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Identification of ApbHLH1 as a Partner Interacting with ApMYB1 to Promote Anthocyanin Biosynthesis during Autumnal Leaf Coloration in *Acer palmatum*

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Abstract: Anthocyanin biosynthesis determines the leaf color of *Acer palmatum* as a widely-planted landscape tree. Previously, ApMYB1 has been characterized as a positive regulator of anthocyanin biosynthesis. To further elucidate the mechanism of leaf coloration, the present study identified a basic helix-loop-helix (bHLH) transcription factor (ApbHLH1) through the phylogenetic analysis of 156 putative bHLH proteins in *Acer palmatum* and eight reference bHLHs which were known to be involved in the anthocyanin biosynthesis of selected plants. Protein structure comparison showed that ApbHLH1 has a conserved bHLH domain, and its N-terminal contains an MYB-interacting region. The expression of *ApbHLH1* in leaves was found to not be correlated with anthocyanin contents either in green, semi-red leaves or during leaf autumnal senescence when anthocyanin content increased. *ApbHLH1* expression in detached leaves was induced by exogenous senescence-promoting chemicals, including H_2O_2, SA, MeJA, ACC and ABA, with certain durations. In particular, either high light or low temperature induced *ApbHLH1* expression significantly, and combination of high light and low temperatures seemed more effective in inducing *ApbHLH1* expression. Luciferase complementation imaging assays confirmed the physical interaction between ApbHLH1 and ApMYB1, which could be abolished by either the truncating MYB-interacting region of ApbHLH1 or the deleting bHLH interacting domain of ApMYB1. The transient expression of *ApbHLH1* could not induce anthocyanin production, while the co-expression of *ApbHLH1* and *ApMYB1* resulted in a higher accumulation of anthocyanins compared to the expression of *ApMYB1* alone in tobacco leaves. Collectively, our results revealed that ApbHLH1 participated in leaf coloration through binding with ApMYB1 and enhancing the ApMYB1 function of promoting anthocyanin biosynthesis during leaf autumnal reddening in *Acer palmatum*. *ApbHLH1* could have the potential for breeding color-leafed plants through co-transformation with *ApMYB1*.

Keywords: bHLH transcription factor; *Acer palmatum*; anthocyanin biosynthesis; ApMYB1; protein interaction

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1. Introduction

Anthocyanin biosynthesis, together with chlorophyll degradation, is a physiological component that is crucial for autumnal leaf coloration in deciduous trees [1]. As a group of secondary metabolites belonging to the flavonoids family, anthocyanin is composed of an anthocyanidin backbone with sugar and acyl conjugates [2]. Although more than 20 anthocyanidins have been identified, only six are most common in plants (i.e., cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin), which differ from each other at the R1 and R2 group on the aromatic benzene ring B [3]. Various anthocyanidins conjugated with sugar and acyl moieties at different positions with different quantities and generated a large number of anthocyanins in nature [2], and different anthocyanins could have a differential color, solubility, reactivity and, thus, physiological activity [4,5].

In plants, anthocyanins not only contributed a purple, blue, red or orange color to certain organs, such as leaves, flowers, fruits, seeds and tubers [6] but also played protective roles under various biotic and abiotic stresses, partially depending on their significant antioxidant activity [7].

Anthocyanins and other flavonoids were synthesized from three molecules of malonyl CoA derived from a fatty acid metabolism and one molecule of p-coumaroyl CoA, which was synthesized from phenylalanine via the general phenylpropanoid pathway [8]. Multiple enzymatic steps of the anthocyanin biosynthetic pathway were well elucidated and could be highly conserved across various plants [6]. The enzymes catalyzing each reaction step were encoded by their respective structural genes which could be separated as early biosynthetic genes [i.e., *CHS* (chalcone synthase), *CHI* (chalcone isomerase), *F3H* (flavonol 3-hydroxylase), *F3′H* (flavonol 3′-hydroxylase) and *FLS1* (flavonol synthase 1)] which catalyze the flavonol biosynthesis and late biosynthetic genes [(i.e., *DFR* (dihydroflavonol 4-reductase), *LDOX* (leucoanthocyanidin oxygenase) and *ANR* (anthocyanidin reductase)] which can catalyze the biosynthesis of both anthocyanin and proanthocyanin [3,9]. Modulating expression of these structural genes represents a primary regulatory level for promoting or suppressing anthocyanin biosynthesis, although positive correlations were more frequently observed between the anthocyanin content and expression levels of late biosynthetic genes [3].

At the transcriptional level, anthocyanin biosynthesis can be activated by a highly conserved MYB-bHLH-WDR (MBW) protein complex in almost all flowering plants [10]. The MBW complex, consisting of DNA-binding R2R3 MYB transcription factors, including the MYC-like basic helix-loop-helix (bHLH) and WD40-repeat (WDR) proteins, activate the targeted structural genes of anthocyanin biosynthesis in multiple species [11,12]. The WD40 proteins of the MBW complex are not thought to bind to DNA and may only serve as a stabilizing function for the interaction between MYB and bHLH proteins [8]. In *Arabidopsis*, a WD40 protein (AtTTG1) was associated with seedling anthocyanin accumulation by forming the MBW complex (AtPAP-GL3/EGL3/TT8-TTG1) [13,14]. Among the three members of the MBW complex, the MYB transcription factor (TF) usually shows the most specificity; thus, it is the activity of MYB TFs that normally determines anthocyanin produced in specific cells or tissues [8]. Many anthocyanin-related R2R3-MYB factors have been identified from a series of plants, including Arabidopsis, grape, eggplant, tomato, populous, and apple [15]. Furthermore, both the amino acid composition of the R2R3 domain in certain MYB TF and the cis-elements in the promoters of anthocyanin biosynthetic genes can determine which specific structural gene(s) are regulated. For example, in *Arabidopsis*, PAP4 (AtMYB114) activates the transcription of *UFGT* (*UDP-glucose:flavonoid-3-O-glucosyltransferase)*, which produces anthocyanins from anthocyanidins, while TT2 (AtMYB123) regulates the expression of *ANR* (*anthocyanidin reductase*), which is specific to proanthocyanin biosynthesis. The alteration of certain amino acids in the R2R3 domain of PAP4 and TT2 has resulted in the modified specificity of PAP4 and TT2 [16].

The bHLH TF in the MBW complex belongs to the conserved subgroup IIIf [17]. Their proteins contain a conserved N-terminal domain that interacts with the specific motif in the R3 domain of R2R3-MYB in the MBW complex [18]. Compared to the MYB partner, bHLH in the MBW complex could have less specificity and show independent functions additional to their regulation of anthocyanin biosynthesis, such as the regulation of proanthocyanidin biosynthesis and the control of trichome and root hair formation in *Arabidopsis* [19]. In *Arabidopsis*, three bHLH transcription factors [i.e., GLABRA3 (GL3), ENHANCER OF GLABRA3 (EGL3), and TRANSPARENT TESTA8 (TT8)] were characterized for their involvement in the regulation of flavonoid biosynthesis [19,20]. Specifically, while AtGL3 and AtEGL3 showed competitive complex formation with some R2R3 MYBs, AtTT8 could interact with AtTTG1 (WD40 protein) and other R2R3 MYBs to form stable MBW complexes [21]. Even the MBW complexes, as combinations of similar but distinct bHLH and MYB, may regulate the expression of different structural genes and determine spatiotemporal anthocyanin production in plants [15]. In addition, a wide variety of proteins (e.g., MYB,

NAC, MADS box, bZIP TFs and JA ZIM domain proteins) and small RNA families can act as repressors of anthocyanin biosynthesis, which, together with those activators, exert the fine regulation of anthocyanin accumulation in different tissue types and in response to different developmental, environmental, and hormonal cues [10,22].

Many species, including *Acer palmatum* in the *Acer* genus, are grown widely as ornamentals in East Asia, North America, and Europe due to their spectacular leaf coloration, especially in autumn [23]. However, few studies have been conducted to elucidate the physiological or molecular mechanisms of leaf coloration. Previously, the R2R3-MYB protein (ApMYB1) was identified in *Acer palmatum*, which was positively associated with anthocyanin production in the leaves [24]. Considering that a putative bHLH binding motif is present in the R3 domain of ApMYB1, it was hypothesized that there could be bHLH protein(s) interacting with ApMYB1. In the present study, through the phylogenetic analysis of protein sequences annotated as putative bHLH proteins in our transcriptomic data, ApbHLH1 was identified and further confirmed to have the capacity to interact with ApMYB1. ApbHLH1 was then characterized by a transient transformation in tobacco leaves to enhance the ApMYB1 function of promoting anthocyanin biosynthesis.

2. Materials and Methods

2.1. Plant Materials

The tree of *Acer palmatum* on the campus of Nanjing Forestry University (32.1° N latitude and 118.8° E longitude, Jiangsu Province, China), which was selected for material collection in a previous study [24], was also chosen for the collection of leaf samples in this study. The tree is grown in natural conditions, and leaves were collected from an individual branch at around 10:30 a.m. from 17 September to 3 December every one or two weeks in 2020 (avoiding rainy days). Each sample had three biological replicates, each of which contained 4–5 individual leaves. The collected leaves were kept in liquid nitrogen first and then stored at −80 °C until further experiments.

Tobacco (*Nicotiana benthamiana*) seeds were surface rinsed with autoclaved ddH_2O and sterilized with 70% ethanol for 1 min and then with 2% NaClO for 10 min. After rinsing 3 times with H_2O, the seeds were sprayed on a $\frac{1}{2}$ MS base medium for 2 weeks in a growth room with 16 h of light at a 150 photosynthetic photon flux density [μmol/(m^2·s)] at 25 °C and 8 h of dark at 22 °C. The uniform seedlings were transplanted in 200-mL plastic pots and filled with a mixture of garden soil and perlite (volume ratio as 4:1). These seedlings were watered regularly to avoid drought stress and were irrigated as needed with a 1/2 strength Hoagland solution. The plants were grown for 4–5 weeks before the appropriate leaves were selected for Agrobacterium injection.

2.2. Measurement of Chlorophyll and Anthocyanin Contents in Leaves

Chlorophylls and anthocyanins in the leaf samples were measured following a previous study [24]. Briefly, chlorophylls were extracted with 80% acetone, and the contents were calculated based on the absorbance at 663, 646 and 470 nm of the extracts [25]. Anthocyanins were extracted with a 1% methanolic HCl solution overnight in the dark at 4 °C [26], and the relative contents were calculated based on their absorbance at 530 nm and 653 nm [27]. One unit of anthocyanin concentration was expressed as an absorbance change of 0.1.

2.3. Phylogenetic Analysis of Putative bHLH Proteins

All putative transcripts encoding bHLH proteins in *Acer palmatum* (ApbHLH) were selected according to the annotation results in a previous study [24]. The peptide sequences of putative ApbHLHs (except those shorter than 60 amino acids) and eight reference bHLHs (i.e., AtTT8, AtGL3, AtEGL3, PhAN1, MdbHLH3, MdbHLH33, MtTT8, VvMYC1) with a known capacity for forming the MBW complex in *Arabidopsis thaliana* (At), *Petunia hybrida* (Ph), *Malus domestica* (Md), *Medicago truncatula* (Mt), and *Vitis vinifera* (Vv) [20,28–30] (Supplemental Table S1) were input to MAFFT (online version) to perform an alignment

with default parameters (https://mafft.cbrc.jp/alignment/server/index.html (accessed on 26 March 2023)). These alignment results were used to produce a maximum-likelihood phylogenetic tree using MEGA5 [31]. The Bootstrap method, with 100 replications, was used to provide confidence levels (reported as a percentage) for the branch points on the phylogenetic tree. Putative bHLH targets in this study were identified from the same clade of a phylogenetic tree containing reference bHLH proteins.

2.4. Isolation of a Putative Target ApbHLH1 and Protein Structure Analysis

Several forward and reverse primers were designed according to the corresponding transcript assembled in previous RNA-Seq results [24]. The total RNA was extracted from senescing leaves, and cDNA was synthesized. The amplified fragment was sequenced, and the open reading frame (ORF) was named *ApbHLH1*. The deduced peptide sequence of ApbHLH1 was aligned with multiple reference bHLH proteins to show conserved domains in protein structure.

2.5. Expression of ApbHLH1 in Various Leaf Samples

The expression of *ApbHLH1* in green and semi-red leaves during autumnal coloration was measured in the samples stored at −80 °C, which were collected in a previous study [24]. The expression of *ApbHLH1* in leaves at five selected time points with an increase in the anthocyanin contents during autumnal senescence was also measured. For chemical treatments, the newly expanded leaves were detached from *Acer palmatum* trees in April and May and placed on filter papers which were wetted with various solutions in Petri dishes under a dim light (40 μmol/(m^2·s)) at 25 °C. The chemical solutions included 20 μM abscisic acid (ABA), 50 μM 1-amino-cyclopropane-1-carboxylic acid (ACC, the direct precursor of ethylene), 50 μM methyl jasmonate (MeJA), 100 μM salicylic acid (SA) and 1% H_2O_2, with H_2O as a control. For environmental treatments, the detached leaves were put on filter papers, wetted with H_2O in Petri dishes and incubated in four environmental conditions: normal light (5000 lux)/25 °C (NL/RT); high light (15,000 lux)/25 °C (HL/RT); normal light (5000 lux)/10 °C (NL/CT); and high light (15,000 lux)/10 °C (HL/CT). The treated leaves were collected at various time points, and *ApbHLH1* expression levels were measured by quantitative RT-PCR.

2.6. Quantitative RT-PCR (qRT-PCR)

As described previously, qRT-PCR was performed with a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The qRT-PCR primers are listed in Supplementary Table S4. The relative transcript levels were normalized to a putative UBQ of *Acer palmatum*, using Equation $2^{-\Delta Ct}$, where Ct represented the threshold cycle for each gene.

2.7. Luciferase Complementation Assay for Protein–Protein Interactions

The interaction between ApbHLH1 and ApMYB1 or their variants [ApbHLH1T (with truncated MYB-interacting region) and ApMYB1D (with deletion of bHLH binding motif)] were detected using the Luciferase Complementation Imaging assay following the protocol published previously [32,33]. In brief, the open reading frames (without stop codon) of *ApbHLH1* and *ApMYB1* were amplified by a PCR and inserted, respectively, into pCAMBIA1300-CCLuc (pCCL-P9) or pCAMBIA1300-HA-NLuc (pHNL-P14), containing a split C-terminal or N-terminal fragment of luciferase, respectively, at both KpnI and SalI sites to generate the C-terminal or N-terminal luciferase-fusion constructs [34]. The resultant constructs were verified by sequencing and transformed into the *Agrobacterium tumefaciens* strain EHA105 by the freeze–thaw method [35], while positive clones were cultured for injecting the leaves of tobacco (*Nicotiana benthamiana*). Equal volumes of Agrobacterium culture, which harbored various constructs of pCCL and pHNL, were mixed as different combinations to a final concentration of OD600 = 0.7. The Agrobacterium mixture was then injected using a needleless syringe into different regions of the same fully expanded leaves of 7-week-old tobacco plants. After the tobacco plants grew

for 2–3 days under normal conditions, the leaves were sprayed with 0.2 mM D-Luciferin solution (Yeasen Biotechnology Co., Shanghai, China) and placed in darkness for 10–20 min. Bioluminescence was detected with a CCD camera (Tanon 5200, Shanghai, China), and representative images were taken. Each experiment was repeated at least three times.

2.8. Transient Overexpression of ApbHLH1 in Tobacco

The open reading frame (2130 bp) of *ApbHLH1* with the addition of BglII and PmeI sites was amplified by a PCR (primers were listed in Supplemental Table S4) and ligated into the pCAMBIA1305 vector through BglII and PmlI sites to generate the *35S::ApbHLH1* construct, which was then transformed into the *Agrobacterium tumefaciens* (EHA105 strain) via a freeze thaw method [35]. The Agrobacterium EHA105 containing the *35S::ApMYB1* construct, which was generated in our previous study [24], was deployed individually or together with Agrobacterium containing *35S::ApbHLH1* for transient overexpression. The transient transformation of tobacco (*Nicotiana benthamiana*) leaves was conducted as described previously [36]. In brief, the newly expanded leaves on 6-week-old *Nicotiana benthamiana* plants were injected using a needleless syringe independently with mixtures of the Agrobacterium culture and containing constructs *35S::ApbHLH1* or *35S::ApMYB1* or an empty vector (*pCAMBIA1305*) at a final OD600 = 0.8. The plants were grown in darkness for 24 h and then under normal growth conditions for 5–7 days until the injected areas exhibited a clear phenotype. The coloration was recorded, and the anthocyanin content was measured in corresponding leaf regions with Agrobacterium injection. The experiment was then repeated three times.

2.9. Statistical Analysis

Values were presented as the mean ± SD of the three biological replicates. The normality distribution of the data was first checked using the Shapiro–Wilk Test of Descriptive Statistics in SPSS version 25.0 (IBM, Armonk, NY, USA). Then, the significant difference between the two means (i.e., between a certain treatment and the control or between two neighboring time points) was analyzed using Student's *t*-test at $p < 0.05$. The statistical difference among multiple means was first estimated by a one-way variance analysis (ANOVA) and then evaluated by Tukey's multiple range test at a 0.05 probability level using SPSS.

3. Results

3.1. Identification of ApbHLH1 According to Phylogenetic Analysis

Out of all 88893 putative transcripts assembled in a previous study [24], there were 172 putative transcripts annotated as encoding bHLH proteins in *Acer palmatum*. Excluding 16 putative peptides with less than 60 amino acids, the deduced 156 peptides were subject to phylogenetic alignment with eight reference bHLH proteins that had a known capacity for interacting with MYB partners (Supplemental Table S1). It was found that the proteins of two transcripts (i.e., Unigene13498_All and CL8881.contig2_All) were grouped in the same subclade of the phylogenetic tree with five reference proteins (i.e., VvMYC1, MdbHLH3, MtTT8, PnAN1 and AtTT8) (Figure 1), indicating that they could have a similar capacity in forming the MBW complex when regulating anthocyanin biosynthesis [37]. Considering that proteins deduced from the transcript "Unigene13498_All" showed a higher residue identity with reference proteins than that deduced from the transcript "CL8881.contig2_All" (Supplemental Table S2), the transcript "Unigene13498_All" was named *ApbHLH1* and selected for further cloning and characterization in this study.

3.2. Cloning ApbHLH1 cDNA and Analyzing Amino Acid Sequences

Seven forward primers and four reverse primers were designed (Supplementary Table S4) according to the corresponding sequence of the transcript "Unigene13498_All", which was assembled in a previous RNA-Seq transcriptomic analysis [24]. A cDNA fragment (2286 bp) was amplified by RT-PCR with a forward primer (F4) paired with a reverse primer (R1). The

open reading frame (ORF) of *ApbHLH1* was 2130 bp (Supplementary Table S3), which could encode a peptide with 709 amino acids (aa). Sequence alignment showed that the ApbHLH1 protein had a conserved bHLH domain at 481–532 aa, inside of which existed the HER motif (H×××E×××R). Similar to five reference bHLHs, the N-terminal of the ApbHLH1 protein contained an MYB-interacting region at 9–211 aa, which was a prerequisite for the formation of MBW activation complexes (Figure 2).

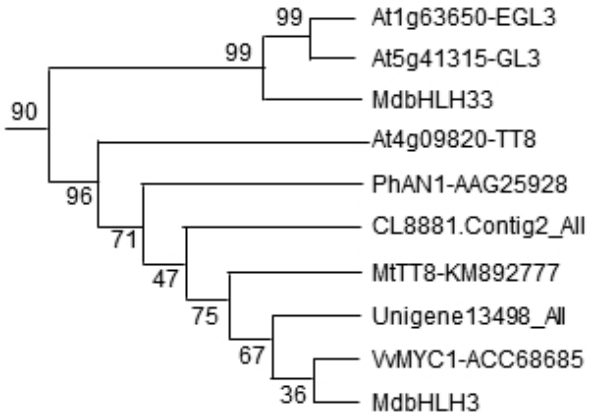

Figure 1. The selected clade of the phylogenetic tree containing eight reference bHLHs and two putative target bHLH proteins (i.e., deduced from transcript Unigene13498_All and CL8881.contig2_All, respectively) in *Acer palmatum*. The numbers besides the branches are bootstrap values. The intact phylogenetic tree is shown in Supplemental Figure S1.

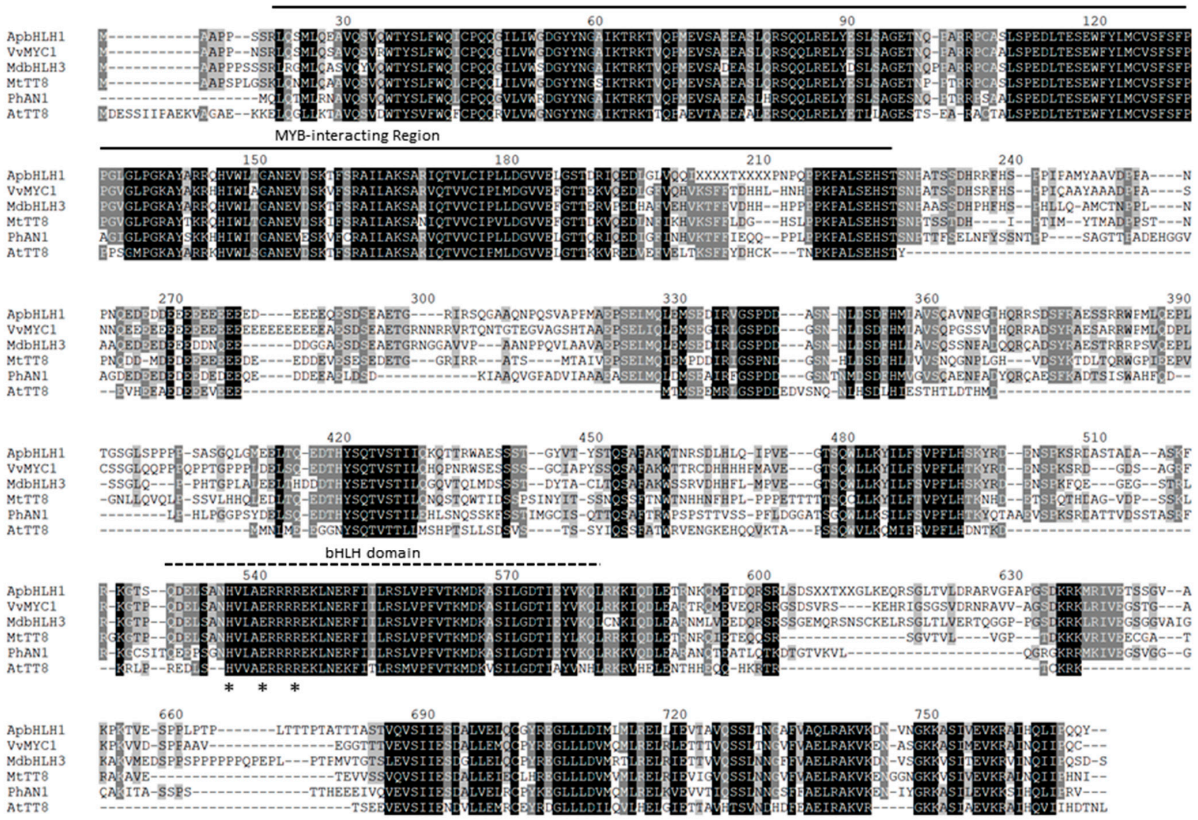

Figure 2. Alignment of amino acid sequences of ApbHLH1 and five reference bHLH TFs with a known function of regulating anthocyanin biosynthesis. Black shading indicates identical amino acids. The bHLH domain is indicated with a dashed line. The HER motif in the bHLH domain is indicated with three asterisks. The MYB-interacting region is indicated with black lines. The five

reference proteins were AtTT8 (Q9FT81) from *Arabidopsis thaliana*, MdbHLH3 (ADL36597) from *Malus domestica*, MtTT8 (KM892777) from *Medicago truncatula*, PhAN1 (AAG25928) from *Petunia* × *hybrida*, and VvMYC1 (ACC68685) from *Vitis vinifera*.

3.3. Contents of Chlorophyll and Anthocyanins and Expression Levels of ApbHLH1 during Leaf Coloration

The contents of chlorophyll and anthocyanins were measured in *Acer palmatum* leaves from the middle of September to the beginning of December. The chlorophyll content was about 4.0 mg/g FW before and on 29 October and decreased significantly afterward until it reached 0.7 mg/g FW on 3 December (Figure 3A). Although anthocyanin content was low both before and on 15 October (around 0.026 U/mg), it increased significantly to 0.112 U/mg on 29 October and to 0.831 U/mg on 3 December (Figure 3B). These results possibly indicate that anthocyanin in senescing leaves could be synthesized prior to chlorophyll degradation.

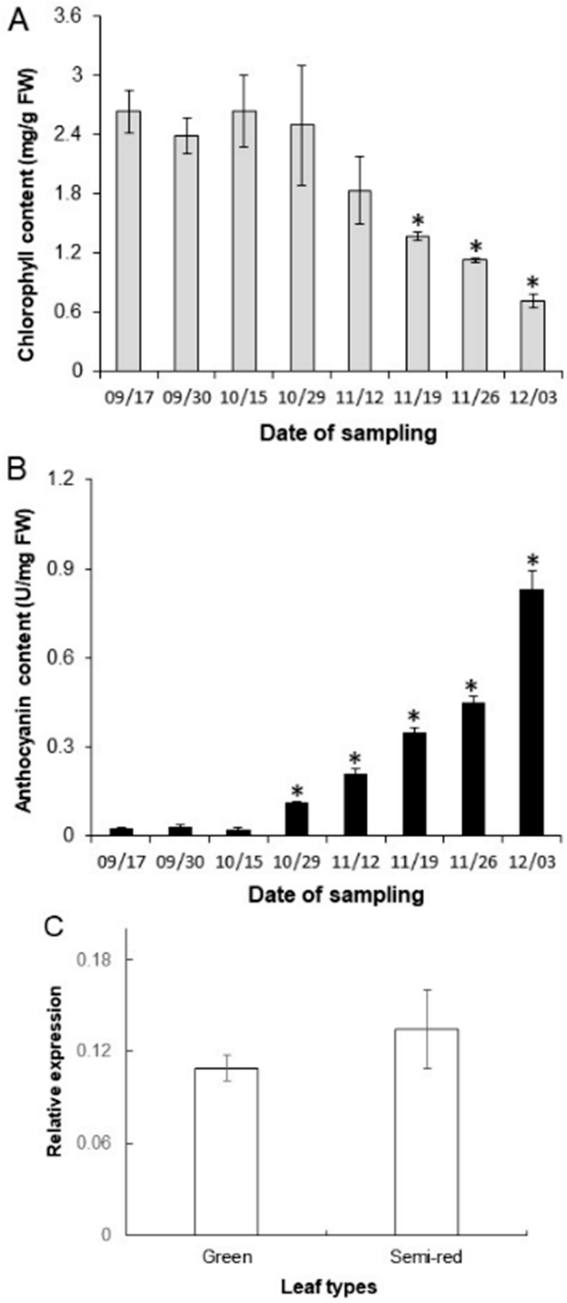

Figure 3. *Cont.*

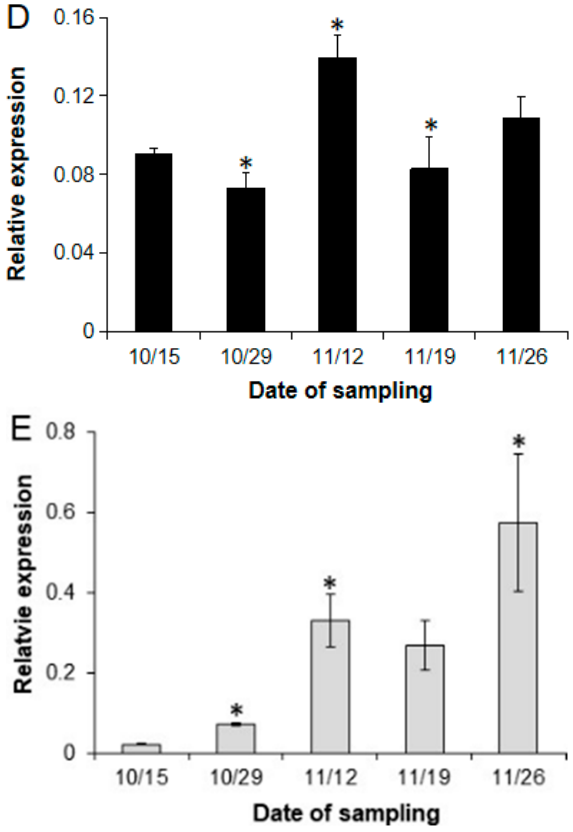

Figure 3. Contents of pigments (chlorophyll and anthocyanin) and expression levels of *ApbHLH1* in leaves. (**A**,**B**) Contents of chlorophyll and anthocyanin, respectively, in leaves sampled from middle September to early December. (**C**) Expression of *ApbHLH1* in green and semi-red leaves which were collected in a previous study [24]. (**D**,**E**) Expression of *ApbHLH1* and *ApMYB1*, respectively, in leaves at five consecutive time points with increasing anthocyanin contents. Values are means ± SD (n = 3). An asterisk indicates the significant difference between the immediate previous time point at $p < 0.05$.

In the previous study [24], anthocyanin content in the semi-red leaves was found to be significantly higher than that in green leaves, with both types of leaves collected inside a canopy in late November. However, as shown in Figure 3C, the expression of *ApbHLH* was statistically similar between the green and semi-red leaves. When the expression of *ApbHLH1* was measured in leaves at five-time points when anthocyanin content showed an obvious increase (15 October, 29 October, 12 November, 19 November and 26 November) (Figure 3B), it was found that *ApbHLH1* expression fluctuated with less than a two-fold variation at these time points (Figure 3D). In contrast, the expression of *ApMYB1*, encoding the positive regulator of anthocyanin biosynthesis [24], exhibited a general trend that increased from 15 October to 26 November (Figure 3E), which was correlated positively with the increase in the anthocyanin content (Figure 3B). These results indicate that *ApbHLH1* alone may not be responsible for leaf coloration at the transcriptional level.

3.4. ApbHLH1 Expression in Detached Leaves Submitted to Exogenous Treatments

To test which exogenous factors could be effective in influencing *ApbHLH1* expression, the detached leaves of *Acer palmatum* were incubated with various chemicals. As shown in Figure 4A, compared to the H_2O control, 4 h, 8 h and 16 h of SA, MeJA, ACC and ABA incubation and 8 h and 16 h of H_2O_2 incubation all induced *ApbHLH1* expression significantly. In particular, 4 h of ABA, SA and MeJA incubation, 8 h of ACC incubation and 16 h of H_2O_2, ACC and ABA incubation resulted in an increased *ApbHLH1* expression which was larger than two folds compared to that of the respective H_2O control.

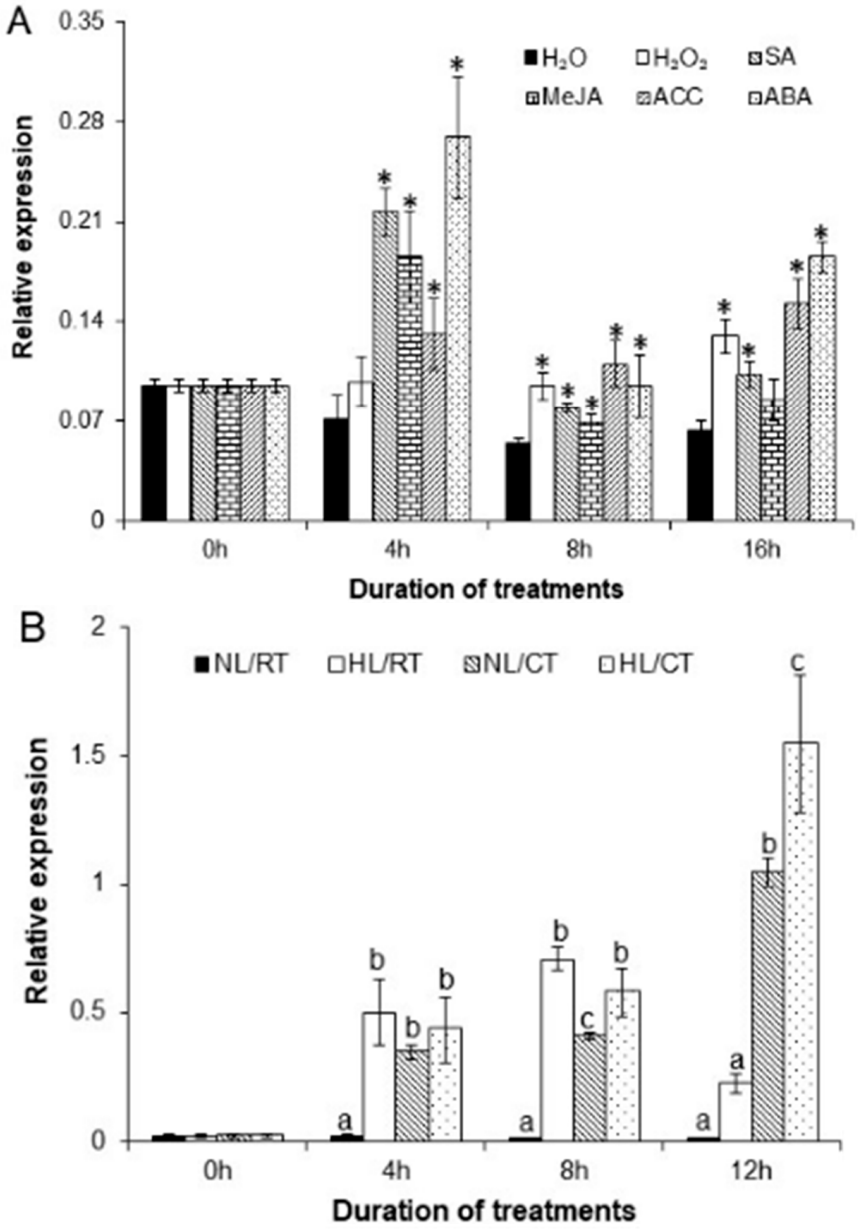

Figure 4. Response of *ApbHLH1* expression in detached *Acer palmatum* leaves with exogenous treatments. (**A**) The relative expression of *ApbHLH1* in detached leaves incubated under dim light with H_2O (negative control), H_2O_2 (1%), SA (100 μM), MeJA (50 μM), ACC (precursor of ethylene, 50 μM) and ABA (20 μM). (**B**) Expression of *ApbHLH1* in detached *Acer palmatum* leaves incubated under four types of environmental conditions: NL/RT, normal light (5000 lux)/25 °C; HL/RT, high light (15,000 lux)/25 °C; NL/CT, normal light (5000 lux)/10 °C; and HL/CT, high light (15,000 lux)/10 °C. Values are means ± SD (n = 3). An asterisk in (**A**) indicates significant differences ($p < 0.05$) between the treatment and control at a certain time point. Different lowercase letters in (**B**) indicate significant differences ($p < 0.05$) between any treatments at a certain time point.

The effect of high light and low temperatures on *ApbHLH1* expression was also tested. Among the four types of incubation conditions, normal light/25 °C (NL/RT) showed the least induction on *ApbHLH1* expression, as the *ApbHLH1* expression under NL/RT incubation was the lowest among the four types of conditions at either time point (Figure 4B). Under high light/25 °C (HL/RT), the expression of *ApbHLH1* was 25.1 folds at 4 h, 56.2 folds at 8 h and 18.9 folds at 12 h, respectively, as high as that under NL/RT. Incubation under normal light/low temperature (NL/CT) resulted in the expression levels

of *ApbHLH1* being 17.3 folds at 4 h, 32.3 folds at 8 h and 86.5 folds at 12 h, respectively, relative to those under NL/RT. Although *ApbHLH1* expression under the incubation of high light and low temperature (HL/CT) exhibited a similar trend of increasing to that under NL/CT, it was found that *ApbHLH1* expression under HL/CT at 8 h was significantly higher than that under NL/CT. With 12 h of incubation, *ApbHLH1* expression under HL/CT was even higher than the sum of both levels under HL/RT and NL/CT (Figure 4B), possibly indicating that a combination of high light and low temperature with a certain duration could have synergistic effects on inducing *ApbHLH1* expression.

3.5. Interacting between ApbHLH1 with ApMYB1

To test if ApbHLH1 could interact with ApMYB1 physically, luciferase complementation imaging assays were conducted [32,33]. As shown in Figure 5A, luminescence signals were detected only in the regions injected with (pCCL-ApbHLH1 + pHNL-ApMYB1) of the *Nicotiana benthamiana* leaf, but not in regions injected with empty vector control (pCCL-P9 + pHNL-P14) and another two kinds of Agrobacterium mixture: (pCCL-ApbHLH1 + pHNL-P14) and (pCCL-P9 + pHNL-ApMYB1), suggesting that ApbHLH1 interacted with ApMYB1 in vivo. However, either the truncating MYB-interacting region of ApbHLH1 (ApbHLH1T) or deleting bHLH interacting domain of ApMYB1 (ApMYB1D) abolished the interaction between ApbHLH1 and ApMYB1 (Figure 5B,C).

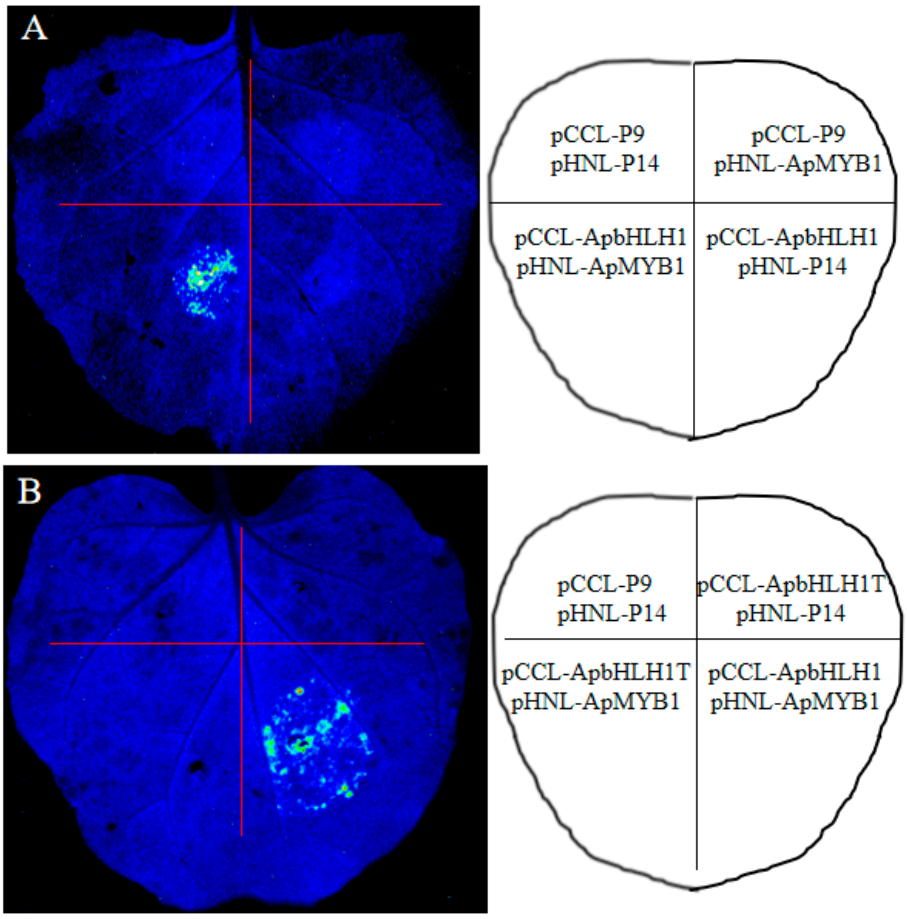

Figure 5. *Cont.*

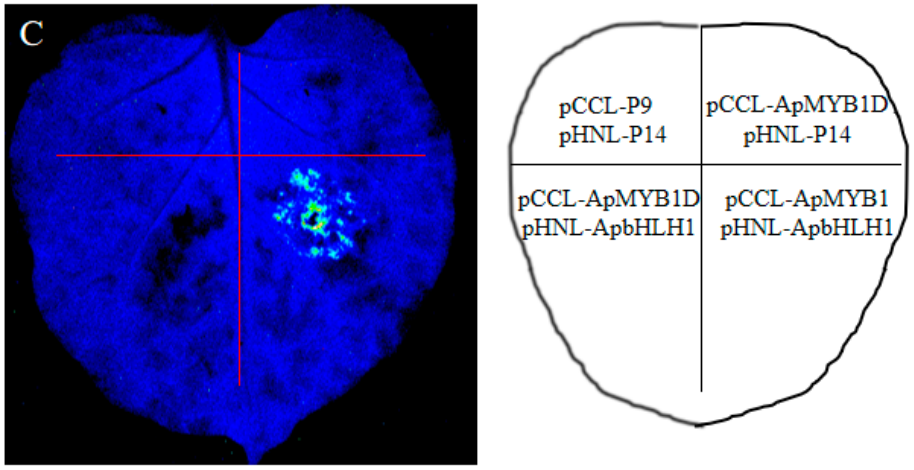

Figure 5. Interaction between ApbHLH1 and ApMYB1 when tested by luciferase complementation imaging assays. (**A**) Luminescence signals detected only in the region of the *Nicotiana benthamiana* leaf injected with an Agrobacterium mixture of pCCL-ApbHLH1 and pHNL-ApMYB1. (**B**) Luminescence signals abolished in the leaf region (**lower left**) injected with an Agrobacterium mixture of pHNL-ApMYB1 and pCCL-ApbHLH1T with truncated MYB-interacting region. (**C**) Luminescence signals abolished in the leaf region (**lower left**) injected with Agrobacterium mixture of pHNL-ApbHLH1 and pCCL-ApMYB1D with the deletion of bHLH interacting domain. The experiments were repeated four times with similar results and the representative images are presented.

3.6. Transient Expression of ApbHLH1 in Tobacco Leaves

To further investigate the function of *ApbHLH1* in regulating anthocyanin biosynthesis, a constitutive construct (*35S::ApbHLH1*) was injected together with an empty vector (EV, *35S*::*GUS*) or *35S*::*ApMYB1* into the leaves of tobacco (*Nicotiana benthamiana*) via Agrobacterium-mediated transformation. The injection of only EV was used as a negative control. Out of our expectations, anthocyanin production was not visualized in the leaf regions injected with either EV or *35S::ApbHLH1* + EV. By contrast, tissue redness was visualized in the leaf regions with the Agrobacterium injection of either *35S::ApMYB1* + EV or *35S::ApbHLH1* + *35S::ApMYB1*. Leaf regions with an injection were then separated, and anthocyanin content was measured. Consistent with phenotypic observation, the anthocyanin contents of the leaf regions injected with EV and with *35S::ApbHLH1* + EV were statistically similar, about 0.035 U/mg FW. However, the anthocyanin content in leaf regions injected with *35S::ApMYB1* + EV was 0.077 U/mg FW, which was significantly higher than that in leaf regions injected with *35S::ApbHLH1* + EV, while injections with *35S::ApbHLH1* + *35S::ApMYB1* resulted in anthocyanin content as 0.142 U/mg FW, which was about two folds of and significantly higher than that with the injection of *35S::ApMYB1* + EV. These results indicated that *ApbHLH1* alone could not, while *ApMYB1* alone could induce significant anthocyanin biosynthesis in tobacco leaves. Notably, the injection of *35S::ApbHLH1* + *35S::ApMYB1* showed a higher capacity to promote anthocyanin production than the injection of *35S::ApMYB1* + *35S*::*GUS*. Together with the aforementioned results, it could be suggested that ApbHLH1 directly interacts with ApMYB1, which could enhance the function of ApMYB1 to promote anthocyanin biosynthesis.

4. Discussion

Acer palmatum is a deciduous tree that is widely planted for landscape ornamentation due to its bright leaf redness during autumn [38], but the regulatory mechanisms of leaf coloration are largely unknown. Leaf color, as the comprehensive exhibition of the various pigments present in leaf tissues, is mainly determined by the contents and constituents of chlorophylls, carotenoids and anthocyanins [1]. For many deciduous trees, although their leaves are normally green in the growing season, their leaf color changes into yellow,

orange and red gradually when the trees enter autumnal senescence, which is usually accompanied by both chlorophyll degradation and anthocyanin biosynthesis. In this study, by measuring the leaf chlorophyll and anthocyanin contents from middle September to early December, it was found that the time point with decreasing chlorophyll content was after 29 October, while the time point with increasing anthocyanin contents was after 15 October (Figure 3A,B), indicating that anthocyanin biosynthesis in the senescing leaves of *Acer palmatum* occurred prior to chlorophyll degradation. However, because of being masked by a high content of chlorophyll, the increase in anthocyanin content in late October did not result in visible leaf reddening. It is of interest to elucidate how the initiation of both anthocyanin biosynthesis and chlorophyll degradation is coordinated during leaf autumnal coloration.

As for the regulatory mechanisms of anthocyanin biosynthesis, the conserved MYB–bHLH–WD repeat (MBW) transcriptional complexes have been revealed to be widespread in the eudicots for the activating expression of anthocyanin biosynthetic genes [39,40]. Those MBW complexes consisting of different MYB or bHLH members usually show diverse functions within and across species for the subtle regulation of pigment production (including quantity, types and patterns) [41]. Furthermore, other transcription factors may interact with the core MBW complex either to repress or to promote its activity [42], thus generating a highly complicated and dynamic regulatory network to fine-tune anthocyanin production in response to developmental and environmental variations.

In our previous study, an R2R3 MYB transcription factor (ApMYB1) was identified as a positive regulator of anthocyanin biosynthesis in the autumnal senescing leaves of *Acer palmatum* [24]. It was found that the R3 domain of ApMYB1 contained a bHLH binding motif, indicating that there may be bHLH factors interacting with ApMYB1 that facilitate its function. To identify such kinds of bHLH factors in *Acer palmatum* in the present study, phylogenetic analysis was first performed with 156 putative bHLH proteins (with lengths larger than 60 amino acids) and eight reference bHLHs which have been characterized to interact with MYB partners and are involved in anthocyanin biosynthesis in other plants (Supplemental Figure S1, Supplemental Table S1). Two putative ApbHLH proteins were grouped with five reference bHLHs in a subclade (Figure 1). The one which was deduced from transcript Unigene13498_All (named ApbHLH1 then) showed a higher amino acid identity with five reference bHLHs (Supplemental Table S2) and was selected for further characterization.

Protein structure analysis confirmed that ApbHLH1 had a conserved bHLH domain containing 52 amino acid residues, which was supposed to be necessary for the formation of dimers [43]. Additionally, an HER motif (H×××E×××R) was found inside the bHLH domain (Figure 2), which is the classic G-box (CACGTG) recognition motif and is critical for DNA binding and regulating the transcription of target genes [44,45]. Especially when compared with reference bHLH proteins, it showed that at the N-terminal of ApbHLH1, there was an MYB-interacting region with 203 amino acid residues (Figure 2), suggesting the putative capacity of ApbHLH1 to bind its MYB partner to form MBW complexes. Then, a luciferase complementation imaging assay, which is highly quantitative and has an extremely low background for studying protein–protein interactions in plants [32], was performed to test the putative interaction between ApbHLH1 and ApMYB1 (Figure 5). It was found that the regions of tobacco leaves with either the injection of Agrobacterium (pCCL-ApbHLH1 + pHNL-ApMYB1) or (pCCL-ApMYB1 + pHNL-ApbHLH1) exhibited luminescence signals, confirming the physical binding of ApbHLH1 and ApMYB1. However, the interaction between ApbHLH1 and ApMYB1 was abolished by either the truncating MYB-interacting region of ApbHLH1 (ApbHLH1T) or the deleting bHLH interacting domain of ApMYB1 (ApMYB1D) (Figure 5B,C), suggesting that an intact MYB-interacting region of ApbHLH1 and presence of the bHLH interacting domain in ApMYB1 are necessary for the physical interaction between these two proteins [17].

Opposite to our expectations, as shown in Figure 3C, *ApbHLH1* expression was similar in green and semi-red leaves [24], meaning that *ApbHLH1* transcription was not positively

correlated with anthocyanin accumulation. This was further supported by the fact that *ApbHLH1* expression did not show a trend of increasing in the leaves from 15 October to 26 November, when anthocyanin content showed a significant increase (Figure 3B,D). In opposite, consistent with its function as a positive regulator gene of anthocyanin biosynthesis, the expression of *ApMYB1* showed an increase over this period, which was positively correlated with anthocyanin contents (Figure 3E). Considering that autumnal leaf coloration coincides usually with leaves' natural senescence in many deciduous trees [1], the responses of *ApbHLH1* expression to those senescence-promoting hormones (including ABA, SA, MeJA, ACC and H_2O_2) were tested [46]. It was found that an increase larger than two folds of *ApbHLH1* expression was induced in detached *Acer palmatum* leaves by ABA, SA, MeJA, ACC and H_2O_2 with certain treatment durations (Figure 4A). Among those environmental factors, low temperature and high light were the two common ones that induced leaf anthocyanin biosynthesis in autumn [47]. Consistent with previous reports [30,48–50], it was found that *ApbHLH1* expression was significantly increased by either high light or low temperature when compared to the control treatment with normal-light and room-temperature (Figure 4B). Especially, the expression of *ApbHLH1* in detached *Acer palmatum* leaves was induced with synergistic effects by a 12 h treatment with high light and low-temperature combination, which was similar to previous results about anthocyanin production in apple calluses and grape skins [51,52]. Considering that many bHLHs also play vital roles in plant responses to abiotic stress [53], it is of interest to elucidate whether *ApbHLH1* might be associated with the adaptability of *Acer palmatum* leaves to high light or low temperatures.

The role of *ApbHLH1* in anthocyanin biosynthesis in vivo was tested by the transient expression in tobacco leaves. Consistent with the previous conclusion that ApMYB1 is a positive regulator of anthocyanin biosynthesis [24], *ApMYB1* alone was found capable of inducing anthocyanin production in injected tobacco leaves (Figure 6). In contrast, *ApbHLH1* alone did not show such a kind of capacity (Figure 6), indicating that ApbHLH1 is not a direct regulator of anthocyanin biosynthesis. This was different from VdbHLH037 in spine grapes (*Vitis davidii*) [54] and FvbHLH9 in strawberries (*Fragaria vesca*) [55], which could promote anthocyanin biosynthesis in respective plants by themselves. Notably, an injection with both *ApbHLH1* and *ApMYB1* together resulted in an increased anthocyanin content, which was even higher than that of the single *ApMYB1* injection, indicating that ApbHLH1 may participate in anthocyanin biosynthesis through interacting with ApMYB1 and enforcing its function (Figure 5), possibly by forming an MBW complex. It would be interesting to further confirm the function of *ApbHLH1* through a stable transformation in future studies. Additionally, it remains to elucidate if the binding of ApbHLH1 could modify the specificity of ApMYB1 targeting downstream structural genes in anthocyanin biosynthesis.

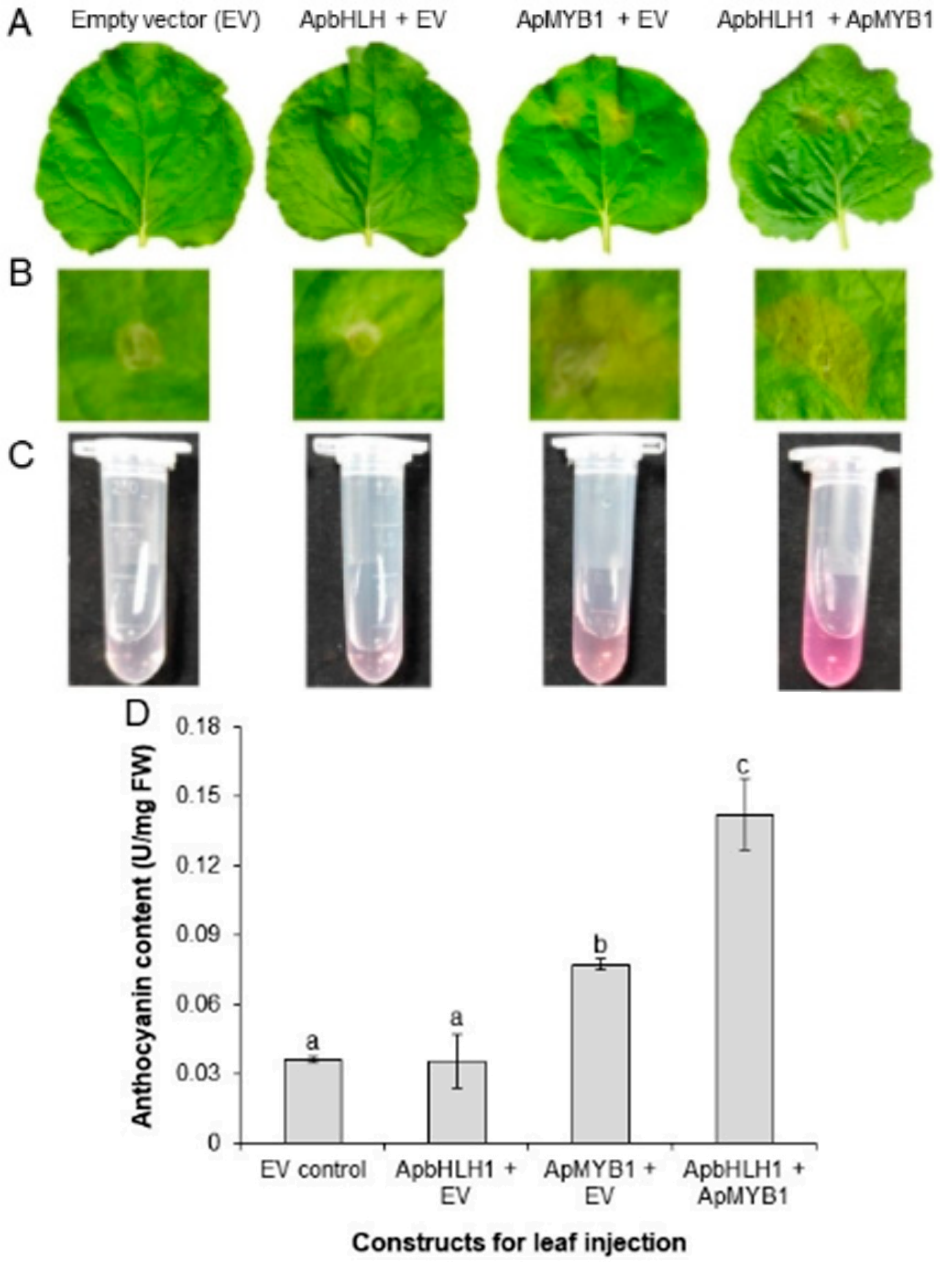

Figure 6. Transient expression of various constructs in tobacco leaves. (**A**) Phenotype of tobacco leaves 6 days after injection with various constructs. (**B**) Phenotype of leaf regions with various Agrobacterium injections. (**C**) Colors of anthocyanin extracts. (**D**) Contents of anthocyanins in leaf regions injected with various constructs. The values in D are the means ± SD (n = 3). The significant difference ($p < 0.05$) is indicated by different lowercase letters above the error bars.

5. Conclusions

This study presents a further understanding of the molecular mechanisms of leaf coloring in the deciduous *Acer palmatum* tree. It was found that a bHLH transcription factor (ApbHLH1) could bind with ApMYB1 and enhance the function of ApMYB1 as a positive regulator in promoting anthocyanin biosynthesis, although ApbHLH1 alone could not promote anthocyanin biosynthesis. The interaction between ApbHLH1 and ApMYB1 could represent a fine-tuning mechanism in autumnal leaf coloration in *Acer palmatum.*

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14061262/s1, including Supplemental Figure S1. The intact phylogenetic tree of 156 putative bHLH proteins from *Acer palmatum* and eight reference bHLHs with known function; Supplemental Table S1. The name list of 156 putative bHLH proteins (longer than 60 amino acids) in *Acer palmatum* and 8 reference bHLHs; Supplemental Table S2. Percentage of residue identity between two putative target bHLH proteins and five reference proteins. Supplemental Table S3. Open reading frame of *ApbHLH1.* Supplemental Table S4. Primers used in this study.

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