**MdBBX21, a B-Box Protein, Positively Regulates Light-Induced Anthocyanin Accumulation in Apple Peel**

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The red coloration of apple (*Malus × domestica* Borkh.) is due to the accumulation of anthocyanins in the fruit peel. Light is essential for anthocyanin biosynthesis in apple. In this study, we performed a transcriptome sequencing (RNA-seq) analysis of apple fruit exposed to light after unbagging. The identified differentially expressed genes included *MdBBX21*, which is homologous to Arabidopsis *BBX21*, suggesting it may be involved in light-induced anthocyanin biosynthesis. Additionally, *MdBBX21* was localized in the nucleus and its gene was expressed earlier than *MdMYB1* in apple peel treated with light. Overexpressing *MdBBX21* in Arabidopsis and apple calli under light increased anthocyanin accumulation. Dual-luciferase and yeast one-hybrid assays confirmed that *MdBBX21* binds to the *MdHY5*, *MdBBX20*, and *MdBBX22*–1/2 promoters and induces expression. At the same time, *MdHY5* can also activate the expression of *MdBBX21*. Furthermore, bimolecular fluorescence complementation and yeast two-hybrid assays demonstrated that *MdBBX21* can interact with *MdHY5*. This interaction can significantly enhance *MdMYB1* promoter activity. These findings clarify the molecular mechanism by which *MdBBX21* positively regulates light-induced anthocyanin accumulation in apple.

**Keywords:** apple, light, anthocyanin, *MdBBX21*, *MdMYB1*

**INTRODUCTION**

Apples are one of the most widely cultivated fruits worldwide. Red-skinned apples are more popular with consumers than green-skinned or yellow-skinned apples. The redness of the peel is mainly determined by anthocyanins, which are natural water-soluble pigments (Kim et al., 2003; Zhang Y. et al., 2014). Under natural conditions, anthocyanins are stored in plant vacuoles in the form of glycosides (Passeri et al., 2016). The diversity in the colors of flowers, stems, leaves, fruits, and other plant organs and tissues is due to the type, content, and distribution of anthocyanins (Lawrence et al., 1939; Zhang H. et al., 2014).
Anthocyanins are produced by the flavonoid pathway in a process that is controlled by a series of structural genes and regulatory factors (Winkel-Shirley, 2001). The structural genes (e.g., early and late biosynthetic genes) encode various enzymes in the plant anthocyanin biosynthetic pathway. The early biosynthetic genes include those encoding chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone 3-hydroxylase (F3H), whereas the late biosynthetic genes include those encoding dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and flavonoid 3-O-glycosyltransferase (UFGT) (Martin et al., 1991; Kubasek et al., 1992). Transcriptional regulators control the expression of structural genes (Quattrocchio et al., 1993, 1998; de Vetten et al., 1997). For example, MYB proteins, which are among the most important transcription factors, can combine with bHLH and WD40 proteins to form an MBW complex that binds to the promoter of structural genes to induce expression and regulate anthocyanin synthesis (Ramsay and Glover, 2005; Li, 2014). In apple, MdMYB1 and MdMYBA are responsible for apple skin coloration (Takos A. M. et al., 2006; Ban et al., 2007). Five direct tandem repeats of the MdMYB10-binding motif in the MdMYB10 promoter are associated with the accumulation of anthocyanins throughout the plant, ultimately resulting in striking phenotypes (e.g., red fruit flesh and red foliage) (Espley et al., 2007, 2009). By aligning the MdMYB1 sequence with the GDDH13 and HFTH1 genomes, a long terminal repeat retrotransposon associated with the red-skinned phenotype was detected upstream of MdMYB1 (Zhang et al., 2019). These results strongly indicate that the MdMYB1/A/10 alleles are the core transcriptional regulatory genes of the anthocyanin biosynthesis pathway.

In addition to being genetically regulated, anthocyanin production is also affected by various environmental factors, including drought (An et al., 2020), low temperatures (Xie et al., 2012), salt stress (Lotkowska et al., 2015), low nitrogen availability (Sun et al., 2018), and light (Cominelli et al., 2008). For the biosynthesis of anthocyanins in the apple peel, light is the most important environmental factor (Merzlyak and Chivkunova, 2000). The mechanism underlying light-induced anthocyanin synthesis has been well characterized as part of photomorphogenesis in the model plant Arabidopsis (Maier et al., 2013). Light signals are perceived by plants and then transmitted by photoreceptor proteins such as phytochrome (PHY) (Sharrock and Quail, 1989), cryptochrome (CRY) (Lin et al., 1995), phototropin (PHOT) (Kasahara et al., 2002), and ultraviolet light receptor (UVR8) (Rizzini et al., 2011). Different photoreceptor proteins can perceive and transmit light of different wavelengths. The blue light receptor gene MdCRY2 and the UV-B photoreceptor gene MdUVR8 have been characterized in apple (Li et al., 2013; Zhao et al., 2016). Downstream of the photoreceptors, LONG HYPOCOTYL 5 (HY5), ubiquitin E3 ligase constitutive photobiochemical enzyme 1 (COP1), and PHYTOCHROME-INTERACTING FACTORS (PIFs) are involved in the complex signaling network mediating the regulation of plant photomorphogenesis (Deng et al., 1992; Ang and Deng, 1994; Ni et al., 1998). In apple, MdHY5 binds directly to the MdMYB1 promoter to induce expression and promote the accumulation of anthocyanins under light conditions (An et al., 2017). However, under dark conditions, MdCOP1 can interact with MdMYB1 to mediate the ubiquitination and degradation of MdMYB1 (Li et al., 2012).

The B-box (BBX) protein is a zinc finger protein containing one or two B-box motifs (Reymond et al., 2001). It is a transcription factor that regulates plant photomorphogenesis along with HY5, COP1, and PIFs. Members of the BBX family have been identified in many species, including Arabidopsis (Kanna et al., 2009), rice (Huang et al., 2012), tomato (Chu et al., 2016), apple (Liu et al., 2018), and pear (Bai et al., 2019). In Arabidopsis, the BBX protein family is divided into five subfamilies according to the protein structures. Subfamily IV comprises eight members (BBX18–25), each with two B-box zinc finger motifs (Kanna et al., 2009). The transcription factors in this subfamily are closely related to the light signaling cascade. They can interact with the COP1 and HY5 transcription factors, and can link the light signal regulatory network with other regulatory networks (e.g., hormone and temperature) (Sarmiento, 2013; Song et al., 2020). In apple, BBX22 positively regulates UV-B-induced anthocyanin synthesis, but its function depends on the synergistic effect of HY5 (Bai et al., 2014; An et al., 2019). Additionally, MdBBX20 can form a complex with MdHY5 and bind directly to the MdMYB1, MdDFR, and MdANS promoters to promote anthocyanin synthesis in apple (Fang et al., 2019b). Moreover, MdCOL4 interacts with MdHY5 to inhibit the expression of MdMYB1, whereas it can bind directly to the MdUFGT and MdANS promoters to suppress expression (Fang et al., 2019a). However, whether there are other BBX proteins involved in the synthesis of anthocyanins in apple and the relationships among these BBX proteins remain unclear.

Although the mechanism mediating light-induced anthocyanin synthesis has been partially characterized, a thorough analysis is still needed. In this study, on the basis of transcriptome sequencing data, we revealed that MdBBX21 is differentially expressed in the peel when unbagged fruit are exposed to light. Additionally, MdBBX21 responds to light and reaches peak expression levels earlier than MdBBX20, MdBBX22-1/2, and MdHY5. The overexpression of MdBBX21 in transgenic Arabidopsis plants and apple callici can induce anthocyanin accumulation under light. Further analyses indicated that MdBBX21 can activate the expression of MdBBX20, MdBBX22-1/2, and MdHY5, which subsequently promotes anthocyanin accumulation. Furthermore, MdBBX21 can interact with MdHY5 and induce the expression of MdMYB1. These findings clarify the transcriptional regulation that occurs upstream of MdMYB1 during light-induced anthocyanin biosynthesis.

**MATERIALS AND METHODS**

**Plant Materials**

Six-year-old “Starkrimson Delicious” apple trees growing at the Baishi Apple Experimental Farm of Northwest A&F University, Shaanxi province, China were used as experimental materials. Fruit were covered with a paper bag (Hongtai, Shanxi, China) at 45 days after blooming and harvested at 135 days after blooming.
The harvested fruit were placed in an incubator at 23°C under continuous white light (200 µmol m⁻² s⁻¹). The peels were excised at 0, 3, 6, 9, 12, 24, 48, and 72 h after initiating the light treatment. Twelve fruit were selected at each time-point, with four fruit considered as one biological replicate.

**Determination of the Anthocyanin Content**

Anthocyanins were extracted from the apple peels as previously described (Xie et al., 2012). Briefly, 0.5 g peel was treated with 5 mL 1% (v/v) HCl-methanol and incubated in darkness at 4°C for 24 h. After centrifuging at 13,000 × g for 10 min, the anthocyanin content in the upper liquid layer was determined using an HPLC system comprising a Waters 2998 detector (Waters, Milford, MA, United States) and a C18 column (5 µm internal diameter, 250 mm × 4.6 mm; Waters) as previously described (Liu et al., 2013). The anthocyanin content of Arabidopsis was determined as previously described (Wang et al., 2016).

**RNA Extraction, Library Preparation, and RNA-seq**

Total RNA was extracted using the TRIzol RNA Plant Plus Reagent (Tiangen, Beijing, China). The integrity of the RNA was checked using the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States) and by agarose gel electrophoresis. The purity of the RNA was determined using the NanoPhotometer spectrophotometer (IMPLEN, Westlake Village, CA, United States). Sequencing libraries were constructed using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina (NE, United States). After verifying the quality of the libraries, they were sequenced using the Illumina Novaseq 6000 sequencer (150-bp paired-end sequencing) (Illumina, San Diego, CA, United States) at Novogene Bioinformatics Technology Co., Ltd., Beijing, China.

After eliminating the reads with sequencing adapters and the low-quality reads from the raw data, the remaining clean reads were aligned to the *Malus* Genome GDDH13 reference sequence (version 1.1) (Daccord et al., 2017) using the HISAT2 software. After the sequence alignment, the raw counts of the mapped reads for each *Malus* gene model in GDDH13 (version 1.1) were determined and then normalized to FPKM per million mapped reads. Using DESeq2, differentially expressed genes (DEGs) were putatively identified according to the following criteria: \( \log_2(\text{fold-change}) > 1 \) and adjusted \( p \text{adj} < 0.05 \).

**Subcellular Localization**

The MdBBX21 coding sequence (CDS) was cloned from the cDNA derived from “Starkrimson Delicious” fruit peel. The full-length MdBBX21 open reading frame without the stop codon was inserted into the pCAMBIA2301-eGFP vector under the control of the CaMV 35S promoter to obtain the 35S:MdBBX21-eGFP construct. The primer sequences used are listed in Supplementary Table 4. Tobacco (*Nicotiana benthamiana*) leaves were infiltrated with *Agrobacterium tumefaciens* strain GV3101 cells carrying 35S:MdBBX21-eGFP or the empty vector control (pCAMBIA2301-eGFP). After 3 days, the eGFP signal in the tobacco leaves were detected using the LSM 710 confocal laser-scanning microscope (Carl Zeiss) at excitation wavelengths of 488 nm for eGFP.

**Analysis of mRNA Expression**

First-strand cDNA was synthesized using the HiScript® II Q RT SuperMix for qPCR (Vazyme, Nanjing, China). A quantitative real-time polymerase chain reaction (qPCR) analysis was conducted using the ChamQ Universal SYBR qPCR Master Mix (Vazyme) and the ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems, Waltham, MA, United States). The *MdActin* gene was used as the internal control. All expression data were examined according to the delta-delta cycle threshold method (Livak and Schmittgen, 2001). The primer sequences used are listed in Supplementary Table 4.

**Generation of Transgenic Plant Materials**

“Orin” apple calli were transformed according to a slightly modified version of a previously reported method (Xie et al., 2012). First, calli were infected with *A. tumefaciens* strain LBA4404 cells carrying 35S:MdBBX21-eGFP for 20 min. The calli were then transferred to MS medium supplemented with 1 mg L⁻¹ 6-benzylaminopurine (6-BA), 1 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g L⁻¹ sucrose, and 8 g L⁻¹ agar. After a 3-day incubation in darkness, the calli were transferred to screening medium (i.e., MS medium supplemented with 30 g L⁻¹ sucrose, 1 mg L⁻¹ 2,4-D, 1 mg L⁻¹ 6-BA, 8 g L⁻¹ agar, 250 mg L⁻¹ carbenicillin, and 50 mg L⁻¹ kanamycin) to screen for transformants. For the light treatment, wild-type (WT) and MdBBX21-oVexpressing (MdBBX21-OX) transgenic apple calli cultivated in darkness were transferred to a light incubator and placed under constant white light (500 µmol m⁻² s⁻¹) for 3 days.

*Agrobacterium tumefaciens* strain GV3101 cells carrying 35S:MdBBX21-eGFP were used to infect the Arabidopsis *bbx21* mutant (SALK_105390) according to the floral-dip method (Clough and Bent, 1998). Seeds of WT and T3 transgenic Arabidopsis plants were chilled at 4°C for 48 h and then placed under white light (500 µmol m⁻² s⁻¹) at 24°C under long-day conditions (16-h light/8-h dark). The anthocyanin contents of 5-day-old Arabidopsis seedlings were determined.

**Yeast Two-Hybrid Assay**

A yeast two-hybrid assay was performed using the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech). The full-length MdBBX21 CDS was inserted into the pGADT7 (AD) vector to construct the AD-BBX21 recombinant plasmid. The *MdHY5* CDS was inserted into the pGBK7 (BD) vector to obtain the BD-HY5 recombinant plasmid. The primer sequences used are listed in Supplementary Table 4. Yeast strain Y2HGgold cells were transformed with AD-BBX21 + BD-HY5, AD + BD-HY5, AD-BBX21 + BD, pGADT7-T + pGBK7-53, or pGADT7-T + pGBK7-Lam and then cultured on medium lacking tryptophan and leucine at 30°C. To screen for interacting proteins, the yeast cells were transferred to medium lacking control (pCAMBIA2301-eGFP). After 3 days, the eGFP signal in the tobacco leaves were detected using the LSM 710 confocal laser-scanning microscope (Carl Zeiss) at excitation wavelengths of 488 nm for eGFP.
leucine, tryptophan, histidine, and adenine (−L/−T/−H/−A), but supplemented with X-α-gal.

**Yeast One-Hybrid Assay**

The *MdBBX20, MdBBX22-1/2, MdHY5*, and *MdMYB1* promoter fragments were inserted into the pHIS2 vector to construct the *MdBBX20*pro-HIS2, *MdBBX22-1*pro-HIS2, *MdBBX22-2*pro-HIS2, *MdHY5*pro-HIS2, and *MdMYB1*pro-HIS2 recombinant plasmids. The primer sequences used are listed in **Supplementary Table 4**. To determine the optimal 3-AT concentration, yeast strain Y187 cells containing the recombinant pHIS2 plasmids were grown on screening medium lacking histidine and tryptophan (−H/−T), but supplemented with different 3-AT concentrations. Next, Y187 yeast cells were co-transformed with *MdBBX21*-AD and individual recombinant pHIS2 plasmids. Interactions were detected on selection medium lacking histidine, tryptophan, and leucine (−H/−T/−L), but supplemented with the optimal 3-AT concentration.

**Transient Dual-Luciferase Assay**

Transient dual-luciferase assays were performed using tobacco (*N. benthamiana*) leaves. The *MdBBX20, MdBBX22-1/2, MdHY5*, and *MdMYB1* promoter fragments were inserted into the pGreenII 0800-LUC vector to construct the *MdBBX20*pro:LUC, *MdBBX22-1*pro:LUC, *MdBBX22-2*pro:LUC, *MdHY5*pro:LUC, and *MdMYB1*pro:LUC recombinant plasmids. The full-length *MdBBX21* CDS was inserted into the pGreenII 62-SK vector. The primer sequences used are listed in **Supplementary Table 4**. *A. tumefaciens* strain GV3101 cells carrying the pSoup vector were transformed with the recombinant plasmids. Leaves from 5-week-old tobacco (*N. benthamiana*) plants were injected according to a previously described method.

**FIGURE 1** | RNA-seq data of “Starkrimson Delicious” apple fruit exposed to light after unbagging. (**A**) Change in color of “Starkrimson Delicious” fruit peel under light. (**B**) Anthocyanin content in the peel under light. (**C**) Pearson correlation between samples. (**D**) Principal component analysis of the RNA-seq data.
In order to maintain the same concentration of Agrobacterium solution, 50 ul of Agrobacterium solution MdBBX21 (OD = 0.8) and MdHY5 (OD = 0.8) were mixed with 50 ul of Agrobacterium solution MdMYB1pro:LUC for tobacco leaf injection, respectively. Also, equal volumes and concentrations of Agrobacterium solution MdBBX21 and MdHY5 were mixed, and then 50 ul of the mixture was mixed with 50 ul of Agrobacterium solution MdMYB1pro:LUC for tobacco leaf injection.

Bimolecular Fluorescence Complementation Assay

The MdBBX21 CDS was inserted into the pSPYNE vector, whereas the MdHY5 CDS was inserted into the pSPYCE vector. A. tumefaciens strain GV3101 cells were transformed with the MdBBX21-NE and MdHY5-CE recombinant plasmids. The primer sequences used are listed in Supplementary Table 4. Equal volumes of the A. tumefaciens cells carrying MdBBX21-NE and MdHY5-CE were mixed. Arabidopsis protoplast cells were infected with the A. tumefaciens cells for 15 min and then incubated at 23°C for 16–20 h. The YFP fluorescence was detected using the LSM 710 confocal laser-scanning microscope (Carl Zeiss) with an excitation wavelength of 514 nm.

RESULTS

Transcriptome Analysis of Apple Peel Exposed to Light

To clarify the response of apple to light, we first measured the anthocyanin content in the peel of unbagged apple fruit exposed to white light for 0, 3, 6, 9, 12, 24, 48, and 72 h (Figure 1A). Anthocyanins were basically undetectable before 12 h, but they started to accumulate significantly in the peel at 24 h (Figure 1B). The peel samples at the following three time-points underwent an RNA-seq analysis: 0 h (G0), 6 h (G6), and 24 h (G24). After the strict data filtering step, the number of clean reads in each library ranged from 37.69 to 45.95 million, and the Q30 values exceeded 92% (Supplementary Table 1). The mapping rate of the clean reads to the reference genome was 91.25–92.51%. Additionally, 88.81–90.27% of the clean reads were uniquely mapped (Supplementary Table 2). All biological replicates were strongly correlated (R² > 0.94) (Figure 1C); this correlation was confirmed by principal component analysis (Figure 1D).

Characterization and Expression Analysis of MdBBX21

Among the DEGs in up-regulated profile 6 (Supplementary Table 3), MD08G1021000 was revealed to be highly homologous to Arabidopsis AtBBX21. Thus, we named this gene MdBBX21. To clarify the relationship between MdBBX21 and MdBBX20, MdBBX21, and MdBBX22-1/2, we compared their amino acid sequences. The sequence identities between MdBBX21 and MdBBX20, MdBBX22-1, and MdBBX22-2 were 37.58, 31.92, and 31.63%, respectively. In the constructed phylogenetic tree, MdBBX21, MdBBX20, and MdBBX22-1/2 were clustered with Arabidopsis subfamily IV members, but they were distributed in different clades (Figure 3A).

The correct cellular localization of a protein is critical for ensuring it functions properly. In tobacco leaf cells transiently transformed with 35S:MdBBX21-eGFP, green fluorescence was detected only in the nucleus, whereas in the control tobacco cells transiently transformed with 35S:eGFP, green fluorescence was observed throughout the cell, including in the cytoplasm and nucleus. Accordingly, MdBBX21 appears to be a nuclear protein (Figure 3B).

To determine whether MdBBX21 affects anthocyanin synthesis, we analyzed MdBBX21 expression in the apple fruit peel irritated with white light for 0, 3, 6, 9, 12, 24, 48, and 72 h. The MdBBX21 gene was responsive to light, and its expression level started to increase at 3 h, peaking at 9 h. The expression of the regulatory genes (MdMYB1 and MdHY5) and structural genes (MdCHS, MdF3H, MdDFR, MdANS, and MdUFGT) related to anthocyanin synthesis peaked after 24 h. The MdWD40 expression level was essentially unchanged (Figure 4). These results imply that MdBBX21 may function upstream of MdMYB1.

Identification of Differentially Expressed Genes and Kyoto Encyclopedia of Genes and Genomes Analysis

To screen for early light-responsive genes, we compared the G0, G6, and G24 expression levels. In total, 11,941 DEGs were revealed in the G6 vs. G0, G24 vs. G6, and G24 vs. G0 pairwise comparisons. More DEGs were detected in the G6 vs. G0 comparison than in the G24 vs. G6 comparison (Figures 2A,B). These results suggest that many genes started to respond to light signals after 6 h. To further elucidate the gene expression patterns, the 11,941 DEGs were classified into eight gene expression profiles (Supplementary Figure 1). Specifically, 4,082 DEGs were classified into three significant profiles (P < 0.05), including two up-regulated profiles (profiles 6 and 7) and one down-regulated profile (profile 0) (Figure 2C). A KEGG enrichment analysis indicated genes related to flavonoid biosynthesis and phenylpropanoid biosynthesis were significantly enriched among the DEGs in profiles 0, 6, and 7 (Figure 2D).

Heterologous Overexpression of MdBBX21 Promotes Anthocyanin Accumulation in Arabidopsis

To confirm MdBBX21 contributes to anthocyanin biosynthesis, MdBBX21 was overexpressed in the bbx21 Arabidopsis mutant. The 35S:MdBBX21/bbx21 line accumulated more anthocyanins in the cotyledons and hypocotyls than the untransformed bbx21 Arabidopsis mutant (Figures 5A–C). The expression levels of anthocyanin-related genes (AtPAP1, AtCHS, AtF3'H, AtDFR, AtLDOX, and AtUFGT) also increased in the 35S:MdBBX21/bbx21 line, which was consistent with the changes in the anthocyanin content (Figure 5D). We also examined the expression of AtHY5 and Arabidopsis BBX subfamily IV members. The AtHY5 and AtBBX22 expression
levels were higher in the 35S:MdBBX21/bbx21 seedlings than in the bbx21 Arabidopsis mutant seedlings. In contrast, there were no differences in the expression levels of the other subfamily IV BBX genes (Figure 5E and Supplementary Figure 2).

**Overexpression of MdBBX21 Promotes Anthocyanin Accumulation in Apple Calli**

To further confirm the MdBBX21 function related to anthocyanin biosynthesis in apple, we overexpressed MdBBX21 in apple calli (Figures 6A,B). Wild-type and MdBBX21-OX transgenic apple calli were cultured on medium in darkness and then transferred to a light incubator for a 3-day exposure to constant light. The MdBBX21-OX apple calli accumulated more anthocyanins than the WT calli (Figure 6C). As expected, the expression levels of the anthocyanin biosynthesis-related genes (MdMYB1, MdCHS, MdF3H, MdDFR, MdANS, and MdUFGT) were significantly higher in the MdBBX21-OX calli than in the WT calli (Figure 6D). These results suggest that MdBBX21 might positively regulate anthocyanin accumulation by promoting the transcription of anthocyanin biosynthesis-related genes. The transcription levels of the previously characterized genes MdBBX20, MdBBX22-1/2, MdCOL4 (MdBBX24), and MdHY5 were also analyzed by qPCR.
FIGURE 3 | Characterization of MdBBX21. (A) Phylogenetic relationship of apple and Arabidopsis subfamily IV members. The red and green genes represent positive and negative regulators related to light signaling, respectively. The phylogenetic tree was constructed using the neighbor-joining method by MEGA 7.0. The bootstrap values of 1000 replicates were calculated at each node. (B) Subcellular localization of MdBBX21 expressed in tobacco leaf cells.

(Figure 6E and Supplementary Figure 3). The data indicated that MdHY5, MdBBX20, and MdBBX22-1/2 were significantly more highly expressed in MdBBX21-OX calli than in WT calli. The MdBBX24 expression level did not differ between the MdBBX21-OX and WT calli.

MdBBX21 Binds Directly to the MdHY5, MdBBX20, and MdBBX22-1/2 Promoters and Induces Expression

The increased MdHY5, MdBBX20, and MdBBX22-1/2 expression levels in MdBBX21-OX calli led us to speculate that MdBBX21 may directly promote the expression of these four genes. Thus, we first searched for the G-box motif, which is a BBX-binding site (Datta et al., 2008; Xu et al., 2016), in the MdHY5, MdBBX20, and MdBBX22-1/2 promoters. The G-box motif was detected in all three promoters (Figure 7A). In yeast one-hybrid assays, the Y187 yeast strains containing MdBBX21-AD + MdBBX20pro-HIS2, MdBBX21-AD + MdBBX22-1pro-HIS2, MdBBX21-AD + MdBBX22-2pro-HIS2, and MdBBX21-AD + MdHY5pro-HIS2 were able to grow on the −H−T−L selection medium containing 100 mM 3-AT. In contrast, the Y187 yeast strains containing AD + MdBBX20pro-HIS2, AD + MdBBX22-1pro-HIS2, AD + MdBBX22-2pro-HIS2, and AD + MdHY5pro-HIS2 did not grow under the same conditions (Figure 7B). This observation suggests that MdBBX21 can
bind to the \textit{MdHY5}, \textit{MdBBX20}, and \textit{MdBBX22-1/2} promoters. The dual-luciferase assay results confirmed that \textit{MdBBX21} can promote the transcription of \textit{MdHY5}, \textit{MdBBX20}, and \textit{MdBBX22-1/2} (Figure 7C). We also analyzed the \textit{MdHY5}, \textit{MdBBX20}, and \textit{MdBBX22-1/2} expression patterns in the peel exposed to light. Compared with the \textit{MdBBX21} expression pattern (Figure 4), the \textit{MdBBX20}, and \textit{MdBBX22-1/2} expression levels increased significantly at 6 h, and their peak expression levels occurred later than that of \textit{MdBBX21} (Supplementary Figure 4). These findings imply that \textit{MdBBX21} functions upstream of \textit{MdBBX20}, and \textit{MdBBX22-1/2} and induces the expression of \textit{MdBBX21}. Thus, we searched for the G-box motif in the \textit{MdBBX21} promoter. There were two G-box motifs present in the \textit{MdBBX21} promoter (Figure 7A). Yeast one-hybrid assay results showed that \textit{MdHY5} can bind to the \textit{MdBBX21} promoter fragment containing these two motifs (Figure 7B). The dual-luciferase assay results confirmed that \textit{MdHY5} can promote the transcription of \textit{MdBBX21} (Figure 7C).

**The Interaction Between MdBBX21 and MdHY5 Can Significantly Enhance MdMYB1 Promoter Activity**

Because \textit{MdBBX20} (Fang et al., 2019b), \textit{MdBBX22-2} (An et al., 2019), \textit{PpBBX16} (Bai et al., 2019a), and \textit{PpBBX18} (Bai et al., 2019b) can interact with \textit{HY5}, we speculated that \textit{MdBBX21}...
Overexpression of MdBBX21 promotes anthocyanin accumulation in Arabidopsis seedlings. (A) Phenotypes of 35S::MdBBX21/bbx21 and bbx21 mutant seedlings. 35S::MdBBX21/bbx21 (1#), 35S::MdBBX21/bbx21 (9#), and 35S::MdBBX21/bbx21 (11#) represent three transgenic lines. (B) The MdBBX21's expression level in 35S::MdBBX21/bbx21 and bbx21 mutant seedlings. (C) Anthocyanin content in 35S::MdBBX21/bbx21 and bbx21 mutant seedlings. (D) Relative expression levels of anthocyanin biosynthesis-related genes in 35S::MdBBX21/bbx21 and bbx21 mutant seedlings. (E) Relative expression levels of AtHY5, AtBBX22, and AtBBX20 in 35S::MdBBX21/bbx21 and bbx21 mutant seedlings. Error bars represent the standard deviation of three biological replicates. **P < 0.01 (Student's t-test).

may also interact with MdHY5. Bimolecular fluorescence complementation assays were conducted to examine whether MdBBX21 can interact directly with MdHY5. Full-length MdBBX21 and MdHY5 CDSs were cloned into pSPYNE and pSPYCE vectors, respectively, to generate MdBBX21-NE and MdHY5-CE. Yellow fluorescence was detected in the nucleus of
Arabidopsis protoplasts co-transformed with MdBBX21-NE and MdHY5-CE. However, yellow fluorescence was not observed in the BBX21-NE + CE and NE + HY5-CE controls (Figure 8A). These results indicate that MdBBX21 can interact with MdHY5. This interaction was verified in yeast two-hybrid assays (Figure 8B).

MdBBX20 can bind directly to the MdMYB1 promoter and induce expression (Fang et al., 2019b). In this study, Y187 yeast strains containing MdBBX21-AD + MdMYB1pro-HIS2 grew on -H/-T/-L selection medium containing 100 mM 3-AT, but the Y187 yeast strains containing AD + MdMYB1pro-HIS2 did not (Figure 8C). Accordingly, MdBBX21 appears to be able to interact with the MdMYB1 promoter. On the basis of the dual-luciferase assay results, MdBBX21 can promote MdMYB1 expression (Figure 8D). Moreover, MdHY5 can also promote MdMYB1 expression, which is consistent with the results of an earlier study (An et al., 2017). We also determined that the MdBBX21–MdHY5 heterodimer enhances...
the MdMYB1 promoter activity more than MdBBX21 or MdHY5 alone (Figure 8D).

**DISCUSSION**

**MdBBX21 Responds to Light and Induces Anthocyanin Biosynthesis in Apple**

Light is required for anthocyanin biosynthesis in the apple fruit peel (Saure, 1990). An exposure to light up-regulates the expression of anthocyanin biosynthesis-related structural and regulatory genes in the apple peel (Takos M. A. et al., 2006; Feng et al., 2013). However, how light signals affect the expression of these genes was unclear. There is increasing evidence that BBX proteins affect plant photomorphogenesis (Gangappa and Botto, 2014). The RNA-seq analysis in this study revealed that MdBBX21 expression in the peel of dark-grown “Starkrimson Delicious” apples is affected by the subsequent exposure to light. Additionally, MdBBX21 and Arabidopsis AtBBX21 sequences are highly similar. Earlier research demonstrated that AtBBX21 is a positive regulator of anthocyanin biosynthesis (Datta et al., 2007). Hence, we predicted that MdBBX21 and AtBBX21 might have similar functions. We analyzed MdBBX21 expression and observed that it increased in response to light. Moreover, MdBBX21 expression peaked before the expression of anthocyanin-related structural and regulatory genes peaked (Figure 4). The overexpression of MdBBX21 in the bbx21 Arabidopsis mutant resulted in a significant increase in the anthocyanin contents of hypocotyls (Figure 5). This is consistent with the fact pear PpBBX18 (homologous to AtBBX21) regulates anthocyanin accumulation in transgenic Arabidopsis plants. In the present study, the
Figure 8 | The interaction between MdBBX21 and MdHY5 can significantly enhance MdMYB1 promoter activity. (A) Bimolecular fluorescence complementation assay. (B) Yeast two-hybrid assay showing interaction between MdBBX21 and MdHY5. (C) Yeast one-hybrid assay showing interaction between MdBBX21 and MdMYB1 promoter. (D) Effects of MdBBX21 and MdHY5 individually and in combination on promoter activity of MdMYB1 as determined by luciferase reporter assay. Error bars represent the standard deviation of three biological replicates. **P < 0.01 (Student’s t-test).

MdBBX21-OX apple calli accumulated more anthocyanins than the WT apple calli under light (Figure 6). These results indicate that MdBBX21 is responsive to light and induces anthocyanin biosynthesis.

MdBBX21 Up-Regulates MdBBX20, MdBBX22-1/2, and MdHY5 Expression

The Arabidopsis BBX subfamily IV members AtBBX20, AtBBX21, AtBBX22, and AtBBX23 positively regulate plant photomorphogenic processes in response to diverse light signals (Datta et al., 2007; Chang et al., 2008; Datta et al., 2008; Fan et al., 2012; Xu et al., 2016; Zhang et al., 2017). In pear, PpBBX16 and PpBBX18, which are subfamily IV BBX proteins, positively regulate anthocyanin synthesis by interacting with PpHY5 (Bai et al., 2019a,b). In apple, MdBBX20 and MdBBX22-1/2 reportedly promote anthocyanin accumulation in response to UV-B irradiation (Bai et al., 2014; An et al., 2019; Fang et al., 2019b). In addition, Plunkett et al. (2019) found that BBX subfamily IV member MdBBX51 and BBX subfamily I member MdBBX1 have very high expression levels during fruit development. Further, they can activate the promoter of MdMYB1 in the presence of some co-factors MYB and bHLH. However, the relationships among these BBX proteins are unknown. In this study, MdBBX21 expression increased significantly after a 3-h light treatment, and subsequently peaked at 9 h. In contrast, the MdBBX20 and MdBBX22-1/2 expression levels peaked at 12 and 24 h, respectively. The expression of structural and regulatory genes related to anthocyanin synthesis increased rapidly and peaked after 24 h (Figure 4 and Supplementary Figure 2). These findings indicate that MdBBX21 responds to light signals relatively early and likely functions upstream of MdBBX20 and MdBBX22-1/2. Dual-luciferase and yeast one-hybrid assays proved that MdBBX21 induces MdBBX20 and MdBBX22-1/2 expression after binding to their promoters. The AtBBX22, MdBBX20, and MdBBX22-1/2 expression levels increased in Arabidopsis and apple calli overexpressing MdBBX21 (Figures 5–7). Therefore, MdBBX21 can up-regulate the expression of AtBBX22, MdBBX20, and MdBBX22-1/2. However, MdBBX21 did not activate the
expression of MdBBX1, MdBBX24, and MdBBX51 in apple calli (Supplementary Figure 3). In Arabidopsis, AtBBX21 can bind directly to the AtHY5 promoter and induce expression (Xu et al., 2016). In the current study, biochemical assays proved that MdBBX21 can bind directly to the MdHY5 promoter and induce expression (Figure 7). The overexpression of MdBBX21 in Arabidopsis and apple calli resulted in up-regulated HY5 expression (Figures 5, 6). These results imply that MdBBX21 and AtBBX21 have similar functions and can directly up-regulate the expression of HY5. At the same time, we found that MdHY5 can also activate the expression of MdBBX21, indicating that a positive feedback regulation mechanism exists between MdBBX21 and MdHY5.

The MdBBX21–MdHY5 Interaction Can Significantly Enhance MdMYB1 Promoter Activity Under Light

The bZIP transcription factor AtHY5 is a positive regulator of plant photomorphogenesis that functions downstream of the photoreceptors and COP1 (Lau and Deng, 2010). Additionally, AtHY5 regulates various physiological processes, including anthocyanin synthesis, lateral root formation, and hypocotyl elongation (Oyama et al., 1997). Earlier studies regarding in vitro DNA–protein interactions demonstrated that AtHY5 can bind directly and specifically to the G-box motif in the promoters of the anthocyanin-related structural genes AtF3H, AtCHS, and AtCHI and the regulatory gene AtPAP1 (Ang et al., 1998; Lee et al., 2007; Shin et al., 2013). In Arabidopsis, AtBBX21 and AtBBX22 can activate transcription. They interact with AtHY5 in vivo and induce downstream gene expression in an AtHY5-dependent and -independent manner to promote plant photomorphogenesis (Datta et al., 2007, 2008). In apples and pears, HY5 can bind directly to the MYB1 promoter (An et al., 2017; Tao et al., 2018). Both PpBBX16 and PpBBX18 cannot bind directly to the MYB1 promoter, but they can interact with HY5 and promote MYB1 expression (i.e., HY5-dependent manner) (Bai et al., 2019a,b). However, in apple, MdBBX20 can bind directly to the MdMYB1 promoter and induce expression (i.e., HY5-independent manner) (Fang et al., 2019b). In this study, we proved that MdBBX21 can bind directly to the MdMYB1 promoter and induce expression (Figure 8). Additionally, the analysis of protein–protein interactions revealed that MdBBX21 interacts with MdHY5 in yeast and plant cells. Furthermore, the interaction between MdBBX21 and MdHY5 can significantly enhance MdMYB1 promoter activity (Figure 8).

In conclusion, we systematically characterized the effect of light on MdBBX21 in the apple peel. We proved that MdBBX21 binds to the promoters of MdBBX20, MdBBX22, and MdHY5. The subsequent up-regulated expression of these genes enhances anthocyanin accumulation. Additionally, MdBBX21 can interact with MdHY5 and induce MdMYB1 expression. The results of our study will form the basis of future investigations on the functions of BBX proteins in apple.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI SRA BioProject, accession no: PRJNA767632.

AUTHOR CONTRIBUTIONS

BZ, H-JY, and Z-YZ conceived the original screening and research plans. BZ, Z-ZZ, B-CW, N-NH, and Y-ZY supervised the experiments. BZ, H-JY, and DQ analyzed the data. BZ and Z-ZZ wrote the manuscript. H-JY revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.774446/full#supplementary-material

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