1 **Novel insight into the mechanism underlying light-controlled anthocyanin**
2 **accumulation in eggplant (*Solanum melongena* L.)**

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Abstract

Eggplant is rich in anthocyanins, which are the major secondary metabolites and beneficial to human health. We discovered that the anthocyanin biosynthesis of eggplant cultivar ‘Lanshan Hexian’ was regulated by light. In this study, we isolated two blue light receptor genes, *SmCRY1* and *SmCRY2*, and negative/positive anthocyanin regulatory factors *SmCOP1* and *SmHY5* from eggplant. In terms of transcript levels, *SmCRY1*, *SmCRY2* and *SmHY5* were up-regulated by light, while *SmCOP1* was down-regulated. Subsequently, the four genes were functionally complemented in phenotype of corresponding mutants, indicating that they act as counterparts of *Arabidopsis* genes. Yeast two-hybrid and bimolecular fluorescence complementation assays showed that *SmCRY1* and *SmCRY2* interact with *SmCOP1* in a blue-light-dependent manner. It also obtained the result that *SmCOP1* interacts with *SmHY5* and *SmMYB1*. Furthermore, using yeast one-hybrid assay, we found that *SmHY5* and *SmMYB1* both bind the promoters of anthocyanin biosynthesis structural genes (*SmCHS* and *SmDFR*). Taken together, blue-light-triggered *CRY1/CRY2-COP1* interaction creates the condition that *HY5* and *MYB1* combine with the downstream anthocyanin synthesis genes (*CHS* and *DFR*) in eggplant. Our finding provides a new working model by which light controls anthocyanin accumulation in eggplant.

Keywords Light; Anthocyanin; Eggplant; Cryptochrome; COP1; HY5

1 **Introduction**

2 Anthocyanins, the largest subclass of water-soluble pigments synthesized via the flavonoid
3 pathway, confer appealing color present in plants [1,2]. Anthocyanins play important roles in a large
4 range of plant functions, such as providing protection against UV radiation, low temperature, drought
5 stress and pathogen attack [3–6]. These pigments also help human to prevent several chronic diseases,
6 such as cancer, cardiovascular disease, obesity and diabetes [7].

7 In the past few decades, the biosynthesis of anthocyanins was characterized in model plants, like
8 *Arabidopsis thaliana* [8]. The biosynthesis process is catalyzed stepwise by chalcone synthase (CHS),
9 chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR) and
10 anthocyanidin synthase (ANS). In addition, these structural genes are under the regulation of several
11 transcription factors. To date, R2R3-MYB, basic-helix-loop-helix (bHLH), and WD Repeat Proteins
12 (WDR), have been found to form a MYB-bHLH-WDR (MBW) complex to activate anthocyanin
13 biosynthesis-related genes [9,10]. The WDR proteins are thought to act as a bridge to stabilize the
14 MBW complex, and are not thought to bind DNA [11]. The bHLH of the MBW complex does not limit
15 anthocyanin accumulation, and they are involved in the determination of epidermal cell fate [12].
16 Recent studies have shown that the R2R3-MYB transcription factors, which directly affect the
17 expression levels of the structural anthocyanin biosynthesis genes, play a central role in anthocyanin
18 biosynthesis regulation [13,14]. In *Arabidopsis*, *AtMYB75*, *AtMYB90*, *AtMYB113* and *AtMYB114*, are
19 essential for anthocyanin biosynthesis [8,15]. A single *AtMYB75*, knockout mutant is sufficient for loss
20 of anthocyanins in seedlings [15].

21 Light is an essential environmental factor for plant growth and development. Previous studies
22 have shown that light can increase the anthocyanin concentration, especially in fruit skin [16–18].

1 There are several photoreceptors for perceiving light signal, including the blue/ultraviolet-A (320 to
2 500 nm)-light-absorbing cryptochromes (CRYs) [19]. Higher plants generally have two cryptochromes
3 with clear functional diversification. In *Arabidopsis*, CRY1 specializes mostly in responses to high
4 light and CRY2 mediates low light responses [20]. Cryptochromes regulate photomorphogenic
5 development by suppression of CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) activity directly
6 or in association with SPA1 [21–24]. Downstream of the photoreceptors, RING-finger type protein
7 COP1 acts as a ubiquitin E3 ligase responsible for targeting several photomorphogenesis-promoting
8 transcription factors, such as ELONGATED HYPOCOTYL 5 (HY5), HYPOCOTYL HOMOLOG
9 (HYH), LONG AFTER FAR-RED LIGHT 1 (LAF1) and LONG HYPOCOTYL IN FAR-RED 1
10 (HFR1), and subsequently mediates their ubiquitination and degradation via the 26S proteasome
11 pathway [25–28]. HY5 is a basic leucine zipper transcription factor (bZIP) that binds directly to the
12 promoters of light-inducible genes, such as anthocyanin structural genes, promoting their expression
13 and photomorphogenic development [29–32]. Recently, MdCOP1 was shown to interact with
14 MdMYB1 that results subsequently in dark-induced degradation of MdMYB1 and in reduced
15 anthocyanin accumulation in the peel of apple [33]. A similar mechanism for COP1-mediated control
16 of a MYB transcription factor was recently reported to be present in *Arabidopsis* [34].

17 Eggplant (*Solanum melongena* L.) is an agronomically important vegetable, and is cultivated and
18 consumed in many countries [35]. Eggplant cultivars with dark purple skin receive more attention for
19 rich in anthocyanin, with concentrations as 2.34- and 7.08- fold more than grape (*Vitis vinifera*) and
20 onion (*Allium cepa*) [36]. Due to the capacity of oxygen radical scavenging brought by anthocyanin
21 included in fruit peel, eggplant is ranked as a member of the top ten vegetables [37]. Despite its
22 commercial importance, few research efforts have been devoted to anthocyanin analysis of eggplant.

1 The anthocyanin synthesis of some eggplant varieties, such as ‘Lanshan Hexian’, is completely
2 regulated by light.

3 In this study, we isolate two blue light receptor genes, *SmCRY1* and *SmCRY2*, and two
4 photomorphogenesis transcriptional regulatory genes, *SmCOP1* and *SmHY5* from eggplant.
5 Complementation assays using *Arabidopsis* mutants are performed to verify the four genes function.
6 Then, we demonstrate that SmCRY1 and SmCRY2 interact with SmCOP1 in a blue-light-dependent
7 manner, and SmCOP1 interacts with SmHY5 and SmMYB1. Furthermore, we discover that SmHY5
8 and SmMYB1 both bind the promoters of anthocyanin biosynthesis structural genes. Taken together,
9 our results suggest a light-induced working model that regulate anthocyanin biosynthesis in eggplant.

10 **2. Materials and methods**

11 **2.1. Plant materials and treatments**

12 The eggplant cultivar ‘Lanshan Hexian’ was grown in the horticultural farm of Shanghai Jiao
13 Tong University, Shanghai, China. Samples of roots, stems, leaves, petals, peel and flesh from the same
14 plants were collected for determining tissue-specific gene expression. The fruit bagging experiment
15 included three treatments: bagging, where paper bags covered the sepals after full bloom; debagging,
16 for which bags were removed after bagged 14 days at 8:00; and control, with fruits not being bagged at
17 any time. The bags were completely light-impermeable. Peel was collected at 0 h, 0.5 h, 4 h, 8 h, 2 d, 4
18 d, 6 d, 8 d and 10 d after bag removal. In addition, peel was harvested from the fruits that remained
19 bagged and never bagged in the same plants. All the freshly collected plant materials were immediately
20 frozen in liquid nitrogen, and then stored at -80 °C until use.

21 **2.2. Genomic DNA and Total RNA isolation**

22 Genomic DNA and total RNA were extracted from 300 mg of leaves with Genomic DNA

1 Isolation Kit and Total RNA Isolation Kit (Sangon, China) following the manufacturer's instructions.
2 The qualities and concentrations of DNA and RNA were determined by agarose gel electrophoresis and
3 NanoDrop 2000 spectrophotometer (Thermo, USA), respectively. First strand cDNA was synthesized
4 using *Primescript II* 1st strand cDNA synthesis Kit (Takara, Japan).

5 2.3. Gene isolation and sequence analysis

6 In order to isolate the ORF of *CRY1*, *CRY2*, *COPI* and *HY5* from eggplant, we designed
7 degenerate primers based on the conserved region from other plant species (Table S1). The PCR
8 products were cloned into pMD19-T Vector (Takara) and sequenced by the Invitrogen Company
9 (Shanghai, China).

10 Homology searches were performed using the basic local alignment search tool (BLAST) from
11 NCBI (<http://www.ncbi.nlm.nih.gov/>). The conserved domain was predicted by CDD software
12 (<http://www.ncbi.nlm.nih.gov/cdd>), and theoretical isoelectric point (pI) and molecular mass were
13 predicted on the ExPASy website (<http://www.expasy.org/tools>). Multiple alignments of amino acid
14 sequences from eggplant and other plant species were performed using DNAMAN 6.0 (Lynnon Biosoft,
15 Canada). Then the phylogenetic trees were constructed by MEGA 6.0, as a consensus of 1000 bootstrap
16 replicates by the Neighbor-Joining method [38].

17 2.4. Expression analysis

18 Expression patterns analysis were investigated using qRT-PCR. Total RNA was treated with
19 DNase to remove traces of DNA. 1 µg RNA was synthesized into cDNA with the PrimeScript RT
20 Master Mix Perfect Real Time Kit (Takara). According to the manufacturer's instructions of SYBR
21 Premix Ex Taq II Kit (Takara), qRT-PCR was performed on FTC-3000 real-time PCR System (Funglyn
22 Biotech, Canada) using the following program: 95 °C for 30 s, followed by 40 cycles of 95 °C for 20 s,

1 58 °C for 30 s, and 72 °C for 30 s. The *Actin* gene (GU984779.1) from eggplant was amplified in
2 parallel as an internal reference gene [39]. The relative expression levels of the amplified products were
3 analyzed using the comparative C_T method based on C_T values [40].

4 2.5. Functional complementation of *Arabidopsis* mutants

5 Each gene was subcloned into pCAMBIA1302 vector under the control of CaMV35S promoter.
6 All mutants were in the Columbia background. *cry1-104*, *cry2-1*, *cop1-4* and *hy5* were described
7 previously [22,23,41–44]. Seeds were sown on Murashige and Skoog (MS) media, cold-treated for 3 d
8 at 4 °C, and then transferred to controlled environment cabinets under LD (16 h light/8 h dark)
9 condition with a fluence rate of 120 μmol m⁻² sec⁻¹ of white light at 22 °C unless stated otherwise.
10 Plants all grew in LD of white light. Transformation of *Arabidopsis* was carried out by floral dip
11 method [45]. The T₁ transgenic plants were placed on 1/2 strength MS agar medium containing 50
12 mg/L hygromycin. Seeds from each surviving T₁ plant were harvested individually. The T₂ seeds were
13 placed on 1/2 strength MS agar plates containing 50 mg/L hygromycin, and the transgenic lines with a
14 3:1 segregation ratio (resistant:sensitive) were selected to produce T₃ seeds. The T₃ lines displaying
15 100% hygromycin resistance and confirmed by RT-PCR analysis were considered homozygous and
16 used for further experiments.

17 2.6. Extraction and quantification of anthocyanins

18 Anthocyanins were extracted from 500 mg of peel and quantified using the pH differential
19 spectrophotometric method as described by Niu et al [46]. The anthocyanin content was calculated
20 using the following formula: TA=A×MW×5×100×V/e, where TA stands for total anthocyanin content
21 (mg/100 g), V stands for final volume (ml), and A= [A_{510 nm} (pH 1.0) – A_{700 nm} (pH 1.0)] – [A₅₁₀
22 nm (pH 4.5) – A_{700 nm} (pH 4.5)] [47]. Three independent biological replicates were performed.

2.7. Yeast two-hybrid assay

For the GAL4 yeast two-hybrid assay, full-length cDNAs of *SmCRY1*, *SmCRY2*, *SmHY5* and *SmMYB1* were inserted into vector pGADT7 (AD) as prey, and the coding sequence of *SmCOPI* was fused to vector pGBKT7 (BD) as bait. The prey and bait vectors were co-transformed into yeast strain AH109 via the lithium acetate transformation procedure according to the manufacturer's instructions (Clontech, USA). Three days after transformation, six yeast clones grown on nonselective media synthetic dropout-Trp-Leu (SD-TL) with even growth were chosen and inoculated into selective media SD-T-L-His (SD-TLH) in continuous blue light ($30 \mu\text{mol m}^{-2} \text{sec}^{-1}$) or darkness. The LexA yeast two-hybrid assay was performed as described previously [23,48]. Briefly, full-length cDNAs of *SmCRY1*, *SmCRY2*, *SmHY5* and *SmMYB1* were inserted into vector pB42AD (AD) as prey, and the coding sequence of *SmCOPI* was fused to vector pLexA (BD) as bait. The prey and bait vectors were co-transformed into yeast strain EGY48 via the lithium acetate transformation procedure. Three days after transformation, six yeast clones grown on media synthetic dropout-Trp-Ura-His (-TUH) with even growth were chosen and incubated in liquid SD-TUH media for 24 h and the culture was inoculated into SD-TUH media with 80 μM X-gal in continuous blue light ($20 \mu\text{mol m}^{-2} \text{sec}^{-1}$) or darkness.

2.8. Bimolecular fluorescence complementation (BiFC)

All constructs for BiFC assay were obtained by PCR amplification and cloning into the corresponding vectors listed in Table S1. The vectors of BiFC (pXY104 and pXY106) were described previously [49,50]. The constructs were co-transformed into tobacco (*Nicotiana benthamiana*) leaf epidermal cells at the indicated combinations, and analyzed by fluorescence microscope (Leica, Germany).

2.9. Promoter isolation and cis-acting regulatory element analysis

Promoter sequences of five anthocyanin biosynthesis structural genes (*SmCHS*, *SmCHI*, *SmF3H*, *SmDFR* and *SmANS*) were cloned using specific primers (Table S1) with a genome walking kit (Takara). The cis-acting regulatory elements were predicted using PLACE (<http://www.dna.affrc.go.jp/PLACE/signalscan.html>) and PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) databases.

2.10. Yeast one-hybrid assay

The yeast one-hybrid assay was performed as described previously [51]. Full lengths of promoter sequences were fused to vector LacZ. The constructs were co-transformed into yeast strain EGY48 at the indicated combinations. Three days after transformation, six yeast clones grown on media synthetic dropout-Trp-Ura (SD-TU) with even growth were chosen and incubated in liquid SD-TU media for 24 h and the culture was inoculated into SD-TU media with 80 μ M X-gal.

2.11. Statistical analysis

Data were analyzed using variance (ANOVA) and the means were compared by the Duncan's New Multiple Range test ($P < 0.05$) using the SPSS 17.0 (SPSS, USA).

3. Results

3.1. Effect of bagging and debugging treatment on fruit color

As shown in Fig. 1, the fruits displayed white peel when they were bagged. Anthocyanins were not detected in the bagged fruit. After the bags removed, the sun-exposed fruits gradually became purple, and anthocyanin levels were gradually increased in the sun-exposed peel, suggesting that light controls anthocyanin biosynthesis in eggplant.

3.2. Isolation and sequence analysis

Since light controls anthocyanin biosynthesis in eggplant, we isolated *SmCRY1*, *SmCRY2*,

1 *SmCOP1* and *SmHY5* by homologous cloning. Sequence data were deposited in the NCBI database
2 with the accession numbers KT895427 (*SmCRY1*), KT895428 (*SmCRY2*), KT895425 (*SmCOP1*) and
3 KT895426 (*SmHY5*). The ORFs of *SmCRY1*, *SmCRY2*, *SmCOP1* and *SmHY5* were 2037, 1944, 2028
4 and 477 bp in length, encoding 678, 647, 675 and 158 amino acids residues. The encoded protein has a
5 predicted molecular weight of 76.95, 73.21, 76.26 and 17.45 kDa, and the theoretical pI was 5.57, 5.91,
6 6.87 and 9.64, respectively.

7 Both *SmCRY1* and *SmCRY2* contained PHR (photolyase-related) domain in the N-terminal and
8 CCE (cryptochrome C-terminal extension) domain in the C-terminal (Fig. S1, Fig. S2). The CCE
9 domain contained three motifs: DQXVP, acidic region (represented by E and D) and STAES. For
10 *SmCOP1*, it had RING-finger domain in the N-terminal, coiled coil in the middle, and five WD40
11 domains in the C-terminal (Fig. S3). For *SmHY5*, it contained BRLZ (basic region leucine zipper)
12 domain in the C-terminal (Fig. S4).

13 To analyze the phylogenetic relationships between the four genes and corresponding genes from
14 other plants, neighbor-joining phylogenetic trees were constructed using the Mega6.0 program (Fig.
15 S5). The phylogenetic trees indicated that all the four eggplant genes were more closely related to
16 proteins from *Solanum tuberosum* and *Solanum lycopersicum*, while they belong to the same family
17 Solanaceae. In contrast, the four eggplant genes were more distantly related to the proteins from
18 monocotyledon plants, such as *Triticum aestivum*, *Zea mays*, *Brachypodium distachyon* and *Setaria*
19 *italica*. The four phylogenetic trees showed an obvious boundary between dicotyledonous and
20 monocotyledonous plants.

21 3.3. Tissue-specific and light-induced expression analysis

22 It has been proved that *SmMYB1* (KF727476) plays an important role in activating anthocyanin

1 biosynthesis in eggplant [52]. To check the tissue-specific expression levels of *SmCRY1*, *SmCRY2*,
2 *SmCOP1*, *SmHY5* and *SmMYB1*, we performed qRT-PCR analysis using total RNAs obtained from
3 different eggplant tissues. As shown in Fig. 2, *SmCRY1* expressed at the highest level in leaves,
4 followed by the petals and peel. *SmCRY2* expressed at the highest level in roots and leaves, followed by
5 the petals and stems. *SmCOP1* expressed at the highest level in petals, followed by the leaves. The
6 lowest expression levels of the three genes were in the flesh. The expression level of *SmHY5* displayed
7 no tissue-specific. *SmMYB1* expressed at the highest level in peel, followed by the petals and stems,
8 and accumulated extremely low in roots and flesh. The tissue-specific expression level of *SmMYB1*
9 showed significant positive correlation with anthocyanin content of tissues (unpublished result),
10 reflecting the close relation between *SmMYB1* and anthocyanin.

11 To study whether the expression of *SmCRY1*, *SmCRY2*, *SmCOP1*, *SmHY5* and *SmMYB1* were
12 induced by light, fruit bagging experiment was conducted. In the same plant, the expression levels of
13 *SmCRY1*, *SmCRY2*, *SmHY5* and *SmMYB1* were higher in normal irradiated peel, while the expression
14 level of *SmCOP1* was higher in bagged peel (Fig. 3A). Furthermore, as shown in Fig. 3B, the
15 expression levels of *SmCRY1* and *SmHY5* increased immediately after bag removal for 0.5 h of light
16 irradiation, and achieved very high values after 4 h of light irradiation. Although the expression levels
17 of *SmCRY2* and *SmMYB1* did not have significant change after bag removal for 0.5 h of light
18 irradiation, they also achieved a high value after 4 h of light irradiation. The expression level of
19 *SmCOP1* decreased after bag removal. These results indicate that the expression of *SmCRY1*, *SmCRY2*,
20 *SmHY5* and *SmMYB1* are induced by light, and the expression of *SmCOP1* is inhibited by light. In
21 addition, *SmMYB1* do not express in bagged peel, and it may be one of the reasons why bagging
22 treatment strongly inhibits anthocyanin accumulation in eggplant.

3.4. Functional identification of *SmCRY1*, *SmCRY2*, *SmCOP1* and *SmHY5*

To verify the function of *SmCRY1*, *SmCRY2*, *SmCOP1* and *SmHY5*, the *Arabidopsis cry1-104*, *cry2-1*, *cop1-4* and *hy5* were used to conduct functional complementation assays. Four expression vectors containing the *SmCRY1*, *SmCRY2*, *SmCOP1* and *SmHY5* genes driven by the cauliflower mosaic virus 35S promoter were constructed. These four constructs were genetically transformed into their corresponding mutants. After selection of T₃ plants on hygromycin and semi-quantitative RT-PCR analysis, the four genes were ascertained as ectopically expressed in each corresponding mutant (Fig. 4A). Under blue light condition, *cry1-104* showed obviously lengthened hypocotyl than wild-type, and *cry2-1* displayed slightly lengthened hypocotyl (Fig. 4B, Fig. 4C). In addition, the flowering time of *cry2-1* was later than wild-type (Fig. 4D, Fig. 4E). The transgenic seedlings ectopically expressing *SmCRY1* and *SmCRY2* partially recovered the hypocotyl length phenotype under blue light conditions, and *SmCRY2* recovered the flowering time (Fig. 4B, Fig. 4C). These results indicate that *SmCRY1* and *SmCRY2* have CRY1 and CRY2 functions.

Unlike wild-type *Arabidopsis* seedling, *cop1-4* showed strong anthocyanin accumulation under light condition (Fig. 4F). Under darkness condition, wild-type *Arabidopsis* seedling showed closed cotyledon and lengthened hypocotyl, while *cop1-4* did not. These phenomena were due to the constitutive photomorphogenic phenotype (Fig. 4G). Under light condition, *hy5* showed lengthened hypocotyl than wild-type, indicating that HY5 inhibited hypocotyl elongation (Fig. 4H). The transgenic seedlings ectopically expressing *SmCOP1* and *SmHY5* inhibited anthocyanin accumulation and recovered the cotyledon and hypocotyl length phenotype, indicating that *SmCOP1* and *SmHY5* genes act as counterparts of *Arabidopsis COP1* and *HY5*, and have COP1 and HY5 functions.

3.5. *SmCRY1* and *SmCRY2* interact with *SmCOP1* in a blue-light-dependent manner in vitro and in

1 vivo

2 To determine whether SmCRY1 interacts with SmCOP1, we prepared a bait construct expressing
3 SmCOP1 and a prey construct expressing SmCRY1 with two systems (GAL4 and LexA), respectively.
4 Then yeast two-hybrid assay was performed in blue light and in the darkness. Strikingly, SmCRY1
5 interacts with SmCOP1 under blue light, but not in the darkness (Fig. 5A, Fig. 5B). Likewise, we
6 observed that SmCRY2 interacts with SmCOP1 in a blue-light-dependent manner in yeast cells (Fig.
7 5A, Fig. 5B).

8 Given the blue-light-dependent SmCRY1- and SmCRY2-SmCOP1 interactions in yeast cells, we
9 further tested the possibility that photoactivated SmCRY1 and SmCRY2 interact directly with SmCOP1
10 in plant cells in BiFC system. The N-terminal half of yellow fluorescent protein (YFP) was fused to the
11 N terminal of SmCOP1, and the C-terminal half of YFP was fused to the C terminals of SmCRY1 and
12 SmCRY2. Cotransformation of nYFP-SmCOP1 and SmCRY1/SmCRY2-cYFP in tobacco leaf
13 epidermal cells produced robust YFP fluorescence in nuclear in blue light, but not in the darkness (Fig.
14 6). These results demonstrate that SmCRY1 and SmCRY2 interact with SmCOP1 in a
15 blue-light-dependent manner in plant cells.

16 3.6. SmCOP1 interacts with SmHY5 and SmMYB1 in vitro and in vivo

17 To test whether SmCOP1 interacts with SmHY5 and SmMYB1, yeast two-hybrid assays with two
18 systems (GAL4 and LexA) were performed. It was found that SmCOP1 interacts with SmHY5 and
19 SmMYB1 in yeast cells (Fig 7A, Fig. 7B).

20 To further characterize the interaction between SmCOP1 and SmHY5/SmMYB1, an in vivo
21 interaction assay using BiFC was carried out. YFP fluorescence was predominantly detected in the
22 nuclear cotransformed with SmCOP1-pXY106 plus SmHY5-pXY104/SmMYB1-pXY104 (Fig. 8). In

contrast, no fluorescence was observed in the control combinations including the empty pXY104 vector plus SmCOP1-pXY106, and the empty pXY106 vector plus SmHY5-pXY104/SmMYB1-pXY104 (Fig. 8). These results indicate that SmCOP1 interacts with SmHY5 and SmMYB1 in vivo in plant cells.

3.7. Isolation and analysis promoters of anthocyanin biosynthesis structural genes

In previous study, we have isolated and characterized four anthocyanin biosynthesis structural genes (*SmCHS*, *SmCHI*, *SmF3H* and *SmDFR*) in eggplant (unpublished result). The sequence data have been deposited in the NCBI database with the accession numbers KT119427 (*SmCHS*), KT119428 (*SmCHI*), KT159285 (*SmF3H*) and KT119429 (*SmDFR*). Together with structural gene EU809469 (*SmANS*), all the promoters of five structural genes have been isolated. A number of *cis*-regulatory elements were found within the five promoter sequences (Fig. S6-S10). Elements involved in light responsive (G-Box), abscisic acid responsive (ABRE), methyl jasmonate responsive (CGTCA-motif) and circadian control (circadian) were identified in all five promoter sequences, indicating that the five promoter activities could be modulated by light, abscisic acid, methyl jasmonate and circadian rhythm. In addition to G-Box, there were a lot of light responsive elements, such as ACE, AE-box, Box I, Box II, CATT-motif, GA-motif, GAG-motif, and so on. Therefore, it provided the reasonable explanation that anthocyanin synthesis is regulated by light in eggplant. Noteworthy, G-Box was the putative HY5 binding site [28]. Regulatory element that was implicated in auxin (TGA-element) was identified in the promoters of *SmCHS*, *SmDFR* and *SmANS*. Low temperature responsive element (LTR) was found in *SmCHS* and *SmCHI*, while heat stress responsive element (HSE) was detected in *SmF3H* and *SmANS*. Strikingly, the putative MYB binding site (MBS) was contained in the promoter region of *SmCHS*, *SmCHI*, *SmF3H* and *SmDFR*, respectively.

3.8. SmHY5 and SmMYB1 both bind the promoters of anthocyanin biosynthesis structural genes

Given that the putative HY5 binding site G-Box and MYB binding site (MBS) was discovered in the promoters, we performed a yeast one-hybrid assay to test whether SmHY5 and SmMYB1 binding to the promoters of anthocyanin biosynthesis structural genes. As shown in Fig. 9, the results reveal that both SmHY5 and SmMYB1 bind to the promoters of *SmCHS* and *SmDFR*.

4. Discussion

In *Arabidopsis*, anthocyanins biosynthesis is a feature of light-grown plants and does not occur in dark-grown plants [33]. The reason is that COP1 acts as a central switch in light signal transduction by interacting with upstream light receptors and downstream target proteins [53,54]. Our study also discovers that the anthocyanin biosynthesis of eggplant is completely regulated by light (Fig. 1). There are three types of light receptors: the blue light receptors cryptochromes CRY1 and CRY2, the red and far-red light receptors phytochromes phyA and phyB, and the UV-B light receptor UVR8. Cryptochromes are the most widely spread photoreceptors that regulate growth and development in plants [55]. The CRY apoprotein contains two domains: the N-terminal PHR domain and the C-terminal CCE domain [56]. In this study, both SmCRY1 and SmCRY2 contain highly conserved PHR and CCE domains at the N and C termini, suggesting that they may have similar functions regarding the regulation of plant growth and development. PHR domain is the chromophore-binding domain that binds chromophores flavin adenine dinucleotide (FAD) and 5,10-methenyltetrahydrofolate (MTHF), whereas CCE domain is a cryptochrome effector domain that interacts with COP1 through its WD40 domain [56–59]. In addition to the WD40 domain, COP1 contains a RING-finger domain that be responsible for the degradation of several photomorphogenesis-promoting transcription factors [60,61]. SmCOP1 has conserved domains, such as the N-terminal RING-finger and C-terminal WD40 domains. Based on the high similarity of predicted structures, SmCOP1 may have E3 ubiquitin ligase

1 activity and the capacity to interact with upstream light receptors. HY5, a bZIP protein, is a regulator of
2 photomorphogenic development and a light-regulated developmental molecular switch. The BRLZ
3 domain of SmHY5 reflects that it is a bZIP transcription factor. It interacts with COP1 through the
4 WD40 domain [62,63].

5 As photoreceptors sensing light signals directly, *SmCRY1* and *SmCRY2* express high in leaves (Fig.
6 2). Furthermore, compared with *SmCRY2*, the expression level of *SmCRY1* in leaves is much higher
7 than other tissues. The expression level of *SmCRY1* increased 19.81-fold in the light (Fig. 3A). At the
8 same time, *SmCRY2* just raised 3.93-fold. These results imply that *SmCRY1* plays dominant role in
9 sensing light signals. However, what is a surprise is that *SmCRY2* also expresses high in roots (Fig. 2).
10 Previous study found *MdCRY2* has a high expression in roots [64]. There is no difference in *SmHY5*
11 expression level among different tissues, implying its ubiquitous functions. *SmHY5* shows only
12 2.76-fold increase between light- and dark-grown peel. In *Arabidopsis*, HY5 protein is 15–20 times
13 more abundant in seedlings grown in white light than in those grown in the dark, while its mRNA level
14 displays only two-to-threefold increase [25]. The mRNA level contributes to the accumulation of the
15 HY5 protein, but translational or post-translational regulation may be primarily responsible for
16 regulating the abundance of HY5. The *SmCOP1* expression level in the light shows 53.76% of that in
17 the dark, indicating that the expression of negative photomorphogenesis regulatory factor *SmCOP1* is
18 inhibited by light. Bagging treatment strongly inhibits anthocyanin accumulation in eggplant (Fig. 3A).
19 Our results give the evidence that this environmental cue has function in anthocyanin biosynthesis via
20 regulating expression of *SmCOP1*. Furthermore, the expression levels of *SmCRY1*, *SmCRY2*, *SmHY5*
21 and *SmMYB1* are increased immediately and obviously after bag removal (Fig. 3B). It explains the
22 colouration of eggplant peel after debagging.

1 *Arabidopsis* mutants are often used for gene function analysis. In *Arabidopsis*, both CRY1 and
2 CRY2 participate in de-etiolation and flowering, and CRY1 plays a more dominant role in de-etiolation
3 and CRY2 in flowering [65]. In *Arabidopsis* complementation assay, both *SmCRY1* and *SmCRY2*
4 involve in inhibition of hypocotyl elongation (Fig. 4B, Fig. 4C). In addition, compared with the WT, the
5 *cry2* mutant plants *cry2-1* exhibits a late-flowering phenotype. COP1 is the negative regulatory factor
6 of photomorphogenesis, and *cop1-4* is able to conduct photomorphogenesis and accumulate
7 anthocyanin in the darkness. HY5 has many functions, such as inhibiting hypocotyl elongation [26].
8 Thus, *hy5* has lengthened hypocotyl than WT. Furthermore, ectopic expression of the four genes
9 complements the photomorphogenetic deficits of their corresponding *Arabidopsis* mutants, indicating
10 that these proteins are the counterparts of *Arabidopsis* proteins in eggplant.

11 The light signal transduction molecular mechanisms have suggested that both CRY1 and CRY2
12 perform their functions largely through the interaction with COP1 in *Arabidopsis* [21,22]. Both of them
13 are activated by blue light, and then their protein structures are altered [56,66]. The activated CRYs
14 interact with COP1 to repress its E3 ligase activity, permitting downstream photomorphogenic genes,
15 such as HY5, HFR1 and CONSTANS (CO), to accumulate and regulate various processes during plant
16 growth and development [25,28,67]. Here, we found that both *SmCRY1* and *SmCRY2* can interact
17 with *SmCOP1* in a blue-light-dependent manner, indicating that blue-light signal transduction may
18 utilize the same pathway in eggplant. Without light-controlled depletion, COP1 can better enforce its
19 ubiquitination activity that represses photomorphogenesis by targeting a subset of transcription
20 activators, such as HY5 [62]. Our finding supports that *SmCOP1* can also interact with *SmHY5*. MYB
21 proteins can be regulated by COP1 protein as well. COP1 interacts with Blue Insensitive Trait 1 (BIT1,
22 a MYB transcription factor) and mediates its degradation in darkness in *Arabidopsis* [68]. One study

1 shows that COP1 interacts with the PAP1 and PAP2 transcription factors that result subsequently in
2 degradation of PAP1 and PAP2 in dark [33]. Another study demonstrates that MdCOP1 interacts with
3 MdMYB1 to regulate light-induced anthocyanin biosynthesis in apple [33]. As SmMYB1 is involved in
4 regulating anthocyanin biosynthesis [52], we tested whether there is an interaction between SmCOP1
5 and SmMYB1. The result confirms the presence of interaction between them. The HY5 signaling in
6 anthocyanin biosynthesis is thought to be through its direct binding to the promoter of structural genes
7 [32,34]. Previous analysis shows that HY5 protein binds to the promoter of CHS in *Arabidopsis* [62].
8 In this study, we found that SmHY5 binds to the promoters of *SmCHS* and *SmDFR* among the five
9 anthocyanin structural genes. Plant MYBs play important roles in many secondary metabolic pathways.
10 MYBs are identified by conserved imperfect repeats in their DNA-binding domain. There are 138
11 R2R3 MYB genes in *Arabidopsis* and 222 in apple [69,70]. One particular subgroup of R2R3 MYB is
12 involved in activation of anthocyanin biosynthesis [14]. One study discovers that PAP1 interacts with
13 DFR promoter that positively regulates the expression of *DFR* [71]. Another study finds that
14 MdMYBA interacts with *MdANS* promoter to regulate anthocyanin biosynthesis in apple [72]. Our
15 study discovers that SmMYB1 and SmHY5 both bind the promoters of anthocyanin biosynthesis
16 structural genes (*SmCHS* and *SmDFR*). G-box (the putative HY5 binding site) and MBS (the putative
17 MYB binding site) were present in the promoter sequences of *SmCHI* and *SmF3H*. However, SmHY5
18 and SmMYB1 failed to bind the promoters of *SmCHI* and *SmF3H*. Site prediction result is not
19 necessarily consistent with the actual situation. In addition, there may be more than one MYB
20 transcription factors to participate in regulating anthocyanin biosynthesis in eggplant, thus the
21 promoters of *SmCHI* and *SmF3H* may interact with other MYBs to control anthocyanin biosynthesis.

22 We provide a new working model describing the possible mechanism underlying the

1 light-controlled anthocyanin biosynthesis in eggplant (Fig. 10). The light receptors, such as SmCRY1
2 and SmCRY2, perceive light signals. In light, activated CRYs interact with COP1 to prevent E3 ligase
3 activity for ubiquitinating and degrading downstream transcription factors. As a result, SmHY5 and
4 SmMYB1 accumulate and directly bind to the promoters of *SmCHS* and *SmDFR* to activate their
5 expression. Finally, anthocyanin biosynthesis leads to coloration in eggplant fruit. In darkness,
6 SmCOP1 interacts with SmHY5 and SmMYB1 and mediates them ubiquitination and degradation.

7 **Conflict of interest**

8 The authors declare that they have no conflict of interest.

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13

Figure Captions

Fig. 1. Effect of bagging and debugging on fruit color. (A) Color changes of ‘Lanshan Hexian’ peel after bag removal, shown at 0 d, 2 d, 4 d, 6 d, 8 d and 10 d. (B) The levels of anthocyanin at 0 d, 2 d, 4 d, 6 d, 8 d and 10 d after bag removal.

Fig. 2. Expression analysis of *SmCRY1*, *SmCRY2*, *SmCOP1*, *SmHY5* and *SmMYB1* in different tissues. Quantifications were normalized by the expression of *SmACTIN* (GU984779.1). Data represent means $\pm$ SD of three biological replicates. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan’s multiple range test.

Fig. 3. Effect of bagging and debugging on expression of *SmCRY1*, *SmCRY2*, *SmCOP1*, *SmHY5* and *SmMYB1* in peel. (A) Effect of bagging on expression of the five genes. Control, peel which were never bagged. (B) Effect of debugging on expression of the five genes. Peel was collected at 0 h, 0.5 h, 4 h and 8 h after bag removal. Data represent means $\pm$ SD of three biological replicates. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan’s multiple range test.

Fig. 4. Functional characterization of *SmCRY1*, *SmCRY2*, *SmCOP1* and *SmHY5*. (A) Expression levels of *SmCRY1*, *SmCRY2*, *SmCOP1* and *SmHY5* in WT, mutants and transgenic lines in mutants background. (B) Phenotypes of WT, *cry1* mutants and transgenic lines in mutants background grown under blue light ($30 \mu\text{mol m}^{-2} \text{sec}^{-1}$). (C) Phenotypes of WT, *cry2* mutants and transgenic lines in mutants background grown under blue light ($3 \mu\text{mol m}^{-2} \text{sec}^{-1}$). (D) The number of rosette leaves before bolting. (E) The flowering phenotype in long days (16 h light/8 h dark). (F) Phenotypes of WT,

1 *cop1* mutants and transgenic lines in mutants background grown under long days (16 h light/8 h dark).
2 (G) Phenotypes of WT, *cop1* mutants and transgenic lines in mutants background grown under
3 darkness. Phenotypes of WT, *hy5* mutants and transgenic lines in mutants background grown under
4 long days (16 h light/8 h dark).

5

6 **Fig. 5. Yeast two-hybrid assays showing blue-light-dependent interactions between SmCRY1 or**
7 **SmCRY2 and SmCOP1 with GAL4 (A) and LexA (B) system in yeast cells.** (A) Yeast cells
8 coexpressing the indicated combinations of constructs were grown on nonselective media synthetic
9 dropout-Trp-Leu (-TL) or selective media synthetic dropout-Trp-Leu-His (-TLH), in continuous blue
10 light (BL, 30 $\mu\text{mol m}^{-2} \text{sec}^{-1}$) or darkness (DK). Yeast growth on the selective media indicated
11 interactions. AD, pGADT7; BD, pGBKT7. (B) Yeast cells coexpressing the indicated combinations of
12 constructs were grown on synthetic dropout-Trp-Ura-His (-TUH), in continuous blue light (BL, 20
13 $\mu\text{mol m}^{-2} \text{sec}^{-1}$) or darkness (DK). The plates show the corresponding β -galactosidase activities
14 represented by blue precipitates. AD, pB42AD; BD, pLexA.

15

16 **Fig. 6. BiFC assays showing blue-light-dependent interactions between SmCRY1 or SmCRY2**
17 **and SmCOP1 in tobacco leaf epidermal cells.** BiFC assays of in vivo interactions of
18 SmCRY1-SmCOP1 (30 $\mu\text{mol m}^{-2} \text{sec}^{-1}$) and SmCRY2-SmCOP1 (3 $\mu\text{mol m}^{-2} \text{sec}^{-1}$) in blue light and
19 darkness. BF, bright field ; YFP, Yellow fluorescent protein field, Merged, overlay of BF and YFP field.

20

21 **Fig. 7. Yeast two-hybrid assays showing interactions between SmHY5 or SmMYB1 and SmCOP1**
22 **with GAL4 (A) and LexA (B) system in yeast cells.** (A) Yeast cells coexpressing the indicated

1 combinations of constructs were grown on nonselective media synthetic dropout-Trp-Leu (-TL) or
2 selective media synthetic dropout-Trp-Leu-His (-TLH) and synthetic dropout-Trp-Leu-His-Ade
3 (-TLHA). Yeast growth on the selective media indicated interactions. AD, pGADT7; BD, pGBKT7. (B)
4 Yeast cells coexpressing the indicated combinations of constructs were grown on synthetic
5 dropout-Trp-Ura-His (-TUH). The plates show the corresponding β -galactosidase activities represented
6 by blue precipitates. AD, pB42AD; BD, pLexA.

7
8 **Fig. 8. BiFC assays showing interactions between SmHY5 or SmMYB1 and SmCOP1 in tobacco**
9 **leaf epidermal cells.** BF, bright field; YFP, Yellow fluorescent protein field; Merged, overlay of BF
10 and YFP field.

11
12 **Fig. 9. Yeast one-hybrid assays indicating SmHY5 and SmMYB1 both bind the promoters of**
13 **anthocyanin biosynthesis structural genes (*SmCHS* and *SmDFR*).** Yeast cells coexpressing the
14 indicated combinations of constructs were grown on synthetic dropout-Trp-Ura (-TU). The plates show
15 the corresponding β -galactosidase activities represented by blue precipitates. AD, pB42AD.

16
17 **Fig. 10. A working model for light-induced anthocyanin biosynthesis in eggplant.** (A) In light,
18 interaction with activated cryptochromes repress function of COP1 that is allowing downstream
19 transcription factors (HY5 and MYB1) to accumulate. The promoters of structural anthocyanin genes
20 (*CHS* and *DFR*) are directly bind to HY5 and MYB1, and induce anthocyanin biosynthesis. (B) In
21 darkness, cryptochromes fail to bind COP1. COP1 targets positive regulators (HY5 and MYB1), for
22 ubiquitination and subsequent protein degradation through a 26S proteasome pathway. Structural

- 1 anthocyanin genes can not be activated in the absence of HY5 and MYB1, thus blocking the anthocyanin
- 2 biosynthesis.
- 3

A **B**

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A

B

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A **B**

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Bait/Prey	-TU	Bait/Prey	-TU	Bait/Prey	-TU
<i>CHS</i> /HY5		<i>CHS</i> /AD		<i>CHS</i> /MYB1
<i>CHI</i> /HY5		<i>CHI</i> /AD		<i>CHI</i> /MYB1
<i>F3H</i> /HY5		<i>F3H</i> /AD		<i>F3H</i> /MYB1
<i>DFR</i> /HY5		<i>DFR</i> /AD		<i>DFR</i> /MYB1
<i>ANS</i> /HY5		<i>ANS</i> /AD		<i>ANS</i> /MYB1
		<i>LacZ</i> /HY5		<i>LacZ</i> /MYB1

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