The Basic Helix-Loop-Helix Transcription Factor SmbHLH1 Represses Anthocyanin Biosynthesis in Eggplant

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Many basic helix-loop-helix transcription factors (TFs) have been reported to promote anthocyanin biosynthesis in numerous plant species, but little is known about bHLH TFs that inhibit anthocyanin accumulation. In this study, SmbHLH1 from Solanum melongena was identified as a negative regulator of anthocyanin biosynthesis. However, SmbHLH1 showed high identity with SmTT8, which acts as a SmMYB113-dependent positive regulator of anthocyanin-biosynthesis in plants. Overexpression of SmbHLH1 in eggplant caused a dramatic decrease in anthocyanin accumulation. Only the amino acid sequences at the N and C termini of SmbHLH1 differed from the SmTT8 sequence. Expression analysis revealed that the expression pattern of SmbHLH1 was opposite to that of anthocyanin accumulation. Yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays showed that SmbHLH1 could not interact with SmMYB113. Dual-luciferase assay demonstrated that SmbHLH1 directly repressed the expression of SmDFR and SmANS. Our results demonstrate that the biological function of bHLHs in anthocyanin biosynthesis may have evolved and provide new insight into the molecular functions of orthologous genes from different plant species.

Keywords: eggplant, anthocyanin biosynthesis, bHLH transcription factor, negative regulation, RNA-seq

INTRODUCTION

Anthocyanins are water-soluble pigments that are responsible for the red, purple, and blue colors of different organs in a wide range of plants (Mol et al., 1998; Koes et al., 2005; Allan et al., 2008). In addition to coloring flowers and fruits to attract pollinators and seed dispersers, anthocyanins can also protect plants from UV damage and promote resistance to low-temperature stress in plants (Ilk et al., 2015). Anthocyanins have been found to be the most powerful free radical scavengers in plants, and they have been used to lower blood pressure, improve vision, reduce inflammation, and prevent cancer (Konczak and Zhang, 2004; Tsuda, 2012).

Anthocyanins are an important branch of the flavonoid biosynthetic pathway and are formed in a stepwise series of enzymatic reactions catalyzed by chalcone synthase (CHS), chalcone...
isomerase (CHI), flavanone-3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), flavonoid 3′′-hydroxylase (F3′′H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and a 3-glucosyltransferase (3GT; Koes et al., 2005). These anthocyanin biosynthetic genes are transcriptionally regulated by a MYB-BHLH-WD40 (MBW) complex, the crucial role of which has been well-documented across many plants, such as *Arabidopsis*, apple, tomato, and so on (Xie et al., 2012; Töhge et al., 2017; Sun et al., 2020a). AcMYB123 and AcbHLH42, the *Actinidia chinensis* cv. Hongyang orthologs of *Arabidopsis* TT2 (MYB) and TT8 (BHLH), respectively, interact to activate the promoters of AcANS and AcF3GT1 and elevate anthocyanin accumulation in *Actinidia arguta* cv. Baby star and transgenic *Arabidopsis thaliana* (Wang et al., 2019). In apple, MdbHLH3 interacts with MdMYB1, MdMYB9, and MdbMYB11 to promote anthocyanin or proanthocyanidin (PA) accumulation (Xie et al., 2012; An et al., 2014).

The bHLH proteins are the second largest class of transcription factors (TFs) in plants; they can be divided into 26 subgroups (Pires and Dolan, 2010), and this classification can be extended to 32 subgroups if the "atypical" bHLHs are included (Carretero-Paulet et al., 2010). Compared with the 11–13 subgroups of the R2R3 MYBs (Li et al., 2012; He et al., 2016; Huang et al., 2018), bHLH family seems to have a more complex role in anthocyanin regulation. In general, the first 200 amino acids of the BHLH protein are involved in the interaction with the MYB partner, whereas the following 200 amino acids interact with the WD40 protein (Montefiori et al., 2015).

The G-box (5′-CACGTG-3′) or E-box (5′-CANNTG-3′) in gene promoter regions is recognized by bHLH family members (Atchley et al., 1999; Montefiori et al., 2015; Wang et al., 2018). Strikingly, the promoters of most flavonoid or anthocyanin biosynthetic genes contain G-box and E-box elements (Massari and Murre, 2000). Therefore, in addition to forming the MBW complex, bHLHs can also directly regulate anthocyanin biosynthesis. For example, MdbHLH3 can activate the expression of MdmMYB1, MdmMYB9, and MdmMYB11 to promote anthocyanin accumulation (Xie et al., 2012; An et al., 2014). TT8 is known to interact with MYB protein to enhance anthocyanin biosynthesis (Baudry et al., 2006; Li et al., 2016b; Wang et al., 2019). However, TT8 orthologs in different species may also perform different functions. Jia et al. (2021) found that DcTT8 from *Dendrobium candidum* could directly activate the expression of *DcF3H* and *DcUFGT* by binding to their promoters. Zhai et al. (2020) found that targeted mutations of *BnTT8* blocked PA-specific deposition, but elevated seed oil content and altered fatty acid (FA) composition in the seed coat of *Brassica napus*. In addition, bHLHs can also negatively regulate anthocyanin biosynthesis. To date, some bHLH genes have been reported as repressors of anthocyanin biosynthesis in plants, including IN1 from maize (Burr et al., 1996), bHLH3/13/′14/17 from *Arabidopsis* (Song et al., 2013), LcbHLH92 from *Leymus chinensis* (Zhao et al., 2018), and CpbHLH1 from *Chimonanthus praecox* (Zhao et al., 2020), but the molecular mechanisms of their negative regulation have been little reported.

Eggplants (*Solanum melongena*) with purple peel are rich in anthocyanins and popular with consumers. They rank fourth in facility vegetable cultivation planting area. Noda et al. (2000) reported that eggplant extracts showed the most potent superoxide anion radical scavenging activity (SOD-like activity) of 16 common vegetables examined, highlighting the important role of anthocyanins in eggplant. Recently, research focused on the molecular mechanisms of anthocyanin biosynthesis in eggplant has increased (Jiang et al., 2016; Li et al., 2017, 2018; Moglia et al., 2020; Zhou et al., 2020), but gene functions have been verified primarily by ectopic expression in *Arabidopsis* or tobacco. The eggplant genome database was updated by Barchi et al. (2019), providing a valuable resource for the molecular breeding of new eggplant germplasm. Based on the updated eggplant genome, Moglia et al. (2020) functionally characterized the genes belonging to the MBW complex (*SmelANT1*, *SmelAN2*, *SmelAF13*, and *SmelAN1*), and identified out an R3 MYB type repressor (*SmelMYBL1*) of anthocyanin biosynthesis.

Based on the two published eggplant genome databases (Hirakawa et al., 2014; Barchi et al., 2019), *SmBHLH1* (*Sm2e_5_00592.1_g00005.1*) and *SmTT8* (*SMEL_009g326640.1*) were identified as two TT8 homologs. SmTT8 has been shown to interact with SmMYB75 to promote anthocyanin biosynthesis in eggplant (Shi et al., 2021). However, no studies on *SmBHLH1* have been reported. Hence, we overexpressed *SmBHLH1* in eggplant, and obtained two stable transformation lines, *SmBHLH1-1* and *SmBHLH1-2*. The anthocyanin content was markedly reduced in both lines, indicating that *SmBHLH1* and *SmTT8* have different functions and molecular mechanisms in the regulation of anthocyanin biosynthesis in eggplant. We used biochemical experiments and transcriptomic analysis to investigate the molecular mechanism by which *SmBHLH1* regulates anthocyanin biosynthesis.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

Six eggplant cultivars with different color and anthocyanin contents in the fruit and stem peels, named No. 44, No. 64, No. 76, No. 108, No. 109, and No. 133 (Supplementary Figure S1), were grown in the solar greenhouse of Shandong Agricultural University to perform phenotypic observations and collect experimental materials. No. 109 was used for genetic transformation.

*Nicotiana benthamiana* was grown in a phytotron under 25/16°C and 12/12 h day/night conditions. Plants with 5–6 leaves were used for bimolecular fluorescence complementation (BiFC), dual luciferase, and transient expression assays.

**Anthocyanins Content Analysis**

The anthocyanin content was measured with the methods described by Neff and Chory (1998).

**Gene Isolation and Sequence Analysis**

The full-length coding sequences (CDSs) of *SmBHLH1* and *SmTT8* were cloned by PCR amplification. The amino acid sequences of *SmBHLH1* and *SmTT8* were translated with DNAMAN 6.0.
Function of SmbHLH1 in Eggplant

Subcellular Localization
The full-length CDS of SmbHLH1 without the stop codon was inserted into the pHB:GFP vector. The fusion construct was transferred into Agrobacterium tumefaciens strain GV3101, and subcellular localization assays were performed as previously reported (Sparkes et al., 2006).

Yeast Two-Hybrid Assay
The full-length CDSs of SmbHLH1 and SmTT8 were inserted into the pGBKTK7 vector as bait, and SmMYB113 was fused to the pGADT7 vector as prey. The Y2H assays were performed as described in Zhou et al. (2020).

Bimolecular Fluorescence Complementation
The full-length CDSs of SmbHLH1 and SmTT8 without stop codons were inserted into the pXY104 vector, and SmMYB113 with the stop codon was subcloned into the pXY106 vector. BiFC assays were performed as described by Li et al. (2016a).

Plasmid Construction and Plant Transformation
The full-length CDS of SmbHLH1 was inserted into the pRI 101 vector with the 35S-CaMV promoter. The fusion vector was transferred into Agrobacterium strain LBA4404 and introduced into No. 109 by Agrobacterium-mediated cotyledon explant transformation based on an earlier report by Vasudevan et al. (2005) with minor modifications.

Dual Luciferase and Transient Expression Assays in N. benthamiana Leaves
The complete coding regions of SmMYB113, SmbHLH1, and SmTT8 were inserted into the pHB vector, and the promoters of anthocyanin biosynthetic genes (SmCHS, SmCHI, SmF3H, SmF3′H, SmF3′5′H, SmDFR, SmANS, and Sm3GT) were amplified and cloned into pGreenII 0800-LUC vectors. The Dual-LUC assay was performed as previously reported (Hu et al., 2019). For the transient expression assays, 1/2x, 1x, and 2x pHB-SmbHLH1 was co-infiltrated with pHB-SmMYB113 or pHB-SmMYB113 + pHB-SmTT8. The anthocyanin content was measured 4 days after infiltration with the methods described in Li et al. (2017).

Quantitative Reverse Transcription PCR
The fruit and stem peels of six eggplant cultivars and the tender stems close to the growing point of No.109 were collected, and total RNA was extracted from the samples using the TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer’s instructions. Then 1 μg RNA was used for cDNA synthesis with the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa). The quantitative reverse transcription PCR (RT-qPCR) assay was performed according to the manufacturer’s instructions of the SYBR Premix Ex Taq II Kit (TaKaRa) on the LightCycler 96 system (Roche, Basel, Switzerland). The Actin gene (GU984779.1) was used as an internal control gene. The relative expression levels of the amplified products were analyzed using the comparative CT method (Livak and Schmittgen, 2001).

RESULTS
Molecular Cloning and Characterization of TT8 Homologs in Eggplant
The CDSs of SmbHLH1 and SmTT8 were amplified based on the two published eggplant genome databases (Hirakawa et al., 2014; Barchi et al., 2019). After sequencing and alignment, we found that the SmTT8 sequence showed some differences from the published sequence (SMEL_009326660.1), whereas SmbHLH1 was identical to Smc2.5_00592100.1 (Supplementary Figure S2). We then aligned the amino acid sequences of SmbHLH1 and SmTT8 (Figure 1) and found that only the regions at the N terminus (amino acids 1–158 and 188–193) and C terminus (amino acids 633–659) differed between SmbHLH1 and SmTT8.

A phylogenetic tree was constructed to analyze the relationships of SmbHLH1 and SmTT8 with all bHLH proteins from Arabidopsis, Solanum lycopersicum, Malus × domestica, Vitis vinifera, Fragaria × ananassa, Petunia × hybrida, Prunus persica, Chimonanthus praecox, and Medicago truncatula known to be involved in the anthocyanin biosynthesis pathway (Supplementary Figure S3; Li et al., 2017; Zhao et al., 2020). As shown in Supplementary Figure S3, SmbHLH1 and SmTT8 were placed in the same clade.

Different Expression Pattern of SmbHLH1 and SmTT8 in Eggplant
To analyze the relationship between SmbHLH1 and SmTT8, the expression pattern of SmbHLH1 and SmTT8 was measured...
in the fruit and stem peels from six eggplant cultivars with different anthocyanin contents (Figure 2A; Supplementary Figure S1). The primers for RT-qPCR were designed from the 5′ termini, which differed between SmbHLH1 and SmTT8 (Supplementary Table S1). As shown in Figure 2A and Supplementary Figure S1, the anthocyanin content in the fruit peel was sorted as No. 76 > No. 64 > No. 44 > No. 108/No. 109/No. 133, while No. 76/No. 109 > No. 44 > No. 64 > No. 108 in the stem peel. The expression patterns of SmTT8 in the fruit and stem peel were both consistent with anthocyanin contents (Figure 2B). However, the expression pattern of SmbHLH1 was opposite with anthocyanin contents in both fruit and stem peel (Figure 2C). These results implied that the function of SmbHLH1 may be different from that of SmTT8.

To confirm the subcellular location of SmbHLH1, the 35S::SmbHLH1-GFP fusion protein was constructed and transiently expressed in tobacco leaves. Compared with the positive control, SmbHLH1-GFP was predominately present in the cytosol and nucleus (Figure 2D).

Overexpression of SmbHLH1 Inhibited Anthocyanin Accumulation in Eggplant

To characterize the function of SmbHLH1 in eggplant, transient VIGS assay, CRISPR-Cas9 system, and overexpression system were all performed on all the six eggplant cultivars. However, the approaches to transform the eggplant with the transient VIGS assay and CRISPR-Cas9 system were failed. So we decided to transform the eggplant with 35S promoter-constructs to probe the overall function of the SmbHLH1 gene. SmbHLH1 was introduced into eggplant by Agrobacterium-mediated transformation. Only the eggplant cultivar No.109 differentiated two SmbHLH1 overexpression transgenic lines (overexpressed by the 35S-CaMV promoter, named as SmbHLH1-1 and SmbHLH1-2), which would be used to probe the overall function of the SmbHLH1 gene. SmbHLH1 was introduced into eggplant by Agrobacterium-mediated transformation. Only the eggplant cultivar No.109 differentiated two SmbHLH1 overexpression transgenic lines (overexpressed by the 35S-CaMV promoter, named as SmbHLH1-1 and SmbHLH1-2), which would be used to probe the overall function of the SmbHLH1 gene.
peels were changed from purple to green, which could be accumulated anthocyanins in cultivar No.109. However, the fruit peels of cultivar No.109 was orange with little anthocyanins, no significant difference was found comparing with the two 35S::SmbHLH1 transgenic eggplant lines. Next, we evaluated the anthocyanin contents and the expression levels of anthocyanin biosynthetic genes (SmCHS, SmCHI, SmF3H, SmF3’5’H, SmDFR, and SmANS) in the stems peel. Anthocyanin contents and expression levels of anthocyanin biosynthetic genes were lower in stems peel of the two SmbHLH1 transgenic lines than in WT stems (Figures 3C, D).

SmbHLH1 Could Not Interact With SmMYB113

We performed yeast two-hybrid (Y2H) assays to determine whether SmbHLH1 regulated anthocyanin biosynthesis through interaction with SmMYB113, which has been reported as a critical anthocyanin regulator and interacted with SmTT8 (Zhou et al., 2020). Positive β-gal activity was observed only in yeast that contained AD-SmMYB113 plus BD-SmTT8 grown on -T/-L/-H/-A screening medium but not in yeast that contained AD plus BD-SmbHLH1, AD plus BD-SmTT8, AD-SmMYB113 plus BD, or AD-SmMYB113 plus BD-SmbHLH1 (Figure 4A). These results indicated that SmbHLH1 could not interact with SmMYB113, whereas SmTT8 could.

Bimolecular fluorescence complementation assays were performed to further characterize the interaction between SmbHLH1/SmTT8 and SmMYB113 in vivo. As shown in Figure 4B, YFP fluorescence signals appeared only when YFPc-SmMYB113 and YFPc-SmTT8 were co-expressed. No fluorescence was detected in cells that contained combinations of YFPc-SmMYB113 plus YFPc-SmbHLH1 and other empty vector controls. In addition, we found that the interaction location of SmMYB113 and SmTT8 complexes was in the nucleus. These results confirmed that SmbHLH1 could not interact with SmMYB113 in plant cells, but SmTT8 could.

SmbHLH1 Is a Negative Transcription Regulator of SmDFR and SmANS

The cis-elements in the promoter sequences of six anthocyanin biosynthetic genes (SmCHS, SmCHI, SmF3H, SmF3’5’H, SmDFR, and SmANS) were analyzed, and E-box or G-box cis-elements recognized by bHLH family members were found in all six promoter sequences (Supplementary Figure S4). Then, we performed a dual-luciferase assay by infiltration of tobacco leaves to analyze whether SmbHLH1 can inhibit the transcription of the anthocyanin biosynthetic genes. As a result, we found that SmDFR and SmANS promoter activities were inhibited by SmbHLH1 (Figure 5A). Because SmMYB113 can bind directly to the SmDFR promoter and activate its expression.

![Figure 2](image-url)
As shown in Figure 5B, the inhibition of the SmDFR promoter activity by SmbHLH1 occurred in the presence of SmMYB113. Another transient expression assay was carried out in tobacco leaves to explore the role of SmbHLH1 in anthocyanin biosynthesis. Agrobacterium tumefaciens GV3101 strains containing the empty vector pHB, SmbHLH1, SmTT8, or SmMYB113 were infiltrated or co-infiltrated into tobacco leaves. As shown in Figure 5C, pigmentation was evident after infiltration with SmMYB113 alone and co-infiltration with SmbHLH1 and SmTT8. Compared with the leaves infiltrated with pHB and SmMYB113 alone, anthocyanin content decreased when SmbHLH1 was added but increased when SmTT8 was added. Next, different ratios of SmbHLH1:SmMYB113 and SmbHLH1:SmMYB113:SmTT8 were used to analyze the effect of SmbHLH1 on SmMYB113-induced anthocyanin biosynthesis (Figures 5D,E). We found that anthocyanin accumulation in infiltrated patches was negatively correlated with the dose of SmbHLH1. These results suggested that SmbHLH1 was a key negative regulator of anthocyanin accumulation in eggplant, directly inhibiting SmDFR and SmANS expression through a SmMYB113-independent pathway.

Overview of RNA-Seq Analysis
To explore how SmbHLH1 regulates gene expression at the whole-genome level, total RNA was extracted from stems of the WT and two SmbHLH1 transgenic lines for RNA-seq analysis. A total of 29,840 genes or transcripts were obtained. Subsequently, we performed pairwise comparisons of transcript abundance to identify differentially expressed genes (DEGs) between the WT and transgenic lines based on an absolute fold change value of |log₂ ratio| ≥ 0.5 with p < 0.001. In total, 2,120 genes were differentially expressed in SmbHLH1-1 stems compared with the WT (933 up; 1,187 down), and 3,467 genes were differentially expressed in SmbHLH1-2 stems compared with the WT (1,811 up; 1,656 down; Figure 6A; Supplementary Table S2). Among these DEGs, only 126 genes were upregulated and 181 genes were downregulated simultaneously in both SmbHLH1-overexpressing lines compared with the WT (Figure 6B).
GO functional enrichment analysis was performed on the 126 upregulated and 181 downregulated genes shared by the two SmbHLH1 transgenic eggplant lines (Figures 6C,D). The 126 upregulated genes were enriched in 29 GO terms, and the 181 downregulated genes were enriched in 27 GO terms. In the “biological process” category, “cell proliferation” was enriched only in the upregulated genes, and “carbon utilization,” “reproduction,” and “reproductive process” were enriched only in the upregulated genes. In the “molecular function” category, “nutrient reservoir activity” was enriched only in the downregulated genes, and “structural molecule activity” was enriched only in the downregulated genes. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of the 126 shared upregulated genes and the 181 shared downregulated genes were identified using a value of p less than 0.05 as the cut-off (Table 1). As shown in Table 1, the “carbon metabolism” and “starch and sucrose metabolism” pathways were enriched in the upregulated genes, and the “flavonoid biosynthesis” and “anthocyanin biosynthesis” pathways were enriched in the downregulated genes. These results suggest that SmbHLH1 inhibited anthocyanin biosynthesis but promoted carbon metabolism. We next examined glucose, fructose, and sucrose contents in stems, functional leaves, and expanding leaves. The two SmbHLH1 transgenic eggplant lines showed significant increases in glucose, fructose, and sucrose content compared with the WT (Supplementary Figure S5). These results are consistent with the transcriptomic analysis.

**DISCUSSION**

The bHLH, R2R3-MYB, and WD repeat proteins can form a ternary MBW complex to modulate the expression of anthocyanin biosynthesis-related genes. Previous studies have revealed that several bHLH TFs, including AtTT8, AtGL3, AtEGL3, PhAN1, PhJAF13, GhMYC1, SlAN1, MdbHLH3, and PbbHLH2, interact with their respective MYB partners AtPAP1, AtTT2, PhAN2, GhMYB10, SlAN2, MdbMYB1, and PbbMYB9/10 to regulate anthocyanin or proanthocyanidin biosynthesis in Arabidopsis, Petunia hybrida, Gerbera hybrida, S. lycopersicum, M. domestica, and Pyrus bretschneideri (Elomaa et al., 2003; Koes et al., 2005; Ramsay and Glover, 2005; Xie et al., 2012; Sun et al., 2020a; Li et al., 2021b). Xie et al. (2012) reported that two regions (amino acids 1–24 and 186–228) at the N terminus of MdbHLH3 were crucial for the interaction between MdbHLH3 and
MdMYB1. In this study, we found that SmbHLH1, SmTT8, and MdbHLH3 were clustered into one clade by phylogenetic analysis (Supplementary Figure S3), and the N terminus (amino acids 1–158 and 188–193) and C terminus (amino acids 633–659) of SmbHLH1 differed significantly from those of SmTT8 (Figure 1). SmbHLH1 did not interact with SmMYB113 in both Y2H and BiFC assays (Figure 4), whereas SmTT8 did which was corresponding to the results reported by Moglia et al. (2020). These results suggested that the N-terminal portion of SmTT8 was crucial for its interaction with SmMYB113. Simultaneously, the pattern of SmbHLH1 expression contrasted with those of SmTT8 expression and anthocyanin accumulation (Figure 2). Previous studies have reported that the enhancement of anthocyanin biosynthesis by SmTT8 is dependent on SmMYB113 (Moglia et al., 2020; Zhou et al., 2020). However, we found that over-expression of SmbHLH1 inhibited anthocyanin accumulation (Figure 3). Dual-luciferase assays revealed that SmbHLH1 directly repressed the expression of SmDFR and SmANS (Figure 5). Because the color of cultivar No.109 tissues was different, the phenotype changes of 35S::SmbHLH1 transgenic eggplant lines were different. For the tissues with anthocyanins accumulation were changed from purple to green, such as

**FIGURE 5** | Dual-luciferase and transient analysis of SmbHLH1 on anthocyanin accumulation. (A) Inhibition of the activity of the SmDFR and SmANS gene promoters by SmbHLH1 in transient expression assay in N. benthamiana leaves; (B) SmbHLH1 decreased the ratio of LUC/REN of the SmDFR promoter activated by SmMYB113; and (C) SmbHLH1 inhibited SmMYB113-induced anthocyanin accumulation while SmTT8 enhanced in N. benthamiana leaves. (D,E) The relative anthocyanin contents induced by different proportions of SmbHLH1:SmMYB113 and SmbHLH1:SmMYB113:SmTT8 in infiltrated regions. *Represents significance at $p < 0.05$ comparing with the control.
stalks, sepals, and stem peels. However, no significant color change was found in the tissues with little anthocyanins content, such as the fruit peels. Taken all above, we could conclude that $SmbHLH1$ was a repressor in anthocyanin biosynthesis. Moreover, the silencing of $SmbHLH1$ in fruit peel tissue to verify the fruit can accumulate more anthocyanins is worthy in the future.

BHLH proteins are the second largest class of transcription factors in plants and can be divided into 26–32 subgroups (Carretero-Paulet et al., 2010; Pires and Dolan, 2010); they play important roles in stress tolerance, reproduction, and secondary metabolite biosynthesis (Sun et al., 2020b; Guo et al., 2021). It is worth noting that $AtTT8$, $AtGL3$, $AtEGL1$, and $AtMYC1$, which are positive regulator of anthocyanin biosynthesis, were placed into subgroup IIIf (Heim et al., 2003). However, $AtMYC1$ orthologs in maize and $C. praecox$, as well as another bHLH TF from subgroup IVd, have been reported to negatively regulate anthocyanin biosynthesis (Burr et al., 1996; Zhao et al., 2018). Here, $SmTT8$ and $SmbHLH1$ are both eggplant orthologs of $AtTT8$, but they played opposite roles in anthocyanin biosynthesis. It is not uncommon for such homologous genes to exhibit different functions in the plant. For example, Li et al. (2021a) reported that the biological function of MS1 in pollen development differed in the evolution of Poaceae plants. The different biological functions of homologous genes probably result from differences in some amino acids. Taking all of our results into consideration, we speculate that the N-terminal front portion of $SmbHLH1$ may be the key to its role in anthocyanin biosynthesis.

RNA-seq analysis revealed that overexpression of $SmbHLH1$ reduced the expression of anthocyanin biosynthetic genes while enhancing that of genes involved in carbon metabolism and starch and sucrose metabolism. Combined with another phenotype of the two $SmbHLH1$ transgenic eggplant lines, which were greener than the WT due to increased chlorophyll content (Figure 3; Supplementary Figure S4), we speculated that more light was absorbed, resulting in significant increases in glucose, fructose, and sucrose content. Anthocyanin and chlorophyll are two major plant pigments. Anthocyanin can protect plants from UV light and oxidative damage, whereas...
chlorophyll is essential for the conversion of light energy into stored chemical energy. Previous studies have reported that anthocyanin often accumulates in young leaves but degrades in mature leaves, which is beneficial for the gradual accumulation of chlorophyll in many plants (Oren-Shamir, 2009; Ren et al., 2019). On this basis, we speculate that the increased chlorophyll content may be directly regulated by SmbHLH1 or caused by a decrease in anthocyanin, suggesting the existence of a much more complex molecular mechanism that is worthy of study in the future.

CONCLUSION

In the eggplant genome, we found that both SmbHLH1 and SmTT8 are homologs of TT8. Their amino acid sequences differed only at the N and C termini, but they had contrasting functions in anthocyanin biosynthesis. SmbHLH1 repressed the expression of SmDFR and SmANS and inhibited anthocyanin biosynthesis through SmMYB113-independent pathway. We identified SmbHLH1 as a new target to silence to produce more anthocyanins in eggplant fruit peel. In addition, RNA-seq analysis revealed that genes involved in carbon metabolism and starch and sucrose metabolism were upregulated by overexpression of SmbHLH1 in eggplant.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI BioProject, PRJNA761256.

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AUTHOR CONTRIBUTIONS

FY, JL, and MW designed the research. ZD, ST, JL, and GY performed experiments. JL and ZD analyzed data. JW wrote the manuscript. FY and JL read and modified the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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