

The molecular mechanism underlying anthocyanin metabolism in apple using the MdMYB16 and MdbHLH33 genes

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Abstract

Key message MdMYB16 forms homodimers and directly inhibits anthocyanin synthesis via its C-terminal EAR repressor. It weakened the inhibitory effect of MdMYB16 on anthocyanin synthesis when overexpressing MdbHLH33 in callus overexpressing MdMYB16. MdMYB16 could interact with MdbHLH33.

Abstract Anthocyanins are strong antioxidants that play a key role in the prevention of cardiovascular disease, cancer, and diabetes. The germplasm of *Malus sieversii* f. *neidzwetzkyana* is important for the study of anthocyanin metabolism. To date, only limited studies have examined the negative regulatory mechanisms underlying anthocyanin synthesis in apple. Here, we analyzed the relationship between anthocyanin levels and *MdMYB16* expression in mature Red Crisp 1–5 apple (*M. domestica*) fruit, generated an evolutionary tree, and identified an EAR suppression sequence and a bHLH binding motif of the *MdMYB16* protein using protein sequence analyses. Overexpression of *MdMYB16* or *MdMYB16* without bHLH binding sequence (LBS*MdMYB16*) in red-fleshed callus inhibited *MdUFGT* and *MdANS* expression and anthocyanin synthesis. However, overexpression of *MdMYB16* without the EAR sequence (LES*MdMYB16*) in red-fleshed callus had

no inhibitory effect on anthocyanin. The yeast one-hybrid assay showed that MdMYB16 and LESMdMYB16 interacted the promoters of *MdANS* and *MdUFGT*, respectively. Yeast two-hybrid, pull-down, and bimolecular fluorescence complementation assays showed that MdMYB16 formed homodimers and interacted with MdbHLH33, however, the LBSMdMYB16 could not interact with MdbHLH33. We overexpressed *MdbHLH33* in callus overexpressing *MdMYB16* and found that it weakened the inhibitory effect of *MdMYB16* on anthocyanin synthesis. Together, these results suggested that MdMYB16 and MdbHLH33 may be important part of the regulatory network controlling the anthocyanin biosynthetic pathway.

Keywords *Malus sieversii* f. *neidzwetzkyana* · First-filial generation · Anthocyanin · *MdMYB16* · *MdbHLH33* · EAR suppression sequence · Homodimers

Abbreviations

CHS	Chalone synthase
FLS	Flavonol synthase
DFR	Dihydroflavonol reductase
C4H	Cinnamate 4-hydroxylase
ANS	Anthocyanin synthase
UFGT	UDP-glucose:flavonoid 3- glucosyltransferase
EAR	ERF-associated amphiphilic repression
GFP	Green fluorescent protein
GST	Glutathione S-transferase
His	Histidine
Anti	Antibody
MS	Murashige and Skoog
-T(-Trp)	No tryptophan
-L(-Leu)	No leucine
-H(-His)	No histidine
-A(-Ade)	No adenine

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AD	Activation domain
BD	Binding domain
CDS	Coding DNA sequence

Introduction

Flavonoid compounds occur widely in most plant species. In addition to their roles in coloring plant matter, acting as signal substances between plants and microbes, and as plant defensins, they also have anti-bacterial, anti-inflammatory, and anti-oxidation effects. Flavonoids have been shown to have a significant effect on the treatment of cardiovascular disease (Cook & Samman et al. 1996; Hollman, Katan et al. 1997; Boyer, Liu et al. 2004; Schaefer et al. 2008; Sun et al. 2013). Anthocyanin synthesis is a branch of the flavonoid biosynthetic pathway that is related to coloration in a variety of plant tissues and organs. Anthocyanin synthesis, which is induced by UV radiation, drought, cold, jasmonic acid, sugar, low nitrogen, and other environmental signals, involves multiple structural genes and transcription factors (Koes et al. 2005; Solfanelli et al. 2006; Bai et al. 2014; An et al. 2014).

The structural genes of the plant flavonoid biosynthetic pathway have been identified and include *CHS*, *FLS*, *UFGT*, and *ANS* among others (Chikako et al. 2002), with *ANS* and *UFGT* playing major roles in anthocyanin synthesis (Kondo et al. 2002; Kim et al. 2006). Anthocyanin biosynthesis is transcriptionally regulated and coordinately modulated by a conserved MBW regulatory complex. The role of MYB and bHLH family members in anthocyanin synthesis has been extensively studied in plant models (Koes et al. 2005; Ballester et al. 2010; Albert et al. 2011). Recently, significant research into fruit anthocyanins has also been undertaken. In grapevine, *VvMYBA1* and *VvMYBA2*, which are homologs of *Arabidopsis thaliana* *AtMYB75*, *AtMYB90*, and *AtMYB144*, have been found to induce anthocyanin biosynthesis (Azuma et al. 2008). Additionally, homologs of these *Arabidopsis* TFs have been detected in peach, strawberry, and pear, including *PpMYB10*, *FaMYB10*, and *PyMYB10*, respectively (Wang et al. 2010; Feng et al. 2010). In apple, Takos et al. (2006) and Ban et al. (2007) found that *MdMYB1* and *MdMYBA* were induced by light and were involved in regulating anthocyanin synthesis. Espley et al. (2007, 2009) determined that *MdMYB10* regulates flesh anthocyanin synthesis and that its promoter had self-activating activity. Bai et al. (2014) determined that UV-B-induced *MdCOL11* expression, thereby promoting anthocyanin synthesis (Bai et al. 2014). Taken together, these studies are indicative of the positive regulatory role of anthocyanin. This positive role, combined with the discovery of the EAR repressor, offers

new perspectives into the negative regulation of anthocyanin biosynthesis.

The EAR transcriptional repressor plays an important role in regulating plant defenses and abiotic stress responses (Hiratsu et al. 2002; Kazan et al. 2006; Ciftci-Yilmaz et al. 2007). In addition to ethylene response factors (ERFs) and auxin response factors, other transcription factor family members, including MYB, ABI3/VP1, and MADS, all contain an EAR motif and have active suppressor functions (Preston et al. 2004; Tsukagoshi et al. 2007; Hill et al. 2008). *AtMYB4*, which was the first negative regulator identified in the Arabidopsis R2R3 class, was found to have a C-terminus pdLNL^D/_E Lxi^G/_S inhibition zone that inhibited the transcription of target genes through direct binding (Jin et al. 2000; Hemm et al. 2001). *PhMYB4* was found that it has the similar function with *AtMYB4*, and it could repress the expression of *C4H*, indirectly controlling the balance of floral volatile benzenoid/phenylpropanoid (FVBP) production (Colquhoun et al. 2011). *ZmMYB42* and *CmMYB1* also have similar EAR suppression sequences, they inhibit the synthesis of phenylpropanoid and flavonoid, respectively. However, the functional data of *ZmMYB42* and *CmMYB1* are still lacking (Sonbol et al. 2009; Zhu et al. 2013).

The first anthocyanin biosynthesis-associated R2R3-MYB inhibitor to be discovered was *FaMYB1*. When *FaMYB1* protein was heterologously expressed in tobacco it was found to inhibit anthocyanin accumulation in tobacco petals, and it also had the EAR at its C-Terminus (Aharoni et al. 2001). A petunia homologue, *PhMYB27* was shown to act upon MBW complexes to repress anthocyanin synthesis (Albert et al. 2014) and similar modes of action had also been demonstrated for *FaMYB1*, *VvMYBC2* and *MtMYB2* (Paolucci et al. 2011; Jun et al. 2015; Cavallini et al. 2015). So these R2R3-MYB repressors that act upon MBW complexes differs from the R2R3-MYB repressors that directly bind the target genes (e.g., *AtMYB4*). *MdMYB16* also had the EAR at its C-Terminus, and it co-transfected into tobacco with transcriptional activators *MdMYB10* and *MdbHLH3* could inhibit the leaf anthocyanin (Lin-Wang et al. 2011). However, the mechanism of inhibiting anthocyanin by *MdMYB16* and the type of *MdMYB16* belonging to *AtMYB4*-like or *FaMYB1*-like are not clear recently.

World-wide, cultivated apple (*Malus domestica*) is derived from the ancestral species *M. sieversii* and its red-fleshed variant (*M. sieversii* f. *niedzwetzkyana*). The rich genetic diversity contained in the germplasm of these varieties, therefore, represents an important resource for quality apple breeding (Chen et al. 2007; Velasco et al. 2010; Nocker et al. 2012). *M. sieversii* resources have, however, been severely damaged and are currently endangered. For this reason, our research group, while conducting research studies on genetic diversity in 2006, built hybrid

segregating populations of “Fuji” × *Malus sieversii* f. *niedzwetzkyana*. Furthermore, because of the high levels of anthocyanin and other healthy ingredients in *M. sieversii* f. *niedzwetzkyana*, the “functional apple” concept was proposed in 2014 (Chen et al. 2014). The hybrid offspring of *Malus sieversii* f. *niedzwetzkyana* have clearly separated red-fleshed, pink-fleshed, and white-fleshed traits. The research team conducted RNA-seq analysis of segregating populations with different flesh colors and screened differentially expressed genes associated with anthocyanin biosynthesis (Wang et al. 2015). However, the factors contributing to the differences in flesh coloration remained unclear. Characterizing this mechanism further may help to improve anthocyanin metabolism and provide a theoretical basis for functional apple breeding.

In the present study, we analyzed the expression of *MdMYB16* in fruit with different flesh colors, cloned *MdMYB16* of Red Crisp 1 apple and expressed it in prokaryotes, performed a phylogenetic analysis of 26 MYB transcription factors that were related to flavonoid biosynthesis, overexpressed *MdMYB16* and *MdbHLH33* in red callus, and verified the relationship of interaction between *MdMYB16* and *MdbHLH33* using yeast two-hybrid, pull-down, and bimolecular fluorescence complementation (BiFC) experiments. The results showed that *MdMYB16* plays an inhibitory role through its own EAR inhibition sequence and forms homodimers. And *MdMYB16* could interact with *MdbHLH33*.

Materials and methods

Plant materials and processing

In this study, we used plants of the apple strains Red Crisp 1–5 and Purple 3 that were first-generation hybrids bred from *Malus sieversii* f. *niedzwetzkyana* and *M. domestica* cv. Fuji. The Red Crisp 1–5 apples were picked from 5 different F1 hybrids of *Malus sieversii* f. *niedzwetzkyana*. The using materials were peel-out flesh during mature period. The flowering and maturation times of the six lines were basically the same. Red Crisp 1–5 were sampled at 125 days after flowering. We depended on firmness to measure the fruit maturity. Based on former results, the firmness of Red Crisp 1–5 apples was approximately 0.78 kg. The unit of firmness was kg measured by machine named TA.XT plus texture instrument (Stable Micro Systems). The samples were immediately frozen in liquid nitrogen and stored at -80°C until use. Red-fleshed calli used in the experiments were induced from young leaves of the superior strain Purple 3, according to Ji et al. (2015). The callus was cultured in MS + 1 mg/L 6-Benzylaminopurine + 0.3 mg/L 1-naphthylacetic acid.

Anthocyanin extraction and absorbance measurements

Anthocyanin extraction was performed according to Jin et al. (2000) with minor modifications. Approximately 0.5 g of plant material was ground into a powder in liquid nitrogen and subjected to extraction in 20 mL of 1% (v/v) HCl-methanol at 4°C in the dark for 24 h. After centrifugation at 12,000 rpm for 10 min, the absorbance of the supernatant was measured at 530 nm using a UV spectrophotometer.

Total RNA extraction and qRT-PCR

The plant RNA extraction kit, reverse transcription kit, and SYBR dyes were purchased from CICC Whole Formula (Beijing, China). The Bole CFX96 (Bio-Rad) system was used for PCR and the SYBR[®] Green system for real-time PCR according to the manufacturer’s instructions. Three replicates were prepared for each sample. Each 20- μL reaction consisted of 10 μL 2.5×Real Master Mix/20×SYBR Solution, 1 μL cDNA ($50\text{ ng } \mu\text{L}^{-1}$), 1 μL each of stream and downstream primers ($5\text{ } \mu\text{mol L}^{-1}$), and 7 μL of ddH₂O. The reaction consisted of an initial denaturation at 95°C for 30 s; followed by 45 cycles of denaturation at 95°C for 5 s, annealing at 58°C for 10 s, and extension at 72.0°C for 30 s; then incubation at 65°C for 20 s, dissolution from 55 to 95°C , increasing by 0.5°C/s ; and, finally, termination of the reaction. *MdActin* was used as the internal control and was amplified simultaneously for each gene amplification. Ct values were read under default conditions and the $2^{-\Delta\Delta\text{CT}}$ method was used for data analysis (Kenneth et al. 2001).

MdMYB16 cloning, bioinformatic analyses

MdMYB16 (HM122617.1) CDS was amplified by PCR, products were separated by 1% agarose gel electrophoresis, and target bands were recovered. The isolated fragments were then connected to a PLB zero background vector (VT205) and sent to the Sangon Company (Shanghai, China) for sequencing. Phylogenetic analysis of related MYB transcription factors was conducted using MEGA5.0. The protein sequences of related MYB transcriptional repressors were compared using Clustal X.

Knockout of *MdMYB16* inhibition sequence and bHLH binding sequence by overlap PCR

Using the overlap PCR technique, the EAR suppression sequence of *MdMYB16* (PDLNLDLQIS) was removed. A detailed description of the specific steps is included in Supplementary Fig. S1. The primers were designed as follows:

F1: 5'-ATGGGAAGATCTCCTTGCTG-3'.

R1: 5'-TGGCAGGGAGGGCACCTTTCCTGA
ACTGG-3'.

F2: 5'-AAAGGTGCCCTCCCTGCCAGCCTCA-3'.

R2: 5'-TCATTTTCATCTCCAAGCTTCTG-3'.

Using the F1 and R1 primers the *MdMYB16-1* fragment was amplified by PCR. Using the F2 and R2 primers, fragment *MdMYB16-2* was amplified. Equal amounts of *MdMYB16-1* and *MdMYB16-2* were then mixed and used as a template for amplification with the F1 and R2 primers. The amplified fragment was identified as *MdMYB16* in which the EAR suppression sequence was removed and was, therefore, named lost EAR sequence of *MdMYB16* (LESMdMYB16).

Using the above method, the bHLH binding sequence of *MdMYB16* (LIKLHSLLG) was removed. The primers were designed as follows:

F1: 5'-ATGGGAAGATCTCCTTGCTG-3'.

R1: 5'-CATTTGTTCTCATCTTCTTCTTCA
GTGAA-3'.

F2: 5'-AGAAGATGAGAACAAATGGTCTTTGAT
AGCTG-3'.

R2: 5'-TCATTTTCATCTCCAAGCTTCTG-3'.

The amplified fragment was identified as *MdMYB16* in which the bHLH binding sequence was removed and was, therefore, named lost bHLH binding sequence of *MdMYB16* (LBSMdMYB16).

Red-fleshed callus transformation

The intact, LESMdMYB16 and LBSMdMYB16 CDS were ligated into the pRI101 vector containing the 35 S promoter and a GFP tag sequence to construct the 35S:*MdMYB16*-GFP, 35S:LESMdMYB16-GFP and 35S:LBSMdMYB16-GFP recombinant vectors, respectively (described in Supplementary Fig. S2a). The intact CDS of *MdbHLH33* was transformed into the pCAMBIA1301 vector that also contains a 35S promoter and GFP tag sequence to generate the 35S:*MdbHLH33*-GFP recombinant vector (described in Supplementary Fig. S2b). The pRI101 vector confers kanamycin resistance in both prokaryotic and eukaryotic cells, and the pCAMBIA1301 vector confers kanamycin resistance in prokaryotic cells and hygromycin resistance in eukaryotic cells. The recombinant vector was transformed into *Agrobacterium tumefaciens* LBA4404. For transformation of the red callus, two-week-old calli grown in liquid media were co-cultured with *A. tumefaciens* LBA4404 carrying the 35S:*MdMYB16*-GFP or 35S:LESMdMYB16-GFP or 35S:LBSMdMYB16-GFP vectors. The calli were co-cultured on MS media containing 0.3 mg/L 1-naphthylacetic acid, 1 mg/L 6-Benzylaminopurine, and 7 g/L agar at 25 °C in the dark for 2 d. Subsequently, the callus was transferred onto MS media supplemented with 250 mg/L carbenicillin and 50 mg/L kanamycin for transgene selection. To

obtain co-transfected *MdMYB16*+*MdbHLH33* or LBSM-*dMYB16*+*MdbHLH33* callus, the same method was used, but the callus was transferred to MS media supplemented with 250 mg/L carbenicillin, 50 mg/L kanamycin, and 20 mg/L hygromycin for transgene selection.

Yeast one-hybrid (Y1H) analysis

Y1H assays were performed using yeast strain Y187 (Clontech) according to the manufacturers' instructions. The *MdMYB16* and LESMdMYB16 genes were cloned individually into the pGADT7 vector (described in Supplementary Fig. S2c). The promoters of *MdANS* and *MdUFGT* were inserted individually into the pHIS2 vector (described in Supplementary Fig. S2d). Different combinations were co-transformed into yeast Y187 and the interactions were examined on media lacking Trp, Leu, and His (SD/-Trp-Leu-His) with an optimal concentration of 3-AT.

Yeast two-hybrid (Y2H) analysis

The intact *MdMYB16* and LBSMdMYB16 CDS were ligated into the pGBKT7 vector to construct the recombinant plasmids MdMYB16-BD and LBSMYB16-BD (described in Supplementary Fig. S2e). The intact CDS of *MdbHLH33* and *MdMYB16* was ligated into the pGADT7 vector to generate the MdbHLH33-AD and MdMYB16-AD recombinant plasmids (described in Supplementary Fig. S2c). These two recombinant plasmids were transformed into Y2H cells according to the instructions of the Yeastmaker™ Yeast Transformation System 2 Kit (Clontech). Initially, cells were cultured in selective media lacking Leu and Trp (-Leu/Trp, Clontech), and putative transformants were transferred to media lacking adenine (Ade), His, Leu, and Trp (-Ade/-His/-Leu/-Trp, Clontech). The substrate X- α -gal was added to media lacking four amino acids (-Ade/-His/-Leu/-Trp) for the detection of β -galactosidase activity.

BiFC assay

The intact *MdMYB16* CDS was ligated into a pSPYNE vector containing the 35S promoter and an nYFP tag sequence and a pSPYCE vector containing the 35S promoter and a cYFP tag sequence to generate the 35S:MdMYB16-NYFP and 35S:MdMYB16-CYFP recombinant plasmids, respectively (described in Supplementary Fig. S2f, g). The *MdbHLH33* CDS was ligated into a pSPYCE vector to construct the 35S:MdbHLH33-CYFP recombinant plasmid (described in Supplementary Fig. S2g). The recombinant plasmids were transformed into *A. tumefaciens* LBA4404. Approximately 30 mL of YEP+Kan (50 μ g/mL)+Rif (50 μ g/ml) culture media containing bacteria with different

recombinant plasmids were cultured to an OD₆₀₀ of 0.6. For each bacterial culture, a 15 mL aliquot was collected and added to centrifuge tubes, the cells were collected by centrifugation, and were then resuspended in 30 mL sterile distilled water supplemented with 100 µmol/L acetosyringone. Onion skin was immersed in the infection solution and cultured for 25–30 min. Upon removal, excess bacteria were absorbed with filter paper and the onion skin was cultured on MS solid medium at 28 °C in the dark for 1 to 2 days. The onion cells were later examined for YFP fluorescence signals using confocal microscopy.

Pull-down assays

The intact CDS of *MdMYB16* was ligated into the pET32a and PGEX-4T-1 vectors while the intact CDS of *MdbHLH33* was ligated into the PGEX-4T-1 vector (described in Supplementary Fig. S2h, i). The recombinant vectors were expressed in *E. coli* BL21 (DE3) to form the MdMYB16-HIS, MdMYB16-GST, and MdbHLH33-GST fusion proteins. The different combinations of mixed proteins were column purified using the His tag before the purified mixed-proteins were detected by western blotting with anti-HIS or anti-GST antibodies (Abmart).

Data analysis

We picked 20 fruits from every strain randomly and mixed them up to freeze samples. All the results were based on the average of three parallel experiments. The statistical analysis was performed with appropriate methods using Duncan's new multiple range test. The significance tests were shown as i, ii, iii, iv. Completely different lowercase roman alphabet on the column chart indicated significant differences ($P < 0.05$).

Results

Fruit anthocyanin levels and associated transcription factors expression differed in the mature first-generation hybrid fruit of *Malus sieversii* f. *neidzwetzkyana*

For the superior Red Crisp 1–5 first-generation *Malus sieversii* f. *neidzwetzkyana* hybrid strains flesh coloration and anthocyanin level differed significantly in mature fruit (Fig. 1a, c). The promoter region for *MdMYB10* was amplified from five different cultivars. The agarose gel electrophoresis showed that their *MdMYB10* promoters divided into two bands, 'a' and 'b' (Fig. 1b). Sequencing of 'a' and 'b' showed that the 'a' band had six repeat sequences

of 23 bp, while the 'b' band had one (Fig. 1d). This indicated that the *MdMYB10* promoters of Red Crisp 1–5 were all the R6R1 type. *MdMYB10* expression in the Red Crisp 1 apples was higher than in Red Crisp 3 and 4, while the anthocyanin content in Red Crisp 1 was significantly lower than in Red Crisp 3 and 4 (Fig. 1c, e). However, in Red Crisp 1–5 apples, higher *MdMYB16* expression was associated with lower anthocyanin levels and *MdbHLH33* expression (Fig. 1c, e), suggesting that MdMYB16 and MdbHLH33 might involved of the anthocyanin biosynthesis in addition to *MdMYB10*.

Phylogenetic tree and sequence analyses

The results of phylogenetic analyses of related MYB transporters in the synthesis of different flavonoids is shown in Fig. 2a. Region 1 included related MYB transcription factors that promote anthocyanin biosynthesis (Liu et al. 2013). Region 2 was composed of related MYB transcription factors that promote procyanidin synthesis (An et al. 2014). Region 4 contained the MYB transcription factors that promote flavonol synthesis (Czemmel et al. 2009). Region 3 and 5 consisted of related MYB transcription factors that has the C2 repressor motif (Xu et al. 2014a, b; Zhu et al. 2013; Cavallini et al. 2015; Albert et al. 2014; Jun et al. 2015). However, region 3 and region 5 were separation, it indicated that these MYB repressors in the two regions are different. The region 3 might be *FaMYB1-like*, while the region 5 might be *AtMYB4-like*. *MdMYB16* is within the *AtMYB4* clade. Analysis of the protein sequence characteristics of *MdMYB16* showed that *MdMYB16* has the bHLH binding motif and EAR inhibition sequence.

MdMYB16, LESMdMYB16, and MdbHLH33 participate in anthocyanin biosynthesis pathways

Overexpression of *MdMYB16* in the red-fleshed callus resulted in a change in callus coloration from red to yellow (Fig. 3a), a decrease in the expression of *MdUFGT* and *MdANS* and anthocyanin content (Fig. 3b, d). The red-fleshed callus overexpressing *LESMdMYB16* remained red (Fig. 3a) despite a significant increase in the expression of *MdMYB16* (Fig. 3d). In this callus, the expression levels of *MdUFGT* and *MdANS*, as well as the anthocyanin levels, remained essentially the same (Fig. 3b, d). Overexpressing *MdbHLH33* in callus overexpressing *MdMYB16* resulted in a coloration change from yellow to pink (Fig. 3a). In this callus, the expression levels of *MdUFGT* and *MdANS* and anthocyanin content was somewhat recovered (Fig. 3b, d). The results of western blotting are shown in Fig. 3c. The use of Anti-Actin showed that all calli had bands. Detection using

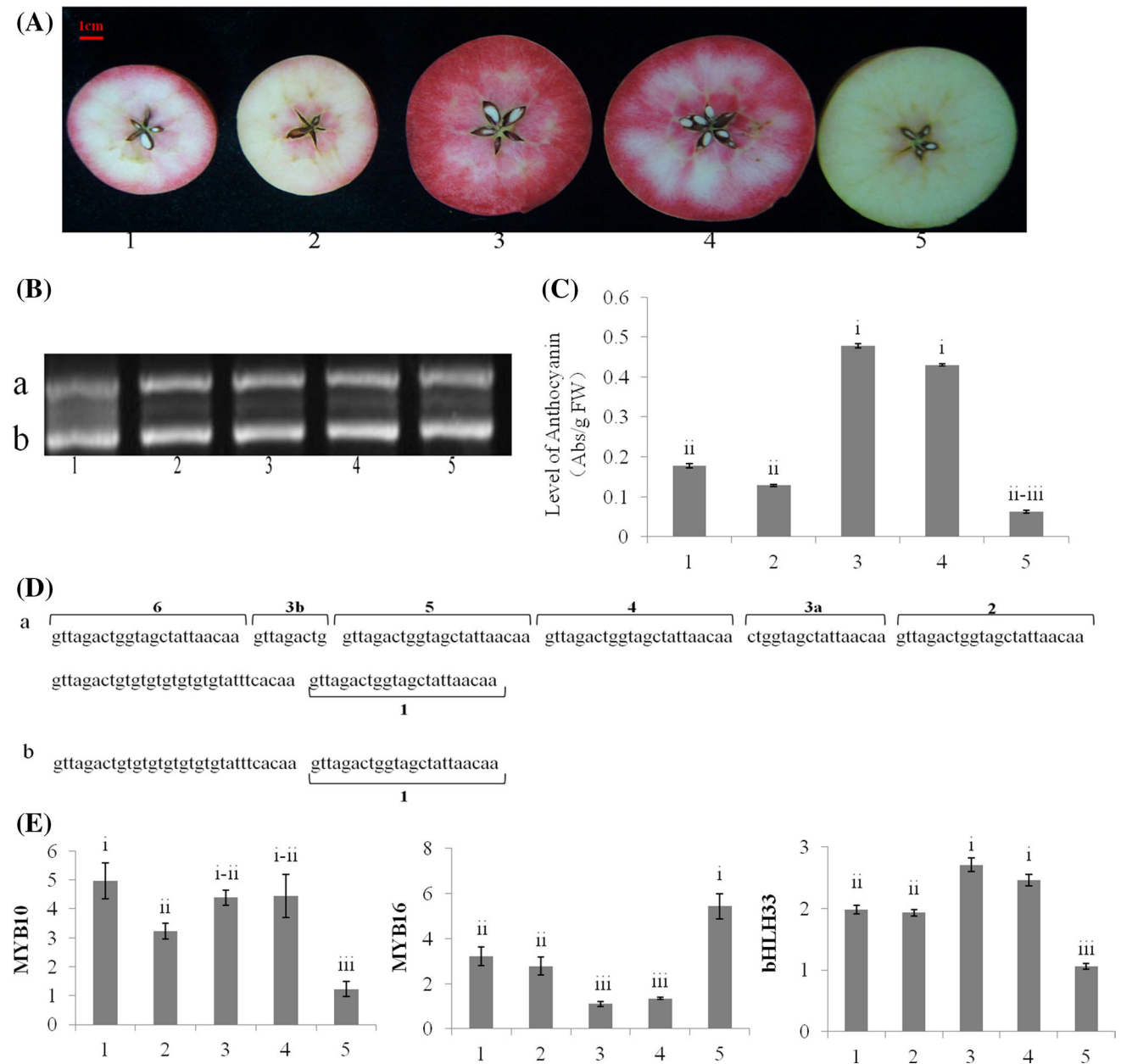


Fig. 1 Analysis of *MdMYB10* promoter type, anthocyanin level, and associated transcription factor expression in the first-generation hybrid fruit of *Malus sieversii* f. *niedzwetzkyana*. The numbers 1, 2, 3, 4, and 5 in panels (a–c, e) represent the first-generation hybrid fruit, Red Crisp 1, 2, 3, 4, and 5. The significance tests are shown as i, ii, iii, iv. Completely different lowercase roman alphabet on the column

chart indicates significant differences ($P < 0.01$). (a) Cross-sectional view of the mature fruit. (b) The promoter region for *MdMYB10* was amplified from 5 different cultivars. (c) Anthocyanin level in mature fruit. (d) *MdMYB10* promoter R6 and R1 sequence of mature fruit. (e) The relative expression of transcription factors *MdMYB10* and *MdMYB16* in mature fruit

anti-GFP showed that wild-type red-fleshed callus had no band. Callus overexpressing both *MdMYB16* and *LESM-dMYB16* showed one band while callus simultaneously overexpressing both *MdMYB16* and *MdbHLLH33* showed two bands.

Y1H assays using the *MdANS* and *MdUFGT* promoters

To analyze the target DNA sequence of *MdMYB16* using Y1H the 1692 bp *MdANS* and 1725 bp *MdUFGT* promoters were chosen (Fig. 4a). The *PHIS2-MdANSPro* and the

pHIS2-*MdUFGTPro* vectors were respectively transformed into Y187 yeast strains that were cultivated in -T-H selective media containing different concentrations of 3-AT. Reduced yeast growth was observed with increasing 3-AT concentration, and yeast could not grow on -T-H selective media with 80 mM 3-AT (Fig. 4b). This suggested that 80 mM 3-AT could inhibit the expression of the reporter gene *HIS3* under the control of the *MdANS* or *MdUFGT* promoters. Different combinations of pGADT7-*MdMYB16* and pGADT7-*LESMdMYB16* with pHIS2-*MdANSPro* and pHIS2-*MdUFGTPro* were respectively co-transformed into Y187 yeast strains and were successfully grown on -T-H-L selective media containing 80 mM 3-AT. Combinations of the untransformed pGADT7 control vector with pHIS2-*MdANSPro* and pHIS2-*MdUFGTPro* did not grow on this media (Fig. 4c). This suggests that *MdMYB16* and *LESMdMYB16* can interact with the promoters of *MdANS* and *MdUFGT*.

MdMYB16 is capable of forming homodimers

The results of the BiFc analysis are shown in Fig. 5a. 35S:*MdMYB16-NYFP* and 35S:*MdMYB16-CYFP* were co-transformed into onion skin, and YFP fluorescence was observed by laser scanning confocal microscopy. Conversely, when 35S:*NYFP* was combined with 35S:*MdMYB16-CYFP* or 35S:*MdMYB16-NYFP* with 35S:*CYFP* fluorescence was not observed. The results of the Y2H assays are shown in Fig. 5b. *MdMYB16-BD* and *MdMYB16-AD* were co-transformed into Y2H yeast strains that grew on -T-L and -T-L-H-A selective media containing the substrate X- α -gal. Yeast co-transformed with *MdMYB16-BD* and the AD or *MdMYB16-AD* and the BD grew on -T-L selective media but not on -T-L-H-A selective media. The results of the His pull-down analysis are shown in Fig. 5c. *MdMYB16-HIS*, *MdMYB16-GST* with *MdMYB16-HIS*, and GST were purified from the columns using HIS tags. Western blotting determined that as *MdMYB16-GST* could be detected by Anti-GST, *MdMYB16-GST* could be pulled down by *MdMYB16-His*, whereas since GST could not be detected by Anti-GST, GST could not be pulled down by *MdMYB16-His*. In summary, *MdMYB16* is capable of forming homodimers both in vivo and in vitro.

MdbHLH33 interacts with MdMYB16

The results of the BiFc analysis are shown in Fig. 6a. 35S:*MdMYB16-NYFP* and 35S:*MdbHLH33-CYFP* were co-transformed into onion skin, and YFP fluorescence was observed by laser scanning confocal microscopy. Conversely, when 35S:*NYFP* was combined with 35S:*MdbHLH33-CYFP* and 35S:*MdMYB16-NYFP* with

35S:*CYFP* no fluorescence was observed. The results of the Y2H assays are shown in Fig. 6b. *MdMYB16-BD* and *MdbHLH33-AD* were co-transformed into Y2H yeast strains that grew successfully on -TLHA and -TL selective media provided with the substrate X- α -gal. Conversely, yeast co-transformed with *MdMYB16-BD* and the AD or *MdbHLH33-AD* and the BD grew on -T-L selective media but not -T-L-H-A selective media. The results of the His pull-down analysis are shown in Fig. 6c. *MdMYB16-His*, *MdbHLH33-GST* with *MdMYB16-His*, and GST were purified from the columns using HIS tags. Western blot analysis indicated that as *MdbHLH33-GST* could be detected by Anti-GST, *MdbHLH33-GST* could be pulled down by *MdMYB16-His*, whereas since GST could not be detected by Anti-GST, GST could not be pulled down by *MdMYB16-His*. In summary, *MdbHLH33* is capable of interacting with *MdMYB16* both in vivo and in vitro. We, therefore, propose that *MdbHLH33* competitively inhibits the formation of *MdMYB16* homodimers or affects the stability of these *MdMYB16* homodimers.

LBSMdMYB16 and MdbHLH33 participate in anthocyanin biosynthesis pathways

The callus overexpressing *LBSMdMYB16* had the similar inhibitory effect on anthocyanin synthesis with the callus overexpressing *MdMYB16* (Fig. 7a, b, d). Overexpressing *MdbHLH33* in callus overexpressing *LBSMdMYB16* resulted in a coloration change from yellow to light pink (Fig. 7a). However, in this callus, the expression levels of *MdUFGT* and *MdANS* and anthocyanin content was lower than those in the callus simultaneously overexpressing *MdMYB16* and *MdbHLH33* (Fig. 7a, c, d). The results of the Y2H assays are shown in Fig. 7b, the *LBSMdMYB16* could not interact with the *MdbHLH33*. These results indicated that *MdMYB16* without bHLH binding sequence could not influence the inhibitory effect of itself on anthocyanin synthesis, however, it could influence the interaction between *MdMYB16* and *MdbHLH33*. Further analysis showed that although *MdbHLH33* was known as a activator of anthocyanin synthesis, it was the interaction between *MdbHLH33* and *MdMYB16* that influenced the inhibitory effect of *MdMYB16* on anthocyanin synthesis.

Discussion

Anthocyanin biosynthesis was regulated by MBW complexes, among which MYB proteins that include activators and repressors were widely studied. The current research shows that MYB repressors may have two types, one acted upon MBW complexes (e.g., Fa-MYB1; Aharoni et al. 2001; Paolocci et al. 2011), the other directly binded the

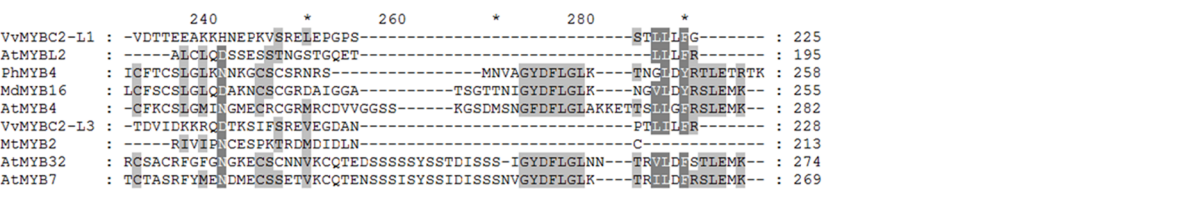
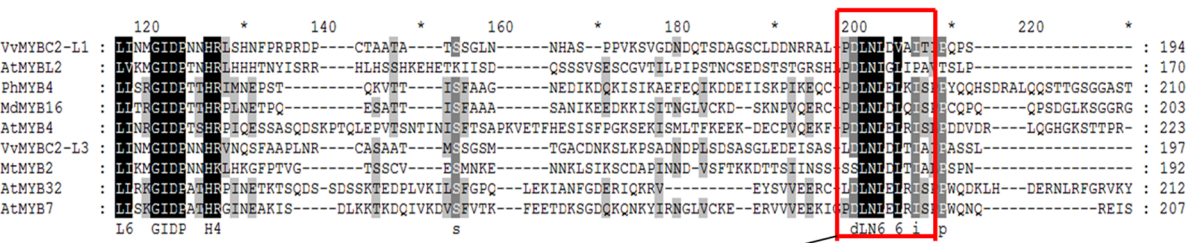
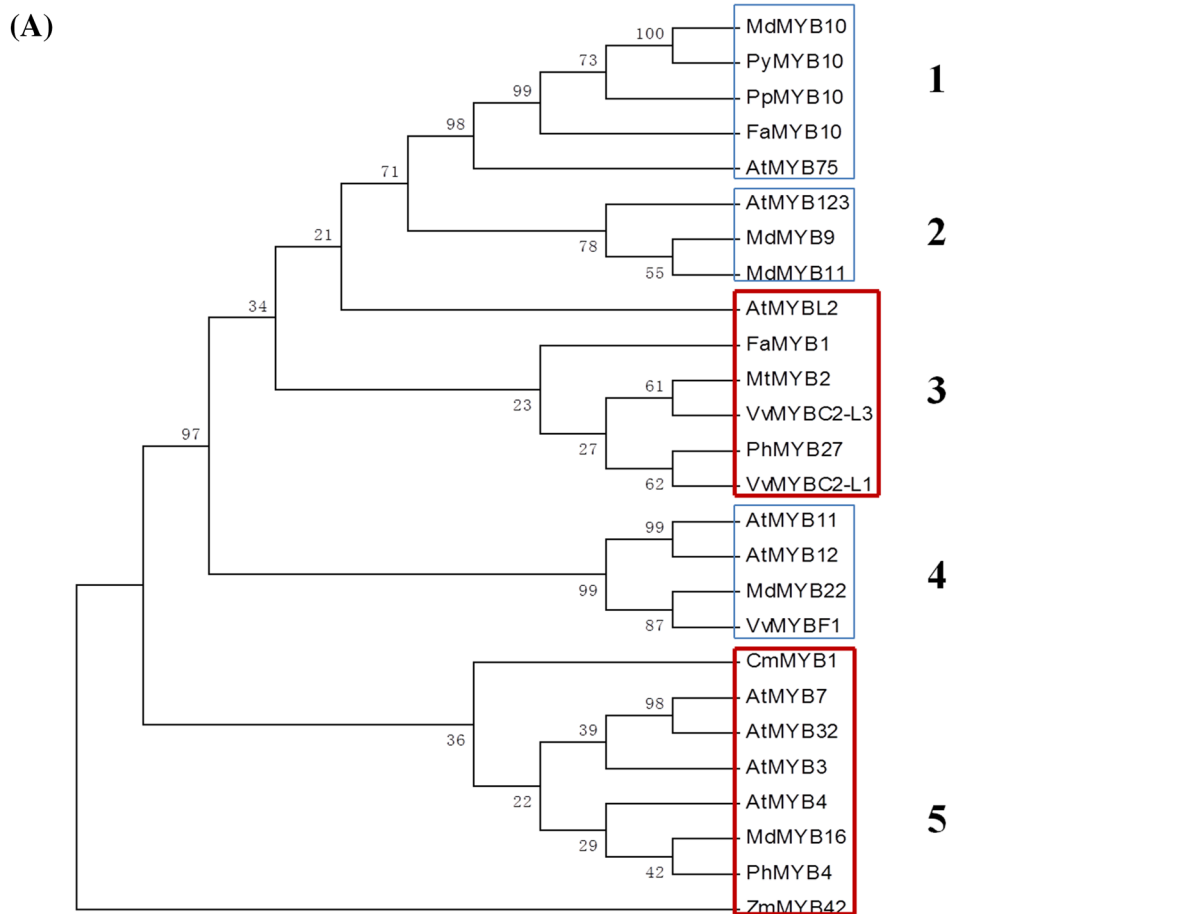


Fig. 2 The analysis of phylogenetic tree and amino acid sequence about MdMYB16 (a) Phylogenetic tree analysis of flavonoid biosynthesis-related MYB transcription factors in different species. (b) Multi-alignment of the amino acid sequences of partial MYB type transcription repressors. The NCBI accessions of MYB transcription factors are AtMYB11 (XM_002876634.1), AtMYB12 (NP_182268.1), MdMYB22 (DQ074470.1), VvMYBF1 (FJ948477.2), ZmMYB42 (NM_001112539.2), CmMYB1 (JF795917.1), MdMYB16 (HM122617.1), FaMYB1 (AF401220.1), AtMYB4 (AB005889.1), AtMYB7(NP_179263), AtMYB32(NP_195225), AtMYB3(AT1G22640), PhMYB4 (ADX33331), PhMYB27(AHX24372), VvMYBC2-L1(ABW34393), VvMYBC2-L3 (KM046932), AtMYBL2(NP_177259.1), MtMYB2(XP_003616388.1), AtMYB123 (NM_122946.2), MdMYB9 (DQ267900.1), MdMYB11 (DQ074463.1) AtMYB75 (NM_104541.3), FaMYB10 (EU155162.1), PpMYB10 (GU936492.1), MdMYB10 (EU518249.2), and PyMYB10 (HM585181.1)

target genes (e.g., At-MYB4; Jin et al. 2000). This study showed that *MdMYB16* is within the *AtMYB4* clade, and could bind the promoter of *MdANS* and *MdUFGT*, thereby directly inhibited anthocyanin biosynthesis via the EAR sequence. Additionally, we also found that *MdMYB16* could form homodimers and interact with *MdbHLH33*.

***MdMYB16* might play a important role in color differences among five Red Crisp first-generation hybrids**

Espley et al. (2007, 2009) found that fruit anthocyanin biosynthesis is mainly regulated by *MdMYB10*, the promoter of which has self-activation characteristics and can appear in two forms, namely R6 and R1, depending on the number of repeats it contains. They also showed that the *MdMYB10* promoter type in red-fleshed apple was R6R6, while the *MdMYB10* promoter type in white-fleshed apple was R1R1. So it might be the different *MdMYB10* promoter type that cause the difference of anthocyanin content. In our study, the *MdMYB10* promoter type in five Red Crisp first-generation hybrids was all R6R1. Therefore, their anthocyanin content should be consistent. However, the significant differences were observed in the flesh color of Red Crisp apple (Fig. 1a), and the expression of *MdMYB10* in each line was not consistent with the anthocyanin levels (Fig. 1c, e). This suggested that there might be other transcription factors in addition to *MdMYB10* involved of the anthocyanin biosynthesis. Lin-Wang et al. (2011) found that *MdMYB16/17/111* could weaken the activation of MYB10-bHLH3 transcriptional activation complex acting on the *DFR* promoter. They also showed that *MdMYB16* is within the *AtMYB4* clade of repressors by phylogenetic tree analysis. However, it was not known how *MdMYB16* inhibit anthocyanin synthesis. Therefore, we took *MdMYB16* as an important candidate gene in our paper. Analysis of *MdMYB16* expression in fruit of the five Red Crisp lines

showed that fruit with high anthocyanin levels had low *MdMYB16* expression levels, while fruit with low anthocyanin levels had high *MdMYB16* expression levels (Fig. 1c, e). Further study showed that overexpressing *MdMYB16* in red callus showed that it could suppress both the expression of anthocyanin structural genes and the accumulation of anthocyanins (Fig. 3a, b, d). So, except for *MdMYB10*, we think *MdMYB16* might play a important role in color differences among five Red Crisp first-generation hybrids.

***MdMYB16* could directly inhibit anthocyanin biosynthesis via the EAR sequence**

The EAR motif was a common feature of EAR-type transcriptional repressors, with changes in the amino acid residues within the EAR motif resulting in a reduction or loss of suppression (Ohta et al. 2001; Tiwari et al. 2001, 2004). Several studies of MYB proteins that has the EAR sequence had a great progress. *AtMYB4* and *PhMYB4* were found that they can repress the expression of *C4H* (Jin et al. 2000; Hemm et al. 2001; Colquhoun et al. 2011). *FaMYB1*, *PhMYB27*, *VvMYBC2* and *MtMYB2* were found that they can act upon MBW complexes (Paolucci et al. 2011; Albert et al. 2014; Jun et al. 2015; Cavallini et al. 2015). So, these MYB proteins were divided into two types of *FaMYB1-like* and *AtMYB4-like* by phylogenetic tree analysis (Jun et al. 2015). In our study, *MdMYB16* is within the *AtMYB4* clade (Fig. 2a). Overexpressing *MdMYB16* in red callus showed that it could suppress both the expression of anthocyanin structural genes and the accumulation of anthocyanins (Fig. 3a, b, d). The YIH assay showed that MdMYB16 could interact with the promoters of *MdANS* and *MdUFGT* (Fig. 4). So *MdMYB16* could directly inhibit anthocyanin biosynthesis by repressing the expression of *MdANS* and *MdUFGT*.

The Arabidopsis full-length AtERF3 and the EAR motif peptide at its C-terminus inhibits the binding of AtERF5 to the GCC box in plants. However, this inhibitory activity is lost when the amino acid residue D is substituted with A in the AtERF3 EAR motif (LDLN-LAP) (Ohta et al. 2001). Overexpression of *ZAT7* genes in Arabidopsis results in increased salt stress resistance, while mutations or deletions in the EAR motif of *ZAT7* proteins reduces this increased resistance in transgenic plants (Ciftci-Yilmaz et al. 2007). SUPERMAN is a transcription factor that has been associated with organ development and contains an EAR motif at its C-terminus. When the EAR motif is absent, the ectopic expression of *SUPERMAN* results in a similar phenotype to the loss-of-function mutant *superman* gene (Hiratsu et al. 2002). Recent studies on the structural and functional aspects of EAR-type transcriptional repression have developed a new suppression tool in the form of chimeric repressor

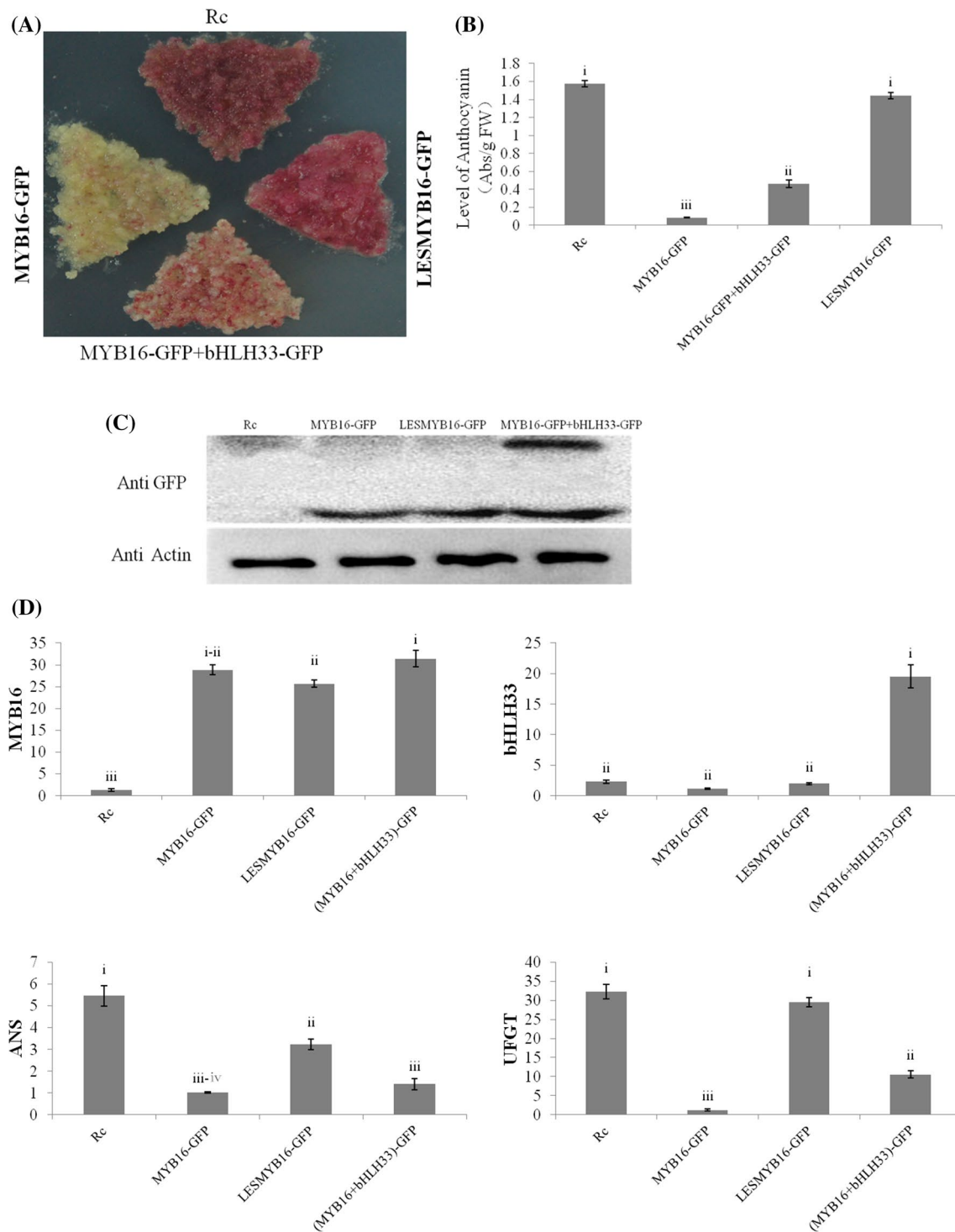


Fig. 3 Transgenic analysis of *MdMYB16*, *LESMdMYB16*, and *MdbHLH33*. **Rc**: red callus, **MYB16-GFP**: overexpressing *MdMYB16* in red callus, **MYB16-GFP+bHLH33-GFP**: overexpressing *MdbHLH33* in callus that overexpressing *MdMYB16* **LESMYB16-GFP**: overexpression of *MdMYB16* without the EAR sequence The significance tests are shown as *i*, *ii*, *iii*, *iv*. Completely different lowercase

roman alphabet on the column chart indicates significant differences ($P < 0.01$). **a** Red calli and three types of transgenic calli. **b** Anthocyanin levels in four kinds of calli. **c** Western blot analysis of four kinds of calli using Anti-GFP and Anti-Actin **d** Expression of anthocyanin biosynthesis structural genes and overexpression of transcription factors in four kinds of calli

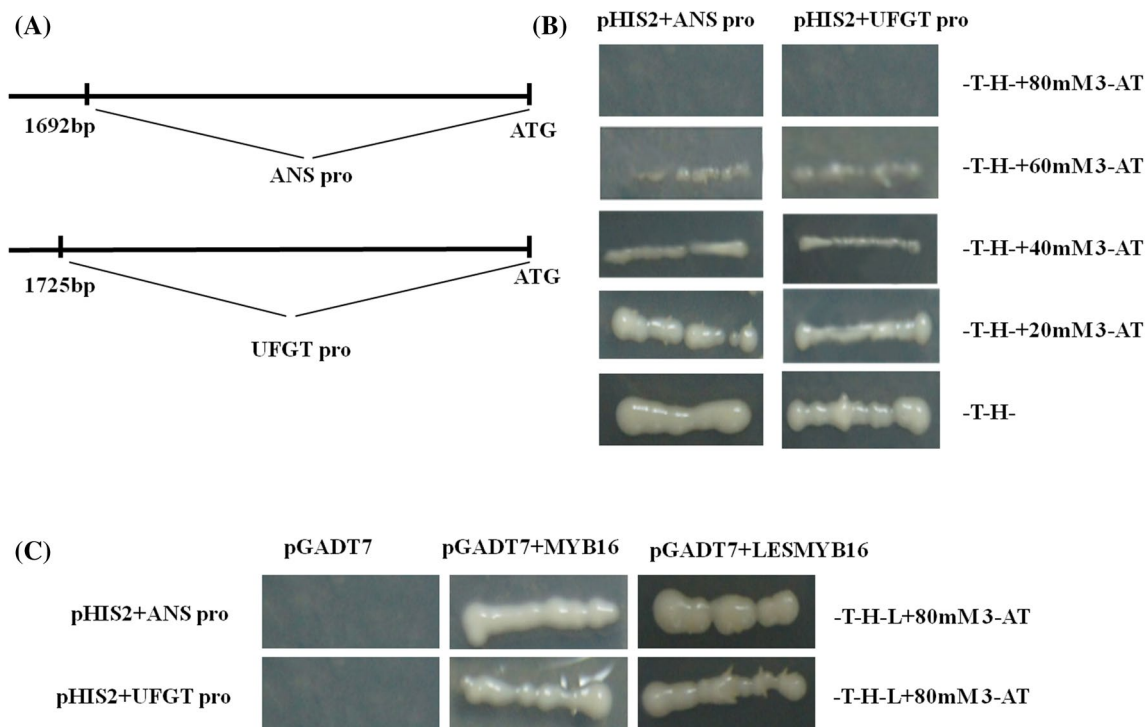


Fig. 4 Yeast one-hybrid (Y1H) assays with the *MdANS* and *MdUFGT* promoters. **a** The 1692 bp long *MdANS* promoter and the 1725-bp long *MdUFGT* promoter chosen to analyze the target DNA sequence. **b** *pHIS2-MdANSPro* and *pHIS2-MdUFGTPro* were

respectively transformed into Y187 yeast strains to screen different 3-AT concentrations for reporter gene inhibition. **c** *MdMYB16* and *LESMdMYB16* could interact with the promoters of *MdANS* and *MdUFGT*

gene-silencing technology. The EAR motif from an EAR-type transcriptional repressor was added to the C-terminus of the activator and fused as a chimeric protein that in turn can be transformed into a highly efficient negative regulator that can be introduced into plants using transgenic methods for the specific and efficient inhibition of the expression of a target gene (Hiratsu et al. 2003; Mitsuda et al. 2006; Kam et al. 2008). In the present study, we analyzed the characteristics of the *MdMYB16* protein sequence and determined that a 10-amino acid EAR suppression sequence (PDLNLDLQIS) was present at the C-terminus (Fig. 2b). We removed the EAR sequence of *MdMYB16* using overlapping PCR and overexpressed the resulting *MdMYB16* gene without the EAR motif (*LESMdMYB16*) in red callus. This showed that the expression of anthocyanin structural genes and anthocyanin levels in the red callus overexpressing *LESMdMYB16* were essentially the same as in the wild-type red-fleshed callus (Fig. 3a, b, d). The Y1H assay showed that *MdMYB16* and *LESMdMYB16* could each interact with the promoters of *MdANS* and *MdUFGT*, suggesting that the *MdMYB16* EAR sequence did not affect the interaction between *MdMYB16* and *MdANS* and *MdUFGT*. Therefore, we concluded that the inhibitory role of *MdMYB16* is achieved by the EAR sequence at the C-terminus.

MdMYB16 could interact with *MdbHLH33* and form homodimers

The interaction between MYB and bHLH transcription factors is crucial for the establishment of a regulatory complex controlling the late enzymatic steps of the anthocyanin (Xu et al. 2014a, b). In the present study, we present a new interaction between MYB and bHLH proteins. *MdMYB16* had the bHLH binding motif and could interact with *MdbHLH33* (Figs. 2b, 6), the relationship of which was similar to the relationship between *AtMYB4* and *AtbHLH* (Zimmermann et al. 2004). Overexpression of *MdbHLH33* in calli overexpressing *MdMYB16* resulted in greater expression of the anthocyanin structural genes, and greater anthocyanin levels, when compared with the calli only overexpressing *MdMYB16* (Fig. 3a, b, d). Further study showed that the expression levels of *MdUFGT* and *MdANS* and anthocyanin content in the callus simultaneously overexpressing *LBSMdMYB16* and *MdbHLH33* was lower than those in the callus simultaneously overexpressing *MdMYB16* and *MdbHLH33* (Fig. 7a, c, d). And the *LBSMdMYB16* could not interact with the *MdbHLH33* (Fig. 7b). This indicated that it was the interaction between *MdbHLH33* and *MdMYB16* that influenced the the inhibitory effect

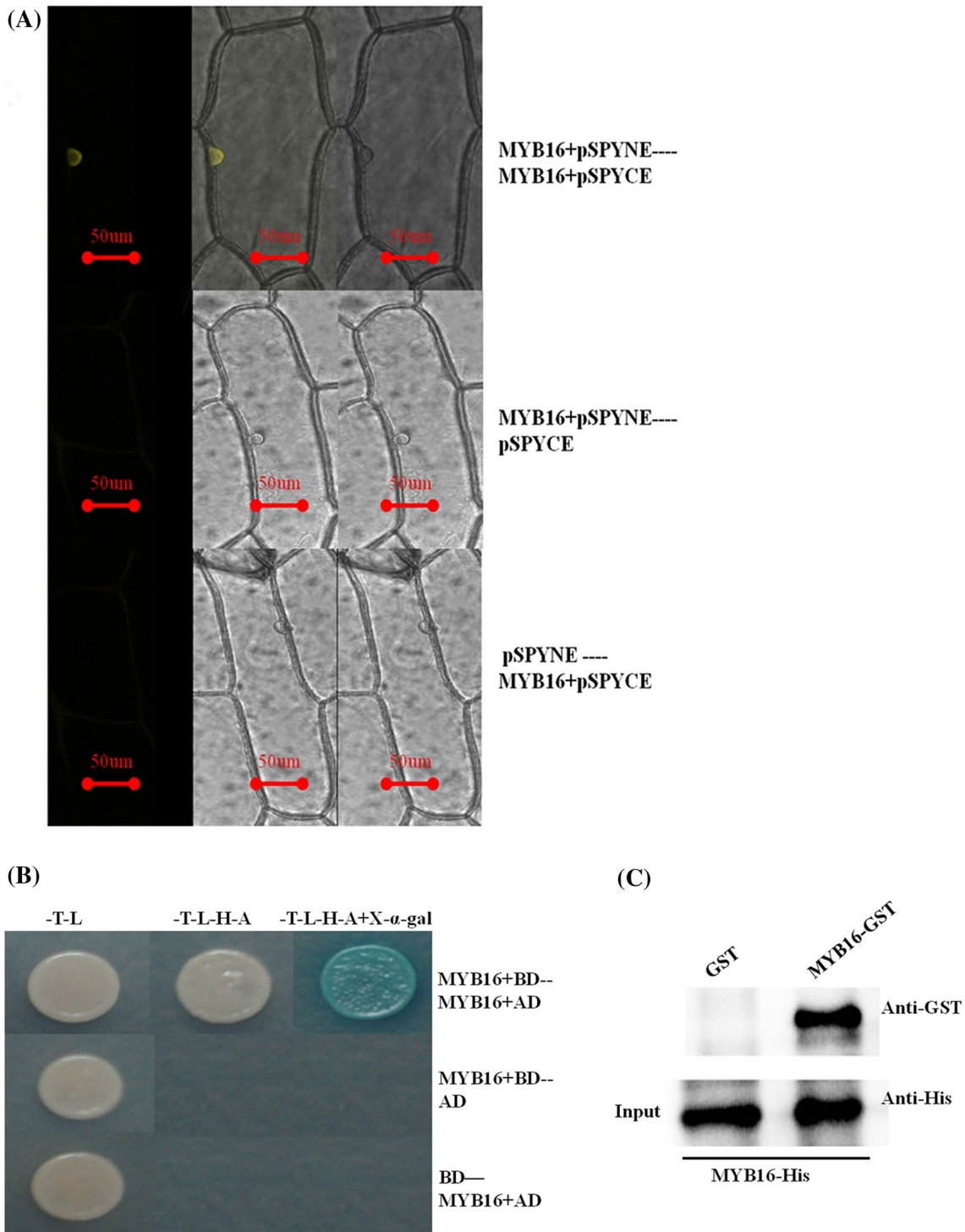
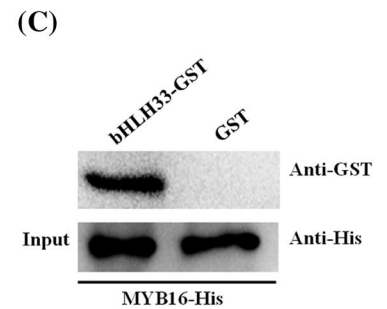
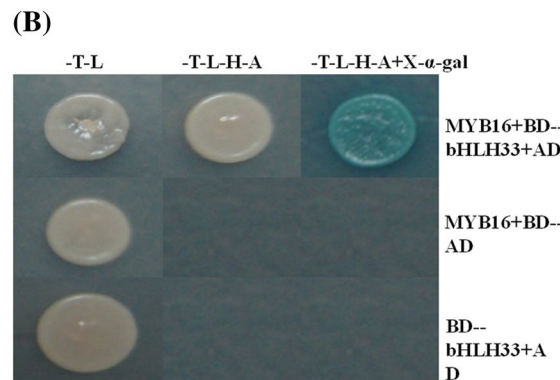
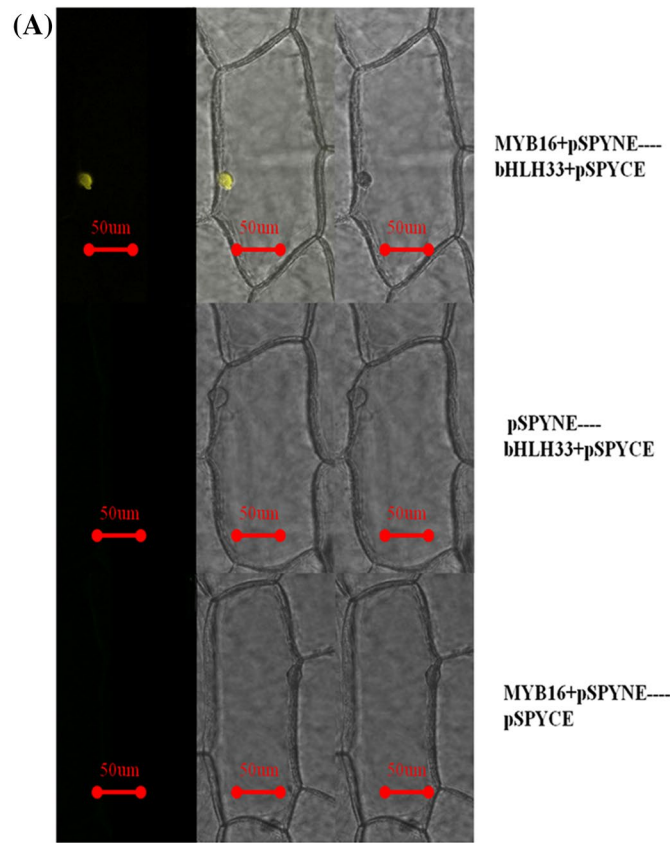


Fig. 5 MdMYB16 could form homodimers. **a** MdMYB16 interacted with itself in BiFC assays. **b** MdMYB16 interacted with itself in yeast two-hybrid assays. **c** MdMYB16 interacted with itself in pull-down assays

of *MdMYB16* on anthocyanin synthesis. Additionally, we also found that MdMYB16 could form homodimers (Fig. 5), we thought the discoveries about MYB–MYB dimerization were very interesting and might help

explain the operation of this class of transcription factor normally. On the basis of these results, we speculated that MdMYB16 could form a large number of stable homodimers by overexpressing *MdMYB16* in red callus,

Fig. 6 MdbHLH33 could interact with MdMYB16. **a** MdbHLH33 interacted with MdMYB16 in BiFC assays. **b** MdbHLH33 interacted with MdMYB16 in yeast two-hybrid assays. **c** MdbHLH33 interacted with MdMYB16 in pull-down assays



and it could directly inhibit anthocyanin biosynthesis via the EAR sequence. However, overexpression of *MdbHLH33* known as a activator of anthocyanin biosynthesis in callus with up-regulated *MdMYB16* resulted in an interaction between MdMYB16 and MdbHLH33, and then affected MdMYB16 repressor complex, thereby ultimately weakened the inhibitory effect of *MdMYB16* on anthocyanins. However, this speculation required further validation, and my research provided a basis for the new regulatory model.

Conclusion

In the present study, we used transgenic approaches and protein interactions to examine the anthocyanin synthesis pathway genes *MdMYB16* and *MdbHLH33*. A putative regulatory network of anthocyanin biosynthesis controlled by MdMYB16 and MdbHLH33 transcription factors was showed in Fig. 8. The results showed that MdMYB16 could directly inhibit anthocyanin biosynthesis via the C-terminal EAR repressor. We also found that MdMYB16 interacted

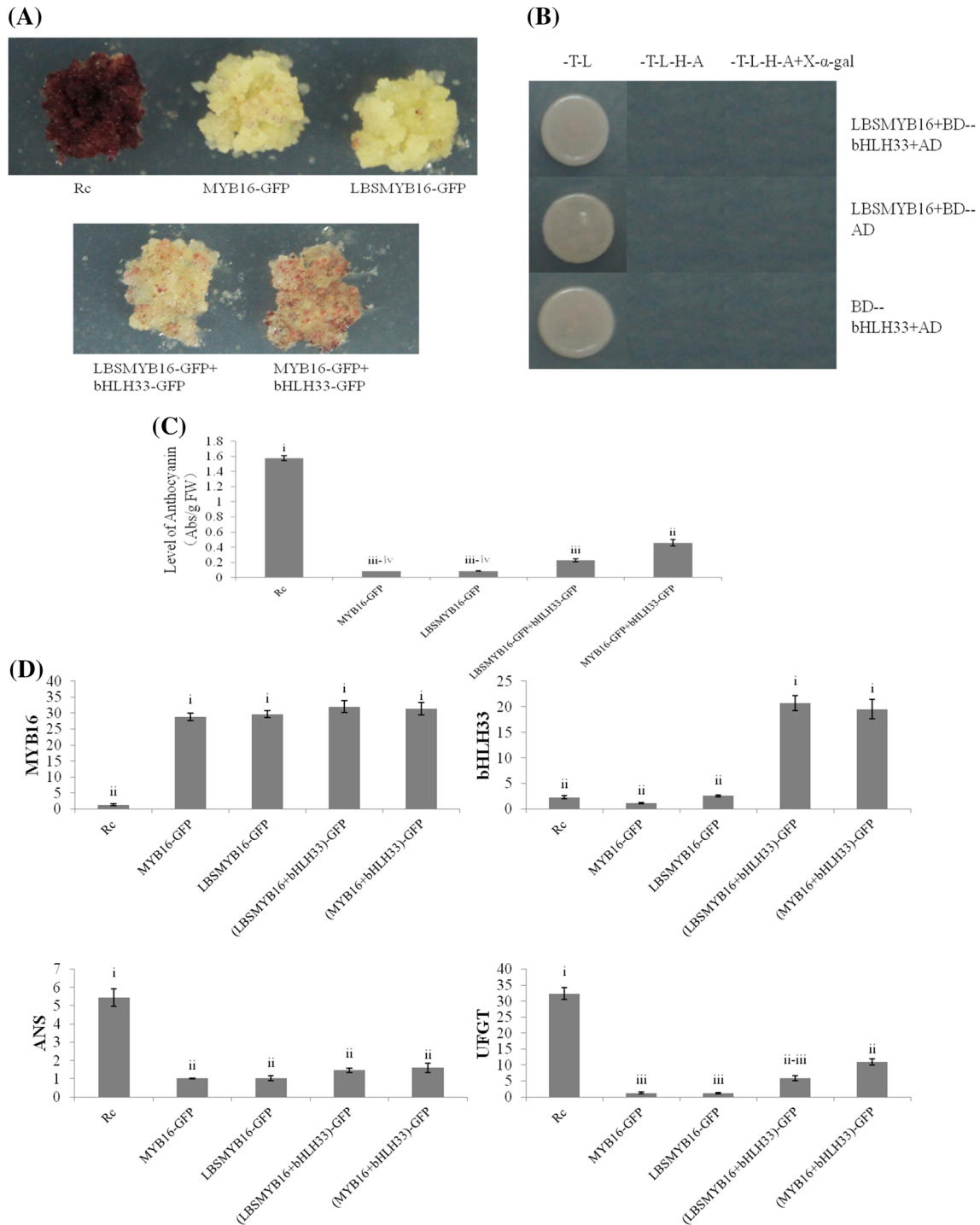
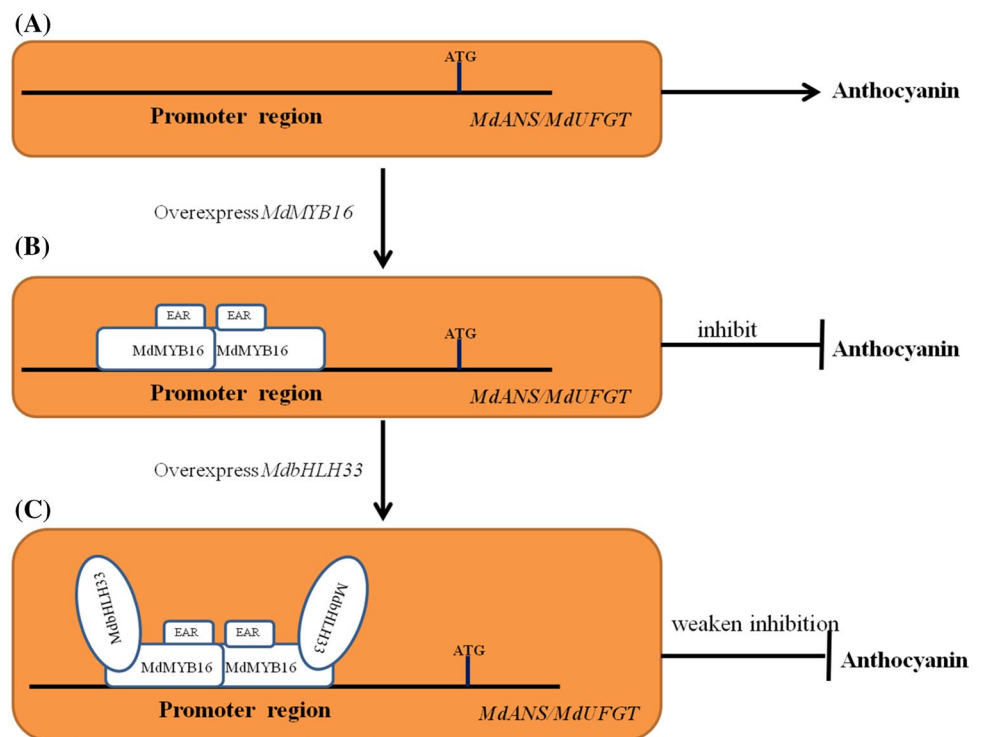


Fig. 7 Transgenic analysis and Y2H assays of *LBSMdMYB16* and *MdbHLH33* Rc: red callus, MYB16-GFP: overexpressing *MdMYB16* in red callus, LBSMYB16-GFP: overexpression of *MdMYB16* without the bHLH binding sequence MYB16-GFP+bHLH33-GFP: overexpressing *MdbHLH33* in callus that overexpressing *MdMYB16* LBSMYB16-GFP+bHLH33-GFP: overexpressing *MdbHLH33* in

callus that overexpressing *LBSMdMYB16*. **a** Red calli and four types of transgenic calli. **b** Y2H analysis between *LBSMdMYB16* and *MdbHLH33*. **c** Anthocyanin levels in five kinds of calli. **d** Expression of anthocyanin biosynthesis structural genes and overexpression of transcription factors in five kinds of calli

Fig. 8 A putative regulatory network of anthocyanin biosynthesis controlled by *MdMYB16* and *MdbHLH33* transcription factors in apple fruit. **a** *MdANS* and *MdUFGT* could control anthocyanin biosynthesis in red callus. **b** *MdMYB16* could form a large number of stable homodimers by overexpressing *MdMYB16* in red callus, and it could directly inhibit anthocyanin biosynthesis via the EAR sequence. **c** Overexpression of *MdbHLH33* in callus with up-regulated *MdMYB16* resulted in an interaction between *MdMYB16* and *MdbHLH33*, and then affected *MdMYB16* repressor complex, thereby ultimately weakened the inhibitory effect of *MdMYB16* on anthocyanins



with *MdbHLH33* and could form homodimer with itself. These results suggested that *MdMYB16* and *MdbHLH33* may be important part of the regulatory network controlling the anthocyanin biosynthetic pathway.

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Author contributions XC and HX: conceived and designed the experiments. HX and NW: performed the experiments. HX: analyzed the data. JL, CQ, YW, SJ, NL, ZZ: contributed reagents/materials/analysis tools. HX and XC: wrote the paper.

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