Journal of Experimental Botany, Vol. 75, No. 22 pp. 7381–7400, 2024
https://doi.org/10.1093/jxb/erae399 Advance Access Publication 20 September 2024

RESEARCH PAPER

A negative feedback regulatory module comprising R3-MYB repressor MYBL2 and R2R3-MYB activator PAP1 fine-tunes high light-induced anthocyanin biosynthesis in Arabidopsis

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Received 5 May 2024; Editorial decision 16 September 2024; Accepted 19 September 2024

Editor: Matt Jones, University of Glasgow, UK

Abstract

Anthocyanins, a group of flavonoids, play diverse roles in plant growth and environmental adaptation. The biosynthesis and accumulation of anthocyanin are regulated by environmental cues, such as high light. However, the precise mechanism underlying anthocyanin biosynthesis under high light conditions remains largely unclear. Here, we report that the R3-MYB repressor MYB-LIKE 2 (MYBL2) negatively regulates high light-induced anthocyanin biosynthesis in Arabidopsis by repressing two R2R3-MYB activators, PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1) and PAP2, which are core components of the MYB–bHLH–WD40 (MBW) complex. We found that MYBL2 interacts with PAP1/2 and reduces their transcriptional activation activities, thus disrupting the expression of key genes involved in anthocyanin biosynthesis, such as *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*) and *TRANSPARENT TESTA 19* (*TT19*). Additionally, MYBL2 attenuates the transcriptional activation of PAP1 and its own expression, but not that of PAP2. Conversely, PAP1 collaborates with TRANSPARENT TESTA 8 (TT8), a bHLH member of the MBW complex, to activate *MYBL2* transcription when excessive anthocyanins are accumulated. Taken together, our findings reveal a negative feedback regulatory module composed of MYBL2 and PAP1 that fine-tunes high light-induced anthocyanin biosynthesis through modulating MBW complex assembly.

Keywords: Anthocyanin biosynthesis, high light, MBW complex, MYB-LIKE 2, PAP1/MYB75, PAP2/MYB90.

Introduction

Anthocyanins, a large group of plant flavonoids, confer vibrant colors such as blue, red, and purple to flowers, fruits, and other organs, which facilitates seed dispersal by attracting insect and avian pollinators and foragers (Grotewold, 2006; Miller *et al.*, 2011; Petroni and Tonelli, 2011). Anthocyanins play pivotal roles in bolstering plant defenses against pathogen attacks and abiotic stresses including high light (Rao *et al.*, 2019; Shi *et al.*, 2022), ultraviolet radiation (Bieza and Lois, 2001), drought (Nakabayashi *et al.*, 2014), salinity (Kim *et al.*, 2017; Li *et al.*, 2022), and cold (Xie *et al.*, 2012; Li *et al.*, 2017). Moreover, an

increasing body of reports has uncovered the beneficial impacts of anthocyanins on human health and their potential application in disease prevention owing to their excellent antioxidant capacity and reactive oxygen species scavenging ability (Petroni *et al.*, 2014; Santos-Buelga *et al.*, 2014).

Over the past few decades, extensive characterization of the anthocyanin biosynthetic pathway has been conducted in various plant species, including Arabidopsis (Carey *et al.*, 2004; Gonzalez *et al.*, 2008), petunia (*Petunia hybrida*; Quattrocchio *et al.*,1999; Spelt *et al.*, 2000, 2002; Albert *et al.*, 2011, 2014), maize (*Zea mays*; Cone *et al.*, 1986; Ludwig *et al.*, 1989), and snapdragon (*Antirrhinum majus*; Goodrich *et al.*, 1992; Schwinn *et al.*, 2006). The common anthocyanin biosynthetic pathway comprises multiple sequential steps. Initially, *p*-coumaroyl-CoA is generated from phenylalanine through a series of reactions catalysed by phenylalanine ammonia-lyase, cinnamate 4-hydroxylase, and 4-coumarate-CoA ligase. Subsequently, four early biosynthetic genes (*EBGs*) encoding chalcone synthase (CHS), chalcone isomerase, flavanone 3-hydroxylase, and flavonoid 3′-hydroxylase facilitate further catalytic processes leading to the production of leucoanthocyanidins such as dihydrokaempferol, dihydroquercetin, and dihydromyricetin. Furthermore, three late biosynthetic genes (*LBGs*) encoding dihydroflavonol 4-reductase (DFR), anthocyanin synthase/leucoanthocyanidin dioxygenase (ANS/LDOX), and anthocyanidin 3-*O*-glucosyltransferase/UDP-glucose:flavonoid 3-*O*-glucosyltransferase (3GT/UF3GT) mediate the conversion to anthocyanidin-3-*O*-glucoside. The cyanidins are subsequently modified with glycosyl, acyl, or methyl groups by corresponding transferases to form stable anthocyanins, and are transported into the vacuole by putative anthocyanin transporters like TRANSPARENT TESTA 19 (TT19), thereby conferring diverse colors to plant organs (Kitamura *et al.*, 2004; Koes *et al.*, 2005; Sun *et al.*, 2012).

The accurate expression of the biosynthetic genes (also known as structural genes) is crucial for anthocyanin biosynthesis and is tightly regulated by positive and negative regulators. In Arabidopsis, the R2R3-MYB transcription factors (TFs) MYB11, MYB12, and MYB111 activate the expression of *EBGs* (Stracke *et al.*, 2010), while the MBW transcription activation complex predominantly positively controls the transcription of *LBGs* and plays a central role in regulating anthocyanin biosynthesis (Borevitz *et al.*, 2000; Koes *et al.*, 2005; Gonzalez *et al.*, 2008). The canonical MBW complex consists of three types of proteins: R2R3-MYB TFs such as MYB75/PAP1, MYB90/PAP2, MYB113, MYB114, and MYB123/TT2; bHLH TFs like ENHANCER OF GLABRA3 (EGL3), GLABRA3 (GL3), and TRANSPARENT TESTA 8 (TT8); and a single WD40 protein, TRANSPARENT TESTA GLABRA 1 (TTG1). Among these components, the specificity and activity of the MBW complex towards target genes are mainly determined by the MYB and bHLH proteins, rather than TTG1; however, TTG1 is essential for assembly and function of the MBW complex (Baudry *et al.*, 2004; Ramsay and Glover, 2005; Gonzalez *et al.*, 2008; Dubos *et al.*, 2010; Jain and Pandey, 2018). Loss-of-function mutations in MBW component genes result in decreased expression of anthocyanin biosynthetic genes and compromised anthocyanin deposition (Gonzalez *et al.*, 2008), while the up-regulation or overexpression of MBW component genes leads to enhanced anthocyanin accumulation (Rowan *et al.*, 2009; S. Li *et al.*, 2016; Lim *et al.*, 2022). Additionally, several negative regulators have been identified that repress anthocyanin biosynthesis by impairing the transcription of these anthocyanin biosynthetic genes. In Arabidopsis, R2R3-MYBs, such as MYB4, MYB7, and MYB32, and R3-MYBs, like CAPRICE (CPC) and MYBL2, have been proposed to compete with PAP1/2 for binding bHLH TFs, thereby interfering with the formation of the MBW complex and suppressing the transcription of anthocyanin biosynthetic genes (Dubos *et al.*, 2008; Matsui *et al.*, 2008; Zhu *et al.*, 2009; Wang *et al.*, 2020). In petunia, the R2R3-MYB protein PhMYB27 functions as a non-canonical component of the MBW complex and a repressor of anthocyanin biosynthesis and accumulation by down-regulating the expression of both the biosynthetic genes and the bHLH TF gene *ANTHOCYANIN1* (*AN1*), which is another crucial MBW component, through its C-terminal EAR motif (Albert *et al.*, 2011, 2014). The R3-MYB TF PhMYBx, a homolog of Arabidopsis CPC, suppresses anthocyanin production by binding to AN1 and disturbing MBW assembly (Albert *et al.*, 2011, 2014). The competitive binding of bHLH proteins between MYB activators and repressors may serve as a balancer of anthocyanin biosynthesis (Albert *et al.*, 2014). Furthermore, emerging evidence indicated that the post-translational (Maier *et al.*, 2013; S. Li *et al.*, 2016; Zheng *et al.*, 2020; H. Zhou *et al.*, 2023) and epigenetic modifications (Fan *et al.*, 2018; Cai *et al.*, 2019; Liao *et al.*, 2022) of both the biosynthetic and regulatory genes provided additional levels of fine regulation of anthocyanin production.

Notably, the precise feedback regulation of MBW components, particularly MYB and bHLH TFs, plays a critical role in anthocyanin biosynthesis and deposition. In Arabidopsis, the R2R3-MYB protein PAP1 activates the transcription of *Trans-Acting SiRNA Gene 4* (*TAS4*). Elevated *TAS4* transcription leads to an increased abundance of *TAS4*-siR81(−) through the *ta*-siRNA pathway, which subsequently down-regulates several *MYB* genes including *PAP1*, *PAP2*, and *MYB113*. A small RNA, miR828, also contributes to the feedback regulation of both *PAP1* and *TAS4* (Luo *et al.*, 2012). Moreover, PAP1 promotes the transcription of *TT8* and R2R3-MYB repressor gene *MYB30* (Baudry *et al.*, 2006; Matsui *et al.*, 2008; H. Zhou *et al.*, 2023). Conversely, MYB30 directly represses *PAP1* expression and inhibits its activity (H. Zhou *et al.*, 2023). Similarly, TT8 positively regulates the expression of R3-MYB repressor gene *MYBL2* as well as its own transcription. As feedback, MYBL2 represses *TT8* transcription (Baudry *et al.*, 2006; Matsui *et al.*, 2008; Xu *et al.*, 2013). In petunia, two R2R3-MYB activators, AN2 and AN4, promote

the expression of bHLH component *AN1* (Quattrocchio *et al.*, 1998; Spelt *et al.*, 2000). The MBW complex activates the expression of two repressor genes, *PhMYB27* and *PhMYBx*. PhMYB27 negatively regulates *AN1* transcription in reverse (Albert *et al.*, 2014). Collectively, these activators, repressors, and MBW components, constitute a flexible regulatory network fine-tuning anthocyanin biosynthesis. The reinforcement and repressive feedback transcriptional regulations may be conserved across different species. However, the intricate details of such regulatory networks still require elucidation even in Arabidopsis.

Anthocyanin biosynthesis is induced by a diverse range of environmental stimuli (Naing and Kim, 2021), including high light (Albert *et al.*, 2009; Rowan *et al.*, 2009; Zheng *et al.*, 2019; Araguirang and Richter, 2022; D. Li *et al.*, 2023), salinity (Li *et al.*, 2022), drought (Nakabayashi *et al.*, 2014; An *et al.*, 2020), temperature (Shao *et al.*, 2007; Yu *et al.*, 2022), and sucrose (Solfanelli *et al.*, 2006). High light is considered as one of the most important triggers of anthocyanin accumulation, which occurs through hierarchical transcriptional regulation of anthocyanin biosynthetic and regulatory genes (Araguirang and Richter, 2022). Under light/high light conditions, the CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1)–SUPPRESSOR OF PHYA-105 (SPA) E3 ubiquitin ligase complex is inhibited by activated photoreceptors such as CRYPTOCHROMES (CRYs), PHYTOCHROMES (PHYs), and UV RESISTANCE LOCUS 8 (UVR8). This inhibition allows the accumulation of ELONGATED HYPOCOTYL 5 (HY5) and PAP1/2 (Maier *et al.*, 2013; Podolec and Ulm, 2018; Bhatia *et al.*, 2021). HY5 directly binds to the promoters of *PAP1* and anthocyanin biosynthetic genes, and activates their transcription (Shin *et al.*, 2013). Additionally, HY5 represses *MYBL2* expression by directly binding to its promoter and indirectly inducing *MYBD* and miR858a expression (Nguyen *et al.*, 2015; Wang *et al.*, 2016). This repression leads to the activation of the MBW complex and the subsequent expression of anthocyanin biosynthetic genes, thus promoting anthocyanin accumulation. The induction of anthocyanin biosynthesis and deposition by light has also been reported in other species like apple (Xing *et al.*, 2023) and eggplant (Y. Li *et al.*, 2023b). However, the precise mechanism underlying this process remains largely unclear.

In this study, we discovered a negative feedback regulatory module controlling high light-induced anthocyanin biosynthesis, comprising MYBL2, PAP1, and TT8. MYBL2 interacts with PAP1, repressing its transcriptional activation of both anthocyanin biosynthetic genes and *PAP1*. Conversely, PAP1 in conjunction with TT8 promotes *MYBL2* transcription. This regulatory module acts as a critical switch, ensuring adequate anthocyanin production to mitigate photo-damage caused by high light while preventing excessive anthocyanin accumulation to maintain normal growth and development.

Materials and methods

Plant materials and growth conditions

In the present study, the Columbia (Col-0) ecotype of Arabidopsis was used as the wild-type (WT), and all mutant and transgenic lines were in the Col-0 background. The *mybl2* (SALK_126807), *MYBL2*-overexpression (*MYBL2-OE*) (Xie *et al.*, 2016), *myb75-c* (S. Li *et al.*, 2016; Zheng *et al.*, 2019), and *pap1-D* (Borevitz *et al.*, 2000) lines have been previously reported. The *mybl2 myb75-c* and *MYBL2-OE pap1-D* lines were generated by genetic crossing. All seeds were surface-sterilized using 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 5 min, followed by five washes with sterile water. Seeds were then sown on Murashige and Skoog (MS) plates [pH 5.8, containing 2% (w/v) sucrose and 0.6% (w/v) agar], and stratified at 4 °C for 3 d. The plates were transferred to a phytotron at 22 °C under a 16 h light/8 h dark photoperiod, with a photosynthetic photon flux density of 80 μmol photons m^{-2} s^{-1} and 70% relative humidity. After 1 week, seedlings were transplanted into soil pots and grown under identical conditions for further analysis. For the light treatment, 3-week-old plants were subjected to continuous high light (1200 μmol photons m^{-2} s^{-1}), growth light (80 μmol photons m^{-2} s^{-1}), or darkness (0 μmol photons m^{-2} s^{-1}) under the same temperature and humidity. The *Nicotiana tabacum* K326 and *N. benthamiana* plants used in this study were grown in the same phytotron.

Measurement of anthocyanin indexes and contents

In Arabidopsis, 3-week-old WT, *mybl2*, *myb75-c*, *pap1-D*, *mybl2 myb75-c*, and *MYBL2-OE pap1-D* plants were subjected to continuous high light or growth light for 24 h. Then the rosette leaves were collected for the determination of anthocyanin indexes and contents. In *N. tabacum* K326, the full-length coding sequences (CDS) of *MYBL2*, *PAP1*, and *PAP2* were individually cloned into the pHB-FLAG vector (Su *et al.*, 2023) using the ClonExpress Ultra One Step Cloning kit (C115-02, Vazyme, China). The recombinant MYBL2-FLAG, PAP1-FLAG, and PAP2-FLAG plasmids as well as the empty pHB-FLAG vector (negative control) were transiently expressed in *N. tabacum* K326 leaves via *Agrobacterium tumefaciens*-mediated transformation. Five days after infiltration, the leaves were collected for imaging and detection of anthocyanin indexes and contents (Bayle *et al.*, 2008).

Anthocyanin content was measured as previously described (Zheng *et al.*, 2019) with minor modifications. In brief, approximately 1 g Arabidopsis rosette leaves or *N. tabacum* K326 leaves were placed in the extraction buffer [1% (v/v) HCl in methanol] at 4 °C in darkness for 24 h; then the supernatants were collected from the extracts after centrifugation at 12 000 *g* for 10 min at 4 °C. The absorbances at wavelengths of 530 nm and 657 nm were measured using the Tecan Spark Microplate Reader (Infinite 200 PRO, Tecan, Switzerland), and the relative anthocyanin content was calculated as $(A_{530}-0.25\times A_{657})$ per gram fresh weight. The anthocyanin indexes were assessed using the PlantExplorer XS system (Phenovation B.V., Netherlands) as previously described (Lazarević *et al.*, 2021). At least three independent biological replicates were performed.

Reverse transcription-quantitative PCR analysis

The indicated plant samples were subjected to total RNA isolation using TRIzol reagent (15596-026, Thermo Fisher Scientific, USA) following the manufacturer's instructions. The first-strand cDNAs were synthesized from 1 μg total RNAs using the HiScript III 1st Strand cDNA Synthesis kit (R312-02, Vazyme, China). Reverse transcription–quantitative PCR (RT-qPCR) was conducted on a LightCycler 480 system (Roche, Switzerland) employing the ChamQ Universal SYBR PCR master mix (Q711-03, Vazyme, China). The relative expression levels of each gene were determined by the $2^{-\Delta\Delta C_t}$ method (Schmittgen and Livak, 2008) normalized to that of the *UBQ10* reference gene. Another

control gene, *ACTIN2*, displayed a stable expression under all experimental conditions, including high light, growth light, and dark, after normalization to *UBQ10*, and vice versa (Supplementary Fig. S1). The gene-specific primers for RT-qPCR are listed in Supplementary Table S1. At least three biological replicates were performed for the expression analysis of each gene.

Yeast two-hybrid assay

The full-length and truncated CDSs of *MYBL2* were amplified and cloned into the pGBKT7 (BD) vector to generate the BD-MYBL2, BD-MYBL2-N (aa 1–34), BD-MYBL2-R3 (aa 35–80), and BD-MYBL2-C (aa 81–196) constructs. The full-length and truncated CDSs of *PAP1* and *PAP2* were cloned into the pGADT7 (AD) vector to generate the AD-PAP1, AD-PAP1-R2 (aa 1–60), AD-PAP1-R3 (aa 61–111), AD-PAP1-R2R3 (aa 1–111), AD-PAP1-C (aa 112–249), AD-PAP2, AD-PAP2-R2 (aa 1–60), AD-PAP2-R3 (aa 61–111), AD-PAP2-R2R3 (aa 1–111), and AD-PAP2-C (aa 111–250) constructs. The primers are listed in Supplementary Table S1. The yeast two-hybrid (Y2H) assays were carried out following the manufacturer's instructions (Takara Bio, USA). In brief, each pair of constructs was co-transformed into yeast AH109 competent cells and screened on synthetic defined (SD) medium lacking Leu and Trp (SD−L/T) at 30 °C for 3 d. Then the co-transformants were transferred onto SD medium lacking Leu, Trp, and His (SD−L/T/H), and that lacking Leu, Trp, His, and Ade (SD−L/T/H/A) with a serial dilution (1×, 10×, 100×, 1000×) and cultured at 30 °C for another 3 d. At least three biological replicates were performed for each pair of proteins/peptides.

Bimolecular fluorescence complementation assay

The full-length CDSs of *MYBL2*, *PAP1*, and *PAP2* were amplified and cloned into the pXY106-nYFP and pXY104-cYFP vectors (Y. Zhou *et al.*, 2023) to generate the MYBL2–nYFP, MYBL2–cYFP, PAP1–nYFP, PAP1–cYFP, PAP2–nYFP, and PAP2–cYFP constructs. The primers are listed in Supplementary Table S1. Bimolecular fluorescence complementation (BiFC) assays were conducted as previously described (Waadt *et al.*, 2008). The different combinations of nYFP- and cYFP-fused proteins, along with the nuclear localization marker H2B–mCherry, were co-expressed in *N. benthamiana* leaves via *A. tumefaciens*-mediated transformation. Three days after infiltration, yellow fluorescent protein (YFP; excitation 514 nm and emission 549 nm) and mCherry (excitation 561 nm and emission 609 nm) fluorescence signals were detected and imaged using a laser confocal microscope (Zeiss, LSM980, Germany). At least three biological replicates were performed for each pair of proteins/peptides.

Dual-luciferase reporter assay

The promoter sequences of *PAP1*, *PAP2*, *DFR*, *TT19*, and *MYBL2* were amplified from Arabidopsis genomic DNA and individually cloned into the upstream of the firefly luciferase reporter gene in pGreen II 0800-LUC to generate the reporter constructs (Hellens *et al.*, 2005). The empty pHB-FLAG vector and the recombinant pHB-MYBL2-FLAG, pHB-PAP1-FLAG, pHB-PAP2-FLAG, and pHB-TT8-FLAG plasmids were used as the effectors. The different combinations of effectors and reporters were co-expressed in *N. benthamiana* leaves via *A. tumefaciens*-mediated transformation. Three days after infiltration, the leaves were collected for LUC imaging using an *in vivo* imaging system (Tanon 5200 Multi, Tanon, China), or for dual-luciferase reporter assays employing the Dual-Luciferase Reporter Gene Assay kit (11402ES60, Yeasen, China) following the manufacturer's instructions. The activities of firefly (LUC) and *Renilla* (REN) luciferases were determined by the Tecan Spark microplate reader. The LUC/REN ratios were used to represent the transcription activities of reporters. At least three biological replicates were performed for each combination of effectors and reporters.

β-Glucuronidase reporter assay

The promoter sequences of *PAP1*, *PAP2*, *DFR*, and *TT19* were cloned into the pBI121 vector to generate the reporter constructs. The same effectors from the dual-luciferase assays were used in transient *β-glucuronidase* (*GUS*) reporter assays. Combinations of effectors and reporters were co-expressed in *N. benthamiana* leaves for 3 d. The transcription activities of the reporters were indicated by measuring the relative *GUS* expression normalized to the *NPT II* reference gene using RT-qPCR analysis (Gu *et al.*, 2019; Rahamkulov and Bakhsh, 2020). At least three biological replicates were performed for each combination of effectors and reporters.

Statistical analysis

Data represent means ±standard deviation (SD) of three biological replicates. Statistical significance was determined using Student's *t*-test (the asterisks indicate statistically significant differences, $\star P<0.05$ and $\star\star P<0.01$) or one-way ANOVA with Duncan's tests (different letters indicate significant differences at $P<0.05$).

Accession numbers

Sequence data described in this article can be accessed through the Arabidopsis Genome Initiative or GenBank/EMBL database under the following accession numbers: *DFR*, AT5G42800; *LDOX*, AT4G22880; *UF3GT*, AT5G54060; *TT19*, AT5G17220; *PAP1*, AT1G56650; *PAP2*, AT1G66390; *TT8*, AT4G09820; *MYBL2*, AT1G71030; *UBQ10*, AT4G05320; *ACTIN2*, AT3G18780.

Results

MYBL2 and PAP1 antagonistically regulate high light-induced anthocyanin accumulation

MYBL2 and PAP1 have been characterized as negative and positive regulators of anthocyanin biosynthesis and accumulation, respectively (Dubos *et al.*, 2008; Gonzalez *et al.*, 2008; Matsui *et al.*, 2008; Rowan *et al.*, 2009). To investigate the effects of MYBL2 and PAP1 on high light-induced anthocyanin biosynthesis, the wild-type Col-0 (WT), *mybl2* (a loss-of-function mutant of *MYBL2*), and *pap1-D* (a dominant gain-of-function mutant of *PAP1*) plants were subjected to continuous growth light and high light irradiations for 24 h. Under both illumination conditions, *mybl2* and *pap1-D* plants displayed more pronounced pigmentation compared with WT plants, and *pap1-D* plants exhibited higher pigmentation than *mybl2* plants (Fig. 1A). The anthocyanin indexes in *mybl2* and *pap1-D* plants were also significantly higher than that in WT plants under both light conditions (Fig. 1B). Specifically, the anthocyanin indexes in *mybl2* and *pap1-D* plants were 1.13- and 2.82-fold that in WT plants under growth light, and 1.45- and 1.99-fold under high light, respectively. Similarly, the relative anthocyanin contents in both mutants were significantly higher than that of WT plants (Fig. 1C). Moreover, *mybl2* plants showed a greater increase in anthocyanin accumulation than WT and *pap1-D*

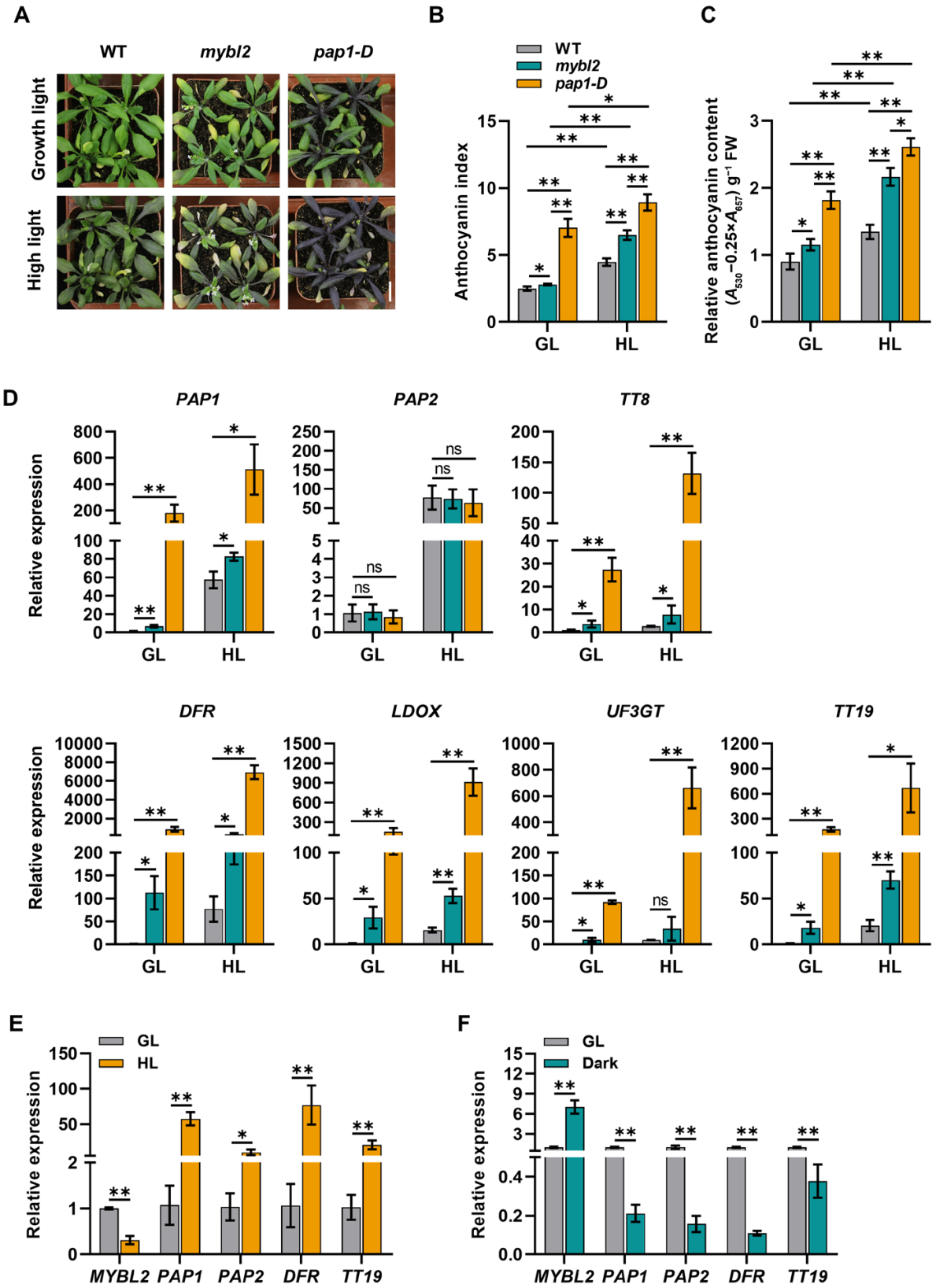

Fig. 1. Antagonistic regulation of anthocyanin biosynthesis under high light by MYBL2 and PAP1. (A) Phenotypes of 3-week-old wild type (WT, Col-0), *mybl2*, and *pap1-D* plants under continuous growth light (80 μmol photons m^{-2} s^{-1}) and high light (1200 μmol photons m^{-2} s^{-1}) for 24 h. Bar: 2 cm. (B, C) Anthocyanin index (B) and relative anthocyanin content (C) of 3-week-old WT, *mybl2*, and *pap1-D* plants under continuous growth light and high light for

24 h. The values are means ±SD of three biological replicates, and each replicate contains 8–10 plants. (D) Relative expression of selective anthocyanin biosynthetic and regulatory genes in indicated plants treated with continuous growth light and high light for 3 h. The expression level of each gene in WT plants under growth light was set as 1. (E, F) Relative expression of *MYBL2*, *PAP1*, *PAP2*, *DFR*, and *TT19* in WT plants transferred from growth light to high light (E) and dark (F) for 3 h. The expression level of each gene in WT plants under growth light was set as 1. For RT-qPCR assays (D–F), *UBQ10* was used as the reference gene. The values are means ±SD of three biological replicates. Asterisks indicate significant differences determined by Student's *t*-test (ns, not significant; **P*<0.05, ***P*<0.01). GL, growth light; HL, high light.

plants when transplanted from growth light to high light, along with more dramatic elevation of anthocyanin index and content, in line with the repressive effect of MYBL2 on high light-induced anthocyanin biosynthesis (Fig. 1A–C). Additionally, all plants exhibited enhanced levels of anthocyanin accumulation, and significantly elevated anthocyanin indexes and contents under high light (Fig. 1A–C). These findings confirm the promotion effect of high light on anthocyanin production and the opposite roles of MYBL2 and PAP1 on high light-induced anthocyanin accumulation.

To elucidate the underlying mechanism, we detected the transcription levels of anthocyanin biosynthesis-related genes. We observed a significantly increased expression of the anthocyanin regulatory genes *PAP1* and *TT8*, as well as the anthocyanin biosynthetic genes *DFR*, *LDOX*, *UF3GT*, and *TT19* in *mybl2* and *pap1-D* plants compared with WT plants under both light conditions (Fig. 1D). Notably, there were no significant differences in *PAP2* expression among *mybl2*, *pap1-D*, or WT plants under either light condition (Fig. 1D). Intriguingly, *TT8* and four anthocyanin biosynthetic genes, *DFR*, *LDOX*, *UF3GT*, and *TT19*, have been reported as the targets of PAP1 (Baudry *et al.*, 2006; Rowan *et al.*, 2009). These results suggest an antagonistic regulation between MYBL2 and PAP1 on the expression of *PAP1* itself and its target genes.

We then investigated the expression alterations of *MYBL2*, *PAP1*, *PAP2*, and the PAP1/2 targets *DFR* and *TT19* under high light and dark conditions. Compared with growth light, high light exposure repressed *MYBL2* expression while enhancing the transcription of *PAP1*, *PAP2*, *DFR*, and *TT19*. Conversely, dark treatment had the opposite effect (Fig. 1E, F). Collectively, these results indicate that MYBL2 and PAP1 antagonistically regulate high light-induced anthocyanin accumulation through opposite modulation of anthocyanin biosynthetic and regulatory genes.

MYBL2 represses high light-induced anthocyanin biosynthesis in a PAP1-dependent manner

To elucidate the genetic relationship between MYBL2 and PAP1 in high light-induced anthocyanin biosynthesis, we generated homozygous *mybl2 myb75-c* and *MYBL2-OE pap1-D* lines by crossing *mybl2* with *myb75-c* (a recessive mutant of *PAP1*) and crossing *MYBL2*-overexpression (*MYBL2-OE*) with *pap1-D*, respectively. Under growth light, only *pap1-D* plants exhibited conspicuous anthocyanin accumulation on the abaxial side (Fig. 2A). However, under high light, both *mybl2* and *pap1-D* plants displayed more pronounced pigmentation, while *myb75-c* plants accumulated fewer anthocyanins than WT, consistent with their respective roles. Furthermore, anthocyanin accumulation was nearly abolished in *mybl2 myb75-c* plants, and anthocyanin deposition in *pap1-D* plants was significantly reduced by the overexpression of *MYBL2* in *MYBL2-OE pap1-D* plants (Fig. 2A). The anthocyanin indexes and content confirm these observations under both light conditions (Fig. 2B, C). These results indicate that the repression of high light-induced anthocyanin biosynthesis by MYBL2 depends on PAP1 function.

To further validate this finding, we evaluated the transcript levels of *DFR* and *TT19*. Despite the overall up-regulation induced by high light, *DFR* and *TT19* showed higher expression levels in *mybl2* and *pap1-D* but decreased levels in *myb75-c* compared with WT. The transcription levels of *DFR* and *TT19* were markedly down-regulated in *mybl2 myb75-c* and *MYBL2-OE pap1-D* plants compared with those in *mybl2* and *pap1-D*, respectively (Fig. 2D, E). However, there were no significant differences observed in *PAP2* transcription levels among these plants (Supplementary Fig. S2). In summary, our findings demonstrate that MYBL2 negatively regulates high light-induced anthocyanin accumulation presumably through the repression of PAP1 function.

MYBL2 interacts with PAP1 and PAP2

MYBL2 has been reported to disrupt the formation of the MBW complex by competitively binding to bHLH proteins such as TT8, GL3, and EGL3 (Dubos *et al.*, 2008; Matsui *et al.*, 2008). Our findings suggest that the repressive effect of MYBL2 on high light-induced anthocyanin accumulation relies on PAP1 function (Figs 1, 2). To investigate the potential interaction between MYBL2 and PAP1, as well as its homolog PAP2, we conducted Y2H assays. We observed the growth of yeast cells harboring both BD-MYBL2 and AD-PAP1 or AD-PAP2 in selective medium, along with the positive control (Fig. 3A), indicating the interactions between MYBL2 and PAP1/2 *in vitro*. Additionally, we performed BiFC assays to validate these interactions. Co-expression of MYBL2 with either PAP1 or PAP2, fused to complementary halves of YFP, resulted in robust fluorescence signals in *N. benthamiana* leaves, which co-localized with the H2B–mCherry nuclear marker (Fig. 3B). This result confirms the interactions between MYBL2 and PAP1/2 *in vivo*.

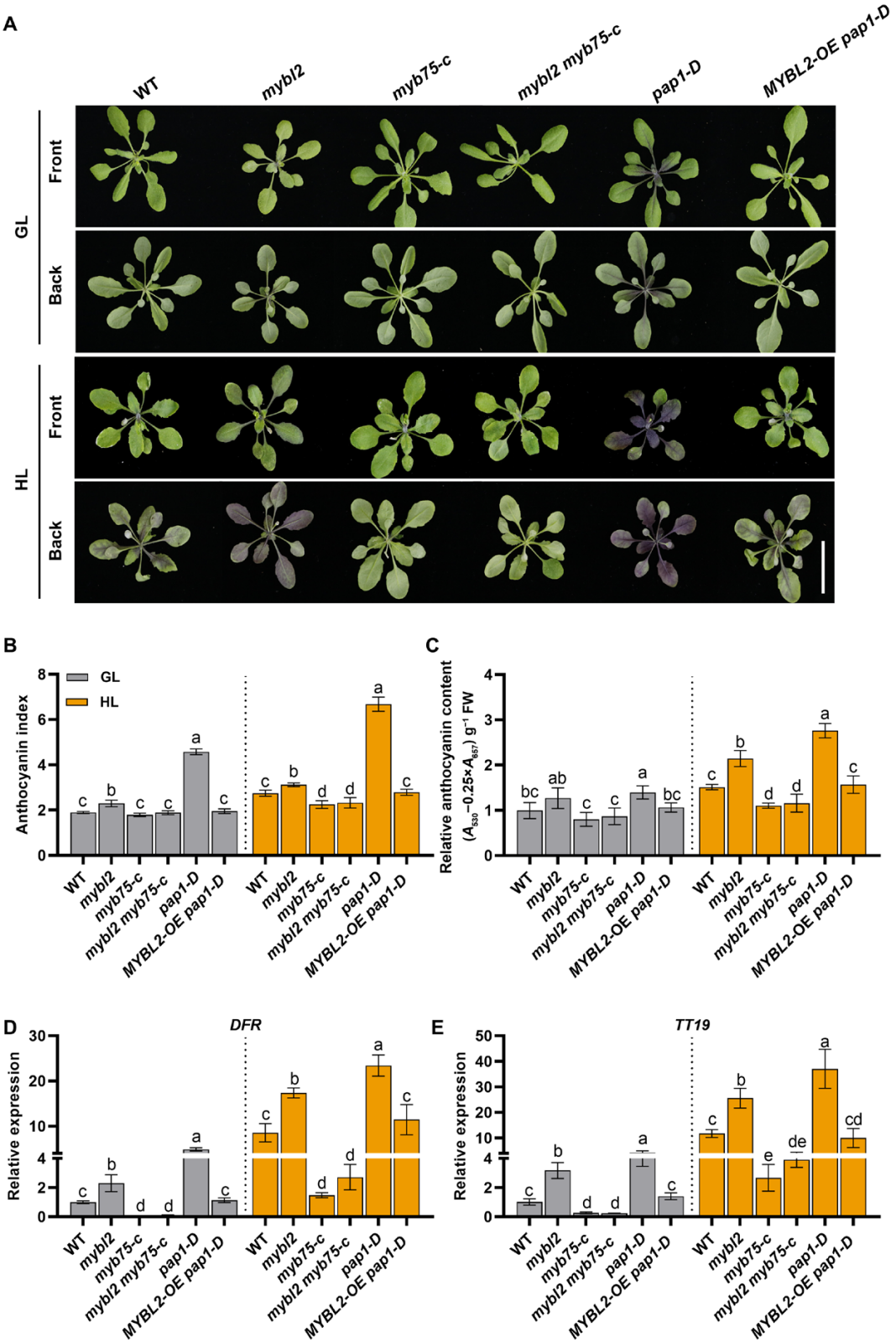

Fig. 2. Repression of high light-induced anthocyanin biosynthesis by MYBL2 is dependent on PAP1. (A) Phenotypes of 3-week-old wild type (WT, Col-0), *mybl2*, *myb75-c*, *mybl2 myb75-c*, *pap1-D*, and *MYBL2-OE pap1-D* plants under continuous growth light (80 μmol photons m^{-2} s^{-1}) and high light (1200 μmol photons m^{-2} s^{-1}) for 24 h. Bars: 2 cm. (B, C) Anthocyanin index (B) and relative anthocyanin content (C) of indicated plants under continuous growth light and high light for 24 h. The values are means ±SD of three biological replicates, and each replicate contains 8–10 plants. (D, E) Relative

expression of *DFR* (D) and *TT19* (E) in indicated plants under growth light and high light for 3 h. The expression levels of *DFR* (D) and *TT19* (E) in WT plants under growth light were set as 1. *UBQ10* was used as the reference gene. The values are means ±SD of three biological replicates. Identical letters indicate no significant differences ($P \geq 0.05$), while different letters indicate significant differences ($P < 0.05$) among corresponding groups, as determined by one-way ANOVA with Duncan's test. GL, growth light; HL, high light.

To further identify the specific domains responsible for these interactions, we performed Y2H assays using truncated forms of MYBL2 (N-terminus, R3, and C-terminus) and PAP1/2 (R2, R3, R2R3, and C-terminus) (Fig. 4A). The R3 and C-terminal regions of MYBL2 interacted with PAP1, while only the C-terminus interacted with PAP2, suggesting that the C-terminus plays a crucial role in protein interactions with MYBL2 (Fig. 4B). Moreover, we observed weaker interactions between the R2R3 domains of PAP1/2 and MYBL2 (Fig. 4C), indicating that the R2R3 domains of PAP1/2 are indispensable for their interactions with MYBL2.

MYBL2 represses the transcriptional activation activities of PAP1 and PAP2

PAP1 and PAP2 are core R2R3-MYB components of the canonical MBW complex, which predominately regulates anthocyanin biosynthesis (Gonzalez *et al.*, 2008; Starkevič *et al.*, 2015, 2020). To investigate the impact of MYBL2-PAP1/2 interactions on anthocyanin biosynthesis, we transiently expressed PAP1 and PAP2 individually or in combination with MYBL2 in *N. tabacum* K326 leaves. Our results showed that either PAP1 or PAP2 alone could induce anthocyanin production (Fig. 5A). However, no discernible pigmentation was observed in leaf sections infiltrated with MYBL2 or the empty vector (EV). Co-expression of MYBL2 with PAP1 or PAP2 markedly suppressed pigmentation and reduced anthocyanin content compared with expression of PAP1 or PAP2 alone (Fig. 5A, B). These results, together with the genetic interaction data (Fig. 2), indicate that MYBL2 represses the promotive effects of PAP1 and PAP2 on anthocyanin biosynthesis.

The MBW complex positively regulates anthocyanin accumulation by transactivating the expression of anthocyanin biosynthetic genes, which relies on the transcription activation activities of the MYB and bHLH components (Gonzalez *et al.*, 2008; Rowan *et al.*, 2009; Xu *et al.*, 2015). To investigate whether MYBL2 affects the transcriptional activation activities of PAP1 and PAP2, we performed dual-luciferase reporter assays and *GUS* reporter assays in *N. benthamiana* leaves. Co-infiltration of the reporters containing the *DFR* and *TT19* promoter regions with PAP1/2 effectors resulted in significantly increased relative luciferase (relative LUC/REN ratio) and *GUS* (relative expression of *GUS*/*NPT II*) activities, compared with co-infiltration with EV. However, no notable differences were observed between co-transformation with MYBL2 or EV (Fig. 5C–E). These results indicate that PAP1/2 rather than MYBL2 can activate the transcription of *DFR* and *TT19*. Furthermore, co-expression of MYBL2 with PAP1 or PAP2 as effectors led to a remarkable reduction in relative luciferase and *GUS* activities compared with expressing PAP1 or PAP2 alone, respectively (Fig. 5C–E), suggesting that MYBL2 represses the transcriptional activation of PAP1/2 on *DFR* and *TT19*. Collectively, these results demonstrate that MYBL2 negatively regulates anthocyanin biosynthesis by repressing the transcriptional activation activities of PAP1/2 on their target genes like *DFR* and *TT19*.

MYBL2 represses the transcriptional self-activation of *PAP1*

Our findings suggest that the antagonistic effects of MYBL2 and PAP1 on anthocyanin biosynthesis and accumulation depend on the divergent regulation of PAP1/2. Next, we investigated the interrelation between MYBL2 and PAP1/2. Using the PlantCARE database, we identified a putative MYB binding site (MBS; TAACCA) near the transcription start site (TSS) of PAP1, and another MBS (CAACCA) in the 1.1 kb upstream region of PAP2 (Fig. 6A), implying potential self-regulation mechanisms for *PAP1/2* transcription. To test this hypothesis, we performed dual-luciferase and *GUS* reporter assays in *N. benthamiana* leaves (Fig. 6B). Co-infiltration of the *PAP1* promoter with PAP1–FLAG effector resulted in a significant increase in the relative luciferase activity by 2.63-fold and GUS activity by 3.92-fold compared with the co-infiltration with EV (Fig. 6C, D), indicating that PAP1 can activate its own expression. However, co-infiltration with MYBL2–FLAG did not affect *PAP1* promoter activity, suggesting that MYBL2 alone does not suppress *PAP1* transcription. Notably, co-expression of MYBL2–FLAG and PAP1–FLAG effectors substantially attenuated the self-activation effect of PAP1 (Fig. 6C, D), consistent with the repressive effect of MYBL2 on PAP1 transcriptional activity (Fig. 5). In contrast, neither PAP2 nor MYBL2 had any notable effect on *PAP2* transcription (Fig. 6C, D.

Since previous studies reported that TT8 is required for the transcriptional activation of PAP1/2 (Gonzalez *et al.*, 2008; Zheng *et al.*, 2019), we co-expressed the reporters together with PAP1/2–FLAG and TT8–FLAG effectors. Co-expression of TT8–FLAG with PAP1–FLAG or PAP2–FLAG significantly enhanced the activities of *DFR* and *TT19* promoters, but had no notable promotion effects on the *PAP1/2* promoters themselves. In contrast, co-expression of PAP1–FLAG and TT8–FLAG resulted in reduced *PAP1* promoter activity compared with the infiltration of PAP1 alone, potentially due to the competition between PAP1 and TT8 for binding to the *PAP1* promoter region, which contains several G-box motifs

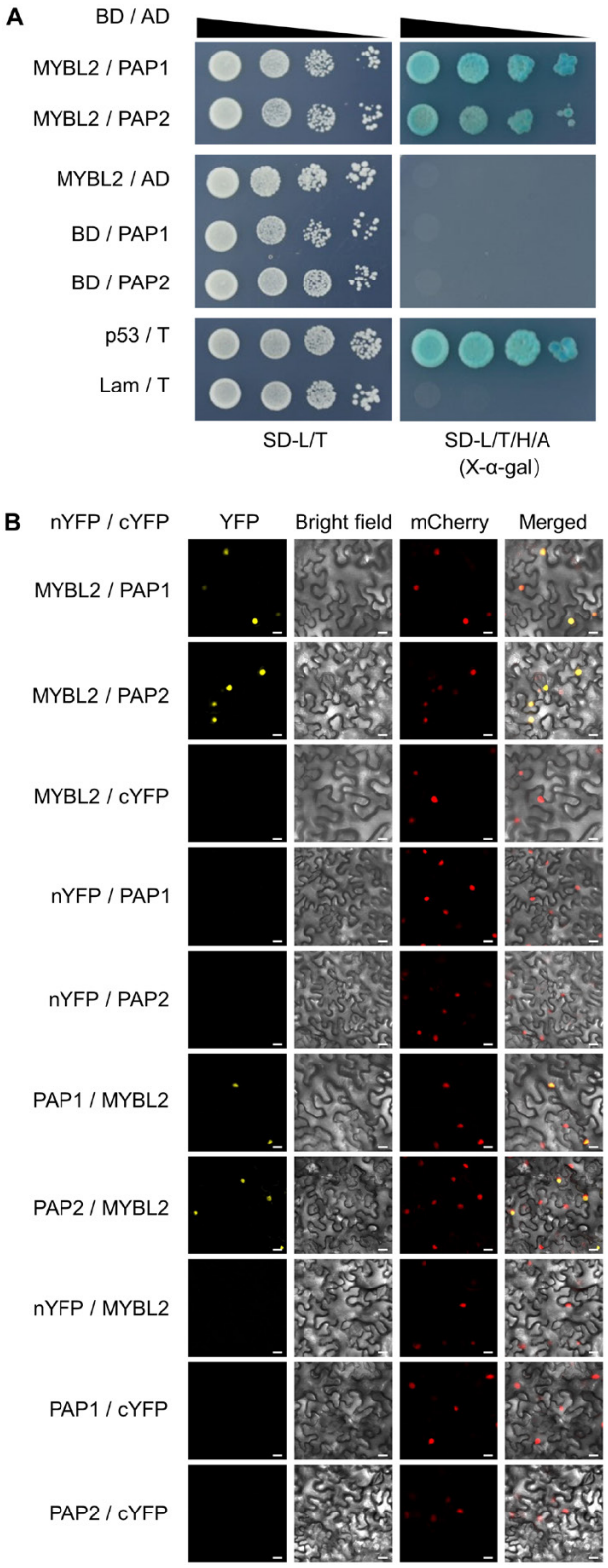

Fig. 3. Interactions between MYBL2 and PAP1/2. (A) Interactions between MYBL2 and PAP1/2 in a yeast two-hybrid assay. The combinations of BD-p53/AD-T and BD-Lam/AD-T were used as positive and negative controls, respectively. SD-L/T, SD medium lacking Leu and Trp; SD-L/T/H/A, SD medium lacking Leu, Trp, His, and Ade; X-α-gal, 5-bromo-4-chloro-3-indoxyl-α-D-galactopyranoside. (B) Interactions between MYBL2 and PAP1/2 in bimolecular fluorescence complementation assay. The 35S:H2B–mCherry vector was used as a nuclear localization marker. Scale bars: 20 μm. At least three independent biological replicates were performed for each pair of proteins/peptides. YFP, yellow fluorescent protein.

responsible for bHLH binding (Fig. 6E–H; Supplementary Fig. S3). The differential regulation of *PAP1* and *PAP2* transcription may be attributed to distinct localization patterns of MBS within their respective promoters. These findings, along with *PAP1* and *PAP2* expression profiles in *mybl2* plants (Fig. 1D), suggest that MYBL2 negatively regulates the expression of *PAP1*, but not of *PAP2*, by repressing the transcriptional self-activation of *PAP1*.

PAP1 and TT8 cooperatively activate *MYBL2* expression

A previous study reported that *MYBL2* transcription is up-regulated in *TT8* overexpression plants, indicating that TT8 activates *MYBL2* transcription (*Matsui et al.*, 2008). Similarly, we observed significantly elevated *MYBL2* expression in *pap1-D* plants, a gain-of-function allele of *PAP1*, while no marked changes were observed in the recessive loss-of-function mutant *myb75-c* (Fig. 7A). Additionally, multiple putative MBSs were identified in the *MYBL2* promoter region, particularly in the 5′ untranslated region, based on *cis*-element predication using the PlantCARE database (Fig. 7B). These results suggest that PAP1 may modulate *MYBL2* expression. To test this hypothesis, we conducted dual-luciferase reporter assays in *N. benthamiana* leaves (Fig. 7C). Co-infiltration of the *MYBL2* promoter with either PAP1–FLAG or TT8–FLAG effectors led to significant increases in reporter activities by approximately 3.10- and 1.63-fold compared with co-infiltration with EV, respectively, indicating that PAP1 and TT8 independently activate *MYBL2* expression. Moreover, co-expression of PAP1–FLAG and TT8–FLAG together resulted in a further elevation in reporter activity (Fig. 7D, E), suggesting a synergistic effect between PAP1 and TT8 on promoting *MYBL2* expression.

Discussion

A growing number of studies have substantiated the pivotal role of the MBW complex in anthocyanin biosynthesis and accumulation (Gonzalez *et al.*, 2008; Albert *et al.*, 2014; Starkevič *et al.*, 2015; Zhang *et al.*, 2019; Zheng *et al.*, 2019; Lim *et al.*, 2022; Broucke *et al.*, 2023). The targeting specificity and transcriptional activity of the MBW complex are predominantly governed by R2R3-MYB components such as PAP1 and PAP2, which typically function as transcriptional activators.

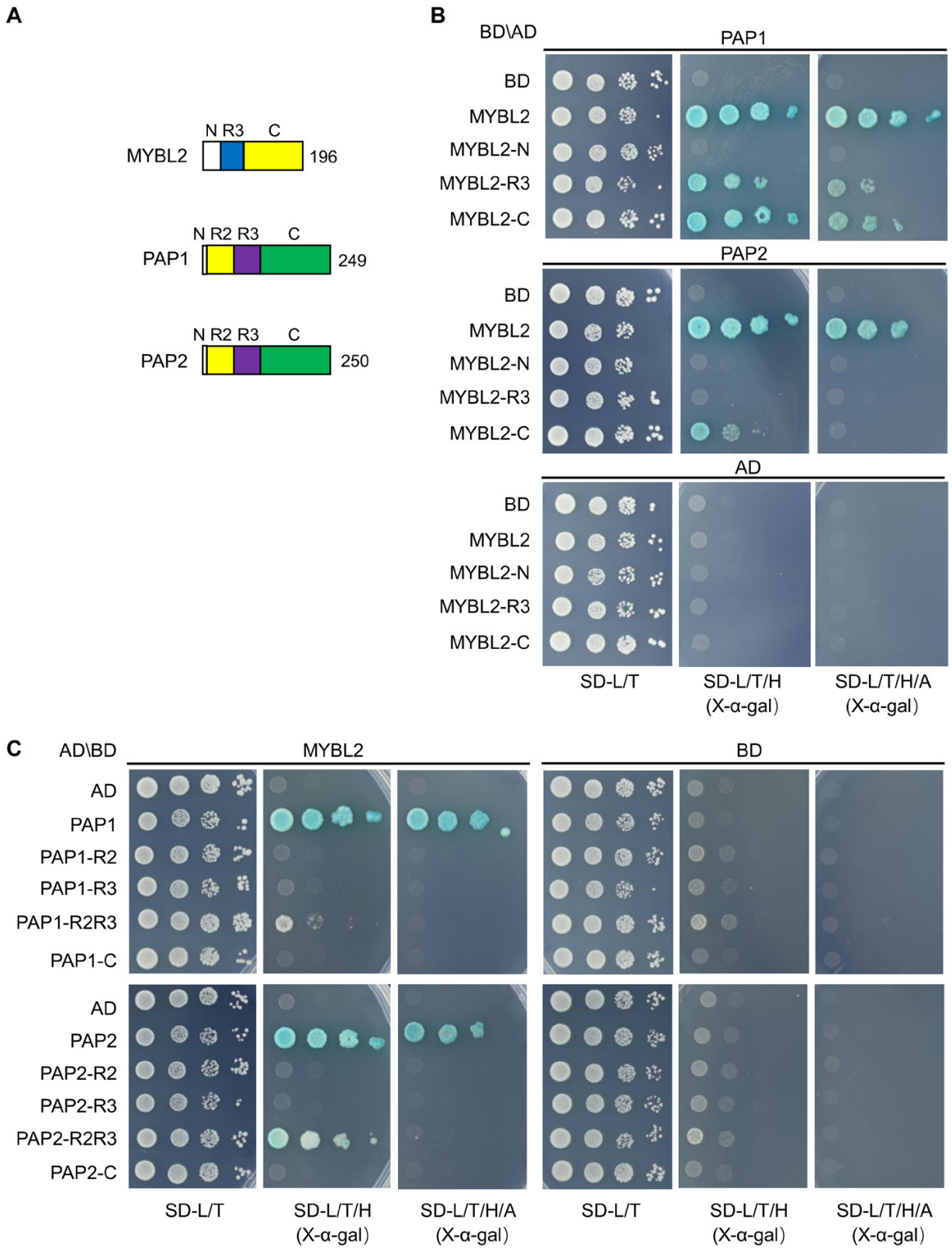

Fig. 4. Determination of the specific domains responsible for the MYBL2–PAP1/2 interactions. (A) Schematic diagrams showing the domain architecture of MYBL2 and PAP1/2 proteins. N, the amino-terminus; R2 and R3, two and three imperfect amino acid sequence repeats (R), respectively; C, the carboxyl-terminus. (B) Interactions of intact or truncated MYBL2 with PAP1/2. (C) Interactions of MYBL2 with intact or truncated PAP1/2. SD-L/T, SD medium lacking Leu and Trp; SD-L/T/H, SD medium lacking Leu, Trp, and His; SD-L/T/H/A, SD medium lacking Leu, Trp, His, and Ade; X-α-gal, 5-bromo-4-chloro-3-indoxyl-α-D-galactopyranoside. At least three independent biological replicates were performed for each pair of proteins/peptides.

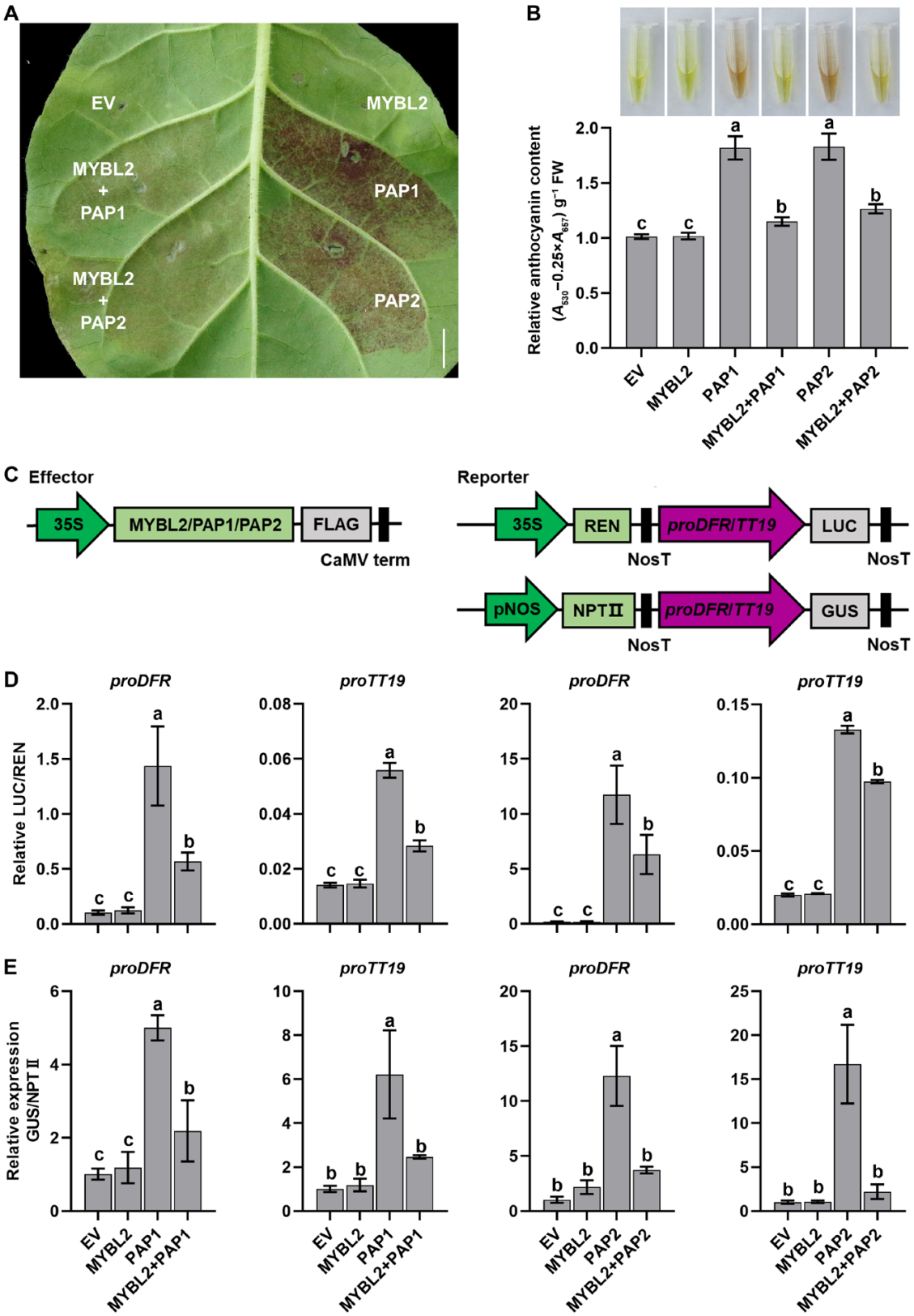

Fig. 5. MYBL2 inhibits anthocyanin accumulation by repressing the transcriptional activation activities of PAP1/2. (A, B) Anthocyanin pigmentation (A) and relative anthocyanin content (B) in *Nicotiana tabacum* K326 leaves ectopically expressing different exogenous genes for 5 d. Scale bar in (A): 1 cm. The values in (B) are means ±SD of three biological replicates, and each replicate contains five leaves. (C) Schematic diagrams of the effector and reporter constructs used in the dual-luciferase and *β-glucuronidase* (*GUS*) reporter assays. (D, E) The relative transcription activities of the *DFR* and *TT19*

promoters in the dual-luciferase (D) and *GUS* (E) reporter assays. The transcription activities of *DFR* and *TT19* promoters co-infiltrated with indicated effectors are indicated as the relative LUC/REN ratio (D) and the relative *GUS* expression normalized to *NPTII* (E). The empty vector (EV) was used as the control effector. The values are means ±SD of three biological replicates. Identical letters indicate no significant differences ($P \geq 0.05$), while different letters indicate significant differences ($P < 0.05$) among corresponding groups, as determined by one-way ANOVA with Duncan's test.

Additionally, several R2R3- and R3-MYB repressors, such as MYB4/7/32 and MYBL2, have been identified as negative regulators of anthocyanin biosynthesis by interacting with bHLH proteins, thus inhibiting MBW assembly. However, the connection between R2R3-MYB activators and R3-MYB repressors remains to be fully understood. Herein, we unveil a precise regulatory loop comprising the R2R3-MYB activator PAP1 and the R3-MYB repressor MYBL2, which fine-tunes anthocyanin biosynthesis under high light conditions.

The antagonistic roles between MYBL2 and PAP1 in high light-induced anthocyanin accumulation

The biosynthesis and accumulation of anthocyanins are regulated by multiple developmental and environmental cues. Light, including high light, is one of the most crucial environmental factors that promote anthocyanin production. On one hand, high light affects the expression of many anthocyanin biosynthetic and regulatory genes at the transcriptional level. For instance, the transcription of several MBW component genes, such as *PAP1*, *PAP2*, and *TT8*, is induced by high light. TTG1 expression is independent of light conditions. Meanwhile, *MYBL2* transcription is repressed under high light (Fig. 1D), *TTG1* expression is independent of light conditions, while *MYBL2* transcription is repressed under high light (Fig. 1E; Cominelli *et al.*, 2008; Dubos *et al.*, 2008). These transcriptional effects may be attributed to the presence of light-responsive elements in their promotor regions (Ban *et al.*, 2007; Shin *et al.*, 2013; P. Li *et al.*, 2016). On the other, the promotion of anthocyanin accumulation by light is entangled with light signaling pathways. The COP1–SPA E3 ubiquitin ligase complex, a major repressor of photomorphogenesis, interacts with PAP1/2 and facilitates their ubiquitination and subsequent post-translational degradation under dark conditions, thereby suppressing anthocyanin accumulation (Maier *et al.*, 2013). Light inhibits the action of the COP1–SPA complex, leading to increased protein levels of PAP1/2 and HY5. HY5 is a photomorphogenesis-promoting transcription factor that activates *PAP1* transcription but suppresses *MYBL2* expression, thereby inducing anthocyanin production (Shin *et al.*, 2013; Nguyen *et al.*, 2015; Wang *et al.*, 2016; Bhatia *et al.*, 2021).

In this study, we investigated the relationship between the R2R3-MYB activator PAP1 and the R3-MYB repressor MYBL2 in high light-induced anthocyanin biosynthesis and accumulation. Our results showed a significant increase in anthocyanin deposition in *mybl2* and *pap1-D* mutants upon high light illumination (Figs 1A–C, 2A–C), in accordance with previous reports (Dubos *et al.*, 2008; Matsui *et al.*, 2008; Zheng *et al.*, 2019). Additionally, we observed a greater increase in anthocyanin deposition in *mybl2* plants than in WT and *pap1-D* plants when transferred from growth light to high light (Fig. 1A–C). This may be attributed to the presence of MYBL2 partially inhibiting high light-induced anthocyanin production in WT and *pap1-D* plants. Furthermore, it was noted that *pap1-D* plants accumulated relatively high levels of anthocyanins under growth light, which may explain its weaker increase in anthocyanin levels compared with the other two genotypes when transferred to high light. Previous studies and our findings demonstrated that MYBL2 acts as an active repressor of *PAP1* and *TT8*. Consistent with this, the expression levels of *PAP1* and *TT8* were elevated in *mybl2* plants but decreased in *MYBL2-OE* plants (Fig. 1D; Dubos *et al.*, 2008; Matsui *et al.*, 2008; Xie *et al.*, 2016). Therefore, the mutation of *MYBL2* in *mybl2* plants might mask anthocyanin accumulation when hybridized with *PAP1*-overexpressing lines like *pap1-D*, whereas the overexpression of *MYBL2* in *MYBL2-OE* plants possibly suppresses anthocyanin production in the hybridization of *MYBL2-OE* with *myb75-c*, posing challenges for elucidating the genetic interaction between MYBL2 and PAP1. However, *mybl2 myb75-c* double mutants exhibited a similar level of anthocyanins as that in *myb75-c* plants, whereas anthocyanin production was lower in *MYBL2-OE pap1-D* plants than in *pap1-D* plants, suggesting that MYBL2 functions upstream of PAP1 in regulating high light-induced anthocyanin accumulation.

The transcriptional regulation of anthocyanin biosynthetic and regulatory genes is a key feature in the control of anthocyanin biosynthesis. Consistent with the observed phenotypes, the expression levels of *DFR*, *LDOX*, *UF3GT*, and *TT19* were significantly up-regulated in both *mybl2* and *pap1-D* plants, indicating that MYBL2 and PAP1 exert antagonistic effects on the transcription of anthocyanin biosynthetic genes. Notably, the transcription levels of *PAP1* and *TT8* were significantly increased in *mybl2* plants, while the transcription of *PAP2* was unaffected by MYBL2 (Fig. 1D), suggesting potential functional divergence among these MBW components. Additionally, we found that high light and dark treatment resulted in opposite changes in the expression patterns of *MYBL2* and another group of genes, including *PAP1*, *PAP2*, *DFR*, and *TT19* (Fig. 1E, F), which is consistent with previous findings by Dubos *et al.* (2008). The contrasting light response between *MYBL2* and *PAP1* may be partly due to differential regulation by HY5, which inhibits *MYBL2* transcription but activates *PAP1* expression (Shin *et al.*, 2013; Nguyen *et al.*, 2015; Wang *et al.*, 2016). However, the underlying mechanisms governing light-responsive expression of anthocyanin biosynthetic

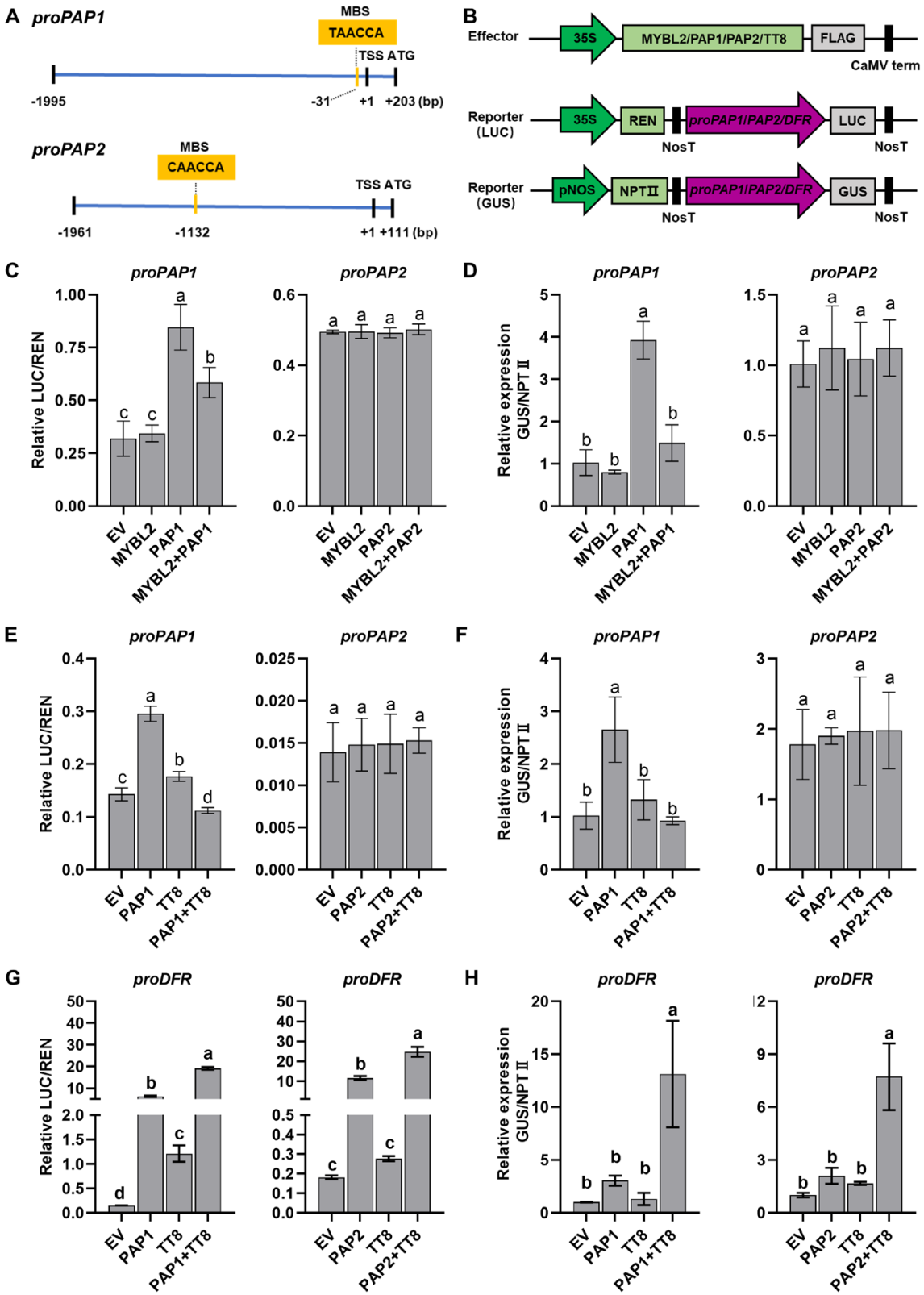

Fig. 6. MYBL2 represses the transcriptional self-activation of PAP1. (A) Putative MYB binding sites in the *PAP1* and *PAP2* promoter regions. ATG, start codon; MBS, MYB binding site; TSS, transcription start site. (B) Schematic diagrams of the effector and reporter constructs for the dual-luciferase and *GUS* reporter assays. (C–F) The relative transcription activities of *PAP1* and *PAP2* promoters in the dual-luciferase (C, E) and *GUS* (D, F) reporter assays.

(G, H) The relative transcription activities of the *DFR* promoter in the dual-luciferase (G) and *GUS* (H) reporter assays. The transcription activities of the indicated promoters co-infiltrated with different effectors are indicated as the relative LUC/REN ratio (C, E, G) and the relative *GUS* expression normalized to *NPTII* (D, F, H). The empty vector (EV) was used as the control effector. The values are means ±SD of three biological replicates. Identical letters indicate no significant differences ($P \geq 0.05$), while different letters indicate significant differences ($P < 0.05$) among corresponding groups, as determined by one-way ANOVA with Duncan's test.

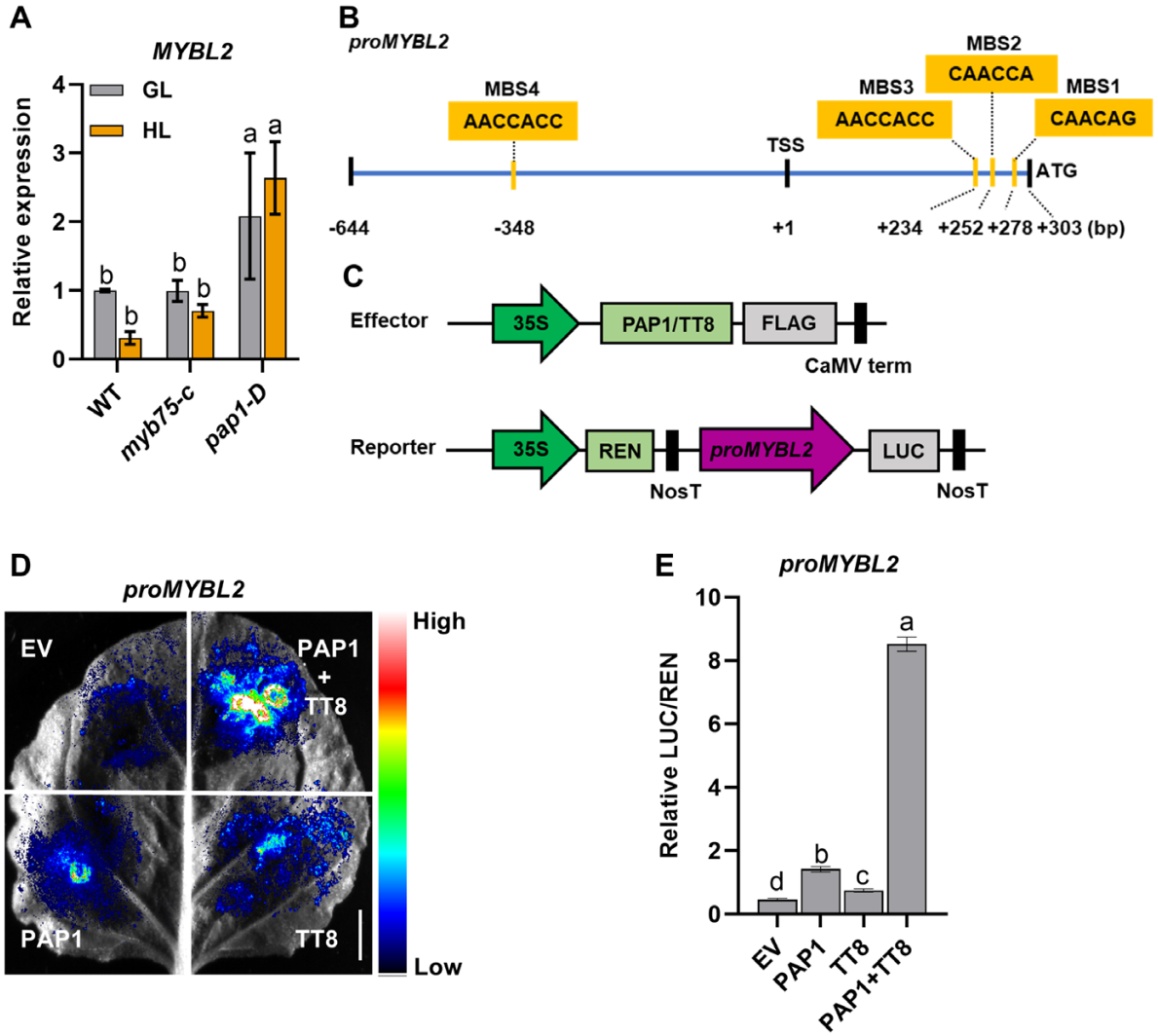

Fig. 7. PAP1 and TT8 cooperatively activate *MYBL2* expression. (A) Relative expression of *MYBL2* in 3-week-old wild type (WT, Col-0), *myb75-c*, and *pap1-D* plants under continuous growth light (80 μmol photons m^{-2} s^{-1}) and high light (1200 μmol photons m^{-2} s^{-1}) for 3 h. *MYBL2* expression level in WT plants under growth light was set as 1. *UBQ10* was used as the reference gene. The values are means ±SD of three biological replicates. (B) Putative MYB binding sites in the *MYBL2* promoter region. ATG, start codon; MBS, MYB binding site; TSS, transcription start site. (C) Schematic diagrams of the effector and reporter constructs in the dual-luciferase reporter assays. (D, E) The luciferase image (D) and the relative transcription activities (E) of the *MYBL2* promoter co-infiltrated with indicated effectors. The empty vector (EV) was used as the control effector. Scale bar in (D): 1 cm. The values (E) are means ±SD of three biological replicates. Identical letters indicate no significant differences ($P \geq 0.05$), while different letters indicate significant differences ($P < 0.05$) among corresponding groups, as determined by one-way ANOVA with Duncan's test. GL, growth light; HL, high light.

and regulatory genes require further investigation. For instance, besides the COP1/SPA–HY5 pathway, the precise roles of photoreceptors in anthocyanin accumulation and the expression of anthocyanin-related genes remain to be elucidated. Strikingly, the transcription levels of *DFR* and *TT19* were significantly decreased in *mybl2 myb75-c* plants compared with that in *mybl2*, and their expression was markedly reduced in *MYBL2-OE pap1-D* plants in comparison with that in *pap1-D* (Fig. 2D, E), suggesting that MYBL2 not only inhibits *PAP1* transcription but also represses the transcriptional activation ability of PAP1 on its target genes like *DFR* and *TT19*. Collectively, our results from the phenotypic and transcriptional regulation analyses

indicate that MYBL2-mediated repression of anthocyanin production is dependent on PAP1 function, encompassing both *PAP1* transcription and its activation of target genes.

The interaction between MYBL2 and PAP1/2

Protein interactions provide another essential regulatory level for anthocyanin biosynthesis and accumulation. The R2R3-MYB TFs PAP1, PAP2, MYB113, and MYB114, the bHLH TFs TT8, GL3, and EGL3, and the WD40 protein TTG1 have been reported to form the MBW complex through alternative protein interactions (Borevitz *et al.*, 2000; Baudry *et al.*, 2004; Koes *et al.*, 2005; Ramsay and Glover, 2005; Gonzalez *et al.*, 2008; Dubos *et al.*, 2010). The MBW complex plays a central role in controlling *LBGs* and anthocyanin biosynthesis. Numerous other proteins have been characterized as interactors of the MBW components to modulate the activity of the MBW complex, thereby participating in regulating anthocyanin biosynthesis. For example, MAP kinase 4 (MPK4), SAP and Miz 1 (SIZ1), TEOSINTE BRANCHED 1, CYCLOIDEA AND PCF TRANSCRIPTION FACTOR 3 (TCP3) and GOLDEN2-LIKE 1 (GLK1) interact with PAP1 and enhance stability of the MBW complex (Li and Zachgo, 2013; S. Li *et al.*, 2016; Zheng *et al.*, 2020; Y. Li *et al.*, 2023a), whereas COP1, HOMEOBOX ARABIDOPSIS THALIANA 1 (HAT1), PHYTOCHROME-INTERACTING FACTOR 4 (PIF4), and EAR MOTIF-CONTAINING ADAPTOR PROTEIN (ECAP) reduce stability of the MBW complex by interacting with PAP1 (Maier *et al.*, 2013; Zheng *et al.*, 2019; Li *et al.*, 2022; Qin *et al.*, 2022). A recent study has demonstrated that the R2R3-MYB repressor MYB30 directly represses *PAP1* transcription and interacts with PAP1 to disrupt the MBW complex assembly (H. Zhou *et al.*, 2023). Several other R2R3-MYB repressors, MYB4/7/32 and PhMYB27, as well as R3-MYB proteins MYBL2, CPC, and PhMYBx, competitively interact with the bHLH components rather than the R2R3-MYBs, and inhibit the formation and activity of the MBW complex (Dubos *et al.*, 2008; Matsui *et al.*, 2008; Zhu *et al.*, 2009; Albert *et al.*, 2014; Zhang *et al.*, 2019; Wang *et al.*, 2020; Zhao *et al.*, 2023). However, petunia R2R3-MYB activator DEEP PURPLE (DPL) failed to interact with the R2R3-MYB repressor PhMYB27 in yeast, unless unlabelled bHLH proteins JAF13 or AN1 were provided as bridging factors (Albert *et al.*, 2014). In contrast, only a limited number of MYBL2-interacting proteins have been identified. In addition to the bHLH subunits of the MBW complex such as TT8, GL3, and EGL3 (Dubos *et al.*, 2008; Matsui *et al.*, 2008), DELLA proteins directly sequester MYBL2 and repress its activity, thereby facilitating the MBW complex formation and promoting subsequent anthocyanin biosynthesis and accumulation under abiotic stresses (Xie *et al.*, 2016). APETALA2 (AP2) not only transcriptionally activates *MYBL2* expression but also physically interacts with MYBL2 protein to form an AP2–MYBL2–TT8/EGL3 complex, thus disrupting MBW complex assembly and repressing proanthocyanidin biosynthesis in seeds (Jiang *et al.*, 2024).

Here, we evidenced that the R3-MYB repressor MYBL2 interacts with the R2R3-MYB activators PAP1/2 in both Y2H and BiFC assays (Fig. 3). This finding differs from the DPL-PhMYB27 interaction that necessitates the bHLH proteins (Albert *et al.*, 2014). One possible explanation for this discrepancy is that PhMYB27, an R2R3-MYB repressor, and MYBL2 exhibit significant differences in their amino acid sequences, sharing only 33.48% similarity, although the R2R3-MYB activator DPL and PAP1/2 display a relatively higher similarity (Supplementary Fig. S4). Phylogenetic analysis further revealed that PhMYB27 belongs to the FaMYB1-like type MYB repressors, while MYBL2 belongs to the AtMYBL1-like clade (Aharoni *et al*, 2001; Yan *et al.*, 2021). A previous study has shown that the interaction between PAP1 and HAT1 requires its R2 domain and the C-terminal region (Zheng *et al.*, 2019). In our case, the interaction between MYBL2 and PAP1/2 relies on the R2R3 domain of PAP1/2. Notably, there is a slight difference between PAP1 and PAP2 in the interaction with MYBL2 (Fig. 4C). On another note, we found that the C-terminal region of MYBL2 is essential for its interactions with PAP1/2 (Fig. 4B), consistent with its interactions with RGA1 and AP2 (Xie *et al.*, 2016; Jiang *et al.*, 2024), while its N-terminal fragment is required for the binding with TT8 (Matsui *et al.*, 2008; Jiang *et al.*, 2024). Given that the interactions between PAP1/2 and MYBL2 are mediated by their R2R3 domains conserved in various R2R3-MYB proteins (Fig. 4), it remains unclear whether other MYBs also interact with MYBL2, or if these specific domains/regions are crucial for such interactions. Conducting Y2H screening and immunoprecipitation–mass spectrometry using intact or truncated MYBL2 may provide insights into these unanswered questions.

Previous studies have demonstrated that the COP1/SPA complex interacts with PAP1/2 and regulates the degradation of PAP1/2 through ubiquitination mediated by the 26S proteasome (Maier *et al.*, 2013). Similarly, it has been reported that a Kelch domain-containing F-box protein, KFB^{CHS}, interacts with CHS and promotes its ubiquitination and degradation (Zhang *et al.*, 2017). Here, we discovered that the interactions between MYBL2 and PAP1/2 repress their activation of PAP1/2 on *DFR* and *TT19* transcription. Surprisingly, their interactions with MYBL2 remarkably diminish the transcription self-activation of *PAP1* but not of *PAP2* (Fig. 6), which may be attributed to their sequence and functional divergence. Taken together, these findings elucidate the diversity and complexity of protein interactions involved in regulating anthocyanin biosynthesis and accumulation.

A feedback regulatory module comprising MYBL2, PAP1, and TT8 fine-tunes high light-induced anthocyanin biosynthesis

Plants have evolved intricate positive and negative feedback regulatory mechanisms to modulate anthocyanin biosynthesis in response to dynamic developmental and environmental cues (Baudry *et al.*, 2006; Petroni and Tonelli, 2011; Gao *et al.*, 2021).

In Arabidopsis, both the R2R3-MYB component PAP1 and the bHLH component TT8 can activate *TT8* transcription (Tohge *et al.*, 2005; Baudry *et al.*, 2006). Here, we observed a remarkable up-regulation of *TT8* transcription in *pap1-D* plants (Fig. 1D). Furthermore, we provided evidence supporting the induction of its own expression by PAP1 (Fig. 6), while *PAP2* transcription remains unaffected by PAP2, TT8, or MYBL2 (Figs 1D, 6; Matsui *et al.*, 2008; Xie *et al.*, 2016), despite the promotion effect on *PAP1/2* transcription by high light. A recent study demonstrated that BRASSINAZOLE RESISTANT 1 (BZR1), a brassinosteroid-responsive transcription factor, promotes *PAP1* transcription and directly interacts with PAP1, thereby cooperatively regulating the expression of anthocyanin biosynthetic genes and anthocyanin accumulation (Lee *et al.*, 2024). The reciprocal regulation among MBW components, along with hierarchical control over anthocyanin biosynthetic genes, may establish positive feedback loops triggering MBW complex assembly for rapid anthocyanin accumulation in response to environmental changes.

On the contrary, TT8 facilitates the transcription of *MYBL2*; MYBL2, in turn, directly represses the expression of *TT8* and *PAP1*, thereby inhibiting the formation and activity of the MBW complex (Figs 1D, 6, 7; Matsui *et al.*, 2008). In the regulation of sugar-induced anthocyanin biosynthesis, *PAP1* transcription is activated by sugars like sucrose, and expressed PAP1 positively regulates *TAS4* transcription; as feedback, increased *TAS4* transcription elevates *TAS4*-siR81(−) abundance, which down-regulates *PAP1*, *PAP2*, and *MYB113* (Luo *et al.*, 2012). Under low sucrose conditions, MYB30 directly represses *PAP1* transcription and interacts with PAP1 to disrupt MBW complex formation; however, when sucrose induces *PAP1* transcription, the expressed PAP1 activates the transcription of a ubiquitin E3 ligase gene *RHA2b* that degrades MYB30, thus releasing PAP1 to promote anthocyanin biosynthesis in response to high sucrose (H. Zhou *et al.*, 2023). Another study revealed that SNF1-related kinase 1 (SnRK1) represses sucrose-induced anthocyanin biosynthesis by suppressing *PAP1* expression but inducing *MYBL2* transcription. Additionally, SnRK1 directly interacts with and phosphorylates PAP1, bHLH2, and TTG1, resulting in impaired MBW complex formation. Nevertheless, sucrose reduces *SnRK1* expression and relieves its repression on the MBW complex (Broucke *et al.*, 2023). In the absence of gibberellin or under abiotic stresses, accumulated DELLA proteins sequester MYBL2 and JAZs, releasing their inhibition of the MBW complex, leading to the formation of MBW complex and the activation of anthocyanin biosynthesis (Xie *et al.*, 2016). A more recent study revealed that WRKY33 represses *DFR* expression and anthocyanin biosynthesis through directly binding to the *DFR* promoter as well as interacting with PAP1 and disturbing the assembly of MBW complex under phosphorus-sufficient conditions (Tao *et al.*, 2024).

In this study, we discovered that high light-induced anthocyanin biosynthesis involves the repression of *MYBL2* expression and the up-regulation of MBW components such as *PAP1*, *PAP2*, and *TT8* (Fig. 1). Strikingly, we revealed that PAP1, but not PAP2, collaborates with TT8 to promote *MYBL2* expression (Fig. 7); however, MYBL2 negatively regulates transcription of *PAP1* and *TT8* as feedback (Fig 1D). Notably, the promotion of *MYBL2* expression by PAP1 seems effective only at high PAP1 levels, as *MYBL2* expression was increased in *pap1-D* plants, but remained unchanged in *myb75-c* plants under both growth light and high light conditions (Fig. 7A). In petunia, two repressor genes, *PhMYB27* and *PhMYBx*, were found to inhibit *AN1* transcription, despite being activated by AN1 and the MBW complex (Quattrocchio *et al.*, 1998; Spelt *et al.*, 2000; Albert *et al.*, 2014). In soybean, GmMYBA2 along with GmTT8a form a positive complex that activates the expression of the MYB repressor gene *GmMYBR*; subsequently, expressed GmMYBR competitively interacts with GmTT8a, thereby interfering with the activity of the GmMYBA2–GmTT8a complex and inhibiting seed coat pigmentation (Gao *et al.*, 2021). These negative feedback loops between MYB repressors and the MBW activation complex may function as a 'rheostat' to prevent excessive anthocyanin accumulation and maintain normal growth and development. The conserved hierarchical and feedback regulatory networks comprising the MBW components, activators, and repressors probably play crucial roles in controlling anthocyanin production.

Based on the integration of previous findings and our results, we propose a working model to elucidate the regulation of anthocyanin production in response to light intensity. This model highlights the pivotal role of MYBL2 and the MBW components PAP1 and TT8, as a core regulatory module. Under low-intensity light conditions, *MYBL2* transcription is activated, and the expressed MYBL2 protein simultaneously represses the expression of *PAP1* and *TT8*. Moreover, MYBL2 physically interacts with TT8 and/or PAP1, thereby disrupting MBW complex formation and inhibiting transcription activation of anthocyanin biosynthetic genes, resulting in restricted anthocyanin production (Fig. 8A). Conversely, high light irradiation suppresses *MYBL2* expression, which alleviates the repression effects on *PAP1* and *TT8* transcription as well as their protein activities. Consequently, expressed PAP1 and TT8 proteins facilitate MBW complex assembly, thereby promoting the expression of anthocyanin biosynthetic genes and enhancing anthocyanin production, which aids plants in adapting to high light stress (Fig. 8B). When plants produce adequate anthocyanins or have high expression levels of *PAP1*, PAP1 and TT8 cooperatively trigger *MYBL2* transcription; MYBL2, in turn, reduces the expression of both *PAP1* and *TT8* and impairs MBW complex formation, serving as a 'brake' to prevent anthocyanin overproduction and to maintain normal growth (Fig. 8B). The dynamic hierarchical and feedback regulation between MYBL2 and PAP1/TT8 allows plants to rapidly, precisely, and flexibly modulate anthocyanin biosynthesis under varying light conditions. Moreover, it is worth noting that additional factors including HY5, HY5-induced *MYBD* and miR858a, DELLAs, AP2, and SnRK1 may contribute to

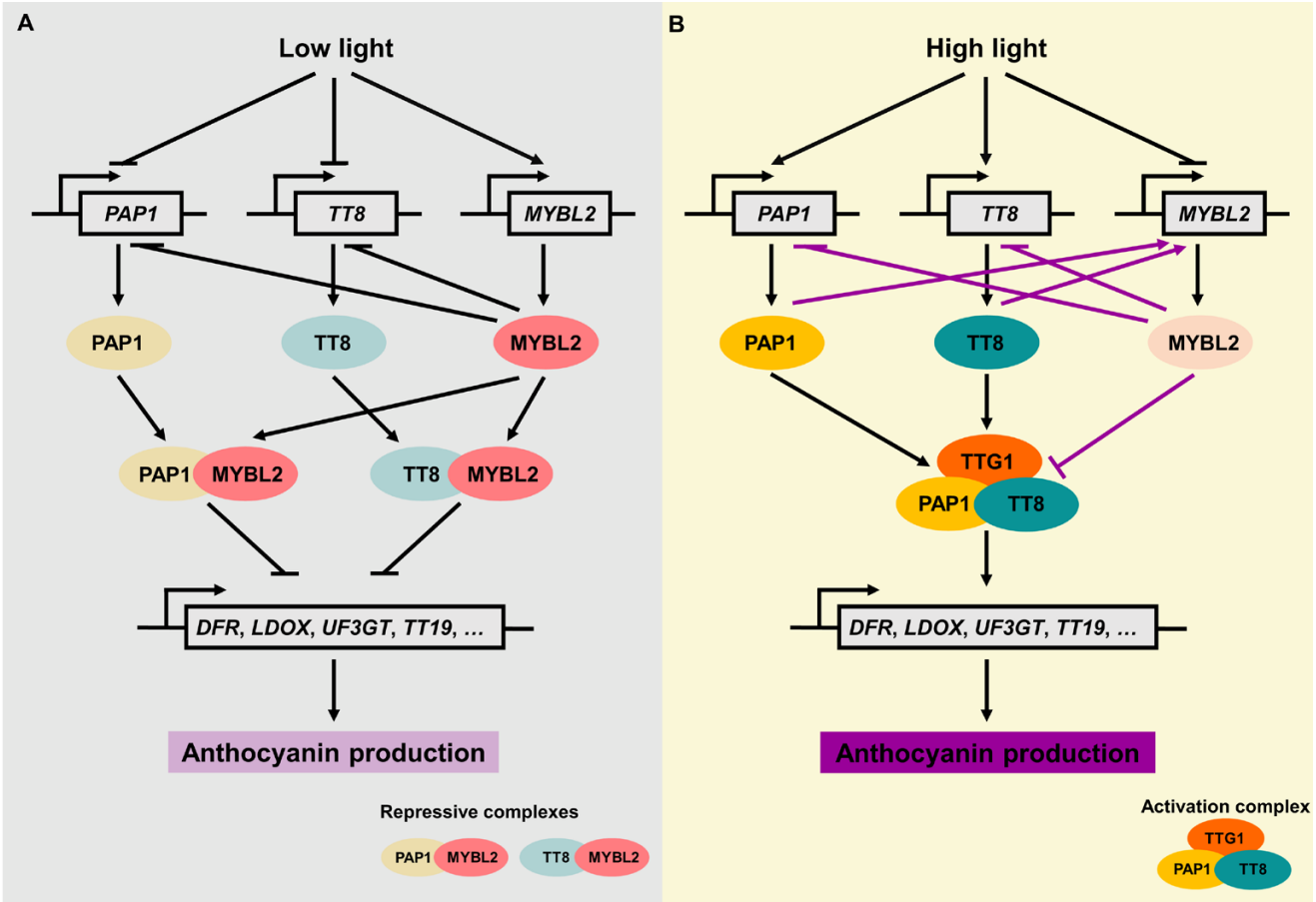

Fig. 8. A possible working model for the MYBL2–PAP1 regulatory module in high light-induced anthocyanin biosynthesis and accumulation. Dynamic anthocyanin production in response to varying light conditions is predominantly controlled by the antagonistic activities between the MYBL2 repressor and the MBW activation complex. (A) Under low light, MYBL2 represses the transcription of *PAP1* and *TT8*, and directly interacts with PAP1 and TT8 proteins, thereby disrupting the MBW complex assembly and inhibiting the transcription of anthocyanin biosynthetic genes, resulting in restricted anthocyanin production. (B) Upon high light irradiation, *MYBL2* transcription is repressed, while the expression of *PAP1* and *TT8* is activated. Expressed PAP1 and TT8 proteins promote the assembly of the MBW complex and trigger the transcription of anthocyanin biosynthetic genes, leading to anthocyanin biosynthesis and accumulation, which enhances plant tolerance to high light stress. Under excessive anthocyanin accumulation or high *PAP1* expression level, PAP1 and TT8 cooperatively activate *MYBL2* transcription; as feedback, expressed MYBL2 represses the transcription of *PAP1* and *TT8* and impairs MBW complex formation, thus preventing anthocyanin over-accumulation and maintaining the balance between growth and high light response. Dashed lines represent possible but unvalidated regulations, solid lines indicate confirmed regulations, and purple lines suggest regulations occurring only under excessive anthocyanin production. DFR, DIHYDROFLAVONOL 4-REDUCTASE; LDOX, LEUCOANTHOCYANIDIN DIOXYGENASE; MBW, MYB–bHLH–WD40; MYBL2, MYB-LIKE 2; PAP1, PRODUCTION OF ANTHOCYANIN PIGMENT 1; TT8, TRANSPARENT TESTA 8 (TT8); TT19, TRANSPARENT TESTA 19; TTG1, TRANSPARENT TESTA GLABRA 1; UF3GT, ANTHOCYANIDIN UDP-GLUCOSE:FLAVONOID 3-*O*-GLUCOSYLTRANSFERASE.

regulating *MYBL2* expression, whereas the transcription regulation of *PAP1* may be associated with HY5, *TAS4*-siR81(−), MYB30, BZR1 and WRKY33 (Supplementary Fig. S5). However, several key issues need to be addressed, such as the precise response of *MYBL2* and *PAP1* expression to varying illumination intensity, the threshold values of anthocyanin content and *PAP1*/*TT8* expression levels triggering the re-activation of *MYBL2* under specific high light conditions, and the interplay between light and other environmental cues in modulating anthocyanin biosynthesis, among others.

Supplementary data

The following supplementary data are available at *JXB* online.

Fig. S1. Relative expression of two validated reference genes, *UBQ10* and *ACTIN2* under different light conditions.

Fig. S2. Relative expression of *PAP2* in different plants under continuous growth light (80 μmol photons m^{-2} s^{-1}) and high light (1200 μmol photons m^{-2} s^{-1}) for 3 h.

Fig. S3. Putative G-box motifs in the *PAP1* promoter regions.

Fig. S4. Multiple sequence alignment of MYBL2, PhMYB27, PAP1, PAP2, and DPL.

Fig. S5. An integrative model for the regulation of high light-induced anthocyanin biosynthesis and accumulation by the MYBL2–PAP1 module and other regulators.

Table S1. Primers used in this study.

Acknowledgements

We thank Dr Jirong Huang (Shanghai Normal University) for kindly providing seeds of *mybl2* (SALK_ 126807) and *MYBL2-OE* lines. We thank Dr Honghui Lin and Dr Dawei Zhang (Sichuan University) for kindly providing seeds of *myb75-c* and *pap1-D* mutants. We thank Dr Xiang Gao (Northeast Normal University) for kindly providing seeds of *Nicotiana tabacum* K326. We thank Dr Yanjie Zhang (Zhengzhou University) for generously providing the pBI121 plasmid and for his valuable advice and helpful comments.

Author contributions

XZ, KC, and MX conceived and designed the research; MX performed most of the experiments; PX, YW, CH, CL, WH, YZ, and XYZ performed some of the experiments; XZ, KC, and MX analysed the data; MX wrote the manuscript; XZ, KC, and MX revised the paper. All authors participated in this research and approved the final draft of the paper.

Conflict of interest

The authors have no conflicts of interest to declare.

Funding

This work was supported by the National Natural Science Foundation of China (32070262) and the Zhongyuan Scholar Workstation Funded Project of Henan Province in China (234400510004).

Data availability

All data generated or analysed during this study are included in this article and its supplementary data.

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