**ORIGINAL ARTICLE**

# A light-responsive transcription factor SmMYB35 enhances anthocyanin biosynthesis in eggplant (*Solanum melongena* L.)

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## Abstract

***Main conclusion*** **SmMYB35, a light-responsive R2R3-MYB transcription factor, positively regulates anthocyanin biosynthesis in eggplant by binding to the promoters of *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS* and enhancing their activities. In addition, SmMYB35 interacts with SmTT8 and SmTTG1 to form a MBW complex, thereby enhancing anthocyanin biosynthesis.**

**Abstract** Eggplant is a vegetable rich in anthocyanins. SmMYB35, a light-responsive R2R3-MYB transcription factor, was isolated from eggplant and investigated for its biological functions. The results suggested that the expression of *SmMYB35* was regulated by SmHY5 through directly binding to G-box in the promoter region, and the overexpression of *SmMYB35* could increase the anthocyanin content in the stems and petals of the transgenic eggplants. SmMYB35 could also bind to the promoters of *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS* and enhance their activities. In addition, SmMYB35 interacted with SmTT8 and SmTTG1 to form a MBW complex which enhanced anthocyanin biosynthesis. Taking together, we firstly verified that SmMYB35 promoted anthocyanin biosynthesis in plants. The results provide new insights into the regulatory effects of SmMYB35 on key anthocyanin biosynthetic genes and advance our understanding of the molecular mechanism of light-induced anthocyanin synthesis in eggplants.

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## Abbreviations

| | |
|---|---|
| ANS | Anthocyanidin synthase |
| CHI | Chalcone isomerasess |
| CHS | Chalcone synthase |
| DFR | Dihydroflavonol-reductase |
| F3H | Flavanone 3-hydroxylase |
| HY5 | ELONGATED HYPOCOTYL5 |
| TF | Transcription factor |
| YFP | Yellow fluorescent protein |

## Introduction

Eggplant (*Solanum melongena* L.) is consumed and cultivated in many countries. Purple eggplants are more attractive to consumers because of its high anthocyanin content. However, low light conditions often result in poor coloration and reduce its consumer acceptance. Anthocyanins are a type of water-soluble pigments, widely detected in plant stems, leaves, petals and fruit peels (Kayesh et al. 2013). Anthocyanins not only supply plants with vivid colors, but

also protect them from various stresses, such as drought, extreme temperature, and high salt conditions (Dixon et al. 1995).

Anthocyanins are synthesized via a branch of the flavonoid biosynthesis pathway (Belwal et al. 2020). Several anthocyanin biosynthetic enzymes have been characterized in different plants, including chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3' 5'-hydroxylase (F3'5'H), dihydroflavonol-reductase (DFR), and anthocyanidin synthase (ANS) (Jiang et al. 2016a; Shoeva et al. 2017). The genes encoding these enzymes are regulated by a group of transcription factors (TFs), including MYB, bHLH and WD40 (Xu et al. 2013). The MYB family is one of the largest groups of transcriptional regulators involving in the regulation of anthocyanin biosynthesis. There are AtMYB75, AtMYB90, AtMYB113, AtMYB114, and AtMYB4 in *Arabidopsis thaliana* (Hemm et al. 2001; Gonzalez et al. 2008), SlANT1, SlAN2 and SlMYBATV in *S. lycopersicum* (Kiferle et al. 2015; Cao et al. 2017), StAN1 and StMYB44 in *S. tuberosum* (Liu et al. 2016, 2019), SmMYB1 and SmMYB86 in *S. melongena* L (Jiang et al. 2016b; Li et al. 2021), and MdMYB10 and MdMYB6 in *Malus×domestica* (Espley et al. 2009; Xu et al. 2020). These MYBs usually promote or inhibit structural genes expression to exhibit their regulatory roles in anthocyanin synthesis. BHLH proteins are the second largest class of TFs (Toledo-Ortiz et al. 2003). Numerous bHLH TFs, such as TT8, GL3, and EGL3, interact with R2R3-MYB proteins to regulate the anthocyanin biosynthesis in Arabidopsis (Feller et al. 2011). In eggplant, SmTT8 binds to the promoter of *SmCHS* and promotes its activation (He et al. 2019). The regulatory capability of SmMYB1 on anthocyanin biosynthetic genes was strengthened when SmTT8 was added (Zhou et al. 2019). So far, a few WD40 proteins and their functionalities in the biosynthesis of anthocyanin, proanthocyanidin and trichome formation have been characterized, including AtTTG1 from Arabidopsis (Shan et al. 2019), PgWD40 from pomegranate (Ben-Simhon et al. 2011), and SmTTG1 from eggplant (Li et al. 2021). The main function of WD40 is to provide a stable platform for MYB and bHLH proteins and promotes their interactions to form a (MYB-bHLH-WD40) MBW complex (Liu et al. 2018a; Tang et al. 2020).

Light is one of the most influential environmental factors not only affecting plant growth and development but also regulating the production of secondary metabolites by regulating the expression of light-response genes. In the light, ELONGATED HYPOCOTYL5 (HY5) acts downstream of photoreceptors to promotes photomorphogenesis (Xu et al. 2018). In addition, SmHY5 directly binds to the promoters of *SmCHS* and *SmDFR*, to promotes anthocyanin biosynthesis in eggplant (Jiang et al. 2016b). What's more, HY5 also directly activates the transcription of several *MYB* genes containing the HY5 potential binding sites in their promoters to regulate physiological processes (Lee et al. 2007; An et al. 2017). Taken together, HY5 promotes anthocyanin accumulation by regulating the expression of *MYB* genes and anthocyanin biosynthetic genes. Although light-induced anthocyanin accumulation has been documented in several plants, the molecular mechanism underlining HY5-dependent was not fully understood.

Our previous study found that the synthesis of anthocyanin in the peel of '*Lanshan Hexian*' eggplant was regulated by light (Jiang et al. 2016b; Li et al. 2017). Our previous work also screened for several genes involving in anthocyanin biosynthesis (Li et al. 2017, 2018). *SmMYB35* was one of the differentially expressed genes in the transcriptome data at 0, 5, and 12 d with up-down-regulated expression pattern in '*Lanshan Hexian*'. Moreover, SmMYB35 affected the anthocyanin accumulation and expression level of structural genes when co-infiltrated with SmMYB1 and SmTT8 in *Nicotiana benthamiana* (Li et al. 2017). However, how SmMYB35 may promote anthocyanin synthesis is still unclear. This study was conducted to investigate the potential regulatory roles of SmMYB35 in eggplant.

## Materials and methods

### Plant materials and growth conditions

The eggplant cultivar '*Lanshan Hexian*' (purple-black peel), '*140*' (white peel), from my own lab, and the *SmMYB35-OE* transgenic eggplant seeds were sown in chambers with a growth condition at 25 °C/22 °C (day/night) and 16 h/8 h photoperiod. The seedlings in the 4-leaf stage were transplanted to the greenhouse of Shanghai Hangyu Seed Base, Shanghai, China.

*Arabidopsis thaliana* of Columbia-0 (Col-0) and *SmMYB35-OE* transgenic *Arabidopsis thaliana* were cultured in 10 cm diameter plastic chambers with a growth condition at 25 °C/22 °C (day/night) and 16 h/8 h photoperiod.

The seedlings of *N benthamiana* were growth under the same condition as that of *Arabidopsis thaliana*.

### Gene isolation and subcellular localization

The coding sequences of *SmMYB35* (Sme2.5_00216.1_g00001.1) were downloaded from the Eggplant Genome DataBase (http://eggplant.kazusa.or.jp/keyword.html). The total RNA was extracted from the peel of photosensitive eggplant cultivar, '*Lanshan Hexian*' using Plant RNA Extraction Kit (TaKaRa, Otsu, Japan). The cDNA was synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa). Total DNA was extracted from eggplant leaves by CTAB method to isolate SmMYB35 promoter.

The online programmer PlantCARE (http://bioinformatics. psb.ugent.be/webtools/plantcare) was used to analyze the number and location of *cis*-elements harbored in the promoter region. For subcellular localization experiments, the PHB-SmMYB35-YFP vector was constructed with homologous recombination method. The GV3101 strains harboring PHB-SmMYB35-YFP or PHB-YFP were transformed into 5-week-old *N. benthamiana* leaves, and the nuclei were stained with DAPI (Sangon Biotech, shanghai, China), as described in my own published paper (Li et al. 2021). All primers of this research were designed by primer 5 and listed in Supplementary Table S1.

## Plant transformation and phenotype analysis

The coding sequences of *SmMYB35* were ligated to the PHB vector containing yellow fluorescent protein and a 35S CaMV promoter to generate the 35S::SmMYB35-YFP plasmids. The recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 and then transfected into *Arabidopsis thaliana* by the floral dip method. Transgenic seedlings were verified by resistance screening and confirmed with PCR, and self-crossed for homozygous selection until $T_3$ lines for analysis. The *Agrobacterium*-mediated transformation was performed using cotyledons of the eggplant cultivar '*140*', as previously described (Jiang et al. 2016a; Li et al. 2021). The phenotypes of *SmMYB35-OE Arabidopsis thaliana*, '*140*' transformed eggplants and their WT were observed at the same growth conditions.

## Extraction and quantification of anthocyanins

Anthocyanins of eggplant tissues were extracted and measured as described by Li et al. (2017). At least three biological replicates were collected and three measurements for each replicate were performed.

## Quantitative real-time polymerase chain reaction (qRT-PCR)

To discover the expression of *SmCHS*, *SmF3H*, *SmANS*, *SmDFR* in both the Col-0 and *SmMYB35-OE* transgenic lines, the expression of *SmMYB35*, *SmCHI*, *SmCHS*, *SmF3H*, *SmF35H*, *SmANS*, *SmDFR* in both the '*140*' and *SmMYB35-OE* transgenic lines were anaylzed. The expression patterns of *SmCHI*, *SmCHS*, *SmF3H*, *SmF35H*, *SmANS*, *SmDFR* in different tissues of '*140*', '*140*' transformed plants and their WT, different tissues of '*Lanshan Hexian*' and '*140*', including roots, stems, leaves, petals, flesh, peel, and sepals were studied. In bagging experiments, the fastest anthocyanin synthesis rate was at around 5 days as shown in our previous study; the time points of sampling for qRT-PCR and RNA-seq were designed to be at 0, 5, and 12 d.

The expression level of *SmMYB35* was differentially in the transcriptome data at 0d, 5d, and 12d, as was found in our previous studies (Li et al. 2017). Then eggplant fruit peel was collected at 0~8 h after bag removal from 8:00 am to 16:00 pm to further explore the expression of *SmMYB35* under light condition. Total RNA was treated with DNase to remove traces of DNA. RNA (1 µg) was used for cDNA synthesis with the PrimeScript RT Master Mix Perfect Real-Time Kit (TaKaRa). RT-qPCR was performed using SYBR Premix Ex Taq II Kit (TaKaRa) on Light Cycler 96 (Roche, Basel, Switzerland). The reaction procedure was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. Three technical replicates were run for the RT-qPCR of each sample using the gene-specific primers shown in Table S1. The Actin (GU984779.1) from the eggplant was amplified in parallel as an internal reference gene. The data were analyzed using the $2^{-\Delta\Delta CT}$ method.

## Yeast one-hybrid assay (Y1H)

The yeast one-hybrid experiment was performed as mentioned earlier (Jiang et al. 2016b). The coding sequences (CDSs) of *SmHY5* (Sme2.5_03211.1_g00004.1) and *SmMYB35* were amplified and ligated to the pB42AD vector. The promoter sequences of anthocyanin structural genes *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS* as reported previously (Jiang et al. 2016a), and three tandem copies of the G-box-1 and G-box-2 motifs from the *SmMYB35* promoter were fused to the pLacZ vector.

## Dual-luciferase assay (Dual-LUC)

The dual-luciferase assay experiment as described in my own paper (Li et al. 2021). The CDS of *SmHY5, SmMYB35, SmTT8,* and *SmTTG1* were inserted into a PHB vector as effectors; the empty PHB vector was used as a negative control. The promoter sequences (*SmMYB35, SmCHS, SmF3H, SmDFR and SmANS*) were cloned and inserted into the cloning sites of the pGreen0800-LUC vector as reporters.

## Yeast two-hybrid assay (Y2H)

The yeast two-hybrid experiment was mentioned earlier (Jiang et al. 2016b; Li et al. 2021). The CDS of *SmMYB35* and *SmTT8* were inserted into the bait vector pGBKT7 (BD) as bait, respectively. *SmMYB35* and *SmTTG1* were fused to the prey vector pGADT7 (AD) as prey, respectively. Because of the strong autoactivation of full-length SmMYB35, the 60 mmol/L 3-AT solution was used for Y2H screening assays whose procedure was described in the Yeast Protocols Handbook (Takara).

## Bimolecular fluorescence complementation (BiFC)

For BiFC assay, the vectors pXY104 and pXY106 were described previously (Jiang et al. 2016b; Li et al. 2021). For the construction of the BiFC vectors, CDS of the *SmMYB35* and *SmTT8* were cloned into pXY106 to produce SmMYB35-nYFP and, SmTT8-nYFP (the N-terminal fragment of YFP) fusion proteins, respectively. CDS of the *SmMYB35* and *SmTTG1* were cloned into pXY104, to obtain SmMYB35-cYFP and, SmTTG1-cYFP (the C-terminal fragment of YFP) fusion proteins, respectively. Infiltration and detection were performed the same as subcellular localization experiments described.

## Results

### SmMYB35 is a nuclear-localized protein and highly expressed in anthocyanin-rich tissues

The subcellular localization of an SmMYB35-YFP fusion protein was determined to discover where the SmMYB35 protein functions within the cell. In contrast to YFP, which was detected in both nucleus and cytoplasm, the SmMYB35-YFP fusion protein was only exclusively observed in the nucleus of *N. benthamiana* leaf cells (Fig. 1a), which was different to YFP detected in both nucleus and cytoplasm, suggesting that SmMYB35 is a nuclear-localized protein.

The spatial and temporal expression patterns of *SmMYB35* were analyzed in different tissues at the same developmental stage of the '*Lanshan Hexian*' and '*140*' by qRT-PCR. The expression level of *SmMYB35* was high in peel, moderate in stems, and very low in roots of '*Lanshan Hexian*' (Fig. 1b). The results also showed that *SmMYB35* was highly expressed in stems and moderately expressed in
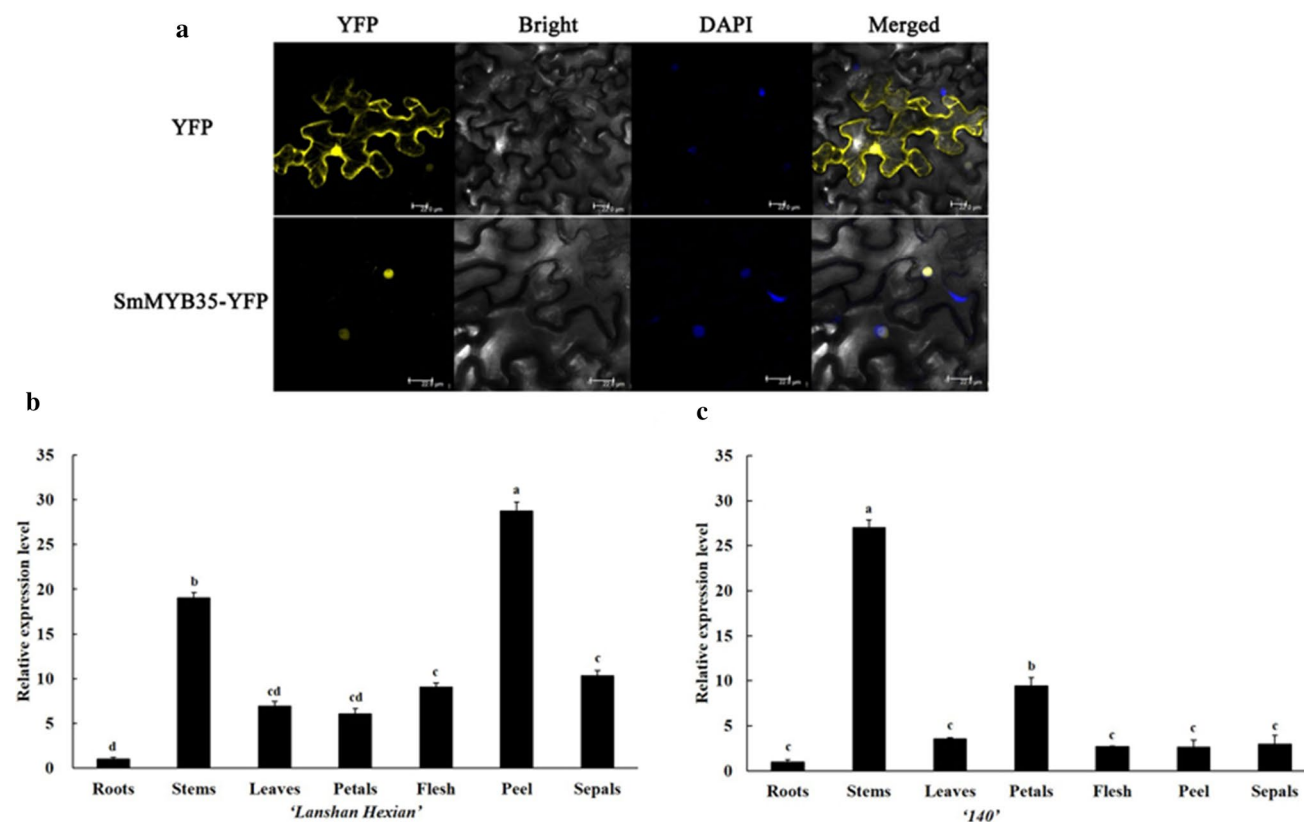
**Fig. 1** Subcellular localization and expression patterns of *SmMYB35*. *a* Subcellular localization of the SmMYB35 protein in *N. benthamiana* leaves epidermal cells. DAPI served as a marker for the nucleus. Scale bars = 22 μm. **b**, **c** The expression level of *SmMYB35* in different eggplant tissues in '*Lanshan Hexian*' and '*140*', respectively. Data are represented by three biological replicates, each of which included three technical replicates. One-way analysis of variance was used to test the statistical significance. which was indicated by different letters ($P < 0.05$)

petals of '*140*' (Fig. 1c). *SmMYB35* was highly expressed in anthocyanin-rich tissues of both '*Lanshan Hexian*' and '*140*'.

## Overexpression of *SmMYB35* increases anthocyanin content and induces the expression of anthocyanin biosynthetic genes in Arabidopsis and eggplant

To study the function of the SmMYB35 in Arabidopsis, the CDS of *SmMYB35* driven by the 35S promoter was over-expressed in Arabidopsis (Fig. S1a). The transgenic plants were identified by basta screening to $T_3$ generation. Three independent transgenic lines harboring 35S::SmMYB35 were then randomly selected for further molecular and phenotypic characterization. Compared to wild-type (WT) plants, *SmMYB35-OE* transgenic plants certified enhanced anthocyanin accumulation in leaves (Fig. S1b). qRT-PCR showed that the expression of key enzyme genes in anthocyanin biosynthesis was increased compared with that of the WT (Fig. S1d). To further verify the function of SmMYB35 in the eggplant cultivar '*140*' (white peel), the $T_1$ *SmMYB35-OE* transgenic eggplant was obtained by the cotyledonary explants mediated by *Agrobacterium tumefaciens*-mediated transformation (Fig. S2a) and was tested by qRT-PCR. Compared to WT, the expression of *SmMYB35* and anthocyanin biosynthetic genes were also increased (Fig. S2b). These results suggested that SmMYB35 induced the expression of anthocyanin biosynthetic genes. To further ensure that SmMYB35 acted as an activator in anthocyanin accumulation, the $T_1$ transgenic plant was self-pollinated to obtain homozygous transgenic eggplants. As a result, we observed a higher anthocyanin accumulation in $T_2$ *SmMYB35-OE* than in WT in stems and petals (Fig. 2a, b). Then, qRT-PCR expression analysis was performed in three randomly selected $T_2$ *SmMYB35-OE* transgenic eggplants. The transcript levels of *SmMYB35* in stems and petals of three transgenic eggplants were considerably higher than in WT (Fig. 2c). The expression of key anthocyanin biosynthetic genes was also drastically increased in three $T_2$ *SmMYB35-OE* compared to WT (Fig. 2d, e). These results indicated that a significant increase in transcript levels in stems and petals of *SmMYB35-OE* compared to that of WT. Above all, over-expressing of *SmMYB35* in eggplant promoted the expression level of anthocyanin biosynthetic genes and increased the anthocyanin content in stems and petals.

## SmMYB35 acts as a positive regulator to activate the transcription of *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS*

To uncover the regulatory mechanism of SmMYB35 on anthocyanin biosynthetic genes (Fig. 3a). We found that SmMYB35 activated the transcription of *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS* in the yeast one-hybrid assay. As shown in Fig. 3b, SmMYB35 bind to the promoters of anthocyanin structural genes, their combinations exhibited blue color on the defective medium (-TU) with x-Gal. However, negative controls failed to express β-galactosidase. These results suggested that SmMYB35 directly attached to the promoters of *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS*, respectively.

To further confirm whether SmMYB35 activates these biosynthetic gene promoters, Dual-LUC was conducted. The promoter of *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS* were fused to LUC luciferase as a reporter. The SmMYB35 driven by 35S promoter as effector (Fig. 3c). The LUC experiment showed that SmMYB35 could activate the expression of *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS*, indicating that SmMYB35 was a positive regulator of anthocyanin synthesis (Fig. 3d–g). The above results demonstrated that SmMYB35 stimulated anthocyanin synthesis through binding to promoters of *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS*, and positively activated the expression of these biosynthetic genes.

## SmTT8 and SmTTG1 enhance the regulatory function of SmMYB35 on anthocyanin biosynthetic genes by physical protein–protein interaction

The proteins interacting with SmMYB35 were screened by yeast two-hybrid, to elucidate the molecular basis for its regulatory role in anthocyanin biosynthesis. Selected genes belonging to bHLH and WD40 family, which have been reported in our previous studies on eggplant, were SmTT8 and SmTTG1, specifically. Y2H assays indicated that both SmTT8 and SmTTG1 interacted with SmMYB35 in yeast cells (Fig. 4a). Then BiFC assays were carried out to further confirm these interactions in *N. benthamiana* leaf cells (Fig. 4b). When SmMYB35-cYFP with SmTT8-nYFP, SmMYB35-cYFP with SmTTG1-nYFP, and SmTT8-cYFP with SmTTG1-nYFP were transiently co-expressed, those YFP fluorescence signals were observed in the nucleus. In contrast, no fluorescence was observed in negative controls. Together, these results demonstrated that two of them interacted with each other in *vitro* and in *vivo*.

To further investigate whether the interactions between SmMYB35, SmTT8, and SmTTG1 affected the transcriptional activation of anthocyanin biosynthetic genes, Dual-LUC assay was conducted. The transcriptional activation of *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS* was significantly enhanced when any combination of two TFs was injected compared to SmMYB35, SmTT8 or SmTTG1 injection alone, and the transcriptional activation was more significant after the co-expression of the three TFs (Fig. 4c). These results indicated that the interaction between these three TFs could facilitate the *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS* expression in varied degrees.
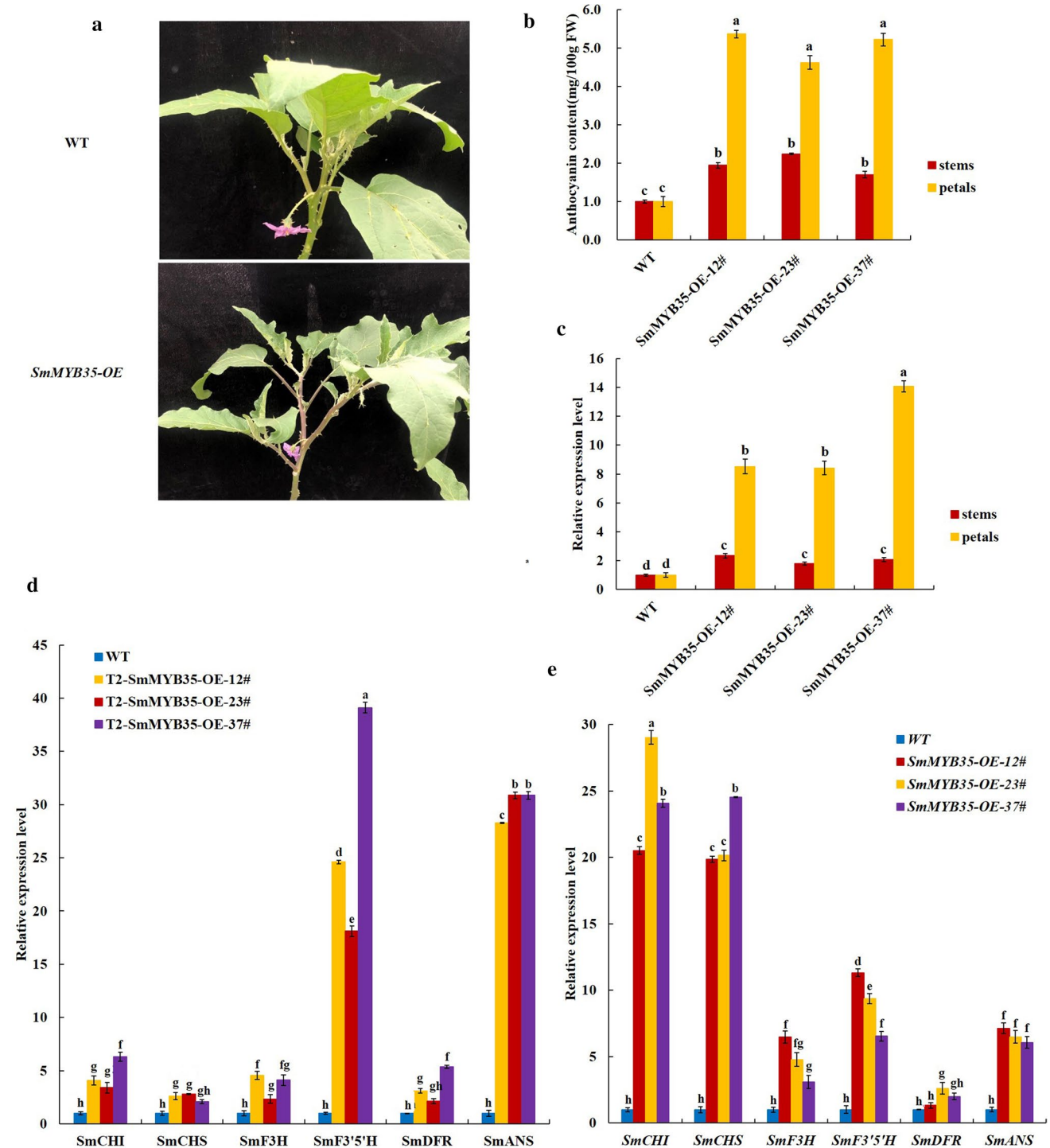
**Fig. 2** Effects of *SmMYB35-OE* on eggplant. **a** Phenotypic difference between WT and *SmMYB35-OE*. **b** Quantitative analysis of anthocyanins contents in the stems and petals from WT and *SmMYB35-OE*. **c** qRT-PCR analysis of the expression of *SmMYB35* in stems and petals of transgenic eggplants. **d** and **e** qRT-PCR analysis of the expression of *SmCHI*, *SmCHS, SmF3H*, flavonoid 3′5′-hydroxylase (*SmF3′5′H*), *SmDFR*, and *SmANS* in stems and petals of transgenic eggplants, respectively. Data are represented by three biological replicates, each of which included three technical replicates. The error bars indicate the standard errors of the means. One-way analysis of variance was utilized to test the statistical significance, different letters on the bars indicate significant differences (*P* < 0.05)
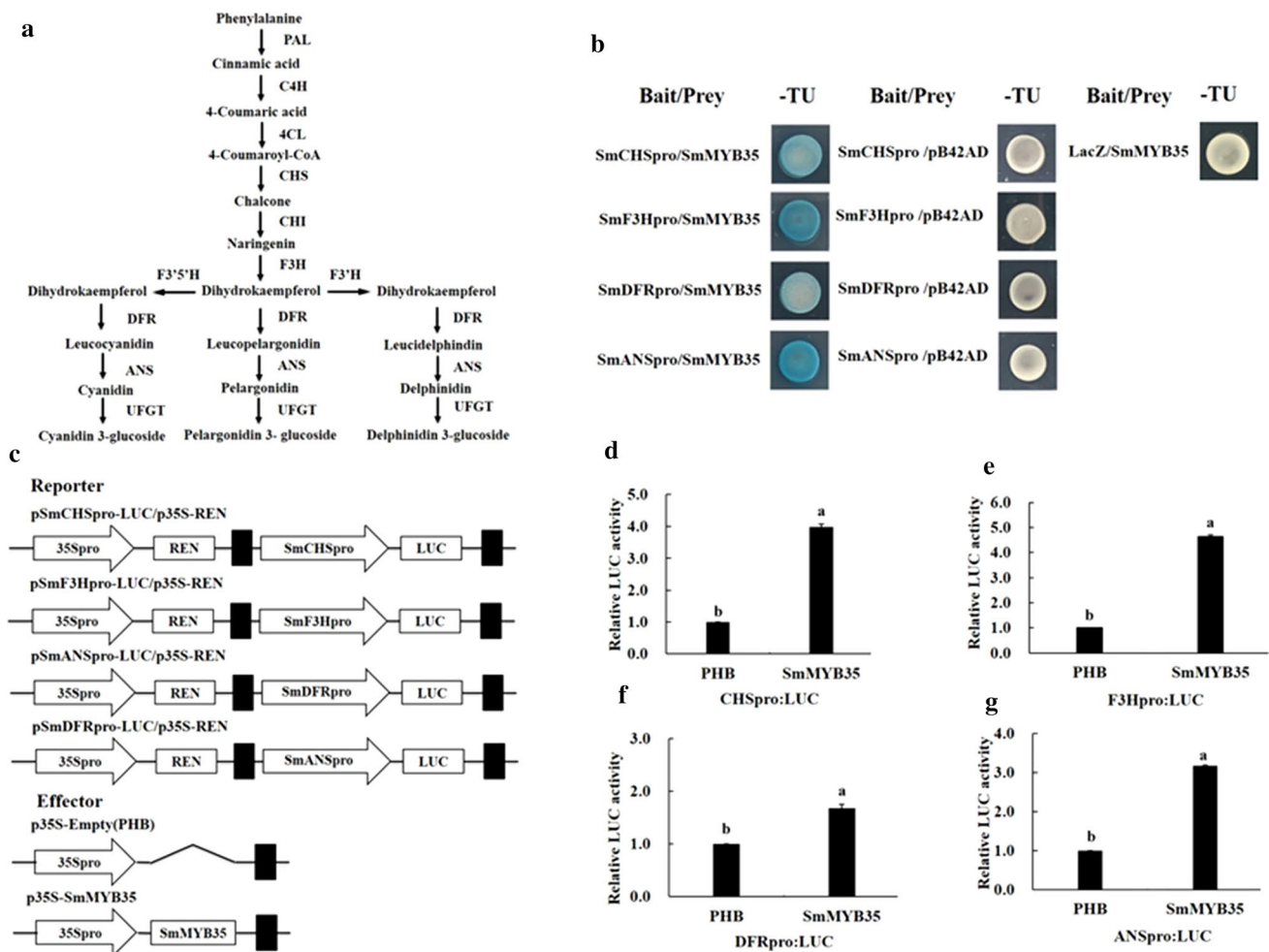
**Fig. 3** SmMYB35 acts as a positive regulator to activate the transcription of *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS* **a** Schematic representation of the anthocyanin biosynthetic pathway. **b** SmMYB35 specifically binded to the promoters of *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS* in one-hybrid system. **c** Schematic diagram of the reporter and effector constructs used in the Dual-LUC assay. **d**, **e**, **f** and **g** Transient Dual-LUC assay in *N. benthamiana* leaves. The promoter activity of *SmCHS, SmF3H, SmDFR,* and *SmANS* was measured by a ratio of LUC to REN. Data are represented by three biological replicates. Significance is indicated by different letters ($P < 0.05$)

## SmHY5 is a transcriptional activator of *SmMYB35*

Our previous bagging experiments demonstrated that the expression level of *SmMYB35* was induced by light in eggplant peel. Based on the differential expression of *SmMYB35* in transcriptome data at 0d, 5d, and 12d, the expression of *SmMYB35* was peaked at 5 d, and downregulated at 12 d. The qRT-PCR and RNA-seq results of *SmMYB35* were highly correlated ($r = 0.994$, Fig. 5a). To explore the relationship between expression of *SmMYB35* and light-exposed time in eggplants, the expression of *SmMYB35* at 0, 1, 2, 3, 4, 5, 6, 7, and 8 h was measured. The expression of *SmMYB35* peaked at 5 h (the highest light intensity of the day) and gradually turned down after that with weakening light intensity (Fig. 5b). Clearly, the expression of *SmMYB35* was triggered when exposed to light.

To understand the light-response mechanism of *SmMYB35*, we analyzed the promoter of *SmMYB35* and found two G-box motifs (G-box-1 and G-box-2) locating at 795 and 1074 bps, upstream of ATG, respectively, which were the potential binding cis-elements of SmHY5 (Fig. 6a, b). To further investigate whether SmHY5 recognizes the G-boxes harbored in the promoter of *SmMYB35*, Y1H assays were conducted. The results demonstrated that the pB42AD-SmHY5 fusion protein could strongly activate the expression of LacZ reporter gene through binding to three tandem repeats of the G-box-1 and G-box-2 motifs, indicating that SmHY5 binds to the G-box-1 and G-box-2 motifs in the *SmMYB35* promoter, respectively. However, SmHY5 did not bind to the full-length promoter (1766 bps upstream ATG) of *SmMYB35* (Fig. 6c). Then, a Dual-LUC assay was performed in *N. benthamiana* leaves to detect whether SmHY5 activated the full-length promoter of *SmMYB35*. The
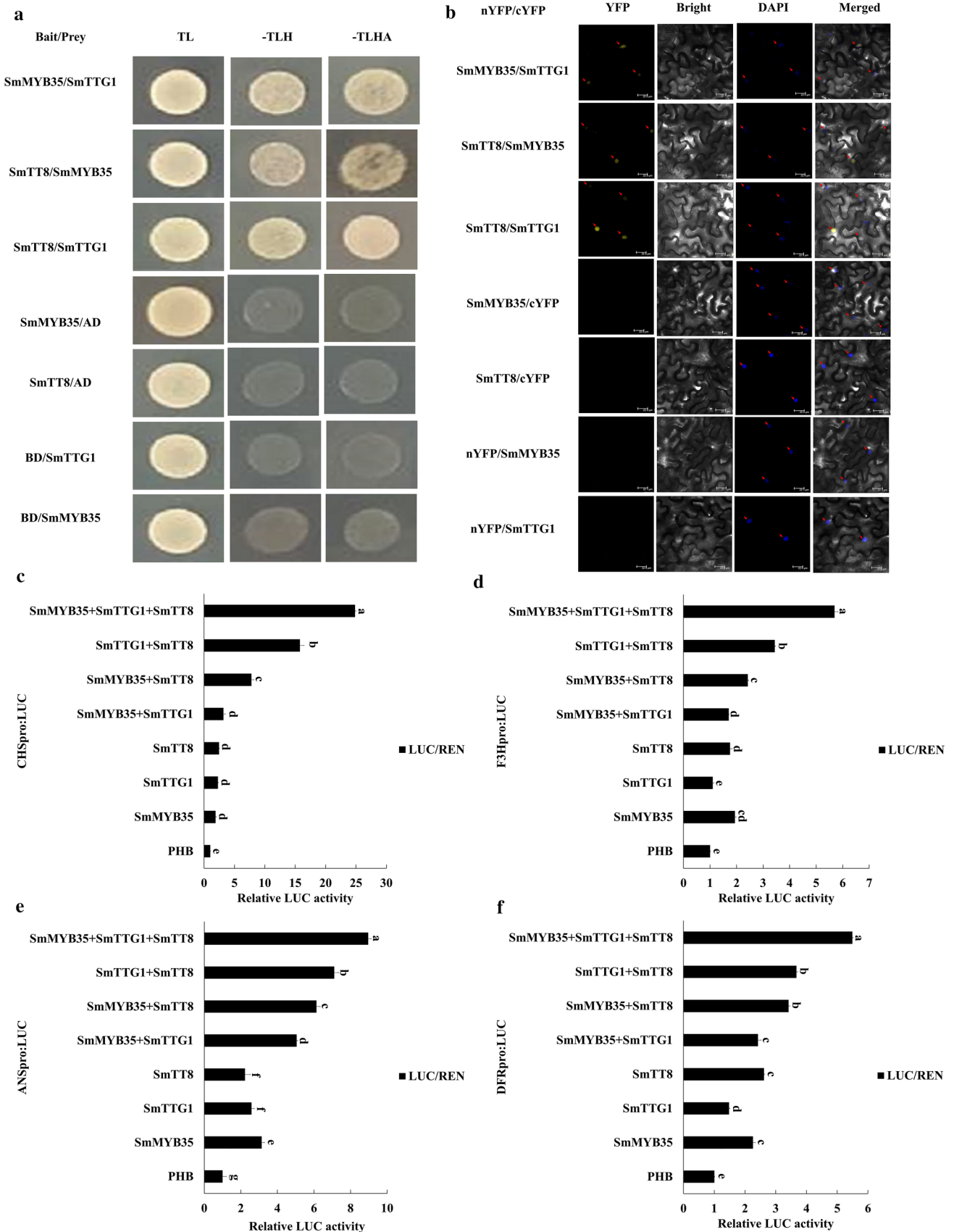
◄**Fig. 4** SmMYB35 interacts with both SmTT8 and SmTTG1, and promotes the transcriptional activation activity of *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS*. **a** Y2H assays to detect the interactions between SmMYB35, SmTT8, and SmTTG1 with GAL4 system in yeast cells. **b** BiFC analysis to detect the pair interactions between SmMYB35, SmTT8, and SmTTG1 in *N. benthamiana* leaves epidermal cells. Scale bars = 22 μm. **c** Transient Dual-LUC assay in *N. benthamiana* leaves. The promoter activity of *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS* was expressed as a ratio of LUC to REN. Data are represented by three biological replicates. Significance is indicated by different letters ($P < 0.05$)

promoter of *SmMYB35* fused to LUC luciferase acting as a reporter, and REN luciferase driven by 35S promoter acting as an inner reference. The effector constructs contained the SmHY5 driven by 35S promoter (Fig. 6d). The results showed that SmHY5 significantly activated the promoter of *SmMYB35* (Fig. 6e). These results indicated that SmHY5 could bind to the promoter of *SmMYB35* and stimulate its expression.

## Discussion

Fruits with uniform coloration conferred by anthocyanin are more attractive to consumers, for the benefits of anthocyanin to the health of the human body. Anthocyanin biosynthesis is determined by a series of enzymes, which gene expressions are regulated by numerous transcription factor families (Gonzalez et al. 2008; Nuraini et al. 2020). Among which MYB, bHLH and WD40 are important for activating the expression of structural genes, which function in a MBW transcription regulatory complex manner (Li et al. 2020). Usually, environmental factors, such as temperature and light, regulate anthocyanin biosynthesis by modulating the expression of anthocyanin-related genes or protein stabilities of MYB and bHLH (Gu et al. 2019). Light is crucial for the coloration of eggplant, which can drastically induce anthocyanin-related genes expression (Jiang et al. 2016b; Li et al. 2017), demonstrating that the light signal participates in the regulation of anthocyanin synthesis in eggplants.

### SmMYB35 is an activator of anthocyanin biosynthesis in eggplant

Many R2R3-MYBs which involved in the regulation of anthocyanin synthesis have been identified at the genetic and molecular levels (Liu et al. 2018b). Based on their well-conserved DNA-binding domains, 73 R2R3-MYB transcriptional factors have been screened in the eggplant database (Wang et al. 2016). SmMYB1 was the earliest discovered and most critical positive-regulator for anthocyanin synthesis (Zhang et al. 2014; Jiang et al. 2016b). The overexpression of *SmMYB1* can drive the rich

anthocyanin accumulation in all tissues except the fruit flesh (Zhang et al. 2016). In the present study, the tissue expression specificity of *SmMYB35* was highly correlative with that of *SmMYB1* (Jiang et al. 2016b) and the anthocyanin content reported in different tissues of eggplant (Jiang et al. 2016a), implying that SmMYB35 may play a similar role as SmMYB1 in regulating anthocyanin synthesis. Interestingly, both Y2H and BiFC showed that SmMYB1 and SmMYB35 could interact with each other (Fig. S3), which illustrated our previous study that SmMYB35 increased the anthocyanin accumulation when co-infiltrated with SmMYB1 and SmTT8 in *N. benthamiana* (Li et al. 2017), suggesting that SmMYB35 acts as an enhancer for SmMYB1 in anthocyanin synthesis in eggplant.

Anthocyanin biosynthesis is generally determined by structural and regulatory genes. Pigment accumulation in plant tissues is mainly controlled by the expression profiles of regulatory genes. SlAN2-like acts as a master regulator that drives rich anthocyanin accumulation both in peel and flesh (Sun et al. 2019). In addition, MdMYB1 and MdMYBA are responsible for red skin coloration (Lin-Wang et al. 2010), while MdMYB10 controls red flesh coloration of apple fruit (Chagné et al. 2007). In the present study, *SmMYB35-OE* transgenic eggplants displayed a higher anthocyanin accumulation ubiquitously in the stems and petals than those in WT, but not in other tissues (Fig. 4). It is worth noting that most structural genes showed the highest expression levels in the stems and petals in *'140'* (Fig. S4). Interestingly, both WT and transgenic plants have white fruit peels and no anthocyanin accumulation. According to previous research, the white peel of eggplant resulted from the mutation and low expression level of a major anthocyanin activator *SmMYB1* (Zhang et al. 2014). Our investigation declared that the expression of *SmMYB1* was low in the peel of WT (Fig. S5), suggesting that overexpression of *SmMYB35* may not produce anthocyanin in the peel. The regulatory effect of SmMYB35 on structural genes is weakened, when SmMYB1, a major anthocyanin activator, was absent or lowly expressed in peels. However, other mechanisms cannot be excluded at present. One study also found that VvMYB5a does not participate in the regulation of anthocyanins, whereas VvMYB5b can activate a subset of late biosynthetic genes of the flavonoid pathway (Cavallini et al. 2014). There are also findings that transcription factors activate a precise pathway on post-transcriptional modification. Such as MYB75 phosphorylation by MPK4 is required for full function of MYB75 in Arabidopsis (Li et al. 2016), and apple MPK4 mediates phosphorylation of MYB1 to enhance light-induced anthocyanin accumulation (Yang et al. 2021).
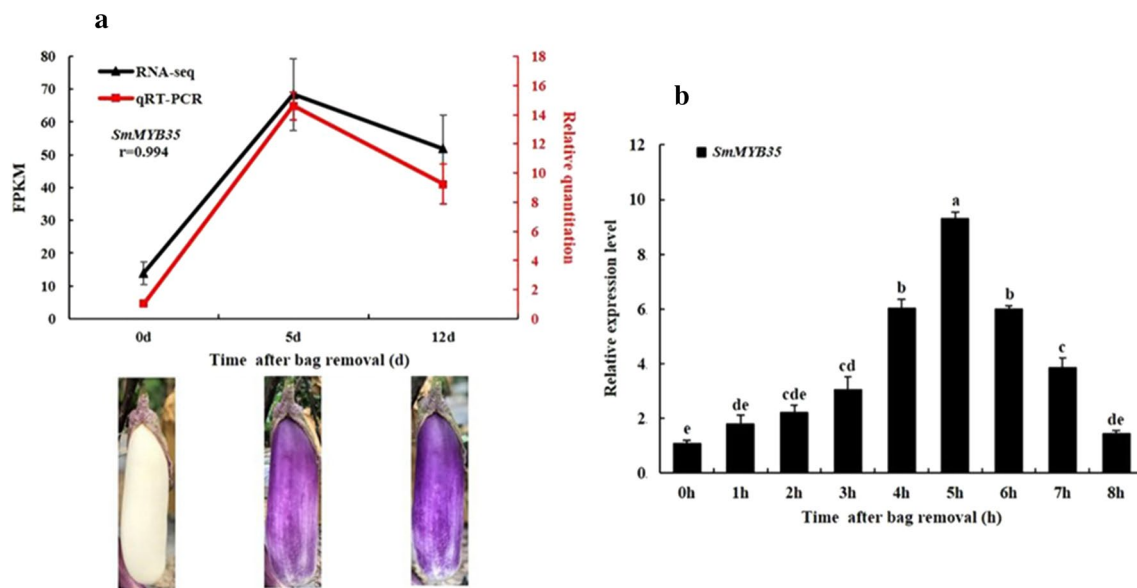
**Fig. 5** Expression patterns of *SmMYB35* in bagging experiment. **a** QRT-PCR was performed to validate the RNA-seq results. **b** The expression patterns of *SmMYB35* from nine-time points at 0, 1, 2, 3, 4, 5, 6, 7, and 8 h after bag removal. Data are represented by three biological replicates, each of which included three technical replicates. One-way analysis of variance was used to test the statistical significance which is indicated by different letters ($P < 0.05$)

## How does SmMYB35 promote anthocyanin accumulation in eggplant

The regulation of anthocyanin biosynthesis in plants has been well documented. There are two mechanisms which MYB activators control anthocyanin biosynthesis by binding to the promoters of the target genes and taking a part in MBW complexes (Koes et al. 2005; Premathilake et al. 2020). Many MYB TFs have also been found to activate the expression of anthocyanin biosynthetic genes in *Rosaceae*, such as apple, grape, and strawberry (Deluc et al. 2008; Espley et al. 2009; Zhang et al. 2020). MBW complexes could more efficiently activate the promoters of anthocyanin biosynthetic genes than MYB alone (Chagné et al. 2013). Other findings in *Arabidopsis thaliana* report that MYBs (such as PAP1, TT2, MYB5 and MYB4) and bHLHs (such as GL3, EGL3, MYC1 and TT8) can directly interact with TTG1 to form a MBW complex and have an important transcription role in the biosynthesis of anthocyanin synthesis (Broun 2005). Another study also indicated that AtTT8 exhibited a strong interaction with AtTTG1 in the presence of all the tested R2R3MYBs including AtMYB61 (Zhang et al. 2019). TTG1 often acts as a s caffold for the combinatorial interactions between MYB and bHLH and is essential for regulating the transcriptional activity of MBW complexes (Shan et al. 2019). The interaction among MYB, bHLH and TTG1 is a necessary condition for anthocyanin accumulation. Overall, the regulation of flavonoid synthesis in plants is considered to be the original function of the MBW complex.

When a R3-MYB type repressor competes with one of the R2R3-MYB partners in the MBW complex, which blocks the formation of MBW, the expression of anthocyanin biosynthetic genes is attenuated (Nguyen et al. 2015). Hence, there is a dynamic balance in the accumulation of anthocyanin. In the present work, SmMYB35 directly binds to the promoters of *SmCHS*, *SmF3H*, *SmDFR*, and *SmANS* to stimulate their activities (Fig. 3). In addition, we found that the interactions of SmMYB35 protein with SmTT8 and SmTTG1 formed a SmMYB35-SmTT8-SmTTG1 ternary transcription complex to participate in the regulation of the anthocyanin pathway (Fig. 4). These results elucidated the phenotype of *SmMYB35-OE* transgenic eggplant, and suggested that the molecular mechanism of MYBs participating in the regulation of anthocyanin biosynthesis is highly conservative among different species.

Light is the most important environmental factors for anthocyanin biosynthesis (Shin et al. 2013). HY5 is a central modulator of light signaling mediating many aspects of light-response by targeting the G-box of the target genes (Lee et al. 2007). Electrophoretic mobility shift assay and ChIP-qPCR assays revealed that SlHY5 could recognize and bind to the G-box and ACGT-containing element contained in the promoters of anthocyanin biosynthesis genes (Liu et al. 2018a, b, c). PyHY5 binding to G-box motifs of the promoters of *PyMYB10* enhanced its expression, and then promoted the accumulation of anthocyanin in red pear (Wang et al. 2020), implying that harbor of the G-box in the promoter might be the potential target
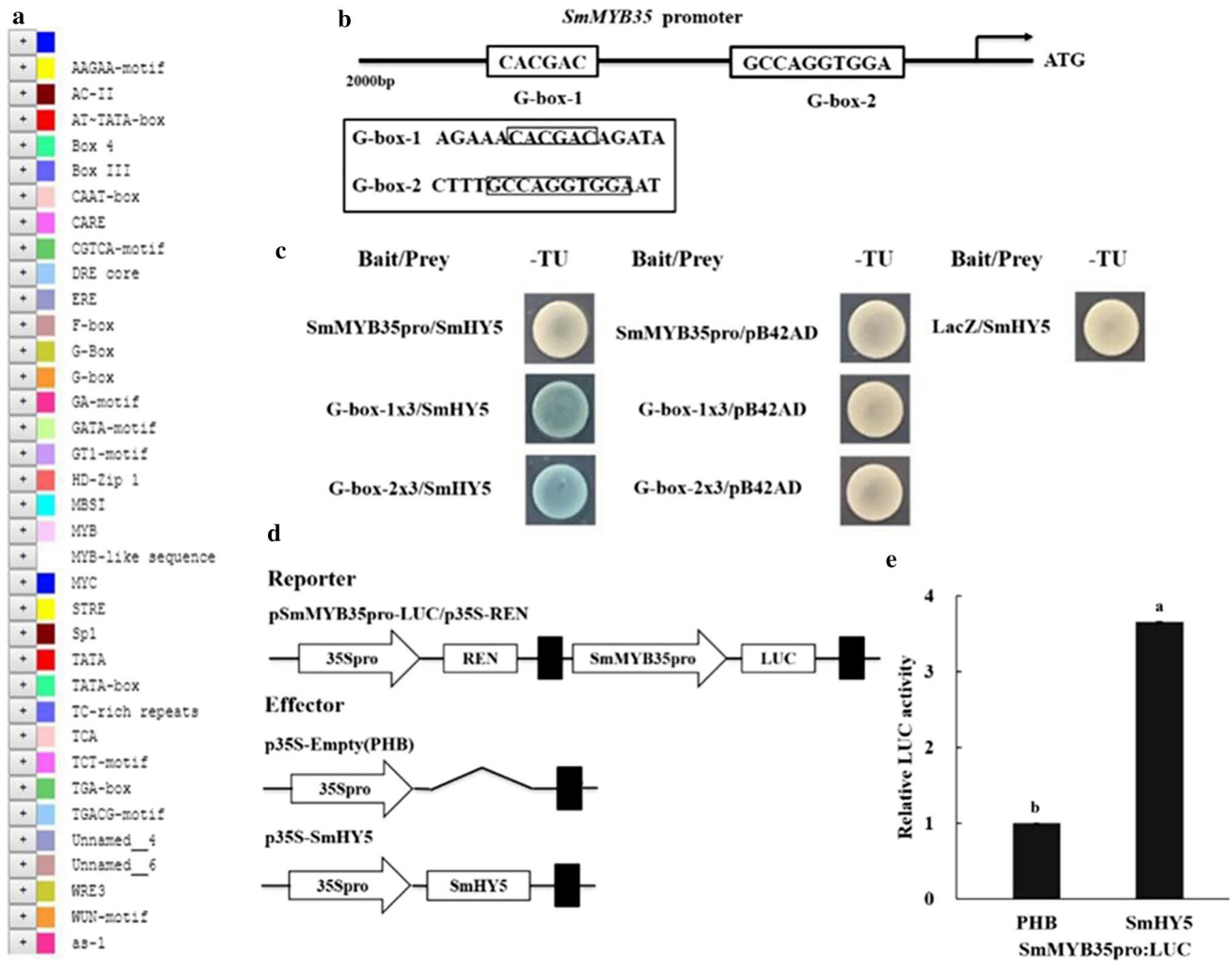
**Fig. 6** SmHY5 intensifies the transcription of *SmMYB35* by binding to its promoter. **a** Cis-acting regulatory elements in *SmMYB35* promoter sequences. **b** Schematic diagrams of the *SmMYB35* promoter. The positions of potential HY5 DNA binding sites (G-box-1, G-box-2) are signed by black boxes. **c** Y1H assays indicate protein-DNA interactions. **d** Schematic diagram of the reporter and effector con-structs used in the Dual-LUC assay. **e** Transient Dual-LUC assay in *N. benthamiana* leaves. The promoter activity of *SmMYB35* promoter is expressed as a ratio of LUC to REN. Data are represented by three biological replicates. Significance is indicated by different letters ($P < 0.05$)

gene of HY5. Jiang Minming's study also found that the expression level of SmHY5 displayed no tissue-specificity in '*Lanshan Hexian*' and increased immediately after bag removal for light irradiation (Jiang et al. 2016b). In the present paper, we found the expression level of SmMYB35 was induced by exposure to light, and was positively cor-related with anthocyanin accumulation at 0 d, 5 d, and 12 d after bag removal. Moreover, the expression of *SmMYB35* gene was affected by light period and light intensity under natural conditions, and the expression level was high-est in the middle of the day (Fig. 5b). Besides, SmHY5 directly binds to the promoter region G-box to induce the transcriptional activation of SmMYB35, thereby mediat-ing light signal modulating the expression of SmMYB35, same as AtMYB75 described in Arabidopsis (Ho et al.

2013). We suggested that anthocyanin biosynthetic genes was regulation via HY5 and via MYB TF.

In conclusion, our findings firstly verified that SmMYB35 enhanced anthocyanin biosynthesis in plants and we established a model diagram for analyzing the mechanism of light-responsive transcription factor SmMYB35 involving in regulating anthocyanin biosyn-thesis in eggplant. In light conditions, nucleus-localized TF SmHY5 binds to the G-box in *SmMYB35* promoter and activates the expression of *SmMYB35*. Then SmMYB35 increases the transcriptional activation of anthocyanin biosynthetic genes by binding to their promoters. Fur-thermore, the regulatory function was enhanced when SmMYB35 interacts with SmTT8, and SmTTG1 to form a SmMYB35-SmTT8-SmTTG1 transcriptional complex
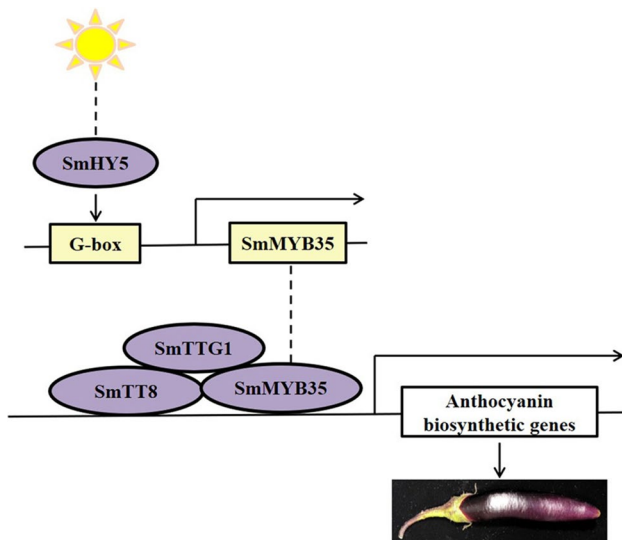
**Fig. 7** Model of light-induced anthocyanin biosynthesis in eggplant. In light conditions, nucleus-localized TF SmHY5 induced expression of *SmMYB35*, then positive-regulators such as SmMYB35, SmTT8, and SmTTG1 form a ternary complex induce expression of anthocyanin biosynthetic genes and increased anthocyanin accumulation. Arrows with polyline lines indicate the promoters of *SmMYB35* and anthocyanin biosynthetic genes. Straight lines indicate promotion

(Fig. 7). This model deepens our understanding of the molecular mechanism of SmMYB35 mediating light modulating anthocyanin biosynthesis in eggplant and provides the basis for innovating anthocyanin-rich eggplant cultivars under low light conditions.

## *Author contribution statement*

HYC, YL, and LZL conceived and designed the research. LZL SHL, HYG, LDL, and SLS performed experiments. LZL analyzed data and wrote the manuscript. All authors read and approved the manuscript.

**Data availability** All data generated or analysed during this study are included in this article [and its supplementary information files].

## Declarations

**Conflict of interest** The authors declare no conflicts of interest.

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