Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2025) **23**, pp. 2083–2094 doi: 10.1111/pbi.70033

Bi-functional transcription factor SlbHLH95 regulates fruits flavonoid metabolism and grey mould resistance in tomato

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Received 28 January 2024;
revised 31 December 2024;
accepted 18 February 2025.
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Keywords: tomato, *SlbHLH95*, bi-functional, flavonoid, fruit quality, SlMYB12.

Summary

Flavonoids are polyphenolic secondary metabolites in tomato fruit with important roles in nutritional quality. Dissecting the transcriptional regulatory network modulating flavonoid metabolism is the first step to improve the nutritional quality of tomato fruits through molecular breeding technology. In this study, we identified a transcription factor SlbHLH95 as a key regulator in flavonoid metabolism through analysis of the MicroTom Metabolic Network (MMN) data set. Functional analyses revealed that knockout of *SlbHLH95* increased the accumulation of naringenin, while the levels of rutin and nictoflorin decreased. Conversely, overexpression of *SlbHLH95* resulted in an opposite pattern of accumulation of flavonoids. Transactivation assays showed that SlbHLH95 positively activated the expression of *SlF3H* and *SlFLS*, two key enzyme-encoding genes in the flavonoid pathway, while repressing the expression of *SlCHS1*. Electrophoretic mobility shift assays (EMSA) demonstrated that SlbHLH95 could directly bind to the promoters of *SlF3H* and *SlFLS*, although it could not bind to the promoter of *SlCHS1*. Furthermore, SlbHLH95 interacted with the transcription factor SlMYB12 and coordinately regulated the expression of *SlF3H* and *SlFLS*. Beyond its role in flavonoid metabolism, SlbHLH95 positively regulated the grey mould resistance in tomato fruits by repressing *SlBG10*. Overall, our findings revealed the important role of bi-functional SlbHLH95 in flavonoid metabolism and grey mould resistance in tomato fruits by acting as both a transcriptional activator and a repressor. This study provides new insights into strategies for improving fruit quality and enhancing fruit disease resistance through targeted genetic modulation.

Introduction

Tomato (*Solanum lycopersicum*) is a widely consumed vegetable with an annual production of 186 million tons in 2022, according to FAO statistics (https://www.fao.org/faostat/en/#data/QCL). Tomatoes are a rich source of health-promoting compounds including carotenoids, vitamins and flavonoids, which contribute to their nutritional value and beneficial effects on human health. In addition, tomatoes serve as an ideal model for research on climacteric fruit development and ripening due to its released genome, ease of transformation, short life cycle and the availability of numerous ripening-related mutants (Giovannoni, 2007; Just *et al*., 2013; Li *et al*., 2018; Matsukura *et al*., 2008; Ranjan *et al*., 2012).

Flavonoids are a diverse class of naturally occurring phenolic compounds with variable structures widely distributed in fruits and vegetables (Panche *et al*., 2016). The fundamental structure of flavonoids consists of a diphenylpropane (C6–C3–C6) skeleton with two aromatic rings (A and B) linked by a heterocyclic pyran ring (C). Variations in the A and/or B rings, degrees of oxidation and substitution patterns in the C ring confer them diverse physiological functions (Farhadi *et al*., 2019). Flavonoids can be classified into six subclasses, including anthocyanins, flavan-3-ols, flavonols, flavanones, flavones and isoflavones (Danihelová *et al*., 2012). These compounds not only play crucial roles in plant growth and development but also exhibit various pharmacological activities, such as antioxidative, free radical scavenging, coronary heart disease prevention and anti-cancer effects (Bondonno *et al*., 2019; Havsteen, 2002; Knekt *et al*., 1997; Middleton Jr. *et al*., 2000; Panche *et al*., 2016; Winkel-Shirley, 2001). Within the plant system, flavonoids contribute to combating oxidative stress and act as growth regulators (Kumar and Pandey, 2013), thus making them a subject of substantial interest in plant biology and human health.

Derived from the phenylpropane metabolic pathway, flavonoids undergo enzymatic conversions catalysed by phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and 4-coumaroyl-CoA ligase, eventually leading to the flavonoid

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biosynthetic pathway (Liu *et al.*, 2021). The biosynthetic and metabolic pathways of flavonoids have been extensively investigated. In the synthesis of nictoflorin, chalcone synthase (CHS) catalyses the initial condensation of 4-coumaroyl-CoA with malonyl-CoA, resulting in the formation of chalcone. Chalcone is subsequently isomerized into naringenin by chalcone isomerase (CHI). Naringenin is then converted into nictoflorin through the actions of flavanone 3-hydroxylase (F3H) and flavonol synthase (FLS). The biosynthesis of rutin, another flavonoid, is produced through enzymatic catalysis involving a series of enzymes, including F3H and FLS, with naringenin serving as the precursor (Grotewold, 2006; Lepiniec *et al.*, 2006; Liu *et al.*, 2021; Owens *et al.*, 2008; Pandey *et al.*, 2016; Saito *et al.*, 2013). Flavonoid accumulation in plants is mainly modulated by the coordinated action of biosynthetic genes and transcription factors (Saito *et al.*, 2013). For instance, overexpression of *SlCHS* and *SlF3H*, two key flavonoid biosynthesis genes, has been linked to higher flavonoid accumulation in citrus fruits (Moriguchi *et al.*, 2001). Transcription factors from the MYB and bHLH families have been shown to play pivotal roles in the transcriptional regulation of flavonoid biosynthesis and metabolism. In *Arabidopsis thaliana*, the MYB transcription factors, AtMYB11, AtMYB12 and AtMYB111 activated the expression of early flavonoid biosynthetic genes such as *AtCHS*, *AtCHI*, *AtF3H* and *AtFLS1*, thereby enhancing flavonoid accumulation (Mehrtens *et al.*, 2005; Stracke *et al.*, 2007). In brassinosteroid signalling, the positive regulatory factor AtBES1 inhibits the transcription of MYB proteins, thereby reducing the biosynthesis of flavonols (Liang *et al.*, 2020). In cucumbers, it has been demonstrated that CsMYB60 can directly regulate the expression of *CsCHS*, thereby promoting the accumulation of flavonols (Li *et al.*, 2020a). Additionally, an MYB–bHLH–WD40 (MBW) transcriptional complex comprising CsaV3_4G001130, CsaV3_1G002260 and CsaV3_5G001800 is responsible for regulating the accumulation of flavonoids in cucumbers (He *et al.*, 2023). Several other MYB transcription factors have been implicated in the regulation of flavonoid biosynthesis in different tissues (Borevitz *et al.*, 2000; Gonzalez *et al.*, 2009; Lepiniec *et al.*, 2006).

Given the rich and diverse flavonoid content in tomato fruits, it has become extensively studied as a model for investigating flavonoid synthesis and metabolism. In addition to its role as a model system, tomato also serves as an excellent biological chassis for the production of flavonoids and other secondary metabolites (Alseekh *et al.*, 2015; Bovy *et al.*, 2007; Schijlen *et al.*, 2006). Using tomato as a model, the important role of MYB12 in regulating the accumulation of flavonoids has been well illustrated (Adato *et al.*, 2009; Ballester *et al.*, 2010; Pandey *et al.*, 2015; Wang *et al.*, 2018; Zhang *et al.*, 2015). For example, the NF-Y transcription factors SlNF-YA, SlNF-YB and SlNF-YC form a complex that binds to the *SlCHS1* promoter, thereby regulating flavonoid synthesis (Wang *et al.*, 2021). In addition, two paralogous genes *SlANT1* and *SlAN2*, encoding homologous R2R3-MYB transcription factors, have been identified as key regulators of flavonoid and anthocyanin biosynthesis across various tomato tissues (Kiferle *et al.*, 2015). Recent studies revealed the involvement of the transcription factor SlEFR.G3-like in regulating flavonoid production in tomato fruit (Li *et al.*, 2020b). Although a lot of transcription factors have been reported to act as key regulators in modulating flavonoid accumulation in tomatoes, the comprehensive regulatory network controlling flavonoid biosynthesis remains largely elusive.

In the present study, we leveraged our previously published tomato spatio-temporal transcriptome and metabolome database (Li *et al.*, 2020b) to identify a basic helix–loop–helix (bHLH) transcription factor, *SlbHLH95*, whose expression was associated with the expression of multiple flavonoid biosynthetic genes and with the accumulation patterns of different flavonoid compounds. Knockout of *SlbHLH95* through CRISPR/Cas9-mediated genome editing system resulted in a down-regulation of *SlF3H* and *SlFLS* genes and a reduced accumulation of downstream flavonoid compounds such as rutin and nictoflorin in ripe tomato fruits. In contrast, overexpression of *SlbHLH95* led to opposite patterns in both expression of structural genes and accumulation of compounds in flavonoid biosynthesis pathway. Furthermore, we found that SlbHLH95 can interact with SlMYB12 to enhance its activation capacity to *SlF3H* and *SlFLS*. Beyond its role in flavonoid metabolism, SlbHLH95 was also found to play an important role in the resistance of tomato fruit against *Botrytis cinerea* infection. Overall, the outcome of this study revealed that SlbHLH95 functions as a novel regulator in flavonoid biosynthesis and grey mould resistance, presenting it as a promising candidate to simultaneously improve fruit nutritional quality and enhance the disease resistance of tomato fruits.

Results

SlbHLH95 acts as a key regulator in flavonoid biosynthesis in tomato

Through mining our previous integrated spatiotemporal transcriptome and metabolome data set (Li *et al.*, 2020b), we observed a strong correlation between the expression levels of *SlbHLH95* and the accumulation of different compounds within the flavonoid metabolic pathway (Figure 1a; Data S1). Moreover, a higher accumulation of *SlbHLH95* transcript was recorded in peel than in flesh tissue (Figure 1b). These results suggested the involvement of this transcription factor in the regulation of flavonoid metabolism. To elucidate the functional significance of *SlbHLH95* in tomato fruit flavonoid metabolism, we generated *SlbHLH95* knockout lines by employing the CRISPR-/Cas9-mediated gene editing technology. This approach produced two *SlbHLH95* knockout lines: *SlbHLH95*-KO1 with a 1-bp deletion and *SlbHLH95*-KO2 with a 4-bp deletion at the gRNA site, both resulting in two truncated protein versions (Figure 1c). A delay in the onset of ripening was observed in *SlbHLH95*-KO lines compared to the wild type (WT) (Figure 1d,e). Transactivation assays further indicated that SlbHLH95 can regulate the transcription of *SlACS2* and *SlACO1*, two key ethylene biosynthesis genes (Figure S1). In addition to the delay of the onset of ripening, fruits of *SlbHLH95*-KO lines displayed a yellow coloration at the red-ripe stage (Br + 7) compared to the WT fruits at the same stage (Figure 1f). The higher hue angle value in *SlbHLH95*-KO fruits at the Br + 7 stage was consistent with the yellowness of *SlbHLH95*-KO fruits (Figure 1g). Since carotenoids play an important role in determining the colour of tomato fruits, we measured the content of carotenoids in fruits of WT and SlbHLH95-KO lines at Br + 7 stage. The results showed that the contents of total carotenoids and the main compounds including lycopene, β-carotene and lutein were all significantly reduced in *SlbHLH95*-KO lines (Figure 1h).

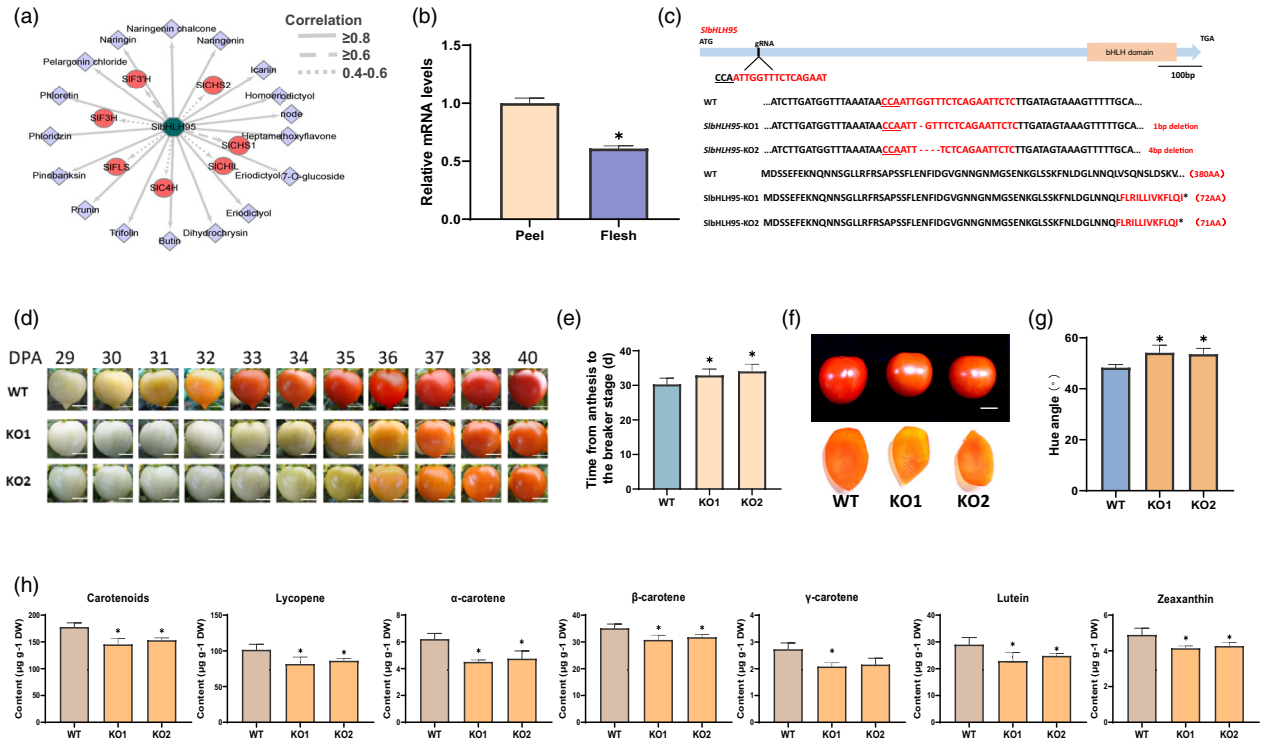

Figure 1 Identification of *SlbHLH95* as a candidate gene associated with flavonoid metabolism in tomato. (a) Correlation network of SlbHLH95 with key structure genes and metabolites in the flavonoid pathway. Flavonoid metabolites, structural genes and transcription factors were represented by purple diamonds, red circles and green circles, respectively. Pearson correlation coefficients were computed to assess the relationship between the expression of *SlbHLH95* and structure genes or metabolites accumulation. (b) Relative expression levels of *SlbHLH95* in the peel and flesh of wild-type (WT) fruits at Br + 7 stage. The relative expression levels were assessed by RT-qPCR. Error bars represent standard deviation (SD, $n = 3$) (*$P < 0.05$; Student's *t*-test). (c) Generation of *SlbHLH95* knockout lines by CRISPR-/Cas9-mediated gene editing technology. The DNA sequence and truncated protein versions of SlbHLH95 in *SlbHLH95* knockout (*SlbHLH95*-KO) lines. (d) Fruit phenotypes at different developmental and ripening stages in WT and *SlbHLH95*-KO lines. DPA, day-post-anthesis. KO1 and KO2 represent two *SlbHLH95* knockout lines. The scale bar represents 1 cm. (e) Time from anthesis to the breaker stage in WT and *SlbHLH95*-KO lines. KO1 and KO2 represent two independent *SlbHLH95*-KO lines. Error bars represent SD ($n = 7$) (*$P < 0.05$; Student's *t*-test). (f) Fruit colour of WT and *SlbHLH95*-KO lines at the Br + 7 stage. (g) Hue angle value of WT and *SlbHLH95*-KO fruits at the Br + 7 stage. KO1 and KO2 represent two independent *SlbHLH95*-KO lines. (h) Contents of the major carotenoids in fruits of WT and *SlbHLHb95*-KO lines at Br + 7 stage. Error bars represent SD ($n = 3$) (*$P < 0.05$; Student's *t*-test).

Knockout of *SlbHLH95* resulted in alteration in flavonoid composition

To explore the regulatory role of *SlbHLH95* in flavonoid biosynthesis in tomato, we assessed the flavonoid contents in both peel and flesh tissues of the *SlbHLH95*-KO lines, given that flavonoids are mainly accumulated in the peel of fruits in tomato (Bovy *et al.*, 2007). As shown in Figure 2a, the flavonoid composition was altered in peel with an increased content of naringenin, whereas the levels of other flavonoids, including eriodictyol, quercetin, astragalin, nicotiflorin and rutin, decreased in peel of fruits of *SlbHLH95*-KO compared to WT at the Br + 7 stage (Figure 2a). In contrast, the contents of these flavonoid compounds in the flesh were relatively low and showed no significant changes between *SlbHLH95*-KO and WT fruits (Figure 2a). Furthermore, the relative expression of enzyme-encoding genes acting upstream of naringenin such as *SlCHS1*, *SlCHS2* and *SlCHI1* was up-regulated in the peels of *SlbHLH95*-KO fruits (Figure 2b), which was in line with the increased naringenin content recorded in these lines. Moreover, despite the increased relative expression of *SlF3'H*, the expression levels of *SlF3H* and *SlFLS*, two key structural genes acting downstream of naringenin, were down-regulated in the peels of *SlbHLH95*-KO fruits (Figure 2b). This down-regulation of the two key downstream genes was consistent with the decreased levels of downstream flavonoid components, especially the two major flavonoids rutin and nictoflorin. Interestingly, we noted that the transcript levels of *SlMYB12* showed no significant changes in either peel or flesh tissues of *SlbHLH95*-KO fruits compared to WT (Figure S2).

Overexpression of *SlbHLH95* leads to an increase in flavonoid contents

To further investigate the regulatory role of *SlbHLH95* in flavonoid metabolism in tomato fruits, we analysed the flavonoid contents in *SlbHLH95* overexpressing lines (OE), which were obtained in our previous study (Chen *et al.*, 2020). Contrasting to the flavonoid composition in the *SlbHLH95* knockout lines, the levels of flavonoid compounds including eriodictyol, quercetin, astragalin, nicotiflorin, and rutin were all significantly increased in the fruit peels of *SlbHLH95*-OE lines at the Br + 7 stage compared to WT fruits (Figure 3a). In contrast, the content of naringenin was decreased in the peels of *SlbHLH95*-OE Br + 7 fruits at the same

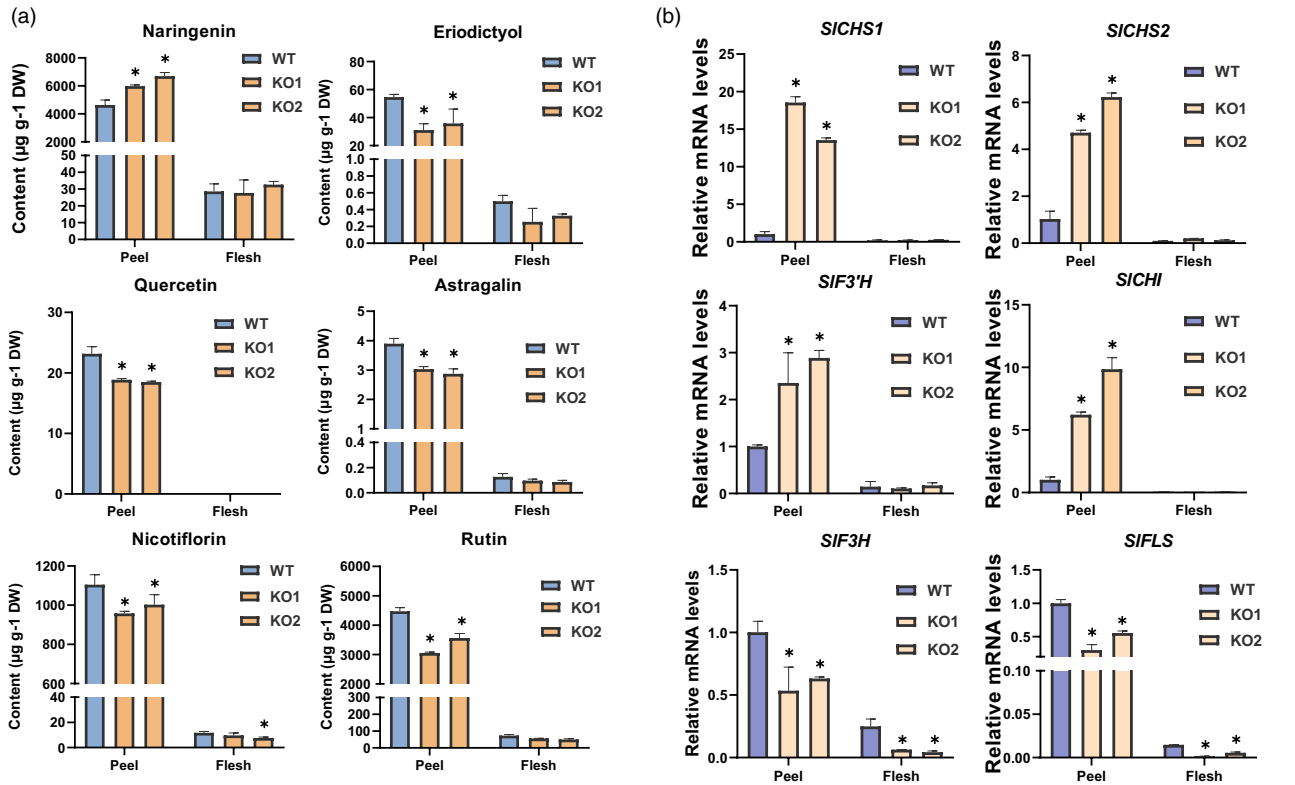

Figure 2 Knocking out *SlbHLH95* leads to a change of flavonoid composition in tomato fruits. (a) Content of different flavonoid compounds in peel and flesh tissues of wild-type (WT) and *SlbHLH95*-KO lines. (b) Relative expression levels of key enzyme encoding genes in flavonoid biosynthesis pathway assessed by RT-qPCR. The relative expression level of each gene in the WT was normalized to 1, with *SlActin* serving as the internal control. KO1 and KO2 represent two independent *SlbHLH95*-KO lines. Tomato fruit samples used for flavonoid content determination and relative expression level examination were collected at the Br + 7 stage. Error bars represent standard deviation (SD, $n = 3$) (*$P < 0.05$; Student's *t*-test).

stage. Furthermore, the expression levels of the *SlF3H* and *SlFLS* genes were increased in the fruits of *SlbHLH95*-OE lines compared to WT (Figure 3b). These results further support the crucial role of SlbHLH95 in modulating flavonoid metabolism in fruit peels of tomato.

SlbHLH95 directly binds the promoter of flavonoid biosynthetic genes to modulate flavonoid metabolism

Given that the relative expression of *SlF3H* and *SlFLS* were significantly down-regulated in *SlbHLH95*-KO fruits but up-regulated in the *SlbHLH95*-OE lines (Figures 2b and 3b), we aimed to investigate whether SlbHLH95 could directly regulate the expression of *SlF3H* and *SlFLS*. Promoter sequence analysis revealed the presence of several E-box motifs, bHLH-type transcription factors binding *cis*-elements, in both the *SlF3H* and *SlFLS* promoter regions (Figure 4a; Data S3). We then cloned the promoter sequences of *SlF3H* and *SlFLS* and fused them to the luciferase (LUC) reporter gene (Figure 4b). Transactivation assays were performed in *N. benthamiana* leaves using a dual-luciferase reporter system to assess the regulatory effects of *SlbHLH95* on these target genes. As shown in Figure 4b, SlbHLH95 could activate the promoter activities of both *SlF3H* and *SlFLS* genes (Figure 4b). Evidence supporting the *in vivo* binding of SlbHLH95 to the promoters of *SlFLS* and *SlF3H* was provided by ChIP-qPCR, which showed significant enrichments of SlbHLH95 at the P1 site of the *SlF3H* promoter and both the P1 and P2 sites of the *SlFLS* promoter (Figure 4c). The direct binding of SlbHLH95 to the specific E-boxes presented in the promoter regions of *SlF3H* or *SlFLS* was further validated by electrophoretic mobility shift assay (EMSA) (Figures 4b,d, S3a).

Notably, *SlCHS1*, a key gene acting at the upstream of flavonoid biosynthesis pathway, displayed an opposite expression pattern compared to *SlF3H* and *SlFLS* genes in *SlbHLH95*-KO lines, which may account for the increase in the content of naringenin in these lines. The up-regulated expression of this gene in *SlbHLH95*-KO lines suggested a repressive effect of SlbHLH95 on *SlCHS1*. Promoter sequence analysis revealed the presence of several E-boxes in the *SlCHS1* promoter region (Figure 4a; Data S3). Nevertheless, ChIP-qPCR and EMSA showed that SlbHLH95 could not bind to these E-boxes in the promoter of *SlCHS1* (Figure S4). However, transactivation assays using a dual luciferase reporter system revealed that SlbHLH95 repressed the promoter activity of *SlCHS1* (Figure 4b), suggesting an indirect regulation of SlbHLH95 on the expression of *SlCHS1*. Collectively, these results indicated that SlbHLH95 acts as a dual-effect transcription factor, simultaneously playing transcriptional activation and repression functions to fine-tune the flavonoid biosynthesis.

SlbHLH95 interacts with SlMYB12 to enhance the regulation on target genes

SlMYB12 has been reported to act as a key regulator in controlling flavonoid accumulation in tomato peel (Ballester *et al.*, 2010; Zhang *et al.*, 2015). To explore whether the regulation of SlbHLH95 on flavonoid metabolism is dependent on SlMYB12, we tested the interaction between SlbHLH95 and

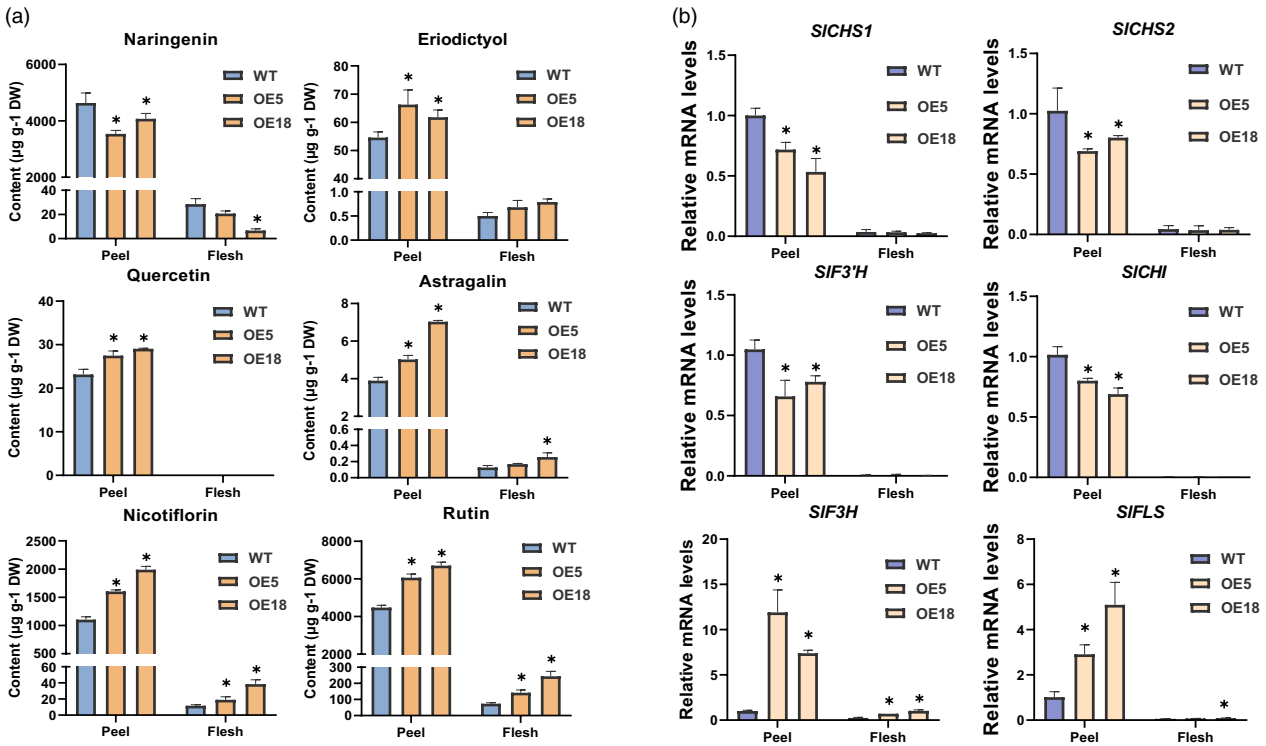

Figure 3 Overexpression of *SlbHLH95* promotes the accumulation of flavonoid compounds in tomato fruits. (a) Content of flavonoids in fruit peel and flesh of wild-type (WT) and *SlbHLH95*-OE lines. (b) Transcript accumulation of key enzyme encoding genes in the flavonoid biosynthesis pathway assessed by RT-qPCR. The relative expression level of each gene in the WT was normalized to 1, with *SlActin* serving as the internal control. OE5 and OE18 represent two independent *SlbHLH95*-OE lines. Error bars represent standard deviation (SD, $n = 3$) (*$P < 0.05$; Student's *t*-test).

SlMYB12 by yeast two-hybrid (Y2H), bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (Co-IP) assays. These results from both Y2H, BiFC and Co-IP validated the interaction between SlbHLH95 and SlMYB12 in both *in vitro* and *in vivo* (Figure 4e–g).

To further investigate the effect of the interaction between SlbHLH95 and SlMYB12 on the regulation of target genes, we co-transformed the vectors containing the promoter sequences of the target genes (*SlF3H* and *SlFLS*), along with vectors harbouring the coding sequence of SlbHLH95 and/or SlMYB12 into *N. benthamiana* leaves for transient expression assays using a dual-luciferase reporter system. Consistent with previous results, both SlbHLH95 and SlMYB12 had activation effects on the two target genes. Moreover, when both transcription factors were present at the same time, the activation effects on the target genes were significantly increased compared to those observed in the single transcription factor alone (Figure 4h). Interestingly, the interaction between SlbHLH95 and SlMYB12 cannot enhance the inhibition of *SlCHS1* (Figure 4h), suggesting the potential involvement of other unknown transcription factors that may collaborate with SlbHLH95 to exert inhibitory functions. These results indicated that SlbHLH95 and SlMYB12 coordinately regulate flavonoid biosynthesis in tomatoes, highlighting a complex regulatory network underlying flavonoid accumulation.

SlbHLH95 regulates fruit resistance to *Botrytis cinerea* by transcriptional suppressing *SlBG10*

Grey mould disease, caused by *Botrytis cinerea*, is a devastating tomato fruit postharvest disease that can cause considerable economic losses. Previous studies have indicated that flavonoids can exert some inhibitory effects on *Botrytis cinerea* (Cotoras *et al*., 2011; Wang *et al*., 2023a, 2023b). To test whether SlbLHL95 is involved in the regulation against *Botrytis cinerea* resistance, fruits from *SlbHLH95*-KO, *SlbHLH95*-OE and WT lines were inoculated with *Botrytis cinerea* at the Br + 2 stage. After 3 days of inoculation, we observed that the lesion area was significantly larger in *SlbHLH95*-KO fruits compared to WT fruits (Figure 5a,b), whereas the lesion area was significantly decreased in *SlbHLH95*-OE fruits (Figure 5a,b). Moreover, the activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were increased in the *SlbHLH95*-OE fruits and also the expression levels of disease resistance related genes including *SlPR1a*, *SlPR2b*, *SlPR5*, *SlMYC2*, *SlPDF1* and *SlPDF2* were significantly up-regulated (Figure 5b,c). In contrast, the *SlbHLH95*-KO lines exhibited reduced activities of SOD, POD and CAT, along with down-regulated expression levels of these resistance-related genes compared to WT (Figure 5b,c).

Notably, we found that the expression of *SlBG10*, a β-1,3-GLUCANASE encoding gene recently reported as a repressor to *Botrytis cinerea* resistance (Pei *et al*., 2023), was up-regulated in *SlbHLH95*-KO lines but down-regulated in the *SlbHLH95*-OE lines (Figure 5d). Dual-luciferase reporter assay showed that SlbHLH95 could repress the expression of *SlBG10* (Figure 5e). To test the putative binding of SlbHLH95 to the promoter of *SlBG10*, we analysed the promoter sequence of *SlBG10* and found the presence of E-box-like sequences instead of the typical E-boxes (Figure 5f). The results of EMSA and ChIP-qPCR experiments showed that SlbHLH95 cannot directly bind to these E-box-like sequences (Figure 5f,g). These data supported that SlbHLH95 positively regulates the resistance to *Botrytis cinerea* by repressing

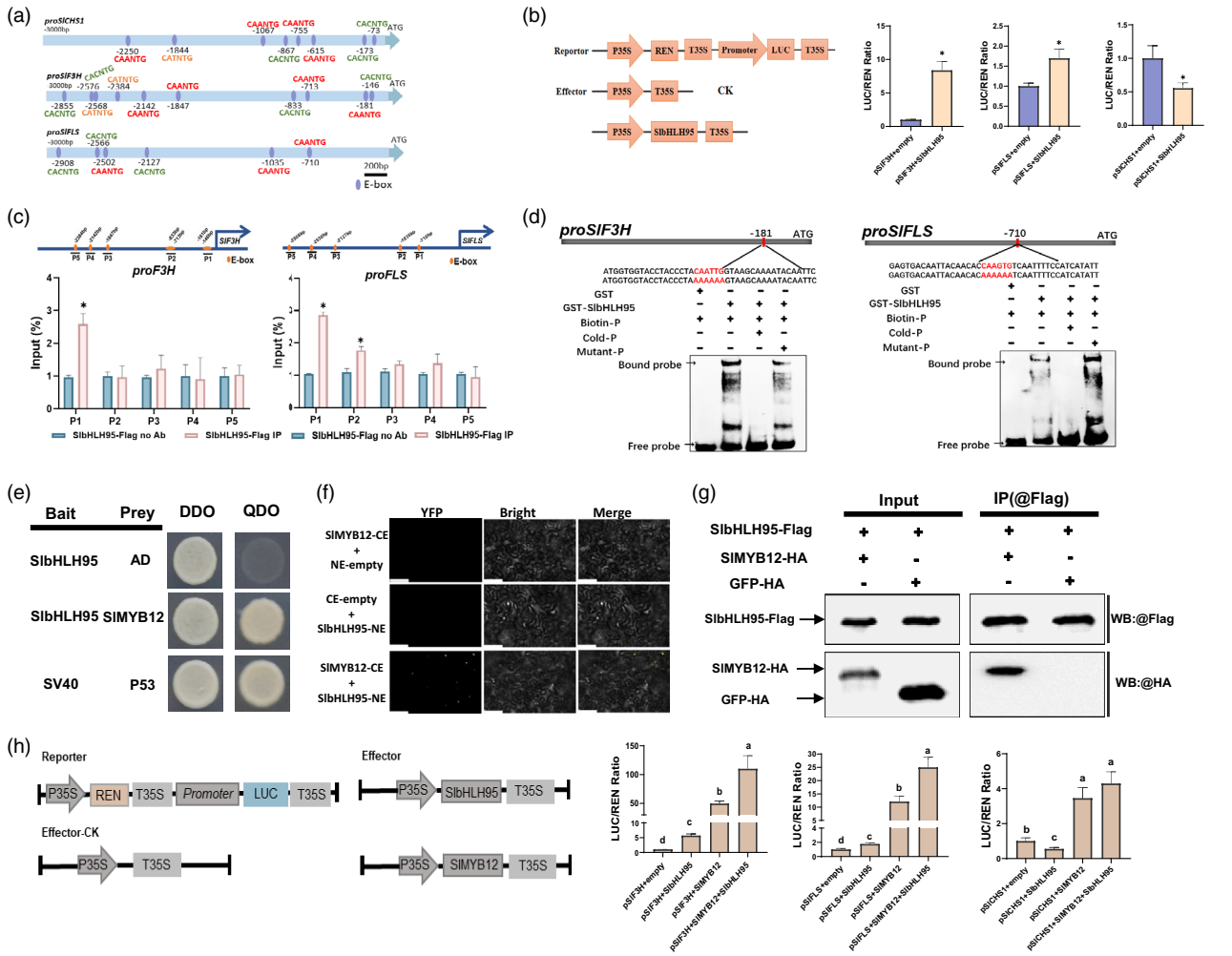

Figure 4 SlbHLH95 regulates key structural genes in the flavonoid biosynthetic pathway and SlbHLH95–SlMYB12 complex enhances the regulation of downstream target genes. (a) The putative bHLH-binding *cis*-elements (E-box, 5′-CANNTG-3′) present in the promoter regions of *SlCHS1*, *SlF3H* and *SlFLS*. The 3.0 kb regions upstream of the predicted translation initiation codon (ATG) was analysed to search the presence of known bHLH-binding *cis*-acting elements in the genomic sequences. (b) The activation of SlbHLH95 on flavonoid biosynthesis genes. Regulatory effects of SlbHLH95 on the promoter activity of *SlCHS1*, *SlF3H* and *SlFLS* were tested in *Nicotiana benthamiana* leaves by dual luciferase reporter assay. (c) Schematic diagram of *proSlCHS1*, *proSlF3H* and *proSlFLS* positions used in ChIP-qPCR assays. ChIP-qPCR of SlbHLH95-Flag levels at five sites (P1–P5) in *SlbHLH95* -OE fruits at Br + 7 stage. Error bars represent standard deviation (SD, $n = 3$) (*$P < 0.05$; Student's *t*-test). (d) Electrophoretic mobility shift assay (EMSA) assessing the binding of SlbHLH95 to flavonoid biosynthesis genes. The probes containing the E-box were labelled with biotin. Competition for SlbHLH95 binding was conducted using unlabelled cold probes containing the E-box or mutant controls (AAAAAA). Symbols – and + represent the absence and presence of SlbHLH95 binding, respectively. Each probe sequence was 42 bp in length, with the E-box at the center of the sequence. (e) Yeast two-hybrid assay was conducted to investigate the interaction between SlbHLH95 and SlMYB12. SlbHLH95 was employed as the bait, while SlMYB12 served as the prey. SV40-BD and P53AD were co-transformed as positive controls. (f) Bimolecular fluorescence complementation (BiFC) assay showed the interaction between SlbHLH95 and SlMYB12 in tobacco leaf cells. Fluorescence signals were shown in the nucleus where SlbHLH95 and SlMYB12 interacted, and controls showed no interaction between SlMYB12-CE and NE-empty, CE-empty and SlbHLH95-NE. (g) Co-immunoprecipitation (Co-IP) assay of *in vivo* interaction between SlbHLH95-FLAG and SlMYB12-HA. SlbHLH95-FLAG and SlMYB12-HA, SlbHLH95-FLAG and GFP-HA. The constructs were transiently co-infiltrated into *Nicotiana benthamiana* leaves as described in the 'Materials and Methods' section. (h) Transactivation assays showed the synergistic action of SlbHLH95 and SlMYB12 in the regulation of promoter activities of *SlCHS1*, *SlF3H* and *SlFLS*. Error bars represent standard deviation (SD) ($n = 3$). Different letters indicate statistically significant differences at $P < 0.05$ (one-way analysis of variance, Tukey's *post hoc* test).

the expression of *SlBG10* in a manner which is independent on these E-box-like sequences in tomato fruit.

Discussion

Improving fruit nutritional quality and enhancing resistance to disease are important aims for high-quality fruit crop breeding. In this study, we uncovered that SlbHLH95 acts as a key regulator in flavonoid metabolism and resistance to *Botrytis cinerea*, a significant pathogen responsible for grey mould in fruit crops. The transcription factor SlbHLH95 was reported to enhance flavonoid biosynthesis by activating the expression of key biosynthetic genes, *SlF3H* (flavanone 3-hydroxylase) and *SlFLS* (flavonol synthase). Concurrently, SlbHLH95 suppressed *SlBG10*,

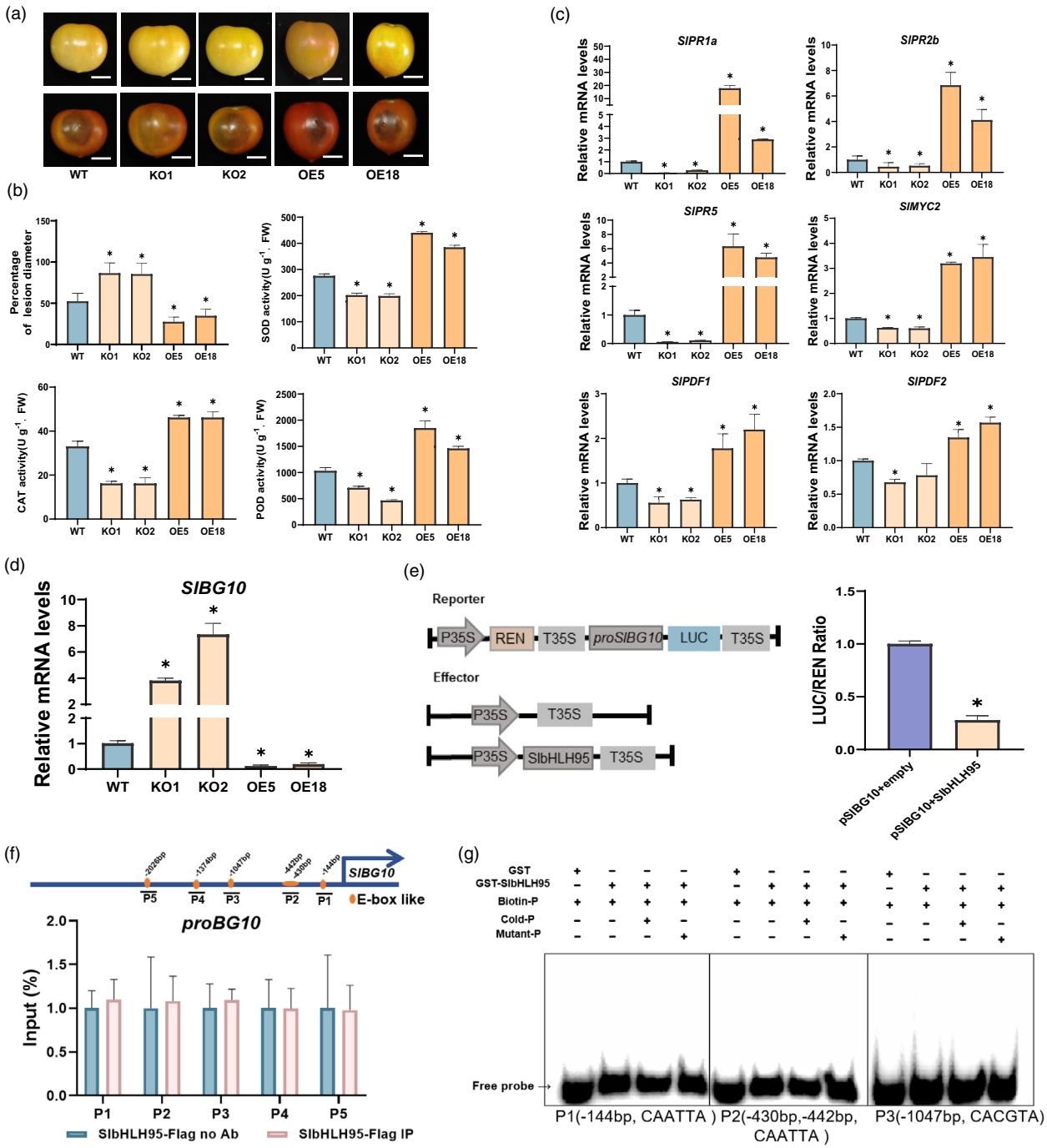

Figure 5 SlbHLH95 regulates fruit resistance to *Botrytis cinerea* infection in tomato. (a) Disease symptoms after inoculation with *Botrytis cinerea* in fruits of wild-type (WT), *SlbHLH95*-KO and OE lines at Br + 2 stage. The white dotted circles represent the lesion area. The scale bar represents 1 cm. (b) Assessment of lesion diameters and antioxidant enzymatic activities. After inoculation with *Botrytis cinerea* mycelium for 3 days, the lesion diameters and antioxidant enzymatic activities of SOD, POD and CAT were assessed in fruit tissues of WT, *SlbHLH95*-KO and *SlbHLH95*-OE lines. Error bars represent standard deviation (SD, $n = 6$) (*$P < 0.05$; Student's *t*-test). (c) Relative expression levels of disease resistance-related genes (*SlPR1a*, *SlPR2b*, *SlPR5*, *SlMYC2*, *SlPDF1* and *SlPDF2*), alongside *SlbHLH95* and *SlMYB12* in WT and *SlbHLH95*-KO, *SlbHLH95*-OE lines after inoculation with *Botrytis cinerea*. Error bars represent SD ($n = 6$) (*$P < 0.05$; Student's *t*-test). (d) Relative expression levels of *SlBG10* in WT, *SlbHLH95*-KO and *SlbHLH95*-OE lines. Error bars represent standard deviationx (SD, $n = 3$) (*$P < 0.05$; Student's *t*-test). (e) The regulation of SlbHLH95 on *SlBG10* revealed by dual-luciferase reporter gene assay. Error bars represent SD ($n = 3$) (*$P < 0.05$; Student's *t*-test). (f) ChIP-qPCR analysis of five sites (P1–P5) of the *SlBG10* promoter in *SlbHLH95*-OE fruits at Br + 7. Bars represent the percentage of input with SlbHLH95-Flag antibody (pink) and without antibody (blue), showing no significant differences among the regions. Error bars indicate standard deviation (SD, $n = 3$). (g) EMSA reveals that SlbHLH95 can't directly bind to the E-box in the promoter of *SlBG10*. The sequences of probes containing the E-box are labelled with biotin. Competition for SlbHLH95 binding is conducted using unlabeled cold probes containing the E-box or mutant controls (AAAAAA). Symbols - and + represent the absence and presence, respectively.

a gene that represses *Botrytis cinerea* infection, thereby contributing to disease resistance. The outcome of this study not only provided new insights into the dissection of the regulatory networks controlling flavonoid metabolism and conferring *Botrytis cinerea* resistance in tomato but also shed new light on simultaneously improving fruit nutritional quality and increasing grey mould resistance in horticulture crops.

Previous studies have shown that SlbHLH95 acts a repressor in gibberellin biosynthesis and trichome formation, while serving as an activator in the process of fruit ripening (Chen *et al*., 2020; Zhang *et al*., 2020). These findings suggest that SlbHLH95 has a dual regulatory capacity, serving as both a transcriptional activator and a repressor depending on the biological context. Interestingly, in line with previous findings, our present study also evidenced that SlbHLH95 worked dually as both an activator and a repressor in different biological processes. Specifically, SlbHLH95 transcriptionally activated the expression of *SlF3H* and *SlFLS*, two key genes acting downstream of flavonoid biosynthesis pathway, thorough direct binding to their promoters. Conversely, SlbHLH95 repressed the transcription of *SlCHS1* and *SlBG10*, two genes involved in flavonoid biosynthesis and *Botrytis cinerea* resistance, respectively. These findings suggested that SlbHLH95 plays multiple functions in diverse developmental processes and stress responses, acting as both an activator and a repressor depending on the target genes. Indeed, several transcription factors have also been reported that can act as transcriptional activators and repressors depending on the specific target genes (Ikeda *et al*., 2009; Nagahage *et al*., 2018; Yant *et al*., 2010). This indicates a broader potentially conserved mechanism by which transcription factors are flexible to modulate gene expression to act both as activators and repressors across diverse biological contexts. Intriguingly, BZR1 has been reported to serve as both an activator and repressor via an EAR motif-dependent mechanism (Oh *et al*., 2014). In contrast, while the EAR motif-mediated repression mechanism is well characterized, the precise underlying mechanisms by which SlbHLH95 confers its transcriptional repression activity remain largely unexplored and warrant further investigation. In addition, our findings revealed that SlbHLH95 exhibited a binding specificity towards DNA sequences. It forms specific interactions with typical E-box sequences when acting as a transcriptional activator (Figure 4g; Figure S3). Interestingly, when it acts a transcriptional repressor, such as repressing *SlCHS1* or *SlGB10*, its regulation is not dependent on E-boxes (Figure 5g; Figure S4). This suggests that SlbHLH95 may not directly bind to E-boxes when acting as a repressor; rather, its inhibitory effects on target genes may be mediated through interactions with other unknown DNA elements or regulatory proteins.

Flavonoids represent a class of potent antioxidants known for their capacity to augment plant resistance to diverse stresses (Agati *et al*., 2011, 2012; Brunetti *et al*., 2013; Mierziak *et al*., 2014; Panche *et al*., 2016). Nevertheless, the direct link between flavonoids and their impact on bolstering the resistance of tomato fruit against *B. cinerea* infection remains largely unclear. It is noteworthy that our findings provided an additional dimension of the influence of SlbHLH95, specifically its capability to heighten the innate capacity of tomato fruits to withstand *B. cinerea* infection. This observation implied that SlbLHLH95 was integral playing a vital role in fruit postharvest disease resistance in addition to its important functions in fruit ripening and nutritional quality regulation. Interestingly, our recent study also showed that the accumulation of flavonoids content enhances resistance to *Botrytis cinerea* in tomato fruits (Wang *et al*., 2023b). Nevertheless, a comprehensive elucidation of the intricate interplay between flavonoids and the resistance of tomato fruits to *Botrytis cinerea* necessitates further investigation to unravel the underlying mechanisms involved.

In this study, we found that transcription factor SlbHLH95 regulates both the biosynthesis of carotenoids and flavonoids in tomato fruits (Figures 1h, 2, 3). Given that tomato fruit colour at the ripe stage is determined by the accumulation of carotenoids in pericarp and flavonoids in the peel (Llorente *et al*., 2016; Yang *et al*., 2023), the change of carotenoids in pericarp and flavonoids in peel in fruits of *SlbHLH95* knockout lines may both account for the yellow coloration in fruits of these lines. However, the precise contribution of the two pigments to the fruit colour at the ripe stage of *SlbHLH95* knockout lines requires further investigation.

Overall, through mining our previously published MMN data set (Li *et al*., 2020b), we identified the bLHLH-type transcription factor SlbHLH95 as a putative regulator in flavonoid biosynthesis. Further studies revealed the involvement of SlbHLH95 in the flavonoid metabolism in tomato fruits, as illustrated in Figure 6. First, SlbHLH95 activated the transcription of *SlF3H* and *SlFLS* by directly binding to the E-boxes presented in the promoter regions of these two genes. Second, SlbHLH95 interacts with SlMYB12 to form a transcriptional complex to enhance the activation of *SlF3H* and *SlFLS*. Third, SlbHLH95 indirectly represses the transcription of *SlCHS1*. In addition, SlbHLH95 confers *B. cinerea* resistance by repressing the expression of a resistance repressor gene *SlBG10* (Figure 6). The outcome of this study not only revealed the bi-functional role of SlbHLH95 as transcriptional activator and repressor on different target genes but also shed lights on fruit quality and disease resistance manipulation in fruit crops.

Materials and methods

Plant materials and growth conditions

The tomato plants (*Solanum Lycopersicum* cv. Micro-Tom) used in this study were cultivated in a controlled growth chamber with the following conditions. The conditions included a 14-h light/10-h dark photoperiod, a temperature regime of 25 °C/20 °C (day/night), 75% relative humidity, and a light intensity of 250 μmol/m^2/s provided by fluorescent lamps (Foshan Electrical and Lighting Co., Ltd, Foshan, China; LED T8, 16 W). To monitor fruit development and ripening, flowers were marked at the anthesis stage. Fruit samples were collected at Br + 7 stage (7 days after the breaker stage), then immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent analysis.

Vector construction and acquisition of transgenic plants

The construction of SlbHLH95 knockout or overexpression vectors was conducted according to the methods described in Deng *et al*. (2022). Briefly, two target sequences specific for SlbHLH95 were designed using the CRISPR P 2.0 online platform (http://cbi.hzau.edu.cn/CRISPR2/). Subsequently, these target sequences were subjected to incubation with a PCR machine at 28 °C to facilitate the formation of double-stranded DNA. The resulting double-stranded DNA fragments were then assembled into the pFASTCas9/ccdB vector using the Golden Gate Assembly method. For the construction of the *SlbHLH95* overexpression vector, the full-length coding sequence of *SlbHLH95* was cloned and ligated into the pBI121 vector, incorporating a 3× Flag tag through homologous recombination. Subsequently, these constructed vectors were transferred into *Agrobacterium* for genetic transformation.

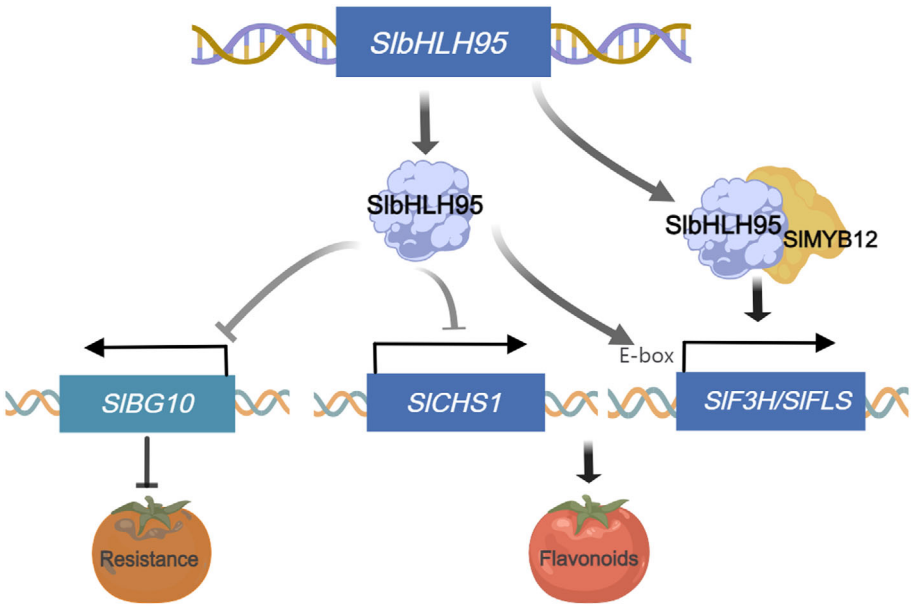

Figure 6 Working model of bi-functional SlbHLH95 in regulation of flavonoids metabolism and grey mould resistance in tomato fruits. On one hand, SlbHLH95 acts as transcriptional activator to activate the expression of *SlF3H* and *SlFLS* by directly binding the E-box elements located in the promoter regions of these genes and modulating the accumulation of the flavonoids. Additionally, the interaction with SlMYB12 further promotes the activation of these target genes by SlbHLH95. On the other hand, SlbHLH95 also acts as transcriptional repressor inhibiting the expression of *SlCHS1* and *SlBG10*. The repression of *SlBG10*, a known repressor of resistance to *Botrytis cinerea*, confers the resistance of tomato fruits against *Botrytis cinerea* infection.

Determination of tomato fruit colour

Fruits colour from different lines at Br + 7 stage was measured using Konica Minolta Chroma Meters (CR-400). Three equidistant points were uniformly selected along the equator of each fruit and six fruits per line were measured. The hue values represented by h were recorded after measurements, and the average value for each line was subsequently calculated.

Determination of carotenoids and flavonoids content

Harvested tomato fruit samples at the Br + 7 stage were ground into a fine powder and then subjected to vacuum lyophilization for flavonoid and carotenoid content determination. The extraction and detection methods of flavonoids were performed as previously described by Zhang *et al*. (2015). For carotenoid analysis, the LC–MS/MS method was used as previously described by Deng *et al*. (2022). Both the WT and each transgenic line were tested in three biological replicates.

RNA extraction and RT-qPCR analysis

Total RNA was extracted from tomato fruit pericarp at Br + 7 developmental stage using the Plant RNA Extraction Kit (BIOFIT, Chengdu, China). The cDNA synthesis was performed according to the HiScript III first-strand cDNA Synthesis Kit (+gDNA wiper) (R312-02; Vazyme, Nanjing, China), following the manufacturer's instructions. After removing the genomic DNA, the reverse transcription was performed to obtain pure cDNA for subsequent RT-qPCR analysis. RT-qPCR was conducted using the Bio-Rad CFX384 Real-Time System, following the manufacturer's instructions and employing 2 × SYBR Green qPCR Mix (BG0014; Baoguang, Chongqing, China). The relative expression levels of each gene were calculated using the ΔCt method, with *SlActin* (Solyc11g005330) as the internal reference gene. The primers used for RT-qPCR in this study are listed in Data S2.

Yeast two-hybrid assay

The coding sequence of *SlbHLH95* was cloned and inserted into the pGBKT7-BD vector to generate the bait construct, while the coding sequence of *SlMYB12* was cloned into the pGADT7-AD vector to create the prey construct. The bait and prey constructs were then co-transformed into yeast strain AH109 using the lithium acetate transformation method. The transformed yeast cells were grown on SD-Leu/-Trp selective medium for 3 days and then transferred to SD-Leu/-Trp/-Ade/-His medium for additional 3 days to facilitate the observation of protein–protein interactions.

Bimolecular fluorescence complementation (BiFC) assay

The coding sequences of *SlbHLH95* or *SlMYB12* were cloned and inserted into the pUC-pSPYCE or pUC-pSPYNE vectors, respectively. The resulting recombinant plasmids were transformed into *Agrobacterium tumefaciens* and then injected into the leaves of 2-week-old *N. benthamiana* plants. After injection, the samples were cultured for 1 day under dark conditions at 25 °C and then cultured for two additional days under normal light conditions. Fluorescence was then observed by fluorescence microscopy.

Dual-luciferase reporter assay

A dual-luciferase reporter assay was performed according to our previous study (Su *et al*., 2022). The vectors were constructed using GoldenBraid 2.0 system, wherein cloned promoters were individually inserted upstream of the Luciferase (*LUC*) gene. Simultaneously, the Renilla (*REN*) gene, driven by the 35S promoter, was incorporated into the same binary vectors as an internal control reporter gene. The coding sequences of *SlbHLH95* or *SlMYB12* were cloned and inserted into pEAQ-HT-DEST vector to serve as an effector. Both effector and reporter plasmids were transformed into *Agrobacterium tumefaciens* and

subsequently injected into the leaves of two-week-old *N. benthamiana* plants following injection, the plants were cultivated in darkness at 25 °C for 1 day and then cultured for two additional days under normal light conditions. The samples were then collected and quick-frozen in liquid nitrogen and ground to a fine powder. LUC and REN activities were measured using a dual LUC assay kit (E1910; Promega, Madison, WI), and the ratio of LUC to REN was calculated to obtain the results.

ChIP-qPCR

Chromatin immunoprecipitation (ChIP) assay was performed in accordance with our previous studies (Deng *et al*., 2022; Zhang *et al*., 2015). In brief, WT and *SlbHLH95*-OE tomato fruits at the Br + 7 developmental stage were harvested and subjected to formaldehyde crosslinking under vacuum conditions using a 1% (v/v) solution. Following crosslinking, the reaction was quenched with 0.125 M glycine, and the quenched samples were rapidly frozen in liquid nitrogen to preserve the chromatin structure. Subsequently, the frozen samples were ground into a fine powder and resuspended in a chromatin lysis buffer composed of Honda buffer and nuclear lysis buffer to facilitate chromatin digestion. The chromatin was then precipitated and resuspended in a nuclear dilution buffer, followed by sonication to shear the DNA into fragments of approximately 200–800 bp. For immunoprecipitation, chromatin was incubated with protein A agarose beads and an anti-FLAG antibody (14793; Cell Signaling Technologies, MA). The beads were then washed with a washing buffer to remove non-specifically bound proteins and contaminants, ensuring that the SlbHLH95 protein and its interacting DNA remained bound to the beads. Finally, the crosslinks were reversed using an elution buffer to recover the DNA fragments. The immunoprecipitated chromatin was dissolved in water for subsequent quantitative PCR analysis, enabling the assessment of specific DNA–protein interactions. Primers used for ChIP-qPCR in this study are listed in Data S2.

EMSA assay

The full-length CDS sequence of SlbHLH95 was cloned into pGEX-4T-1 vector to create a GST fusion construct. The SlbHLH95-GST fusion protein was then expressed in BL21-competent cells, and the protein was purified according to the manufacturer's instructions (P2262; Beyotime, Beijing, China). Biotin-labelled probes containing E-box motifs were synthesized, with unlabelled probes used as competitors. In addition, E-box mutations were introduced to create non-competitive probes with the sequence AAAAAA. The EMSA reactions were conducted following the manufacturer's instructions (GS002 & GS009; Beyotime). Each probe sequence is 42 bp in length, with the E-box at the centre of the sequence. The probe sequences used in the EMSA assay in this study are listed in Data S2.

Co-IP assays

The immunoprecipitation (Co-IP) assays were performed as described by Su *et al*. (2022). The full-length coding sequences (CDS) of SlbHLH95 and SlMYB12 were cloned and inserted into the pBTEX-FLAG or pBTEX-HA vectors, respectively. The pBTEX-FLAG and pBTEX-HA constructs were co-injected into the leaves of *N. benthamiana* plants. Total protein was extracted and incubated with anti-FLAG M2 magnetic beads (M8823; Sigma, MO) for immunoprecipitation. Western blot analysis was then performed to detect the expression of pBTEX-FLAG and pBTEX-HA proteins.

Botrytis cinerea inoculation assay

Botrytis cinerea was cultured on potato dextrose agar medium for 2 days before inoculation. Tomato fruit pericarps with the same ripening stage were punctured with an inoculation needle and then *B. cinerea* was inoculated at the same site. The size of lesions on the fruit after inoculation was observed and measured. The inoculated fruit samples were then harvested and frozen in liquid nitrogen for subsequent experiments.

Antioxidant enzymatic activities

According to the extraction kit method, a 0.1 g aliquot of the powdered tissue from inoculated fruits was weighed and added to 1 mL of extraction solution for homogenization in an ice bath, with three biological replicates for each line. The mixture was then centrifuged at 8000 ***g*** for 10 min at 4 °C, and the supernatant was collected and placed on ice for further analysis. The activities of catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD) were measured using a Thermo Scientific Multiskan® GO full-wavelength spectrophotometerfollowing the protocols outlined in the respective assay kits: SOD activity assay kit (BC0175; Solarbio, Beijing, China), POD activity assay kit (BC0095; Solarbio) and CAT activity assay kit (BC0205; Solarbio).

Acknowledgements

This work was supported in part by the National Natural Science Foundation of China (Nos. 32372780, 32172643 and 32172271), the Institutional Research Funding of Sichuan University (No. 2022SCUNL105), the Applied Basic Research Category of Science and Technology Program of Sichuan Province (2021YFQ0071, 2022YFSY0059-1 and 2021YFYZ0010-5-LH), the Technology Innovation and Application Development Program of Chongqing (No. cstc2021jscx-cylhX0001) the Natural Science Foundation of Sichuan Province, China (No. 2023NSFSC1991).

Conflict of interest

All authors declare no competing interests.

Author contributions

M.L. designed the experiments. D.S., M.W., S.Y. and H.W. performed the experiments; D.S., P.S., H.S., H.D. and Y.Z. analysed data; M.L. and D.S. wrote the manuscript, P.G. and M.B. helped improve the manuscript.

Data availability statement

The authors confirm that all experimental data can be obtained from the main text and/or supplementary data.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1 The levels of the correlation network of SlbHLH95 with flavonoid-related genes and substances in different tissues and developmental stages.
Data S2 List of primers used in this study.
Data S3 The E-box sequences speculated to exist in the promoter regions of *SlCHS1*, *SlF3H*, *SlFLS* and *SlBG10*.
Figure S1 Regulatory effect of SlbHLH95 on the promoters of key ethylene biosynthesis genes *SlACS2* and *SlACO1* showed by dual-luciferase reporter gene assay.
Figure S2 Relative *SlMYB12* transcript levels.
Figure S3 EMSA shows the direct binding of intact SlbHLH95.
Figure S4 SlbHLH95 cannot directly bind to the promoter of *SlCHS1* via the E-box.

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