RESEARCH PAPER

Dynamic regulation of anthocyanin biosynthesis at different light intensities by the BT2-TCP46-MYB1 module in apple

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Abstract

Teosinte branched1/cycloidea/proliferating (TCP) transcription factors play a broad role in plant growth and development, but their involvement in the regulation of anthocyanin biosynthesis is currently unclear. In this study, anthocyanin biosynthesis induced by different light intensities in apple (Malus domestica) was found to be largely dependent on the functions of the MdMYB1 and MdTCP46 transcription factors. The expression of MdTCP46 was responsive to high light intensity, and under these conditions it promoted anthocyanin biosynthesis by direct interactions with MdMYB1 that enhanced the binding of the latter to its target genes. MdTCP46 also interacted with a bric-a-brac/tramtrack/broad (BTB) protein, MdBT2, that is responsive to high light intensity, which ubiquitinated MdTCP46 and mediated its degradation via the 26S proteasome pathway. Our results demonstrate that the dynamic regulatory module MdBT2-MdTCP46-MdMYB1 plays a key role in modulating anthocyanin biosynthesis at different light intensities in apple, and provides new insights into the post-transcriptional regulation of TCP proteins.

Keywords: Anthocyanin accumulation, apple, high-light intensity, Malus domestica, post-transcriptional regulation, TCP transcription factor.

Introduction

Anthocyanins are a type of water-soluble pigment that are found in plant flowers, fruits, stems, leaves, and seeds. In recent decades, anthocyanin biosynthesis has been studied extensively due to the important antioxidant properties of these compounds and their effects on the appearance of commercial plant produce. Anthocyanins are synthesized through the phenylpropanoid pathway, in which a series of enzymes play key roles, including dihydroflavonol 4-reductase (DFR), UDP flavonoid glucosyl transferase (UF3GT), chalcone isomerase (CHI), and chalcone synthase (CHS) (Winkel-Shirley, 1999; Hichri et al., 2011). It is well established that anthocyanin biosynthesis is regulated by the WD40-bHLH-MYB complex at the transcription level through the direct mediation of the expression of its biosynthetic genes (Carbone et al., 2009; Hichri et al., 2011). External stimuli, such as temperature, light, water, nutrients, and exogenous hormones, also play key roles in the regulation of anthocyanin biosynthesis (Winkel-Shirley, 2001, 2002; Carbone et al., 2009; Jaakola, 2013; Honda and Moriya, 2018).

Light is one of the most important environmental factors regulating anthocyanin biosynthesis (Takos et al., 2006; Jaakola, 2013). Numerous studies have demonstrated that light stimulates the accumulation of anthocyanin and that its biosynthesis does not occur in the dark (Meng et al., 2004; Azuma et al., 2012; Zoratti et al., 2014; Guan et al., 2016; Jiang et al., 2016). The light quality also affects biosynthesis, especially ultraviolet
and blue light (Ordidge et al., 2012; Henry-Kirk et al., 2018; Tao et al., 2018; Zhang et al., 2018a). Studies on apple, strawberry, pear, and peach have shown that ultraviolet and blue light treatments promote the expression of transcription factors (TFs) such as MYB, BBX, ERF, and HY5, which in turn activate the expression of anthocyanin biosynthetic genes and ultimately lead to increased anthocyanin levels (Ubi et al., 2006; Gong et al., 2015; Henry-Kirk et al., 2018; Zhang et al., 2018a; An et al., 2019a; Fang et al., 2019; Ni et al., 2019). Light intensity also affects anthocyanin biosynthesis (Jaakola, 2013; Trojak and Skowron, 2017; Zhang et al., 2018b). Previous studies have suggested that TCP and MYB TFs may play roles in anthocyanin accumulation and may be mediated by conditions of different light intensity (Takos et al., 2006; Viola et al., 2016).

In many species, MYB1 and its orthologs (MYB10 and MYBA) play key roles in the regulation of anthocyanin biosynthesis, and they are known to be positive regulators of accumulation that is mediated by various environmental and hormone signaling pathways (Takos et al., 2006; Ban et al., 2007; Espley et al., 2007; Lin-Wang et al., 2011; Niu et al., 2010; Telias et al., 2011; Schwinn et al., 2016; Yi et al., 2018). In apple (Malus domestica), overexpression of MdMYB1 promotes accumulation by activating the transcripts of anthocyanin biosynthetic genes such as MdDFR and MdUF3GT (Takos et al., 2006; Ban et al., 2007; Espley et al., 2007). Previous studies have demonstrated that MdMYB1 can coordinate with MdbHLH3, MdBFR3, MdBZIP44, MdwRKY40, and MdBFR3 to regulate the biosynthesis of anthocyanin that is promoted by low temperature, ethylene, abscisic acid (ABA), wounding, and drought stress, respectively (Xie et al., 2017; An et al., 2018c, 2018d, 2019c, 2020). In addition, MdMYB1 undergoes ubiquitination and degradation, that is mediated by MdCOP1 and MdBTA2 in response to multiple hormonal and environmental signals (Li et al., 2012; Wang et al., 2018).

The teosinte branched1/cycloidea/proliferating (TCP) family encodes specific TFs that feature a TCP domain with a bHLH motif (Cubas et al., 1999; Manassero et al., 2013; Nicolas and Cubas, 2016). In Arabidopsis, 24 TCP proteins have been identified and categorized into two classes based on the presence of the TCP motif (Cubas et al., 1999; Martín-Trillo and Cubas, 2010; Manassero et al., 2013). TCP family proteins have also been discovered in other plant genomes, including rice and apple (Yao et al., 2007; Xu et al., 2014). In apple, they are organized into three classes based on differences in their sequences (Xu et al., 2014). As TFs, TCP proteins regulate the expression of target genes by binding to specific promoter sequences [e.g. GGNCCAC, GGNCC, GCCCR, or G(T/C)GGNCCC; Aggarwal et al., 2010]. TCP proteins have also been documented to function by directly interacting with other proteins have been widely studied, this is not the case for their transcriptional and post-transcriptional regulation mechanisms. There are just a few studies that have shown that TCP proteins may be regulated by miR319 (Schommer et al., 2014; Bresso et al., 2018; Palatnik and Weigel, 2019, Preprint).

In the present study, we found that MdTCP46 acted as a positive regulator of anthocyanin biosynthesis induced by high light intensity in apple. MdTCP46 expression was induced and degradation of the protein was delayed after treatment with high light intensity. MdTCP46 was also found to interact with MdMYB1, and this was essential for anthocyanin biosynthesis induced at high light intensity. MdTCP46 enhanced the binding activity of MdMYB1 to its target gene promoters. In addition, MdTCP46 directly interacted with MdBTA2, which is a negative modulator of anthocyanin biosynthesis. The expression of MdBTA2 was suppressed by high light intensity at both the transcriptional and post-translational levels. MdBTA2 suppressed the role of MdTCP46 by inducing its ubiquitination, resulting in subsequent degradation via the 26S proteasome pathway. Our findings uncover a potential regulatory mechanism of anthocyanin biosynthesis in apple based on the functioning of the BT2-TCP46-MYB1 protein module under different light intensities.

Materials and methods

Plant material and treatments

Uncolored fruit of apple (Malus domestica) ‘Red Delicious’ at 120 d after full bloom were collected from mature trees in an orchard located in Tai’an (Shandong, China). To examine the effects of different light intensities on anthocyanin accumulation in the peel, the fruit were divided into four groups and stored in incubators at 22 °C under different light conditions. The first group was stored with no light. The other groups were stored with 16/8 h photoperiods at either low light (~80 μmol m$^{-2}$

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Preparation of callus and apple tissues

Callus and apple tissues were prepared as described previously (An et al., 2018b, 2019a). Detached leaves and fruits were collected from different light conditions: low light (~50 μmol m−2 s−1), moderate light (~150 μmol m−2 s−1), and high light (~300 μmol m−2 s−1). The tissues were frozen in liquid nitrogen and stored at −80°C for subsequent experiments.

Plasmid construction

The ORFs of MdTCP3, MdTCP12, MdTCP46, and MdTCP46 were fused to pGAD424 to generate MdTCP3-pGAD, MdTCP12-pGAD, MdTCP46-pGAD, and MdTCP46-pGAD. The ORFs of MdMYB1 and MdBT2 were fused to pET32a or pGEX 4T-1 to generate MdTCP46-pGEX4T-1, MdTCP46-pET32a, MdMYB1-pET32a, and MdBT2-pGEX4T-1. The ORFs of MdTCP3, MdMYB1, and MdBT2 were fused to YFP & with the antitope (Antit). MdMYB1-Anti, and MdBT2-Anti, the sequences of MdTCP3, MdMYB1, and MdBT2 were inserted into the pCXSN vector. All primers are listed in Supplementary Table S3.

Measurement of anthocyanin contents

Anthocyanin was extracted using an extraction buffer containing anhydrous ethanol and hydrochloric acid as previously described (An et al., 2018a, 2019a). In brief, samples were placed in the extraction buffer for 5 h. After centrifugation, the supernatant was collected, and its absorption values at 530, 620, and 650 nm were determined using a spectrophotometer (Soptop, Shanghai, China) (An et al., 2018a, 2019a).

Quantitative real-time PCR

RNA extraction was performed using a RNAplant plus Reagent (Tiangen) as previously described (An et al., 2018a, 2020). Quantitative real-time PCR (qRT-PCR) was conducted to identify transgenic material and to detect MdTCP46 and MdBT2 transcripts. To determine the light responses of MdTCP46 and MdBT2, fruit of ‘Red Delicious’ were sampled at 120 d after full bloom and kept in the dark for different light-intensity conditions. Three biological replicates and three technical replicates were used in all qRT-PCR reactions. All primers are listed in Supplementary Table S3.

Yeast two-hybrid assays

The pGAD424 and pGBT9 vectors (Clontech) were used to perform yeast two-hybrid (Y2H) assays. The pGAD424 vector contained a GAL4 activation domain, and the pGBT9 vector contained a GAL4 binding domain. The ORFs of the MdTCP46 and MdMYB1 sequences were cloned into the pGAD424 and pGBT9 vectors, respectively. MdTCP3-pGAD, MdTCP12-pGAD, MdTCP21-pGAD, MdTCP46-pGAD, MdMYB1-pGAD, MdBT2-pG AD, MdBT2-N-pGAD, and MdBT2-C-pGAD were constructed as described above. The genetic transformation of Y2H Gold yeast cells (Clontech) was accomplished by PEG induction. Transformed yeast cells were cultured on a selective medium for 3 d, and Y2H assays were performed as previously described (An et al., 2019b, 2020). The pGAD424 and pGBT9 empty vectors were used as negative controls.

Pull-down assays

The pET32a and pGEX4T-1 vectors (Novagen) were used to perform pull-down assays. The pET32a vector contained a HIS tag, and pGEX4T-1 contained a GST tag. MdTCP46-pGEX4T-1, MdTCP46-pET32a, MdMYB1-pET32a, and MdBT2-pET32a were constructed as described above. The fusion proteins of MdTCP46-GST, MdTCP46-YFP, MdMYB1-GST, and MdBT2-YFP were constructed as described above. The fusion proteins of MdTCP46 and MdBT2 were expressed and purified as described above. The eluted solution was treated with HIS and GST antibodies (Abmart, Shanghai, China) and pull-down assays were performed as previously described (An et al., 2019b, 2020).

Bimolecular fluorescence complementation assays

The pSPYNE-35S/pUC-SPYNE and pSPYCE-35S/pUC-SPYCE vectors (Clontech) were used to perform bimolecular fluorescence complementation (BiFC) assays. The pSPYNE-35S/pUC-SPYNE vector contained a YFP & domain, and the pSPYCE-35S/pUC-SPYCE vector contained a YFP β domain. MdTCP46-YFP & , MdTCP46-YFP β, MdMYB1-YFP & , and MdBT2-YFP β were constructed as described above. Onion epidermis was incubated with an Agrobacterium carrying recombinant plasmids and visualized by fluorescence microscopy. The BiFC interactions were confirmed by fluorescence microscopy.

Screening of proteins interacting with MdMYB1

An apple gene library and the MdMYB1-pGAD plasmid were prepared to screen MdMYB1-interacting proteins using a Y2H system, as previously described (An et al., 2018a). Specific protein interactions were identified using a Y2H assay. The interacting proteins were listed in Supplementary Table S3.

Cloning of MdMYB1

The MdMYB1 cDNA was cloned from apple leaf cDNA using specific primers (An et al., 2018b, 2019a). The MdMYB1 cDNA was generated by RT-PCR using specific primers and was cloned into the pGAD424 vector. The MdMYB1 expression plasmid was transformed into yeast cells, and the interactions were confirmed by Y2H assays.

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one-to-one Y2H assays. Only MdTCP46 was found to be a direct interacting protein with MdMYB1.

Electromobility shift assays

Electromobility shift assays (EMSAs) were conducted to determine the effects of MdTCP46 on the binding activity of MdMYB1 to the promoters of MdDFR and MdUF3GT. MdTCP46-HIS and MdMYB1-HIS fusion proteins were obtained by IPTG-mediated induction. The probe was labeled with biotin by the Sangon Biotech Co. (Shanghai, China). Designated proteins and probes were mixed in the binding buffer for 25 min. The binding and free probes were separated by acrylamide gel, and EMSAs were performed as previously described (An et al., 2018d).

 Detection of protein ubiquitination

DMSO for 0.5 h. DMSO alone was used as the blank control. Proteins from calli were pre-treated with 100 µM MG132 dissolved in DMSO for 0.5 h. DMSO alone was used as the blank control. Samples were taken at 0–6 h of incubation and protein residues were determined using a GST antibody (Abmart). Protein degradation assays in vitro

To determine the effects of different light intensities on the stability of the MdTCP46 and MdBT2 proteins, fruit of ‘Red Delicious’ (120 d after full bloom) were subjected to the low- and high-light treatments for 3 d. The peels were collected, and total proteins were extracted and incubated with the MdTCP46-GST and MdBT2-HIS fusion proteins. Samples were taken at 0–6 h of incubation and protein residues were determined using HIS and GST antibodies (Abmart).

To determine the effects of MdBT2 on the stability of the MdTCP46 protein, WT and transgenic apple calli extracts were incubated with the MdTCP46-GST protein. Samples were taken at 0–6 h of incubation and protein residues were determined using a GST antibody (Abmart). Protein degradation assays in vitro were performed as previously described (An et al., 2019b, 2020). Briefly, calli were ground in liquid nitrogen, soaked with protein extraction liquid, and the supernatant was obtained following centrifugation.

To examine the effects of the MG132 proteasome inhibitor, total proteins from calli were pre- treated with 100 µM MG132 dissolved in DMSO for 0.5 h. DMSO alone was used as the blank control.

Detection of protein ubiquitination in vivo

Detection of protein ubiquitination in vivo was performed using a Pierce™ Co-Immunoprecipitation Kit (ThermoFisher Scientific). In brief, the MdTCP46-GFP protein was extracted from apple calli using a GFP antibody (Abmart) and immunoprecipitated proteins were examined using ubiquitin and GFP antibodies (Abmart). Protein ubiquitination detection in vivo was performed as previously described (An et al., 2019c).

Statistical analyses

All experiments were conducted three times with consistent results, and data are presented for one representative experiment. Experimental results were analysed using the DPS v7.05 software by one-way ANOVA and LSD post hoc tests.

Accession numbers

Sequence data in this study can be obtained from the apple gene function and gene family (https://gdfb.sdu.edu.cn; https://www.rosaceae.org/node/1), TAIR (https://www.arabidopsis.org/), and NCBI (https://www.ncbi.nlm.nih.gov/) databases under the following accession numbers: MdTCP3 (MDP0000243495), MdTCP12 (MDP0000173048), MdTCP46 (MDP0000351994), MdTCP21 (MDP0000581695), MdBT2 (MDP0000151000), MdMYB1 (MDP0000259614), MdDFR (MDP0000454976), and MdUF3GT (MDP0000405396).

Results

MdMYB1 acts as a positive regulator of anthocyanin accumulation under high light intensity

It has been demonstrated that light promotes anthocyanin accumulation in apple, strawberry, pear, and peach (Ubi et al., 2006; Gong et al., 2015; Henry-Kirk et al., 2018; Zhang et al., 2018b; An et al., 2019a; Fang et al., 2019; Ni et al., 2019). In this study, uncolored ‘Red delicious’ apple fruit were stored in incubators under different conditions of light intensity. Compared to storage under constant dark conditions, fruit consistently turned red after treatment with light, and higher light intensities clearly up-regulated the expression of genes related to anthocyanin biosynthesis, resulting in higher contents (Supplementary Fig. S1A–C).

MdMYB1 is known to be involved in anthocyanin biosynthesis, and overexpression of MdMYB1 contributes to its accumulation in apple (Takos et al., 2006; Ban et al., 2007; Espley et al., 2007). We constructed a MdMYB1-antisense suppression plasmid and transformed it into apple calli (Supplementary Fig. S2A). In the wild-type (WT) plants, moderate and high light intensities progressively and significantly increased anthocyanin accumulation compared to low-light conditions (Supplementary Fig. S1D, E). In contrast, MdMYB1–Ant plants showed no difference between low and moderate light, and only a relatively small increase in accumulation under high-light conditions. These results indicated that MdMYB1 was essential for the induction of anthocyanin biosynthesis under high light intensity.

MdTCP46 interacts with MdMYB1

We conducted a search for proteins that interact with MdMYB1 in a yeast screening library. The MdMYB1 protein with the autonomously activated domains removed was fused to pGBT9 to generate MdMYB1△-pGAD. An apple TCP protein was obtained (An et al., 2018b) and a NCBI BLAST search showed it to be a TCP15-like protein (Supplementary Fig. S3). We named it as MdTCP46, according the systematic classification of the apple TCP family (Xu et al., 2014).

The physical interaction between MdTCP46 and MdMYB1 was confirmed by three different assays (Fig. 1). First, MdTCP46–pGAD and MdMYB1△-pGBD were constructed and transformed into Gold yeast cells for Y2H assays. Only yeast cells with both MdTCP46 and MdMYB1 grew normally on selective medium (–T/–L/–H/–A) (Fig. 1A), suggesting a direct interaction. Second, fusion proteins were prepared for pull-down assays and the results indicated that MdTCP46-GST was pulled-down by MdMYB1-HIS (Fig. 1B). Third, BiFC assays were developed that linked MdTCP46 with the N-terminus of YFP (MdTCP46-YFP) and MdMYB1 with the C-terminus of YFP (MdMYB1–YFP). The resulting
fluorescence signals showed that MdTCP46 interacted with MdMYB1 in the nucleus (Fig. 1C). Collectively, these data indicated that MdTCP46 physically interacts with MdMYB1.

**MdTCP46 promotes anthocyanin biosynthesis**

MdTCP46 is a TCP family protein and was found to contain a conserved bHLH domain at the N terminus (Fig. 2A). Compared to TCP proteins from different species, it exhibited the highest homology with the *Pyrus bretschneideri* protein, PbTCP.

When apple fruit were stored under different light intensities, qRT-PCR analysis indicated that moderate and high light significantly induced the expression of *MdTCP46* (Fig. 2B). The abundance of the *MdTCP46* protein was also examined. *MdTCP46*-GST, an *E. coli* fusion protein, was probed with GST antibodies under either low or high light treatment. The results indicated that high light delayed *MdTCP46* protein degradation (Fig. 2C). Furthermore, inclusion of the MG132 proteasome inhibitor blocked degradation completely, indicating that *MdTCP46* is degraded by the 26S-proteasome machinery. These results suggested that *MdTCP46* was responsive to high light intensity at both the transcriptional and post-translational levels.

To assess the biological role of *MdTCP46* in the regulation of anthocyanin biosynthesis, transgenic apple fruit and Arabidopsis were generated (Supplementary Fig. S2B). The *MdTCP46*-pIR construct was used to generate *MdTCP46*-overexpression (−OX) lines and the *MdTCP46*-TRV construct was used to generate *MdTCP46*-antisense (−Anti) lines. Compared to the WT controls, overexpression of *MdTCP46* in both apple fruit and Arabidopsis seedlings clearly increased anthocyanin accumulation whilst antisense suppression decreased it (Fig. 2D–I). These results indicate an essential and positive role of *MdTCP46* in anthocyanin accumulation.

**MdTCP46 is essential for anthocyanin biosynthesis mediated by high light intensity**

We generated stable transgenic apple calli and transient transgenic leaves with either overexpression of *MdTCP46* (*MdTCP46*-OX) or with antisense suppression of *MdTCP46*.
Fig. 2. Overexpression of MdTCP46 promotes anthocyanin biosynthesis in apple. (A) Multiple alignment of the bHLH domain in 10 TCP proteins obtained from the NCBI database. PbTCP, Pyrus × bretschneideri (XP_009360352.1); PnTCP, Prunus mume (XP_008221641.1); RcTCP, Rosa chinensis (XP_024199581.1); FvTCP, Fragaria vesca (XP_004297311.1); PaTCP, Parasponia andersonii (PON70380.1); JrTCP, Juglans regia (XP_018817718.1); SITCP, Sesamum indicum (XP_011071008.1); TcTCP, Theobroma cacao (XP_017969954.1); MdTCP46, Malus × domestica (MDP0000319941); AtTCP15, Arabidopsis thaliana (AT1G69690.1). The bHLH motif is indicated. (B) Transcription of MdTCP46 under different light intensities using qRT-PCR. Plants were treated for 3 d and expression was normalized to the actin gene. Values are relative to the low-light treatment, which was set as 1. (C) Detection of the MdTCP46-GST fusion protein after dark and moderate light treatments. ‘Red Delicious’ apple fruit were treated with low or high light for 3 d. Total proteins were extracted from the peels and were incubated with purified MdTCP46-GST protein for 0–6 h. For treatment with the proteasome inhibitor MG132, total proteins of fruit subjected to low light were pre-treated with 100 µM MG132 for 0.5 h before the sampling began. ACTIN was used as an internal reference. (D) Apple peel injection assays. Uncolored ‘Red Delicious’ fruit were injected with mixed vectors or A. tumefaciens solutions and stored in a phytotron under the high-light treatment for 3 d. pIR, IL60-1+IL60-2; MdTCP46-pIR, IL60-1+MdTCP46-IL60-2; TRV, TRV1+TRV2; MdTCP46-TRV, TRV1+MdTCP46-TRV2. pIR are overexpressors (OX); TRV are antisense suppressors (Anti). (E) Anthocyanin contents of the fruit peels shown in (D). Contents are expressed relative to pIR, the value of which was set as 1. (F) Expression of genes related to anthocyanin biosynthesis in the fruit peels shown in (D) as determined by qRT-PCR. Expression was normalized to the actin gene. Values are expressed relative to pIR, which were set as 1. (G) qRT-PCR detection of MdTCP46 expression levels in Arabidopsis Col-0 seedlings and in overexpressing transgenic lines. The lower panels show the ACTIN gene, which was used as the internal control. (H) Phenotypes of Arabidopsis Col-0 and MdTCP46-overexpressing lines and (I) relative expression of MdTCP46. Expression was normalized to the ACTIN gene. Values are expressed relative to Col-0, which was set as 1. All experiments were performed three times with similar results, and representative data from one experiment are shown. Data are means (±SD), n=6. Different letters indicate significant differences as determined by one-way ANOVA and LSD tests (P<0.05). (This figure is available in colour at JXB online.)
(MdTCP46-Anti) (Supplementary Fig. S2C, D). Compared to the WT controls, overexpression of MdTCP46 promoted anthocyanin accumulation in both the calli and leaves, whilst antisense suppression of MdTCP46 reduced it (Fig. 3). The expression of five genes related to anthocyanin biosynthesis were examined in the calli, namely MdMYB1, MdDFR, MdUF3GT, MdCHI, and MdCHS. All five were expressed at higher levels when MdTCP46 was overexpressed (Fig. 3C). The effect of MdTCP46 expression on anthocyanin accumulation was more obvious as light intensity increased (Fig. 3A, B, D, E), indicating that it was essential for accumulation mediated by high light intensity.

**MdTCP46 promotes anthocyanin biosynthesis when MdMYB1 is suppressed**

Since both MdTCP46 and MdMYB1 play positive roles in anthocyanin biosynthesis induced by high light intensity in apple, we examined the functional relationship between them. Transgenic apple calli were generated to overexpress MdTCP46

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**Fig. 3.** MdTCP46 promotes light-induced anthocyanin biosynthesis in apple. Transgenic apple calli (15 d old) and leaves of MdTCP46-overexpressing (-OX) and MdTCP46-antisense (-Anti) were subjected to different light intensities for 5 d. (A) Phenotypes of calli of the transgenic lines and the wild-type (WT) and (B) their anthocyanin contents. The content of low light-treated WT was used as a reference and set to 1. (C) The expression of genes related to anthocyanin biosynthesis in apple calli as determined by qRT-PCR. The expression of the WT was used as a reference and set to 1. (D) Phenotypes of transient transgenic leaves the empty-vector control (EV) and (E) their anthocyanin contents. The content of low light-treated EV was used as a reference and set to 1. All experiments were performed three times with similar results, and representative data from one experiment are shown. Data are means (±SD), n=4. Different letters indicate significant differences as determined by one-way ANOVA and LSD tests (P<0.05). (This figure is available in colour at JXB online.)
in a MdMYB1-suppression background (i.e. MdTCP46-OX/MdMYB1-Anti) (Supplementary Fig. S2A). Control samples expressing the MdTCP46-OX or MdMYB1-Anti constructs individually showed increased and decreased anthocyanin accumulation, respectively (Fig. 4A, B), whilst samples expressing both the constructs showed increased accumulation, but to a lesser extent than observed in MdTCP46-OX. These results indicated that MdTCP46 promoted anthocyanin biosynthesis when MdMYB1 was suppressed.

MdTCP46 enhances the binding activity of MdMYB1 to its target gene promoters

To determine the functions of MdTCP46 and MdMYB1 during anthocyanin biosynthesis, we examined their roles in binding to the promoter sequences of target genes. First, EMSAs were developed using the MdTCP46-HIS and MdMYB1-HIS fusion proteins and the biotin-labeled probes of MdDFR and MdUF3GT (Fig. 4C, D; Supplementary Tables S1, S2). Mutated forms of the MdDFR-Mut and MdUF3GT-Mut probes were used as negative controls. The results showed that MdMYB1-HIS alone was able to bind to the MdDFR-Probe and MdUF3GT-Probe, which was consistent with previous studies (Takos et al., 2006; Ban et al., 2007; Espley et al., 2007). However, the binding became stronger in the presence of MdTCP46-HIS fusion proteins (Fig. 4C, D), indicating that MdTCP46 could improve the binding activity of MdMYB1 to the MdDFR and MdUF3GT promoters.

Dual-luciferase assays were also conducted to support the EMSA observations. MdTCP46 and MdMYB1 were expressed separately under the control of 35S promoters on the pGreenII 62-SK plasmid vector as effectors, and LUC was used as a reporter behind the MdDFR or MdUF3GT promoter in the pGreenII 0800-LUC plasmid vector (Fig. 4E). It was clear that MdMYB1 alone was able to drive the expression of the luciferase gene, while MdTCP46 could not (Fig. 4F, G). LUC expression was increased significantly when the constructs expressing both proteins were present, suggesting that MdTCP46 enhanced the binding activity of MdMYB1 to the MdDFR and MdUF3GT promoters.

MdBT2 physically interacts with MdTCP46

We next attempted to identify the potential protein interacting partners of MdTCP46. The bric-a-brac/tramtrack/broad (BTB) family protein MdBT2 has been identified and widely investigated as a repressor of anthocyanin accumulation (An et al., 2018a, 2018d, 2019a, 2019x, 2020; Wang et al., 2018). We therefore conducted three assays to verify the physical interaction between the MdTCP46 and MdBT2 proteins. First, MdTCP46 was cloned into the pGAD424 vector. MdBT2, its N-terminus (MdBT2-N), and C-terminus (MdBT2-C) were individually cloned into the pGBT9 vector (Fig. 5A). The results of Y2H assays revealed that only yeast cells co-transformed with both the full-lengths of MdTCP46 and MdBT2 survived on selective medium (Fig. 5A), indicating a direct interaction between MdTCP46 and MdBT2 in the cells. Second, pull-down assays were performed that utilized the E. coli expression of MdBT2-HIS and MdTCP46-GST fusion proteins (Fig. 5B). The polyhistidine binding resin pulled-down the MdBT2-HIS and MdTCP46-GST fusion proteins, but not when the GST tag was present alone (Fig. 5B). Thus, heterologously expressed MdTCP46 and MdBT2 proteins could interact in vitro. Third, BiFC assays were performed using onion epidermal cells, and the expression system was transformed with MdTCP46 linked to the C-terminus ofYFP (MdTCP46-YFPc), MdBT2 linked to the N-terminus ofYFP (MdBT2-YFPN), or both. The results showed that MdTCP46–YFPc interacted with MdBT2-YFPN in the nucleus (Fig. 5C). Collectively, these results suggested that MdTCP46 interacts with MdBT2 directly in vivo and in vitro.

MdBT2 is a negative regulator in anthocyanin biosynthesis induced by high light

MdBT2 has previously been identified to repress anthocyanin biosynthesis (An et al., 2018a; Wang et al., 2018). Here, we specifically examined the involvement of MdBT2 in anthocyanin biosynthesis mediated by different light intensities in apple. MdBT2-OX and MdBT2-Anti calli were generated (Supplementary Fig. S2E). The overexpression of MdBT2 reduced the accumulation of anthocyanin under high light, while suppression of MdBT2 increased anthocyanin levels under all the treatments but particularly under high light (Fig. 6A, B). These results confirmed that MdBT2 functions as a negative regulator of anthocyanin biosynthesis in apple and is especially important under high light conditions. Interestingly, the expression of MdBT2 seemed to be regulated by the different light intensities, and it was noticeably downregulated under the high intensity treatment (Fig. 6C). In vitro protein degradation assays using E. coli expressed MdBT2-HIS fusion proteins indicated that the high-light treatment accelerated the degradation of the MdBT2 protein (Fig. 6D), and this appeared to be mediated by 26S proteasome as the presence of the MG132 proteasome inhibitor completely abolished the degradation of the MdBT2-HIS fusion protein. Thus, high light clearly suppressed the expression of MdBT2 at both the transcriptional and post-translational levels.

MdBT2 suppresses the role of MdTCP46 in anthocyanin biosynthesis induced by high light by inducing its degradation

Given that MdTCP46 played a positive role and its interacting partner MdBT2 played a negative role in anthocyanin accumulation induced by high light (Figs 3, 6A, B), we examined the physiological relationship between these two genes. MdTCP46-OX was co-expressed with MdBT2-OX or MdBT2-Anti in apple calli and leaves under high light (Supplementary Fig. S2F–H). Compared with the control, all the transgenic plants showed significant increases in anthocyanin content in both calli and leaves (Fig. 7). The greatest increases were observed when expression of MdBT2 was suppressed, whilst overexpression of MdBT2 and MdTCP46 together resulted in anthocyanin levels that were lower than when MdTCP46 was overexpressed alone (Fig. 7B, D). These
Fig. 4. MdTCP46 enhances the transcriptional activity of MdMYB1 on MdDFR and MdUF3GT. (A, B) Apple calli (15 d old) of the wild-type (WT), MdTCP46-overexpression (-OX), MdMYB1-antisense (-Anti), and MdTCP46-OX/MdMYB1-Anti (overexpression of MdTCP46 in the background of MdMYB1-Anti) were subjected to high light for 5 d. (A) Phenotypes and (B) anthocyanin levels. The content of the WT was used as a reference and set to 1. (C, D) Electromobility shift assays using MdDFR and MdUF3GT promoter probes. The MBS sequences for MdMYB1 binding and their equivalent mutant forms (-Mut) are underlined. + indicates the presence of corresponding proteins or probes, and − indicates the absence of corresponding proteins. 2× and 3× indicate increased protein contents. (E) Schematic representation of the LUC reporter vector containing the MdDFR and MdUF3GT promoters and effector vectors expressing MdTCP46 or MdMYB1 under the control of the 3SS promoters. The ORFs of MdTCP46 and MdMYB1 were fused to the pGreenII 62-SK vector. The promoter sequences of MdDFR and MdUF3GT were cloned into the pGreenII 0800-LUC vector. (F, G) LUC/REN activities detected by the reporter systems described in (E), which tested the effects of MdMYB1, MdTCP46, and MdMYB1-MdTCP46 on the expression of (F) MdDFR and (G) MdUF3GT. Empty vector, pGreenII 62-SK + pGreenII 0800-LUC; pMdDFR, pGreenII 62-SK + proMdDFR-pGreenII 0800-LUC; pMdUF3GT, pGreenII 62-SK + proMdUF3GT-pGreenII 0800-LUC; MdMYB1-pGreenII 62-SK + proMdDFR-pGreenII 0800-LUC; MdMYB1-pMdUF3GT, pGreenII 62-SK + proMdUF3GT-pGreenII 0800-LUC; MdMYB1+pMdDFR, pGreenII 62-SK + proMdDFR-pGreenII 0800-LUC; MdMYB1+pMdUF3GT, pGreenII 62-SK + proMdUF3GT-pGreenII 0800-LUC; MdTCP46+pGreenII 62-SK + proMdDFR-pGreenII 0800-LUC; MdTCP46+pMdUF3GT, pGreenII 62-SK + proMdUF3GT-pGreenII 0800-LUC; MdTCP46+MdMYB1+pMdDFR, pGreenII 62-SK + proMdDFR-pGreenII 0800-LUC; MdTCP46+MdMYB1-pMdUF3GT, pGreenII 62-SK + proMdUF3GT-pGreenII 0800-LUC. LUC/REN activities of the empty vector were used as references and set to 1. All experiments were performed three times with similar results, and representative data from one experiment are shown. Data are means (±SD), n=3. Different letters indicate significant differences as determined by one-way ANOVA and LSD tests (P<0.05). (This figure is available in colour at JXB online.)
results suggested that \textit{MdTCP46} plays a positive role whilst \textit{MdBT2} plays a negative role in anthocyanin accumulation under high-light conditions. In addition, \textit{MdBT2} suppressed the positive role played by \textit{MdTCP46}.

To further explore the interaction mechanisms between \textit{MdTCP46} and \textit{MdBT2}, in \textit{vitro} protein degradation assays were conducted. The \textit{MdTCP46-GST} fusion protein was incubated with total protein extracts from \textit{MdBT2-OX} and \textit{MdBT2-Anti calli}. Consistent with the patterns of anthocyanin accumulation (Fig. 7A, B), degradation of the \textit{MdTCP46-GST} fusion protein was facilitated by \textit{MdBT2} overexpression, but delayed by \textit{MdBT2} suppression (Fig. 8A). This effect was abolished by the presence of MG132, indicating that the protein degradation was through the 26S proteasome pathway. Indeed, when the \textit{MdTCP46-GFP} fusion protein was incubated with protein extracts from \textit{MdBT2-OX} calli, strong ubiquitination patterns were observed using Ubi and GFP antibodies with various forms of ubiquitinated \textit{MdTCP46-GFP} proteins being detected (Fig. 8B), indicating that a considerable amount of the fusion proteins had been partially degraded and lost the GFP tag. This was consistent with the fact that \textit{MdBT2} suppressed the function of \textit{MdTCP46} by facilitating its degradation through the 26S proteasome pathway.

\textbf{MdTCP46 specifically interacts with the \textit{MdMYB1} and \textit{MdBT2} proteins}

There are several TCP family proteins, some of which may have similar or opposing regulatory functions in the same signaling pathways (Li and Zachgo, 2013; Viola \textit{et al.}, 2016). When we screened the \textit{MdMYB1}-interacting proteins, \textit{MdTCP3}, \textit{MdTCP12}, and \textit{MdTCP21} were screened out (Xu \textit{et al.}, 2014). Therefore, we cloned these three TCP proteins. In contrast to \textit{MdTCP46}, none of them exhibited direct interactions with \textit{MdMYB1} and \textit{MdBT2} in the Y2H assays (Supplementary Fig. 5).
An et al. S4). Hence, MdTCP46 seems to specifically interact with the MdMYB1 and MdBT2 proteins.

**Discussion**

Anthocyanin biosynthesis is regulated by various environmental stimuli (Winkel-Shirley, 2001, 2002; Carbone et al., 2009; Jaakola, 2013; Honda and Moriya, 2018), and light, especially at high intensity, has been implicated in promoting biosynthesis in many species including apple (Jaakola, 2013; Trojan and Skowron, 2017; Zhang et al., 2018a). A positive correlation exists between anthocyanin accumulation and light intensity (Jaakola, 2013; Trojan and Skowron, 2017; Zhang et al., 2018a). Although many proteins have been shown to participate synergistically in light-modulated anthocyanin accumulation, including MYB, BBX, ERF, and HY5 (Ubi et al., 2006; Gong et al., 2015; Henry-Kirk et al., 2018; Zhang et al., 2018a; An et al., 2019a; Fang et al., 2019; Ni et al., 2019), the underlying molecular mechanisms are not fully understood. Here, we found that MdTCP46 and MdMYB1 play positive roles in anthocyanin biosynthesis induced by high light. In addition, the BTB-domain protein MdBT2 acted as a repressor of anthocyanin biosynthesis under different light intensities by regulating the stability of the MdTCP46 protein.

MdMYB1 is known to be responsive to light treatment in apple (Takos et al., 2006; Ban et al., 2007; Espley et al., 2007). The importance of MdMYB1 in light-induced anthocyanin biosynthesis has been well established, and it functions by directly activating the expression of genes related to biosynthesis,
such as DFR and UF3GT (Takos et al., 2006; Ban et al., 2007; Espley et al., 2007; An et al., 2018d, c 2019c, 2020). In our current study, the promotion of anthocyanin accumulation by different light intensities was confirmed in apple fruit and calli (Supplementary Fig. S1). MdMYB1 was shown to play a key role, as suppression of MdMYB1 resulted in a significant decrease in the accumulation of anthocyanin in apple calli under different light intensities.

In Arabidopsis, overexpression of TCP15 decreases anthocyanin biosynthesis and high light inactivates the TCP15 protein (Viola et al., 2016), whilst TCP3 plays a positive role in the regulation of biosynthesis by interacting with MYB proteins (Li and Zachgo, 2013). Here, we demonstrated that MdTCP46 interacted with MdMYB1 in Y2H, pull-down, and BiFC assays (Fig. 1).

The transcription of MdTCP46 was stimulated as light intensity increased (Fig. 2B), and the high light treatment dramatically increased the stability of MdTCP46 (Fig. 2C). This indicates that high light intensity affects the expression of MdTCP46 at both the transcriptional and post-transcriptional levels. We found that MdTCP46 positively regulated anthocyanin accumulation in response to different light intensities by up-regulating the expression of biosynthesis genes (Fig. 2D–I; Fig. 3). Overexpression of MdTCP46 did not interfere with MdMYB1 expression (Fig. 2F, 3C), which prompted us to examine whether MdTCP46 affected the binding activity of MdMYB1 to its target genes. Previous reports have indicated that MdMYB1 is able to bind to the promoter fragments of DFR and UF3GT (Takos et al., 2006; Ban et al., 2007; Espley et al., 2007; An et al., 2018d, 2019c, 2020). As expected, we found that interaction with MdTCP46 did indeed facilitate the binding activity of MdMYB1 to its target genes (Fig. 4C–G), providing evidence that this interaction contributes to anthocyanin biosynthesis induced by high light. Combining our results with those of previous studies, it appears that MdMYB1 integrates the accumulation of anthocyanin in response to multiple stresses by interacting with different hormones and environmental signal–response factors, such as the ABA–response factor MdbZIP44, the wounding–response factor MdWRKY40, the drought–response factor MdERF38, and the light–response factor MdTCP46 (Takos et al., 2006; Ban et al., 2007; Espley et al., 2007; An et al., 2018d, 2019c, 2020; Fig. 9B), in each of which MdMYB1 plays a core regulatory role. Therefore, it seems likely that there may be functional redundancy in these different response factors.

In Arabidopsis, AtTCP15 is a repressor of anthocyanin biosynthesis and may act by inducing the expression of a PAP1 repressor and other anthocyanin regulatory genes (Viola et al., 2016). Although MdTCP46 is a TCP15-like protein, it exhibits a different function to AtTCP15 as it is a positive regulator of anthocyanin biosynthesis and is involved in regulating synthesis by interacting with the MBW complex (Viola et al., 2016). Thus species differences in function may exist.

To date, the transcriptional and post-transcriptional regulation of TCP proteins have rarely been investigated. Several reports have showed that TCP proteins may be regulated by
miR319 (Schommer et al., 2014; Bresso et al., 2018; Palatnik and Weigel, 2019, Preprint). Since our high light intensity treatment improved the stability of the MdTCP46 protein (Fig. 2C), we examined interacting partners that might have potential roles in regulating this stability. The BTB protein MdBT2 was found to interact with MdTCP46 (Fig. 5). The expression of MdBT2 was inhibited as light intensity increased at both the transcriptional and post-transcriptional levels (Fig. 6C, D), which contrasted with the response pattern of MdTCP46 (Fig. 2B, C). We found that MdBT2 negatively regulated the biosynthesis of anthocyanin under high light intensity by degrading the MdTCP46 protein directly (Figs 7, 8). Based on these findings, we propose that MdBT2 mainly functions under low light conditions while high light intensity triggers the accumulation of MdTCP46, which is probably due to the degradation of MdBT2 that is promoted by high light.

A proposed model that summarizes the functioning of MdTCP46 in light-induced anthocyanin biosynthesis is shown in Fig. 9A. MdTCP46 improves the binding activity of MdMYB1 to the promoters of MdDFR and MdUF3GT by directly interacting with it, and thereby promotes the biosynthesis of anthocyanin that is induced by high light. Under low light intensity, MdBT2 ubiquitinates and degrades the MdTCP46 and MdMYB1 proteins, thus decreasing MdTCP46-promoted accumulation of anthocyanin. As light intensity increases, the expression of MdBT2 is inhibited while that of MdTCP46 is activated, thereby triggering high light intensity-induced anthocyanin biosynthesis. Thus, we speculate that MdBT2 may act as a dose-responder of light intensity. Our discovery of this ‘MdBT2–MdTCP46’ ubiquitination regulation module provides new insights that should aid future studies on the post-transcriptional regulation of TCP proteins.

BT2 is a key component of the BT2-CUL3-RBX1 ubiquitin ligase complex, and multiple proteins have been confirmed to be targets for ubiquitination by BT2 (Figueroa et al., 2005; Mandadi et al., 2009; Zhao et al., 2016; An et al., 2018; Wang et al., 2018). In addition to MdTCP46, different types of MdBT2-interacting proteins have been identified.

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**Fig. 8.** MdBT2 degrades the MdTCP46 protein. (A) Detection of MdTCP46-GST fusion proteins in stability assays. Protein extracts were taken from 15-d-old calli grown under dark conditions for the wild-type (WT) and transgenic lines of MdBT2-overexpression (-OX) and MdBT2-antisense (-Anti) and were treated for 0.5 h with either 100 µM of the proteasome inhibitor MG132 dissolved in DMSO or in DMSO alone (blank control), before being incubated with MdTCP46-GST protein for 0–6 h. ACTIN was used as an internal reference. (B) MdBT2 promotes the ubiquitination of the MdTCP46 protein in vivo. MdTCP46-GFP was immunoprecipitated using the GFP antibody from the two transgenic calli MdTCP46-GFP and MdTCP46-GFP/MdBT2-OX. The immunoprecipitated proteins were examined using antibodies for ubiquitin (left) and GFP (right).
Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Mediation of anthocyanin biosynthesis at different light intensities is dependent on MdMYB1.

Fig. S2. Identification of transgenic plant material by qRT-PCR.

Fig. S3. Protein sequence alignment of TCP proteins in apple and Arabidopsis.

Fig. S4. Yeast two-hybrid assays showing that MdTCP46 specifically interacts with MdMYB1 and MdBT2.

Table S1. The promoter sequence of MdDFR.

Table S2. The promoter sequence of MdUF3GT.

Table S3. Primers used for gene expression analysis and vector construction.

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Including MdMYB1, MdMYB9, MdMYB23, MdbHLH93, MdbHLH104, MdbBBX22, MdbZIP44, MdbWRKY40, and MdERF38 (Zhao et al., 2016; An et al., 2018a, 2018b, 2018d, 2019a, 2019b, 2019c, 2020; Wang et al., 2018). MdBT2 regulates the stability of these proteins through direct interactions and participates in multiple stress–response processes, suggesting that it may be a multifunctional fine-tuning protein that integrates a post-transcriptional regulatory network in response to multiple stress signals. The observations that different transcription factors function as the interaction proteins of both MdBT2 and MdMYB1 indicate that the ‘BT2-interacting proteins–MYB1’ module may be a central component of anthocyanin biosynthesis induced by multiple stresses (Fig. 9B).
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