The basic leucine zipper (bZIP) transcription factor HY5 plays a multifaceted role in plant growth and development. Here the apple MdHY5 gene was cloned based on its homology with Arabidopsis HY5. Expression analysis demonstrated that MdHY5 transcription was induced by light and abscisic acid treatments. Electrophoretic mobility shift assays and transient expression assays subsequently showed that MdHY5 positively regulated both its own transcription and that of MdMYB10 by binding to E-box and G-box motifs, respectively. Furthermore, we obtained transgenic apple calli that overexpressed the MdHY5 gene, and apple calli coloration assays showed that MdHY5 promoted anthocyanin accumulation by regulating expression of the MdMYB10 gene and downstream anthocyanin biosynthesis genes. In addition, the transcript levels of a series of nitrate reductase genes and nitrate uptake genes in both wild-type and transgenic apple calli were detected. In association with increased nitrate reductase activities and nitrate contents, the results indicated that MdHY5 might be an important regulator in nutrient assimilation. Taken together, these results indicate that MdHY5 plays a vital role in anthocyanin accumulation and nitrate assimilation in apple.

ARTICLE
The bZIP transcription factor MdHY5 regulates anthocyanin accumulation and nitrate assimilation in apple
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INTRODUCTION
Apple (Malus × domestica) is an important fruit crop that is widely cultivated worldwide. Peel color is an important trait that determines apple market value; red fruits are more attractive to consumers. As a kind of secondary metabolite, anthocyanins are responsible for the red coloration in apple peel.¹,² In general, anthocyanins are synthesized via the phenylpropanoid pathway, and multiple enzymes are involved in this pathway, including phenylalanine ammonia lyase, chalcone isomerase, chalcone synthase, flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), UDP glucose: flavonoid 3-O-glucosyltransferase (UF3GT) and anthocyanidin synthase (ANS).³,⁴ Three main protein families—MYB, bHLH and WD40—are involved in the regulation of anthocyanin biosynthesis by forming the MYB-bHLH-WD40 protein complex.⁵,⁶ In apple, three MYB transcription factors (TFs) (MdMYB1, MdMYB10 and MdMYBA) have been functionally identified and confirmed to be responsible for anthocyanin accumulation, as these TFs directly regulate the expression of anthocyanin biosynthesis structural genes.⁷,⁸

In addition to genetic components, nutritional components such as nitrogen, phosphorus and potassium can affect fruit coloration as well as plant growth.⁹,¹⁰ Among these nutritional components, nitrogen is the most important; NO₃⁻ and NH₄⁺ are the major sources of nitrogen in aerobic and anoxic soils, respectively.¹¹ In recent years, the physiological process and genetic mechanism of nitrogen uptake and transport have been studied in depth, and protein families, such as NRT1/2, NAR2 and AMT1/2, participate in the absorption and transport of NO₃⁻ and NH₄⁺.¹²⁻¹⁵ In addition, NO₃⁻ is widely regarded as a crucial signal molecule that modulates multiple aspects of plant growth and development, such as nitrate-mediated root development,¹⁶,¹⁷ stress tolerance¹⁸ and crop yield and quality.¹⁹,²⁰ Therefore, identification of the functions of genes involved in nitrogen uptake and transport in apple is essential.

TFs are a group of key regulatory proteins that play important roles in controlling the expression of signal response genes. They can be divided into many families, such as the basic leucine zipper (bZIP), bHLH, MYB, zinc-finger and NAC families, according to their conserved domains. In addition, different types of TFs play diverse roles in plant growth and development. An important TF in plants is the bZIP protein HY5, which was first identified to positively regulate plant photomorphogenesis based on the light insensitivity of the hy5 mutant.²¹,²² This factor has also been subsequently implicated in abscisic acid (ABA) signaling.²³

HY5 acts as a master regulator that integrates signals from multiple pathways to coordinate plant stress tolerance and development.²⁴ HY5 binds the T/G-box (CACGTT), E-box (CAATTG), GATA-box (GATGATA), ACE-box (ACGT), Z-box (ATACGGT) and C-box (GTCANN) as well as the hybrid C/G⁻ (G) and C/A-boxes in the promoters of many genes that are involved in various signaling pathways, such as light signaling,²⁵ anthocyanin and chlorophyll biosynthesis,²⁶,²⁷ nutrient signaling²⁸ and defense signaling.²⁹ In addition, HY5 regulates the expression of microRNAs by directly binding to their promoters,³⁰ which indicates that HY5 regulates gene expression at both the transcriptional and post-transcriptional levels. The HY5 protein is unstable and is degraded in darkness by COP1 via the 26S proteasome pathway,³¹,³² but HY5 stability is indirectly promoted in blue light by SPA1 by dissociating with COP1 and associating with CRY1.³³ Moreover, recent studies have shown that low-temperature and short-heat shock treatments also stabilize the HY5 protein.²⁶,³⁴ Thus, HY5 is dynamic in plants and plays a central role in the hub of transcriptional networks.

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In the present study, we cloned and functionally characterized the apple bZIP protein MdHY5. MdHY5 may be involved in anthocyanin biosynthesis by directly activating MdMYB10 and nitrogen signaling by promoting the expression of nitrate reductase (NR) genes and nitrate uptake genes. In brief, these results indicate that MdHY5 plays a vital role in anthocyanin accumulation and nitrate assimilation in apple.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

Calli of the ‘Orin’ apple (wild type) were subcultured on Murashige and Skoog (MS) medium containing 0.5 mg L\(^{-1}\) indole acetic acid and 1.5 mg L\(^{-1}\) 6-butyric acid (BA) at room temperature (24 °C) in continuous darkness; calli were subcultured at 20 days intervals. For high-light and low-temperature treatments, transgenic and wild-type (WT) apple calli were transferred to a phytotron at 15 °C under constant high light (photon flux density of ~100 µmol s\(^{-1}\) m\(^{-2}\)).

For light treatment, apomictic crabapple (*Malus hupehensis*) seedlings grown in darkness for 3 days at room temperature were transferred to white light conditions. For ABA treatment, apomictic crabapple seedlings were treated with 50 µM ABA. RT-qPCR and semi-quantitative RT-PCR were performed to monitor the expression level of *MdHY5*.

**Gene cloning of *MdHY5***

Using the NCBI database with the Basic Local Alignment Search Tool (BLAST) program, we found two potential homologous genes (MDP0000586302 and MDP0000264514) that differed by only one amino acid. On the basis of these results, we speculate that these two genes might have similar functions and possibly be allelic. MDP0000586302 was selected for functional identification.

**RT-qPCR and semi-quantitative RT-PCR**

Apple seedlings and 4 g of apple calli collected from three plates were used for RNA extraction. RNAs were extracted from the apple seedlings and calli using RNA plant plus Reagent (Tiangen, Beijing, China) followed by reverse transcription using a PrimeScript first-strand cDNA synthesis kit (Takara, Dalian, China). RT-qPCR assays were conducted with the UltraSYBR mixture (SYBR Green I) (Takara) using an ABI7500 RT-qPCR system. The concentration of cDNA was diluted to 1–10 ng µL\(^{-1}\). One microliter of diluted cDNA was used for RT-qPCR, and the 2\(^{−\Delta\Delta Ct}\) calculation method of RT-qPCR was used. The results were normalized to those of *MaACTIN*. A minimum of three biological replicates per sample were used for RT-qPCR.

**RT-PCR**

RT-PCR was conducted to examine the expression levels of *MdHY5* in response to light and ABA. Each PCR mixture contained 200 ng of cDNA, 1x Taq buffer, 2.5 mM dNTPs, 0.5 µL of Taq DNA polymerase (Trans, Beijing, China) and each primer at 10 µM in a total volume of 25 µL. The reactions were performed as follows: denaturation at 95 °C for 10 min; 25–28 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s; and a final cycle at 72 °C for 5 min.

**Measurement of anthocyanins**

Total anthocyanins were extracted as described by Lee and Wicker.\(^{37}\) Three grams of apple calli were incubated in an anthocyanin extraction solution for 12 h at 4 °C in darkness. The absorbance of each sample was measured at 530, 620 and 650 nm using a spectrophotometer (Shimadzu UV-2450, Kyoto, Japan). The relative anthocyanin contents were normalized according to the following formula: optical density (OD) = (A530 – A620 – 0.1x[A650 – A620]). One unit of anthocyanin content was expressed as a change of 0.1x OD (units × 10\(^{-7}\) g\(^{-1}\) of fresh weight (FW)). Measurements were performed in triplicate.

**MdHY5-GST fusion protein expression and purification**

The open reading frames of *MdHY5* were cloned into the pGEX-4T-1 vector, which contained a glutathione (GST) tag sequence, and the recombinant vector was then transformed into *Escherichia coli* BL21 (DE3). The BL21 bacteria were subsequently treated with 3 mM isopropyl β-D-thiogalactoside (IPTG) in order to induce the production of the *MdHY5*-GST fusion protein. The fusion protein was then added to a cobalt chelate affinity resin containing the immobilized GST-tag and incubated at 4 °C for 2 h under rotation. After three washings, the proteins were collected and detected via western blot with GST antibodies (Abcam, Cambridge, UK).

**Electrophoretic mobility shift assay (EMSA)**

EMSA was conducted using a LightShift Chemiluminescent EMSA Kit (Thermo, Waltham, MA, USA). Briefly, biotin-labeled probes were incubated in a 1 x binding buffer containing 2.5% glycerol, 10 mM EDTA, 5 mM MgCl\(_2\) and 50 mM KCl with or without proteins at 24 °C for 25 min. An unlabeled probe was added to the reactions for unlabeled probe competition.

**Transient expression assay in *Nicotiana benthamiana* leaves**

Transient expression assays in *N. benthamiana* leaves were performed as previously described.\(^{38,39}\) The *MdHY5* and *MdMYB10* promoters were amplified and cloned into pGreenII 62-SK vector. A charge-coupled device imaging apparatus (NightOWL II LB983 in conjunction with Indigo software) was used to collect the LUC images and quantify luminescence intensity. Transformed leaves were sprayed with and soaked in 100 mM luciferin, after which they were placed in darkness for 6 min before luminescence examination.

**Measurement of NR activity**

NR activity was measured as previously described.\(^{40}\) Five-hundred-milligram samples (apple calli) were transferred to test tubes after being washed with distilled water and weighed; 1 mL of trichloroacetic acid served as the control. Then, 9 mL of 0.1 M phosphate buffer (pH = 7.5) mixed with 3% propanol and 0.1 M KNO\(_3\) was added to the tubes, and the samples were vacuum infiltrated until the samples sunk to the bottom of the tubes. The reactions were performed at 30 °C in the dark for 30 min, and 1 mL of trichloroacetic acid was added to stop the reaction. After standing for 2 min, 4 mL of sulfanilamide mixed with 3 M HCl and 4 mL of 0.2% N-1-naphthyl-ethylene-diamine were added to the 2 mL of supernatant, which was transferred into a new tube. Finally, after 30 min, the absorbance was measured at 540 nm. Next, 0–2 g of NaNO\(_3\) per reaction was used to generate a standard curve. A regression equation was calculated according to the standard curve. NR activity is presented as
nanomoles of nitrite produced per hour per gram of FW (nmol nitrite h\(^{-1}\) g\(^{-1}\) FW). Measurements were performed in triplicate.

Measurement of nitrate content
Nitrate content was measured according to the salicylic acid method\(^{41,42}\). First, samples of ~1 g (apple calli) were frozen in liquid nitrogen and milled into powder. Next, 10 mL of deionized water was added to the tubes. The samples were boiled at 100 °C for 20 min and centrifuged at 15 000 \(g\) for 10 min, after which 0.1 mL of the supernatant was transferred to a new tube. Then, 0.4 mL of a 5% salicylic acid–sulfuric acid solution was then added to the tubes. The reactions occurred at room temperature for 20 min, after which 9.5 mL of 8% NaOH was slowly added to the reactions. After the samples cooled to room temperature, the optical density at 410 nm (OD\(_{410}\) value) was measured; deionized water served as the control. The nitrate content was calculated according to the following equation: 

\[
N = \frac{C \times V \times W}{N_{\text{KNO}_3}}
\]

Concentrations of 10–120 mg L\(^{-1}\) \(\text{KNO}_3\) were used to generate a standard curve. The regression equation

![Figure 1. Sequence alignment and phylogenetic analysis of MdHY5. (a) Protein alignment of MdHY5 and its homologs in Arabidopsis. The zinc-finger domain is boxed. (b) Phylogenetic analysis of MdHY5 and 35 other plants HY5 protein sequences obtained from the NCBI database. MdHY5 is denoted by the red asterisk, and the scale bar indicates the branch length. RsHY5: Raphanus sativus, XP_018445811.1; AtHY5: Arabidopsis thaliana, AT5G1260.1; bHY5: Brassica rapa, XP_009121971.1; ThHY5: Tarenaya hassleriana, XP_010541629.1; PeHY5: Populus euphratica, XP_011039711.1; MdHY5: Malus domestica, MDP0000586302; PbHY5: Pyrus bretschneideri, XP_009355719.1; FvHY5: Fragaria vesca, XP_004291469.1; ZjHY5: Ziziphus jujuba, XP_015885857.1; PmHY5: Prunus mume, XP_008219477.1; FaHY5: Fragraaria ananassa, AKGS58815.1; PpHY5: Prunus persica, ON134365.1; ChHY5: Citrus clementina, XP_006450470.1; BpHY5: Betula platyphylla, AHY20043.1; EgHY5: Eucalyptus grandis, XP_010048982.1; JcHY5: Jatropha curcas, XP_012076602.1; CaHY5: Camptotheca acuminata, APD29065.1; PpHY5: Populus trichocarpa, XP_002308656.1; CmHY5: Cucumis melo, NP_001284656.1; TcHY5: Theobroma cacao, XP_007013841.2; RcHY5: Ricinus communis, XP_002515537.1; VvHY5: Vitis vinifera, XP_010646848.1; MnHY5: Morus notabilis, XP_011010356.1; SiHY5: Sesamum indicum, XP_011081579.1; NnHY5: Nelumbo nucifera, XP_010250337.1; NcHy5: Nocoea caerulescens, JAUL18721.1; EsHY5: Extrema salugsineum, XP_006399627.1; StHY5: Solanum tuberosum, XP_006361723.1; MIHY5: Medicago truncatula, XP_013459310.1; SiHY5: Solanum lycopersicum, NP_001234820.1; PdHY5: Phoenix dactylifera, XP_008785002.1; AcHY5: Ananas comosus, XP_020097860.1; NaHY5: Nicotiana attenuate, XP_019265660.1; DcHY5: Daucus carota, XP_017229054.1; GsHY5: Gentiana rigescens, AIC64080.1; CsHY5: Camelina sativa, XP_010419684.1.](image)
was calculated according to the standard curve. Measurements were performed in triplicate.

Statistical analysis
Statistical analysis was conducted as previously described using appropriate methods and R (3.0.2) software with the R Commander package. Differences were considered statistically significant when \( P < 0.05 \) and \( P < 0.01 \). The results were analyzed in triplicate.

RESULTS

Identification of the \( HYS \) gene in apple

Regarding the identification of the \( HYS \) gene in the apple genome, the \( Arabidopsis \) \( HYS \) gene was used as a query to search similar sequences in apple by mining the NCBI database with the BLAST program. Two potential homologous genes were found: GenBank accession numbers MDP0000586302 and MDP0000264514, which differed by only one amino acid (Supplementary Figure S1). The gene MDP0000586302 was selected for functional identification. This gene contained a 495-bp open reading frame that encoded a protein containing 164 amino acids and was named \( MdHY5 \). In addition, the protein secondary structure of \( MdHY5 \) showed that it contained a bZIP domain on its C-terminal side (amino acids 90–141) (Figure 1a), which indicated the correlation between structure and function as observed in other bZIP proteins.

To analyze the phylogenetic relationship between \( MdHY5 \) and \( HYS \) proteins from other plant species, a phylogenetic tree of 36 plant \( HYS \) proteins was constructed using MEGA 5.0. Figure 1b shows that \( MdHY5 \) was most closely related to and exhibited the highest homology with \( PpHY5 \) and \( PmHY5 \) from \( Prunus persica \) and \( Prunus mume \), respectively.

Expression analysis of the \( MdHY5 \) gene

\( Arabidopsis \) \( HYS \) plays a vital role in light signaling and ABA responses.\(^{23,25} \) To investigate the functions of \( MdHY5 \) in planta, the expression patterns of \( MdHY5 \) were determined by RT-qPCR and semi-quantitative RT-PCR using cDNA isolated from apomictic crabapple seedlings treated with light or subjected to ABA. These results demonstrated that the expression levels of \( MdHY5 \) increased in response to light and ABA treatments. The expression level increased 1 h after light treatment and peaked at 3 h (Figure 2a). In addition, treatment of apple seedlings with ABA led to a peak in the expression of \( MdHY5 \) at 3–6 h (Figure 2b). Therefore, \( MdHY5 \) plays an important role in light signaling and ABA responses.

\( MdHY5 \) positively regulates its own transcription

\( HYS \) and its homolog \( HYH \) can regulate \( HYS \) gene induction in \( Arabidopsis \).\(^{44} \) To test whether \( MdHY5 \) could also directly interact with the \( MdHY5 \) promoter in vitro, we expressed and purified recombinant \( MdHY5 \)-GST fusion protein and prepared \( MdHY5 \) promoter fragments containing versions of the E-box motif. As predicted, EMSAs demonstrated that \( MdHY5 \) bound to the oligonucleotide sequence of the CAATTG-box of \( MdHY5 \) (Figures 3a and b).

To investigate whether \( MdHY5 \) could activate its own transcription, we carried out a transient assay to compare the activation effect of \( MdHY5 \) on the expression of \( MdHY5-pro-Luc \) and \( MdHY5-pro(Mut)-Luc \) reporters containing the \( MdHY5 \) promoter fragments or mutated \( MdHY5 \) promoter fragments fused with \( LUC \) genes. As shown in Figures 3c and d, co-expression of \( MdHY5-pro-Luc \) with 35S\(_{pro}:MdHY5 \) markedly increased the luminescence intensity. In contrast, 35S\(_{pro}:MdHY5 \) failed to induce the expression of \( MdHY5-pro(Mut)-Luc \). This result suggests that \( MdHY5 \) is also capable of positively regulating its own expression.

\( MdHY5 \) promotes anthocyanin accumulation by directly binding to the \( MdMYB10 \) promoter

\( Arabidopsis \) \( HYS \) promotes anthocyanin biosynthesis by inducing the expression of \( PAP1 \).\(^{27} \) To investigate this in apple, we investigated the DNA–protein interaction between \( MdHY5 \) and \( MdMYB10 \) promoter fragments containing G-boxes. As shown in Figure 4a, the upstream region of the \( MdMYB10 \) gene contained two \( MdHY5 \)-binding sites (G-box-1 and G-box-2). Interestingly, the EMSA assays showed that \( MdHY5 \) interacted with an \( MdMYB10 \) promoter fragment (G-box-2) (Figure 4b). In addition, the transient assay suggested that \( MdHY5 \) could induce the transcriptional activation of \( MdMYB10 \) in apple.

![Figure 2](image-url) Effects of light and ABA on the transcript level of \( MdHY5 \). (a) Apomictic crabapple (\( Malus hupehensis \)) seedlings grown in darkness for 3 days at room temperature were treated with continuous white light for 1, 3, 6, 9 and 12 h. The transcript levels of \( MdHY5 \) were determined using RT-qPCR (above) and RT-PCR (below); the value for dark-treated plants was set to 1. (b) Apomictic crabapple (\( M. hupehensis \)) seedlings were treated with 50 \( \mu \)M ABA at the indicated time intervals. The expression levels of \( MdHY5 \) were monitored by RT-qPCR (above) and RT-PCR (below); the value for untreated plants was set to 1.
To examine whether MdHY5 regulates anthocyanin accumulation, we transformed the overexpression construct MdHY5-pCAMBIA1300 into 'Orin' apple calli through Agrobacterium-mediated genetic transformation (Supplementary Figure S2). The observation of the appearance of the apple calli revealed that the MdHY5 overexpression calli (MdHY5-L1 and MdHY5-L2) appeared redder in color under low-temperature and high-light conditions than did the WT control (Figure 5a).
Spectrophotometric analysis demonstrated that the apple calli overexpressing *MdHY5* produced much higher contents of anthocyanins (Figure 5b). In addition, the expression levels of flavonoid structural genes in the WT and transgenic calli were analyzed by RT-qPCR. The overexpression of *MdHY5* significantly upregulated the expression of primary genes involved in the anthocyanin biosynthesis pathway, including *MdMYB10*, *MdDFR*, *MdUF3GT*, *MdF3H*, *MdCHI* and *MdCHS* (Figure 5c). This result suggests that *MdHY5* affects the accumulation of anthocyanins. Taken together, these results demonstrate that *MdHY5* binds to the G-box-2 site of the *MdMYB10* promoter to induce anthocyanin biosynthesis in apple.
Arabidopsis expression of the NR gene detected. As shown in Figure 6, MdHY5 positively regulated the contained potential MdHY5-binding sites. The expression levels of nitrogen signaling (Supplementary Figure S3 shows that four genes related to cis elements in the NR genes and nitrate uptake genes in apple. The possible target genes of MdHY5; we therefore investigated the in regulate nitrate uptake genes. To examine whether MdHY5 been previously reported in apple or Arabidopsis. MdCOP1: apple ubiquitin E3 ligase CONSTITUTIVE PHOTOMORPHOGENIC 1; Ubi: ubiquitination. (1) Li et al. and Maier et al. (2) Ang et al. (3) Takos et al. and Ban et al. MdHY5 is involved in nitrate assimilation Arabidopsis HY5 and HYH upregulate NR genes but negatively regulate nitrate uptake genes. To examine whether MdHY5 influenced nitrogen signaling in apple, we attempted to identify the possible target genes of MdHY5; we therefore investigated the cis elements in the NR genes and nitrate uptake genes in apple. Supplementary Figure S3 shows that four genes related to nitrogen signaling (MdNIA1, MdNRT1.1, MdNRT2.4 and MdNRT2.7) contained potential MdHY5-binding sites. The expression levels of a series of NR genes and nitrate uptake genes were subsequently detected. As shown in Figure 6, MdHY5 positively regulated the expression of the NR gene MdNIA2, which is in agreement with the observation in Arabidopsis. In contrast to the results for MdNIA2, MdHY5 negatively regulated the expression of the nitrate uptake gene MdNRT1.1 in apple. In addition, overexpression of MdHY5 promoted the expression of MdNRT2.1, MdNRT2.4 and MdNRT2.7, which might help coordinate the acquisition of plant carbon and nitrogen.

We also examined NR activity and nitrate content. As shown in Figure 7a, NR activities increased in MdHY5-overexpressing apple calli that were grown under low-temperature and high-light conditions, which indicates that MdHY5 might be a positive regulator of NR in apple. In addition, the nitrate contents of transgenic apple calli markedly increased (Figure 7b), which might be associated with increased demand for metabolites during plant growth.

DISCUSSION
The bZIP TF HY5 is a central regulator of plant growth and development and has been functionally characterized to be involved in multifaceted developmental processes such as cell elongation and proliferation, chloroplast development, photomorphogenesis, pigment accumulation, nutrient assimilation and carbon/nitrogen balance. Other HY5-mediated response pathways, such as hormone signaling, reactive oxygen species and defense signaling, as well as temperature responses have recently been uncovered. Among these responses, pigment accumulation and nutrient assimilation are economically important characteristics that influence apple quality and yield.

Here we cloned and isolated a gene from the apple genome that contains a highly conserved bZIP motif in its C terminus and is highly homologous to HY5 proteins from other species; this finding indicated that MdHY5 might have similar functions as other HY5 proteins. As we predicted, our molecular and genetic analyses implied that MdHY5 was highly similar in structure and function to Arabidopsis HY5 in terms of the conserved bZIP domain (Figure 1) and response to light and ABA signaling (Figure 2). In addition, EMSA assays showed that MdHY5 could bind to the E-box motif of its promoter (Figure 3), which suggests a potential autoregulatory loop for MdHY5 transcription. These results indicate that the functions of MdHY5 are conserved across different species.

To further investigate the functions of MdHY5 in planta, we used Agrobacterium-mediated transformation to obtain transgenic apple calli overexpressing MdHY5 under the control of the 35S promoter (Supplementary Figure S2). HY5 is a positive regulator of flavonoid biosynthesis by modulating the expression of MYB75/PAP1 in Arabidopsis. We also investigated the role of MdHY5 in the regulation of flavonoid biosynthesis on the basis of EMSA-binding assays and apple calli coloration detection. As hypothesized, the EMSA results showed that MdHY5 was able to bind the G-box site of the MdMYB10 promoter (Figure 4). Furthermore, the MdHY5-overexpressing apple calli produced much higher amounts of anthocyanins than did the WT control, and the RT-qPCR analysis indicated that anthocyanin biosynthesis genes, including MdDFR, MdUF3GT, MdF3H, MdCHI and MdCHS, were clearly upregulated in the MdHY5 transgenic lines (Figure 5). These results suggest that MdHY5 promotes anthocyanin accumulation by directly binding to the MdMYB10 promoter.

In addition, HY5 positively regulates NR activity and coordinates plant carbon and nitrogen acquisition by affecting the expression of both NR genes and nitrate uptake genes in Arabidopsis. To determine whether MdHY5 is also required for nitrogen
assimilation in apple, we measured the transcripts of a series of NR genes and nitrate uptake genes in transgenic and WT apple calli. MdHY5 positively regulated the expression of MdNIA2, MdNRT2.1, MdNRT2.4 and MdNRT2.7 but negatively regulated the expression of MdNRT1.1, which was in agreement with observations in Arabidopsis (Figure 6). In addition, the upstream region of the MdNIA1 gene may contain a putative MdHY5-binding site (G-box) (Supplementary Figure S3), and overexpression of MdHY5 slightly induced the transcription of MdNIA1, despite AtHY5 having no impact on the expression of MdNIA1.28 The function of MdHY5 might be distinct from that of Arabidopsis HYS, or the function of MdHY5 might be a secondary metabolic effect caused by the upregulation of MdNIA2. In addition, the increased NR activities and nitrate contents in MdHY5-overexpressing apple calli might be associated with the increased demand for metabolites during plant growth (Figure 7). Therefore, MdHY5 may play a vital role in nitrogen assimilation in apple. However, these results are not sufficient and require further investigation.

On the basis of our results and previous studies in model plant species, we propose a hypothetical working model for MdHY5-regulated anthocyanin accumulation and nitrate assimilation (Figure 8). MdHY5 is released from MdCOP1-mediated degradation after detection of light signaling. MdHY5 then directly binds to the G-box site of the MdMYB10 promoter to induce its expression to regulate anthocyanin biosynthesis. In addition, MdHY5 may also be involved in nitrate assimilation by regulating the transcription of both NR genes and nitrate uptake genes. At the same time, MdHY5 induces its own expression in all light conditions by directly binding to the E-box site of its promoter. Improved comprehension of MdHY5 function and signaling in apple could be highly useful for improving fruit traits, such as pigment accumulation and nitrogen use efficiency, for producing high-quality fruits.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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