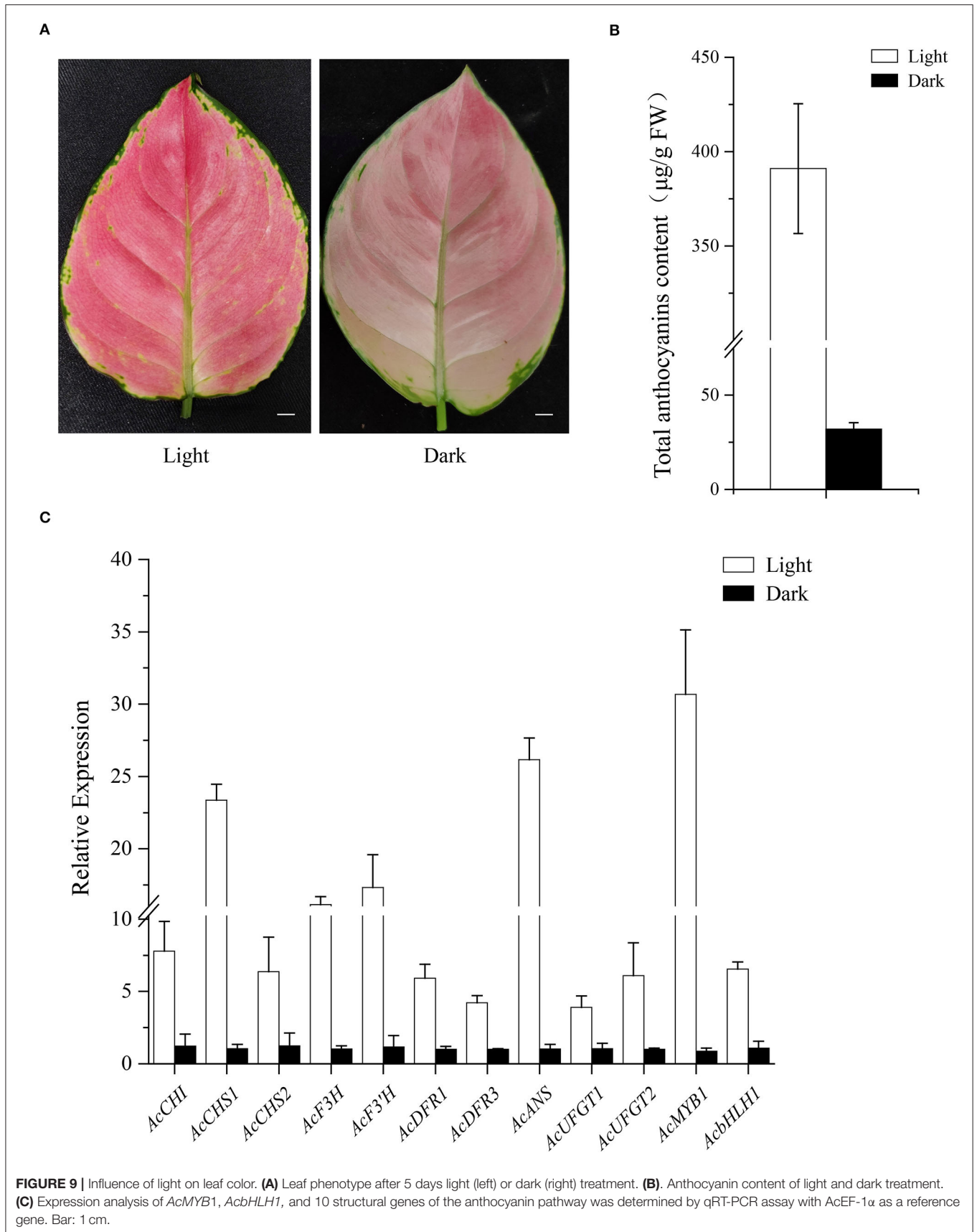


FIGURE 7 | Expression analysis of anthocyanin biosynthesis pathway genes in the leaves and corollas of *Nicotiana tabacum* cv. NC89 using qRT-PCR. The expression patterns of *AcMYB1* in the **(A)** leaves and **(B)** corollas. Relative expression levels of anthocyanin pathway-related genes in the leaves **(C)** and corollas **(D)**. L1, L5, and L7 represent the three transgenic tobacco lines. The relative expression was calculated based on the $2^{-\Delta\Delta CT}$ method using the *NtActin* gene as the reference gene.



structural genes in light-grown conditions were all noticeably higher in comparison with the seedlings growing in dark (Figure 9C). The results reveal that light can strongly induce

the expression of anthocyanin-related genes and promote the anthocyanin accumulation in *A. commutatum* “Red Valentine” leaf.



DISCUSSION

Recently, more attention has been given to the cultivation of ornamental plants with colored leaves; a phenotype that is mainly obtained by the accumulation of anthocyanins (Lightbourn et al., 2008; Li et al., 2021; Huang et al., 2022). Multiple R2R3-MYB and bHLH TFs have been reported as key regulators of anthocyanin biosynthesis (Allan et al., 2008; Petroni and Tonelli, 2011; Qin et al., 2022). *A. commutatum* is an excellent foliage plant with abundant leaf coloration; therefore, acquiring increasing importance for breeding desirable leaf color traits. However, few studies have focused on the molecular mechanisms underlying anthocyanin biosynthesis and regulation in *A. commutatum*.

Many R2R3-MYBs involved in the regulation of anthocyanin biosynthesis have been identified at the genetic and molecular levels (Nuraini et al., 2020; Yin et al., 2021), such as PeMYB2, PeMYB11, and PeMYB12 in *Phalaenopsis* spp. (Hsu et al., 2015); MaAN2 and MaMYBA in *Muscari armeniacum* (Chen et al., 2017, 2019); MdMYB10 and MdMYB6 in *Malus × domestica* (Espley et al., 2009; Xu et al., 2020); and FhPAP1 from *Freesia hybrida* (Li et al., 2020). In this study, AcMYB1 had the typical characteristics of an R2R3-MYB transcription factor. The AcMYB1 protein contains a highly conserved R2 and R3 repeat in the N-terminal region and a bHLH-interacting motif in the R3 domain. Phylogenetic analysis indicated that AcMYB1 was grouped into the AN2 subgroup, which is represented by PhAN2 and AtPAP1 (Allan et al., 2008). Besides, AcMYB1 was closest to the AaMYB2 transcription factor. AaMYB2 was reported to be a key regulator of anthocyanin biosynthesis in the spathes and leaves of *Anthurium andraeanum*, another member of the Araceae (Li et al., 2016). Moreover, based on the expression patterns, the anthocyanin biosynthetic genes, except for AcF3H, in *A. commutatum* share similar trends with those of *AcMYB1* in the different tissues. These results suggest a potential role for AcMYB1 in regulating anthocyanin accumulation in *A. commutatum*.

The enzymes of anthocyanin structural genes are mainly regulated at the transcriptional level by the interaction between R2R3-MYB and bHLH transcription factors (Koes et al., 2005; Xu et al., 2014). Examples include the ZmC-ZmLc (R2R3-MYB-bHLH) complex in *Zea mays* (Dooner et al., 1991), PhAN2-PhAN1 complex in *Petunia* spp. (Spelt et al., 2000), and NtAn2-NtAn1 complex in *N. tabacum* (Bai et al., 2011). In this study, AcMYB1 formed a heterodimer with AcbHLH1 and could play a key role in the regulation of leaf color in *A. commutatum* (Figure 5). Additionally, in the three lines of OE-AcMYB1 tobacco leaves, the expression levels of both NtAn1a and NtAn1b were significantly upregulated (Figure 7C), suggesting that AcMYB1 may activate anthocyanin-related bHLH in tobacco.

Heterologous expression in model plants can quickly provide the basis for functional identification of target genes. Tobacco is one of the most widely studied model plants in verifying the functions of anthocyanin regulators (Vimolmangkang et al., 2013; Huang et al., 2016; Zhao et al., 2022). For example, HtMYB2 (Gao et al., 2020), EsMYB9 (Huang et al., 2017), AaMYB2 (Li et al., 2016), and IbMYB1a (An et al., 2015)

were found to regulate tobacco anthocyanin biosynthesis to varying degrees. Similarly, in this study, overexpression of AcMYB1 in tobacco displayed striking changes in anthocyanin accumulation in both vegetative and reproductive tissues at various developmental stages (Figures 6A–L). A remarkable increase in anthocyanin was also observed in OE-AcMYB1 lines (Figures 6M,N). We further investigated the function of AcMYB1 in regulating anthocyanin biosynthesis by examining the expression patterns of anthocyanin biosynthesis pathway-related genes in tobacco. In the leaves of the three OE-AcMYB1 lines, all anthocyanin structural genes and endogenous bHLH genes were highly expressed, whereas little or no mRNA abundance was detected in control tobacco. In the corolla, the activation of *AcMYB1* was not as strong as that of leaves; only *NtCHI*, *NtANS*, *NtUFGT*, and *NtAn1b* were significantly upregulated in the three OE-AcMYB1 lines. Overall, the heterogeneous expression analysis strongly supports the notion that AcMYB1 plays an important role in determining red leaf coloration in *A. commutatum*.

It has been reported in a variety of plants that strong light can increase the expression of anthocyanin-related genes and promote the accumulation of anthocyanins, while under dark or weak light conditions, the expression and the biosynthesis of anthocyanins were both inhibited, usually showing white color or pale phenotype (Cominelli et al., 2008; Azuma et al., 2012; Zhang et al., 2018). After the dark treatment for 6 days, the color of the lily flower became lighter with a decrease of anthocyanin content, and the expression of the structural gene *LhDFR* and the regulatory gene *LhbHLLH2* was only one-fifth of that under light conditions, while another anthocyanin-related gene *LhbHLLH1* was not affected by shading (Nakatsuka et al., 2009). Similarly, the anthocyanin content and the expression abundance of anthocyanin genes, including *MrF3H*, *MrF3'H*, *MrDFR*, *MrANS*, *MrUFGT*, and *MrMYB1* of the Chinese bayberry fruit were significantly inhibited after bagging treatment (Niu et al., 2010). In *A. commutatum*, both structural and regulatory genes were expressed at a high level under light conditions compared with the significant reduction in dark conditions. It can be seen that light is a vital environmental factor for the red coloration of *A. commutatum* leaf. Further studies are needed to reveal the potential mechanism of how light regulates anthocyanin biosynthesis in *A. commutatum*.

CONCLUSIONS

In this study, novel R2R3-MYB and bHLH transcriptional factors were identified in *A. commutatum* “Red Valentine” leaves and named *AcMYB1* and *AcbHLLH1*. Expression pattern analysis showed that the transcript abundances of *AcMYB1* and *AcbHLLH1* are similar to those of several structural anthocyanin genes and correlate with anthocyanin distribution. *AcbHLLH1* interacts with *AcMYB1* to form a transcriptional complex. Moreover, overexpression of *AcMYB1* in tobacco results in excessive accumulation of anthocyanins in tobacco leaves and other tissues and upregulates anthocyanin regulatory genes. Furthermore, light can significantly promote anthocyanin accumulation, and

anthocyanin-related genes were strongly up-regulated in *A. commutatum* leaves. Therefore, we believe that *AcMYB1* is a key gene in regulating anthocyanin production in *A. commutatum* “Red Valentine.” Our study may be useful for modifying leaf color in ornamental breeding and provide a basis for further research and development in the plant breeding industry.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JL, LF, and SZ conceived the research project, designed the research, and wrote the manuscript. JL and

LF performed the research. JL, LF, LL, and GM analyzed the data. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2022.886313/full#supplementary-material>

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