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HY5 regulates anthocyanin biosynthesis by inducing the transcriptional activation of the MYB75/PAP1 transcription factor in *Arabidopsis*

Dong Ho Shin [a,1], MyungGoo Choi [a,1], Keunhwa Kim [b], Geul Bang [a], Misuk Cho [a], Sang-Bong Choi [c], Giltsu Choi [b], Youn-Il Park [a,*]

[a] Department of Biological Sciences, Chungnam National University, Daejeon 305-764, Republic of Korea
[b] Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea
[c] Division of Bioscience and Bioinformatics, Myongji University, Yongin 449-728, Republic of Korea

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ABSTRACT

Several positive transcription factors regulate *Arabidopsis* anthocyanin biosynthesis. HY5, a component of light-signaling pathways, and PAP1, an R2R3-MYB transcription factor, share common regulatory targets on anthocyanin biosynthesis genes. The epistatic interactions between the two transcription factors are currently unknown. To address this problem, we analyzed crosses between *hy5* and *pap1* mutants (*hy5pap1*) or *pap1D* overexpressors (*hy5pap1D*), performed chromatin immunoprecipitation-qPCR, and determined the *PAP1* promoter region through deletion analysis. The results show that HY5 regulates *PAP1* expression via direct binding to G- and ACE-boxes in the promoter region, which suggests bifurcate regulation of anthocyanin biosynthesis by HY5 via transcriptional activation of PAP1.

1. Introduction

Anthocyanins are water-soluble, vacuolar pigments in plants that belong to the family of flavonoid compounds. Anthocyanins are involved in red, blue, and purple coloring of vegetative and reproductive organs. These compounds attract pollinators and promote seed dispersal. They also have roles in protection from UV damage and pathogens. The biosynthesis of anthocyanins is mediated by multiple enzymes in the phenylpropanoid pathway. The coding genes of these enzymes are grouped into two classes: early biosynthetic genes (EBGs; *CHS*, *CHI*, *F3H*), which are common in flavonoid pathways, and late biosynthetic genes (LBGs; *DFR*, *LDOX*, *UF3GT*), which are induced after expression of the EBGs [1].

Expression of LBGs in *Arabidopsis* involves dual regulation by the positive transcription factors PAP1 and HY5. PAP1 is one of four R2R3 MYB activators (the other three are PAP2, MYB113, and MYB114) involved in anthocyanin biosynthesis. PAP1 interacts with one of the three bHLH activators (TT8, EGL3, or GL3) to form transcription complexes with the WD-repeat transcription factor, TTG1 [2,3]. The primary targets of the canonical R2R3-MYB/bHLH/WD-repeat combinatorial transcription complex (MBW) are LBGs, because overexpression of *PAP1* in a *pap1* dominant mutant (*pap1D*) preferentially upregulates the transcripts of *DFR*, *LDOX*, and *UF3GT*, whereas EBGs remain unchanged [4,5]. Preferential expression of LBGs by PAP1 occurs through its binding to the 10 bp CCACG-containing PAP1 *cis*-regulatory element (PCE) within their promoter regions [6]. The transcription of PAP1 is strongly dependent upon light, and is positively regulated by sugars and cytokinins and negatively regulated by ethylene [7]. The leucine-zipper transcription factor, HY5 (a downstream component of phytochrome (PHY), cryptochrome (CRY), and UV-B (UVR8) photoreceptor-mediated light signaling), is also involved in anthocyanin biosynthesis [8,9]. HY5 regulates expression of anthocyanin structural genes through direct binding to the promoters of both EBGs and LBGs such as *CHS*, *CHI*, *F3H*, *F3′H*, *DFR*, and *LDOX* [8,10–12]. Light-dependent expression of LBGs in *Arabidopsis* appears to be regulated by both PAP1 and HY5. However, it is not known if epistatic interaction occurs between these two regulatory factors.

We noticed that the *PAP1* promoter region contains putative HY5 binding sites comprising G- and ACE-boxes. This strongly

Abbreviations: Bc, continuous blue light; EBG, early anthocyanin biosynthesis genes; FRc, continuous far-red light; HY5, long hypocotyl 5; LBG, late anthocyanin biosynthesis genes; MBW, R2R3-MYB/bHLH/WD-repeat combinatorial transcription complex; PAP1, production of anthocyanin pigment 1; Rc, continuous red light

* Corresponding author. Tel: +82 42 821 5493; Fax: +82 42 821 9690.
E-mail address: yipark@cnu.ac.kr (Y.-I. Park).

[1] Both authors contributed equally to this work.

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suggests that HY5 regulates *PAP1* expression by binding to these elements. Thus, the present study examined both the expression of gene transcripts associated with *PAP1* and anthocyanin biosynthesis and anthocyanin pigment levels using *pap1hy5* and *pap1Dhy5* double mutants (crosses between the loss-of-function *pap1* mutant or the *pap1D* gain-of-function mutant and the *hy5* mutant). Chromatin immunoprecipitation (ChIP)-qPCR and promoter analyses were carried out. We report that *PAP1* expression is strongly dependent on HY5, which suggests that HY5 regulates anthocyanin biosynthesis via a bifurcate regulation mechanism.

2. Materials and methods

2.1. Plant materials and growth conditions

The *Arabidopsis thaliana* plant lines used for these studies included the following: wild-type (WT) Col-0, No-0, and Ler plants; *PAP1* loss-of-function (*pap1*, line *pst16228* from RIKEN Biological Resource Center in No-0 background) and *PAP1* gain-of-function (*pap1D* from the Arabidopsis Biological Resource Center in the Col-0 background) mutants; the light-signaling mutants *hy5* (*hy5-215*) [13], *phyA211* (M548T) [14], *cry1* (*hy4-2.23N* in Ler background) [15]; and the *HY5*-overexpressing transformant, *HY5ox,* in *hy5* mutant background [11]. Seeds were surface-sterilized and cold-stratified at 4°C for 3 d on solidified 1/2-strength MS media supplemented with 60 mM sucrose; then, plants were grown in a growth room with a photoperiod of 16-h light/8-h dark at 22±1°C. Growth boxes equipped with blue ($_{max}$ = 464 ± 23.3 nm), red ($_{max}$ = 650 ± 19.4 nm), and far-red ($_{max}$ = 720 ± 19.4 nm) LEDs (Good Feeling, Sungnam, Korea) were used for growth under different light conditions. Light intensities for white, blue, red, and far-red were 70, 20, 15, and 5 μmol m^{-2} s^{-1}, respectively.

2.2. Construction of pap1hy5 and pap1Dhy5 double mutants

The *pap1hy5* and *pap1Dhy5* double mutants were obtained by crossing the *hy5* mutant to *pap1* and *pap1D* mutants, respectively (Figs. S1 and S2). Selection of the respective mutants was performed under constant low white light by sequential selection of F2 segregants for long hypocotyls, which is a marker that indicates homozygosity for *hy5*, followed by PCR analysis to select *pap1* and *pap1D* homozygotes.

2.3. Anthocyanin measurement

Anthocyanin was extracted with methanol acidified with 1% HCl (v/v) and then dechlorophyllized in chloroform [16]. The anthocyanin yield was calculated by subtracting the A_{657} from the A_{530} as previously described [17].

2.4. PCR analysis

For quantitative real-time PCR analysis, total RNA was extracted with TRI reagent (Molecular Research Center), followed by DNaseI (MB) treatment. cDNA was synthesized from 1 μg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad) [14]. Quantitative PCRs were performed with the SYBR Green qPCR Kit (TOYOBO) using the CFX96™ Real Time System (Bio-Rad, USA) according to the manufacturer's instructions, and using primers (Table S1) for regulatory and structural genes of anthocyanin biosynthesis [16]. *Protein phosphatase 2A* (*PP2A*), and *ACTIN2* or *18S rRNA* were used as internal controls for qPCR and RT-PCR, respectively.

2.5. ChIP analysis

ChIP was performed essentially as described previously [18]. Eluted DNA was purified with a PCR purification kit (Solgent) and subjected to real-time qPCR.

2.6. Transient transformation and fluorometric assays

Five different fragments (P1 to P5; Fig. 2A) from the *PAP1* promoter region were cloned and fused to the *β-glucuronidase* (*GUS*) reporter gene in *pCAMBIA3301* using the primers listed in Supplemental Table 1. To test HY5 activation of *PAP1* expression, *HY5* cDNA-containing constructs were transiently transformed into *Arabidopsis* WT or *pap1hy5* double mutants. Four-day-old seedlings grown in darkness were vacuum-infiltrated with *Agrobacterium* twice for 1 min [19], followed by 1 d incubation in darkness, and then transferred to white-light growth conditions for 2 d. GUS activities were measured using the substrate 4-methylumbelliferyl-β-D-glucuronide (Duchefa, Netherlands) [20] and a spectrofluorometer (LS-55, Perkin-Elmer). For calibration, 4-methylubelliferone (Sigma-Aldrich) was used; protein content was determined using BSA as standard [21].

3. Results

3.1. HY5 and PAP1 redundantly regulate expression of LBGs

ChIP analyses [10–12] indicated that transcript levels of both EBGs and LBGs, including *CHS*, *DFR*, *LDOX*, and *UF3GT*, were positively regulated by HY5 via its direct binding to G-box and ACE elements. Reporter assays [6] revealed that PAP1 preferentially targets LBGs to EBGs by binding to the *cis*-regulatory element (PCE). We tested this by performing genetic crosses between *hy5* and *pap1* mutants. The *hy5* seedlings with long hypocotyls had decreased anthocyanin levels under blue, red, and far-red light, but not under dark conditions because the transcript levels of most EBGs and LBGs were directly controlled by HY5 [10–12]. The *pap1* mutant, which was generated by insertion of the dissociation (DS) transposon in the third exon of the *PAP1* gene in No-0 background [4], displayed an anthocyanin-deficient phenotype even in sucrose (Suc)-containing medium [5]. *Arabidopsis* seedlings were grown on half-strength MS media containing 60 mM (2.16%) sucrose under continuous far-red (FRc) or blue (Bc) light conditions; WT Col-0, No-0 and Ler plants accumulated anthocyanins, but anthocyanins were not observed in *phyA* and *cry1* mutants (Fig. 1A). This result was consistent with previous reports [22] that anthocyanin accumulation was mediated by PHYA and CRY1 under FRc and Bc, respectively. As expected, *hy5* mutants failed to respond to both FRc and Bc and did not accumulate anthocyanin. This confirmed an earlier report that HY5 is a downstream component of PHY and CRY signaling pathways [8]. Under white-light growth conditions, *PAP1* expression is linked to photosynthesis-dependent light signaling [16]; therefore, the accumulation of anthocyanin is not expected to be hindered by *pap1* mutations under FRc or Bc conditions in which PHY and CRY signaling, rather than photosynthesis-derived signaling, play a dominant role in anthocyanin pigmentation. Contrary to our expectations, *pap1* mutants were unable to respond to FRc or Bc (Fig. 1A). Because *HY5* was not genetically altered in *pap1* mutants, the inability of *pap1* mutants to respond to both FRc and Bc strongly suggested that PAP1 was a downstream component for both PHY and CRY signalings. We generated *pap1hy5* double mutants by crossing *pap1* with *hy5* (Fig. SI). The resulting transformants lacked functional PAP1 and HY5, and were unable to respond to both FRc and Bc (Fig. 1A).

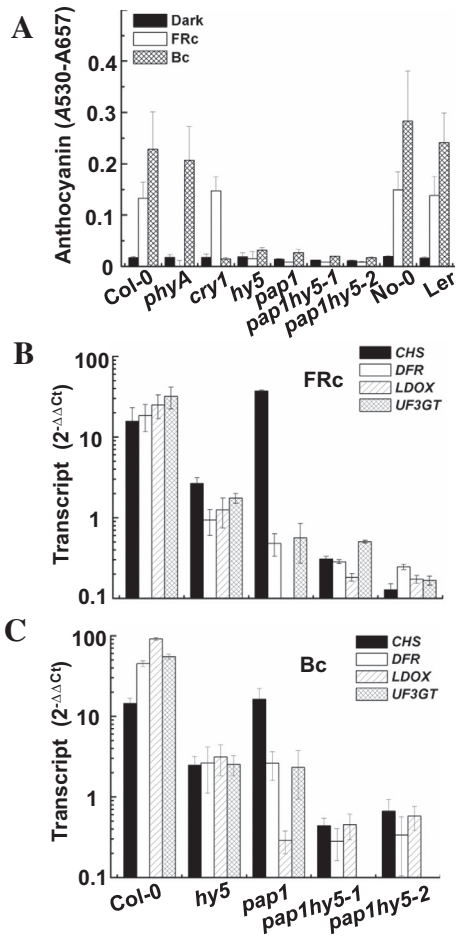

Fig. 1. HY5 and PAP1 co-regulate the expression of LBGs. (A) Anthocyanin accumulation by FRc and Bc in Col-0, No-0 and Ler was reduced in the *phyA*, *cry1*, *hy5* and *pap1* single and *pap1hy5* double mutants. LBG transcripts were significantly lowered in the *hy5* and *pap1* single mutants and *pap1hy5* double mutants grown under FRc (B) and Bc (C) conditions compared with that in WT seedlings. Error bars ± SD (n = 3 ~ 5).

To correlate the regulation of anthocyanin pigment levels with structural genes, transcripts of representative sugar- and light-inducible genes, including *CHS, DFR, LDOX*, and *UF3GT* [16], were determined by using quantitative RT-PCR. Transcript levels of both EBGs and LBGs were significantly decreased in the *hy5* mutant plants under FRc and Bc light conditions (Fig. 1B and C). In contrast to HY5 regulation and consistent with a previous report [5], PAP1 predominantly regulated transcript levels of LBGs, because *CHS* expression was not significantly influenced by the loss-of-function mutation in *PAP1*. Dramatic changes in the transcript levels of LBGs were observed in *pap1* mutants (Fig. 1B and C). In *pap1hy5* mutants, light induction of the transcript levels of both EBGs and LBGs was severely inhibited compared to those of the *hy5* and *pap1* single mutants (Fig. 1B and 1C) under both FRc and Bc.

3.2. HY5 directly binds to the PAP1 promoter in vivo

PAP1 is not thought to be a putative target for HY5, even though 51 genes belonging to the MYB (or MYB-like) family of transcription factors have been identified as HY5 targets [10,12]. We found putative HY5 binding sites in the promoter region of *PAP1*, which included a G-box (CACGTG) and two ACE elements (Fig. 2A). To test the *in vivo* binding of HY5 to the *PAP1* promoter, we performed ChIP analysis using myc-tagged-*HY5* transgenic lines [11], and determined the enrichment in the immunoprecipitates of genomic DNA fragments from the promoter of the *PAP1* gene to the 3′-UTR (untranslated region; A to E; Fig. 2A) by using five primer pairs (Table S1). Primer pairs that amplified the genomic region around the *5S rDNA* and *CHS*-D, which contains the ACE-box [11], were included as negative and positive controls, respectively. ChIP analysis of the immunoprecipated HY5-myc demonstrated that the *PAP1* promoter fragments C and D and the *CHS*-D promoter were enriched 5.1-, 4.5-, and 6.4-fold, respectively, compared to the negative control (*5S rDNA*; Fig. 2B). By contrast, fragment E, intragenic control fragment B, and the 3′-end region A of *PAP1*, were less enriched than fragments C and D (Fig. 2B). Consistent with this, DNAs immunoprecipitated by an anti-GFP antibody showed no specific enrichment of the G-box/ACE element containing the *PAP1* fragments or the *CHS*-D or *CHI*-C promoters [11] (Fig. S3), indicating that HY5 binds directly to the *PAP1* promoter *in vivo*.

To investigate the role of ChIP-enriched promoter regions *in vivo*, we cloned five fragments from the *PAP1* promoter region that had lengths ranging from 167 bp to 1.2 kb, fused them to a *GUS* reporter gene in *pCAMBIA3301*, isolated five constructs (P1 - P5), and transformed them alone or with *HY5* into *Arabidopsis* WT and *pap1hy5* double-mutant plants. In *pap1hy5* double mutants, GUS activity of the promoter from 1,196 to 850 (P1) was 35% lower than that from the WT promoter (Fig. S4). Cotransformation of the P1-GUS construct with *HY5* in *pap1hy5* double-mutant lines resulted in a statistically significant partial recovery of GUS activity (1.2-fold increase, p<0.05) (Fig. S4). To determine the importance of the C region in HY5 recognition, the *PAP1* promoter fragments P1 - P5 were cotransformed with *HY5* in *pap1hy5* double-mutant lines. The fragments P1, P2, P3, and P4, which contained the C region, responded to *HY5* co-expression; the fragment P5, which lacked the C region, failed to show significant HY5-dependent recovery of GUS activity (Fig. 2C). These results suggested that HY5 bound directly to the *PAP1* promoter *via* the C region that contained both the G-box and the ACE element.

3.3. HY5 regulates the expression level of PAP1

If *PAP1* is indeed a target for HY5, then the *hy5* mutation should affect *PAP1* transcript levels. Compared with those in dark-grown seedlings, *PAP1* transcript levels increased 2.6-, 7.0-, and 3.5-fold under Rc, FRc, or Bc light conditions, respectively (Fig. 2D). By contrast, mutation of *hy5* almost completely inhibited the light-dependent induction of *PAP1* transcripts; *PAP1* transcript levels were 63 - 70% lower in *hy5* mutant plants than in dark-grown Col-0 WT plants (Fig. 2D). Overexpression of *HY5* in the *hy5* mutant background (*HY5ox*) restored the *hy5* mutant phenotype to the WT phenotype, accompanied by 1.8-, 2.7-, and 2.0-fold increases in *PAP1* transcript levels under Rc, FRc, and Bc conditions, respectively (Fig. 2D). In *pap1* single and *pap1hy5* double-mutant plants, *PAP1* transcript levels were not detectable. Taken together, these results strongly indicated that HY5 regulated *PAP1* transcript levels.

We next investigated whether HY5 modulates *PAP1* transcript levels by introducing the *hy5* mutation into the gain-of-function *pap1* mutant, *pap1D*. The *pap1D* (*production of anthocyanin pigment 1-dominant*) mutant displayed strongly increased *PAP1* transcript levels due to the insertion of the CaMV-35S enhancer approximately 5.1 kb downstream of the transcription start site, which resulted in elevated anthocyanin levels due to increased transcript levels of LBGs [4,5]. As expected, introduction of the *HY5*-null mutation into *pap1D* (Fig. S2) led to noticeable reductions in both *PAP1* transcript levels and anthocyanin accumulation (Fig. 3). For example, the *PAP1* transcript levels observed in *pap1D* mutants

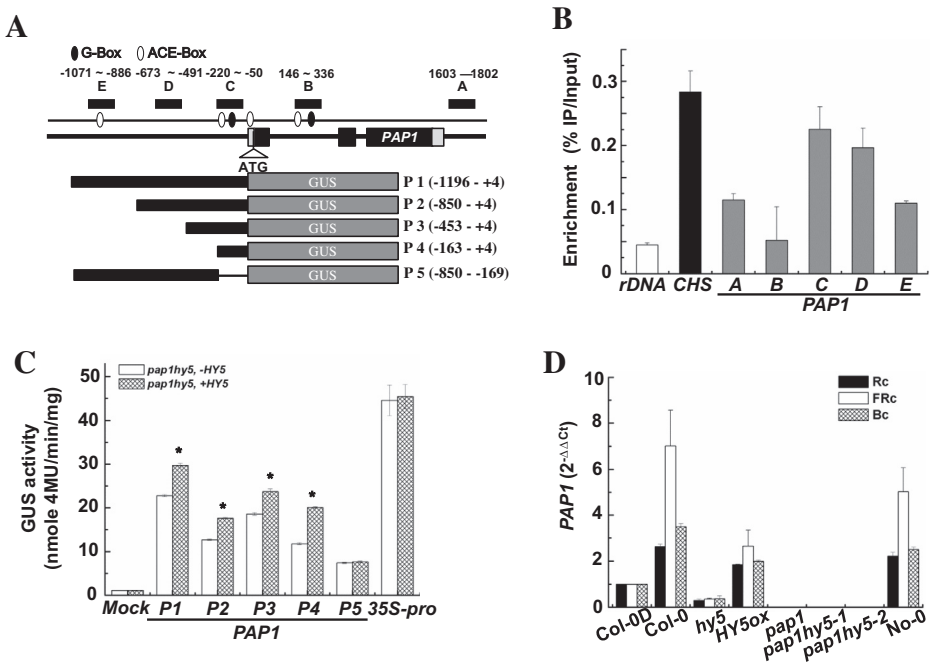

Fig. 2. HY5 directly binds to the *PAP1* promoter at G- and ACE-boxes *in vivo*. (A) Diagram of the *PAP1* promoter fragments (A–E) and chimeric *PAP1* promoter-GUS constructs including ACE and G-boxes (P1–P5). Each element is indicated by a base number as counted from the translation start site. (B) ChIP analysis shows the direct binding of HY5 to the *PAP1* promoter *in vivo*. A to E indicate genomic DNA fragments of the *PAP1* promoter tested for enrichment by quantitative PCR (SD, $n = 3$). *5S rDNA* and *CHS* were used as negative and positive controls, respectively. The ChIP values were normalized to the respective DNA inputs. (C) GUS activity in *pap1hy5* double mutants transiently transformed with *PAP1* promoter fragment–GUS fusions (*P1–P5*) either with (*+HY5*) or without HY5 (*-HY5*). Mock and 35S Pro indicate non-transfected and 35S promoter *pCAMBIA3301*-transfected *pap1hy5* seedlings, respectively. (D) *PAP1* transcript levels were significantly lowered in the *hy5* and *pap1hy5* mutants grown under Rc, FRc, and Bc conditions. The levels of *PAP1* transcription are shown relative to those of dark-grown WT (Col-0D). Error bars ± SD ($n = 3 \sim 5$, $p<0.05$).

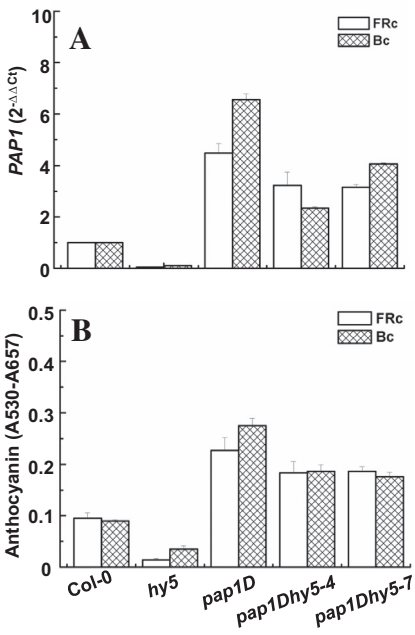

Fig. 3. The *HY5* mutation down-regulates *PAP1* transcription and reduces the accumulation of anthocyanin. *PAP1* transcription (A) and anthocyanin content (B) were investigated in WT, *hy5* and *pap1D* single mutants and in *pap1Dhy5* double mutants grown under FRc and Bc conditions. The levels of *PAP1* transcripts in the mutants are shown relative to those in the WT (Col-0) grown under FRc and Bc conditions. Error bars represent the standard deviation ($n = 3$).

decreased in *pap1Dhy5-4* mutants by 30% and 65% under FRc and Bc light conditions, respectively, compared to those of the WT controls (Fig. 3A). Accordingly, decreases in the anthocyanin levels by 20% and 35% were observed in *pap1Dhy5-4* mutants grown under FRc and Bc conditions, respectively (Fig. 3B). Therefore, HY5 appeared to regulate *PAP1* expression by binding at the 5′ promoter region via the G-box and ACE elements.

4. Discussion

The only known mechanism for HY5 signaling in anthocyanin biosynthesis was thought to be through its direct binding to the G-box promoter region of structural genes [10,12]. The present study describes a detour pathway for HY5 regulation of anthocyanin biosynthesis via PAP1. HY5 was required for light-induced accumulation of the *PAP1* transcript, because transcript levels decreased in response to *HY5* mutation and were complemented by *HY5* overexpression. ChIP-qPCR and transient analysis showed that activation of *PAP1* transcription by HY5 appeared to occur via

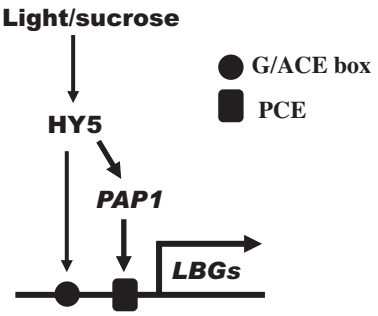

Fig. 4. Schematic diagram for the HY5-dependent bifurcate regulation of anthocyanin biosynthesis.

direct binding to the G-box and ACE element promoter region near the translation start site. Therefore, HY5 appeared to regulate anthocyanin biosynthesis in two ways: via direct binding to the promoter regions of the structural genes, and by positive regulation of *PAP1* transcription (Fig. 4).

The LBGs involved in anthocyanin biosynthesis were regulated by the HY5 and PAP1 transcription factors [6,11]. We noted the presence of both HY5 and PAP1 binding sites in the promoter region of LBGs such as *DFR*, *LDOX*, and *UF3GT*, but the number of (and distance between) the respective elements were variable (Fig. S5). First, two G/ACE and PCE elements were found in the *DFR* promoter region, but the *LDOX* and *UF3GT* promoter regions contained only one element. Second, the HY5-binding G-box and ACE element were located either very close to the PCE sequences for the promoters of *DFR* and *LODX*, or were far apart for the *UF3GT* promoter. We found that the reduction of LBG transcripts in *hy5pap1* double-mutant plants was approximately the sum of the reduction of each gene transcript level observed in the single mutant plants of *hy5* and *pap1* (Figs 1 and 3). Thus, the binding of each transcription factor to its own sites in the promoter regions of LBGs would act in an additive way. However, HY5 and PAP1 did not appear to be equally effective in the activation of LBG transcription. PAP1 appeared to play a more important role than HY5, because accumulation of LBG transcript levels was more severely inhibited by mutation of *PAP1* than mutation of *HY5* (Fig. 1). The mechanism of how these two transcription factors cooperated in transcriptional activation of LBGs, specifically for *DFR* and *LDOX*, was not clear. The near overlap of G/ACE and PCE elements in the promoter region would preclude simultaneous binding of HY5 and PAP1. Regardless of the mechanism underlying co-activation, the binding of HY5 to its binding site may facilitate PAP1 binding to the PCE element during sugar-induced anthocyanin pigmentation, a process that requires light to activate the transcription of LBGs [16].

We showed the R2R3-MYB PAP1 was transcriptionally activated under continuous FRc and Bc light. *PAP1* expression was highly dependent on the presence of HY5, suggesting that PAP1 was a downstream target of HY5. We recently reported the presence of a light signal that was dependent on photosynthesis but independent of HY5-mediated PHY and CRY signaling cascades [16]. The implication of this plastid-derived signal for *PAP1* expression should be further explored within the context of HY5-mediated PHY and CRY signaling.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.03.037.

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