

CmBt and CmBr synergistically regulate cucurbitacin B biosynthesis in melon

Shiqi Wang^{1‡}, Xue Zhao^{1‡}, Xinru Wang^{1‡}, Mingyan Wang^{1,3‡}, Chuangjiang Wang¹, Mengke Liu¹, Yaxuan Zhu¹, Tingting Liu¹, Yichen Wang¹, Xinxiu Chen¹, Yuanchao Xu², Fei Cheng¹, Xinhui Che¹, Xiaofeng Liu¹, Bingsheng Lv¹, Sen Chai¹, Kuipeng Xu¹, Zhonghua Zhang^{1*}, and Huimin Zhang^{1*}

¹Engineering Laboratory of Genetic Improvement of Horticultural Crops of Shandong Province, Laboratory of Quality & Safety Risk Assessment for Fruit (Qingdao), Ministry of Agriculture and Rural Affairs, National Technology Centre for Whole Process Quality Control of FSEN Horticultural Products (Qingdao), College of Horticulture, Qingdao Agricultural University, Qingdao 266109, China

²Shenzhen Branch, Guangdong Laboratory for Lingnan Modern Agriculture, Shenzhen Key Laboratory of Agricultural Synthetic Biology, Genome Analysis Laboratory of the Ministry of Agriculture and Rural Affairs, Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen 518120, China

³College of Horticulture, China Agricultural University, Beijing 100193, China

*Corresponding authors: Zhonghua Zhang, College of Horticulture, Qingdao Agricultural University, No. 700 Changcheng Road, Chengyang District, Qingdao 266109, China. Email: zhangzhonghua@qau.edu.cn; Huimin Zhang, College of Horticulture, Qingdao Agricultural University, No. 700 Changcheng Road, Chengyang District, Qingdao 266109, China. Email: zhanghm@qau.edu.cn

‡These authors contributed equally to this study.

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Abstract

Cucurbitacin B (CuB) is the primary compound responsible for bitterness in melon (*Cucumis melo* L.) and negatively affects fruit quality and consumer acceptance. Although CuB biosynthesis is regulated by multiple transcription factors, the regulatory mechanisms underlying CuB biosynthesis across different tissues remain unclear. Here, we generated 2 independent knockout lines (*Cmbt-18* and *Cmbt-19*) of the basic helix-loop-helix (bHLH) transcription factor gene *CmBt* (*Bitter fruit*) using CRISPR-Cas9. Loss of *CmBt* reduces CuB levels in fruits and roots and produces non-bitter leaves, indicating that *CmBt* predominantly regulates CuB accumulation in leaves. By contrast, its paralog *CmBr* (*Bitter root*) predominantly controls CuB accumulation in fruits and roots. These results redefine *CmBt* as a leaf-predominant regulator and *CmBr* as a fruit/root-predominant regulator of CuB biosynthesis, revising previous findings. Furthermore, we demonstrate that *CmBt* physically interacts with *CmBr*. The *CmbtCmbr* double mutants exhibit a more severe reduction in CuB content in fruits, leaves, and roots compared with either single mutant, confirming a synergistic interaction between *CmBt* and *CmBr*. Reverse transcription quantitative PCR and tobacco dual-luciferase assays further show that *CmBt* and *CmBr* synergistically activate the CuB biosynthetic gene *CmBi*. Additionally, the *CmbtCmbr* double mutant completely prevents CPPU-induced fruit bitterness. Our findings reveal that *CmBt* and *CmBr* exert tissue-specific, partially redundant, and synergistic roles in activating *CmBi* and promoting CuB biosynthesis. These results identify the *CmBt-CmBr* locus as a promising target for breeding non-bitter melon cultivars.

Introduction

Plant secondary metabolites are produced in various plant as natural defensive compounds against herbivores attacks and environmental stresses (Yang et al. 2012; Wink 2016; Jan et al. 2021; Divekar et al. 2022). In the Cucurbitaceae, cucurbitacins are a class of triterpenoids that serves either as protection against herbivores and microbes or Chinese herbal medicine with anti-cancer effects (Cai et al. 2015; Zieniuk and Pawelkiewicz 2023). However, cucurbitacins are the principal bitter compounds in cucurbit crops, an undesirable trait that reduces fruit quality and consumer acceptance (Zhang et al. 2013; Shang et al. 2014; Zhao et al. 2019).

Melon (*Cucumis melo* L.) is an important cucurbit crop that is highly favored by consumers due to its sweet fruit. Cucurbitacin B (CuB) is the main bitter compound in melon (Chen et al. 2009; Mei et al. 2016; Zhou et al. 2016). By contrast, cucurbitacin C (CuC) (Rice et al. 1981; Balkema-Boomstra et al. 2003; Horie et al. 2007) and cucurbitacin E (CuE) (Matsuo et al. 1999; Abdelwahab et al. 2011; Kim et al. 2018) predominate in cucumber and watermelon, respectively.

Biosynthetic and transport pathways for cucurbitacins have been elucidated in cucurbits. In cucumber, the CuC biosynthetic pathway comprises an oxidosqualene cyclase (OSC; *CsBi*) (Zhang et al. 2013),

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7 cytochrome P450 monooxygenases (CYPs), and an acyltransferase (ACT) (Shang et al. 2014). In melon, analogous studies have identified a CuB pathway composed of an OSC (*CmBi*), 6 CYPs (*Cm160*, *Cm170*, *Cm180*, *Cm710*, *Cm890*, *Cm490*), and an ACT (*CmACT*). In this pathway, *CmBi* cyclizes the 2,3-oxidosqualene to cucurbitadienol. *Cm890* catalyzes subsequent oxidations to produce 11-carbonyl-20 β -hydroxycucurbitadienol. *Cm180* introduces a C2 hydroxyl to yield 11-carbonyl-2 β , 20 β -dihydroxycucurbitadienol. In the last step, *CmACT* acetylates cucurbitacin D (CuD) at the C25-hydroxyl position to generate the CuB (Zhou et al. 2016). In addition, the transporter *CmMATE1*, which is coexpressed with biosynthetic genes, contributes to CuB translocation from the roots into the soil and modulates the rhizosphere microbiome (Zhong et al. 2022).

The transcriptional regulation of cucurbitacin biosynthesis has been investigated extensively. In cucumber, tissue-specific basic helix-loop-helix (bHLH) transcription factors *CsBl* and *CsBt* control CuC production in leaves and fruit, respectively (Qi et al. 2013; Shang et al. 2014). In melon, classical genetic studies identified 3 loci associated with fruit bitterness (*Bif-1*, *Bif-2*, and *Bif-3*) (Lee and Janick 1978; Parthasarathy and Sambandam 1981; Dogimont 2011), and more recent QTL mapping implicated 2 dominant loci on chromosomes 2 and 5 (Shang et al. 2020). Comparative genomics identified a syntenic bHLH gene cluster in melon that includes 2 paralogs, *CmBt* (Bitter fruit) and *CmBr* (Bitter root), in melon. *CmBt* shows fruit-specific expression and was thought to regulate CuB biosynthesis in fruits, while *CmBr* is predominantly expressed in roots, suggesting a role in regulating CuB biosynthesis in roots. *CmBt* and *CmBr* directly bind to and activate the expression of CuB biosynthetic genes (*CmBi*, *Cm160*, *Cm170*, *Cm180*, *Cm490*, *Cm710*, *Cm890*, and *CmACT*) and activate their expression (Zhou et al. 2016). More recently, *CmBt* and *CmBr* have been identified involved in N-(2-chloro-pyridin-4-yl)-N'-phenylurea (CPPU)-induced CuB accumulation in fruits (Luo et al. 2020; Wang et al. 2024). However, the tissue-specific genetic roles of *CmBt* and *CmBr* have not been fully investigated, and how they coordinate to regulate CuB biosynthesis remains unresolved.

In this study, we investigate the function of *CmBt* using CRISPR-Cas9 and show that *CmBt* (Bitter fruit) predominantly regulates CuB accumulation in leaves, whereas its paralog *CmBr* (Bitter root) exerts a more prominent role in fruits and roots. Our results redefine *CmBt* as a leaf-predominant regulator and *CmBr* as a fruit-/root-predominant regulator of CuB biosynthesis, correcting previous findings. We further demonstrate that *CmBt* physically interacts with *CmBr*, and the *CmBt*-*CmBr* module synergistically activates the CuB biosynthetic gene *CmBi* to promote CuB accumulation across tissues. Notably, the *CmBtCmBr* double mutant exhibits a complete loss of fruit bitterness after CPPU treatment in field trials. In summary, these findings reveal the tissue-specific and synergistic regulation of cucurbitacin biosynthesis and identify the *CmBt*-*CmBr* module as a promising genetic target for breeding non-bitter melon varieties.

Results

CmBt knockout reduces CuB biosynthesis in fruits, leaves, and roots

Previous studies have reported that *CmBt* is a major regulator of CuB biosynthesis in fruits (Zhou et al. 2016; Zhao et al. 2019). However, the genetic evidence for *CmBt* regulating CuB biosynthesis in fruits remains unclear. Phylogenetic analysis indicated that *CmBt* belongs to

the bHLH Clade Ib subfamily (Zhang et al. 2018; Gao and Dubos 2024). *CmBt* shares 36.41% amino acid identity with the Arabidopsis bHLH118 protein (AT4G25400) and contains the conserved basic helix-loop-helix domain (Fig. S1a, b). *CmBt* has an additional homolog in the melon genome *MELO9W045720* (*CmBr*) (Fig. S1a). RNA-seq data from the public database Melonet-DB showed that *CmBt* is expressed in young fruits (0, 2, and 4 d after flowering [DAF]) and exhibits higher expression in young leaves (Fig. S1c).

To validate the function of *CmBt* in CuB biosynthesis, we generated 2 Cas9-free independent knockout lines (*Cmbt-18* and *Cmbt-19*) in the bitter cultivar ivf05 using clustered regularly interspaced short palindromic repeats (CRISPR) CRISPR-associated nuclease 9 (Cas9)-mediated gene editing with 1 single guide RNA (sgRNA). The *Cmbt-18* line carries a 1-bp insertion at the target site, whereas the *Cmbt-19* line harbors a 1-bp deletion (Fig. S2a, b). The 2 lines are predicted to produce truncated proteins of 155 and 164 amino acids, respectively (Fig. 1a). No mutations were detected at potential off-target sites in mutants by Sanger sequencing (Fig. S3).

Both *Cmbt-18* and *Cmbt-19* displayed overall plant morphology similar to wild-type ivf05. We analyzed CuB content in young fruits (0 d after anthesis [DAA]), leaves, and roots of *Cmbt* mutants and wild type (WT) using liquid chromatography mass spectrometry (LC-MS). *Cmbt-18* and *Cmbt-19* mutant lines exhibited significantly reduced CuB content in fruits compared with WT (Fig. 1b, c). Notably, the CuB levels in leaves of the *Cmbt-18* and *Cmbt-19* mutants were markedly decreased, leading to non-bitter leaves (Fig. 1d, e). Similarly, the root CuB content was also lower in the *Cmbt-18* and *Cmbt-19* mutant lines than in WT (Fig. 1f, g). The results are consistent with the expression pattern of *CmBt* (Fig. S1c). Collectively, these results demonstrate that *CmBt* plays a predominant role in regulating CuB accumulation in leaves, while also contributing to CuB biosynthesis in fruits and roots.

CmBr plays a predominant role in regulating CuB accumulation in fruits and roots

We previously identified *CmBr* as a major regulator of CPPU-induced CuB accumulation in fruits (Wang et al. 2024). To further investigate the regulatory role of *CmBr* in CuB accumulation across different tissues, we utilized the previously generated *CmBr* introgression lines (*Cmbr^{IL}*) and quantified CuB content in young fruits (0 DAA), leaves, and roots of *Cmbr^{IL}* mutant and WT. *Cmbr^{IL}* mutant exhibited significantly reduced CuB content in fruits compared with WT, resulting in a non-bitter fruit phenotype (Fig. 2a, b). Similarly, the root CuB content in the *Cmbr^{IL}* mutant was also significantly lower than that in WT (Fig. 2e, f), consistent with the previously reported role of *CmBr* in roots CuB biosynthesis (Zhou et al. 2016). Additionally, CuB levels in the leaves of the *Cmbr^{IL}* mutant were decreased relative to WT (Fig. 2c, d). The results are consistent with the expression pattern of *CmBr* (Fig. S1c). These results confirmed that *CmBr* plays a predominant role in regulating CuB accumulation in fruits and roots, while also contributing to CuB accumulation in leaves.

CmBt physically interacts with *CmBr*

Given that both *CmBt* and *CmBr* regulate CuB accumulation in fruits, leaves, and roots, we first investigated the regulatory relationship between *CmBt* and *CmBr*. Transcript levels of *CmBr* in *Cmbt-18* did not differ significantly from those in WT (Fig. 3a), and *CmBt* expression was unchanged in the *Cmbr^{IL}* mutant relative to WT (Fig. 3b). These results indicate that *CmBt* does not appear to regulate *CmBr* at the

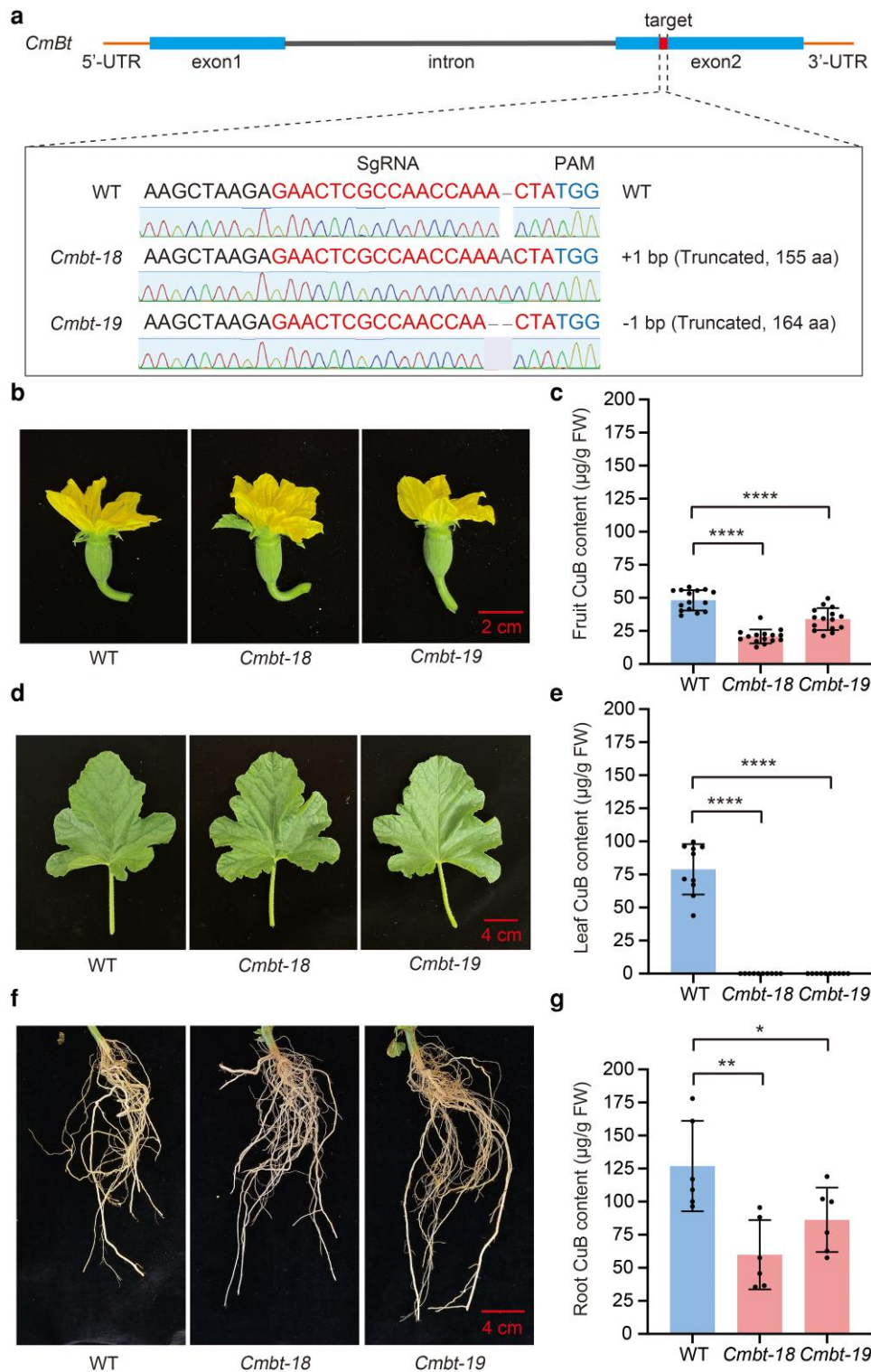


Figure 1 CRISPR-Cas9 engineering of *Cmbt* reduces CuB content in fruits, leaves, and roots. **a**) Target site in *Cmbt* and Sanger sequencing of the CRISPR-Cas9-edited alleles in the *Cmbt*-edited line in *ivf05* background. Wild-type *ivf05* is shown as control. sgRNA-targeted sequences are highlighted in red and the protospacer adjacent motif (PAM) is shown in blue. Deletions and insertions are indicated by dashed lines and grey text, respectively. UTR, untranslated region. **b**) Representative fruits of WT and *Cmbt* mutants at 0 DAA. Scale bar = 2 cm for all images. **c**) CuB content in young fruits (0 DAA) of *Cmbt* mutants and WT, measured by LC-MS and expressed in $\mu\text{g/g}$ fresh weight (FW). Data are given as mean $\pm$ SEM ($n = 15$ biological replicates). Statistical significance by 2-tailed t test: **** $P < 0.0001$. **d**) Representative leaves of WT and *Cmbt* mutants. Scale bar = 4 cm for all images. **e**) CuB levels in leaves of *Cmbt* mutants and WT, measured by LC-MS and expressed in $\mu\text{g/g}$ FW. Data are given as mean $\pm$ SEM ($n = 10$ biological replicates). Statistical significance by 2-tailed t test: **** $P < 0.0001$. **f**) Representative roots of WT and *Cmbt* mutants. Scale bar = 4 cm for all images. **g**) CuB levels in roots of *Cmbt* mutants and WT, measured by LC-MS and expressed in $\mu\text{g/g}$ FW. Data are given as mean $\pm$ SEM ($n = 6$ biological replicates). Statistical significance by 2-tailed t test: * $P < 0.05$; ** $P < 0.01$.

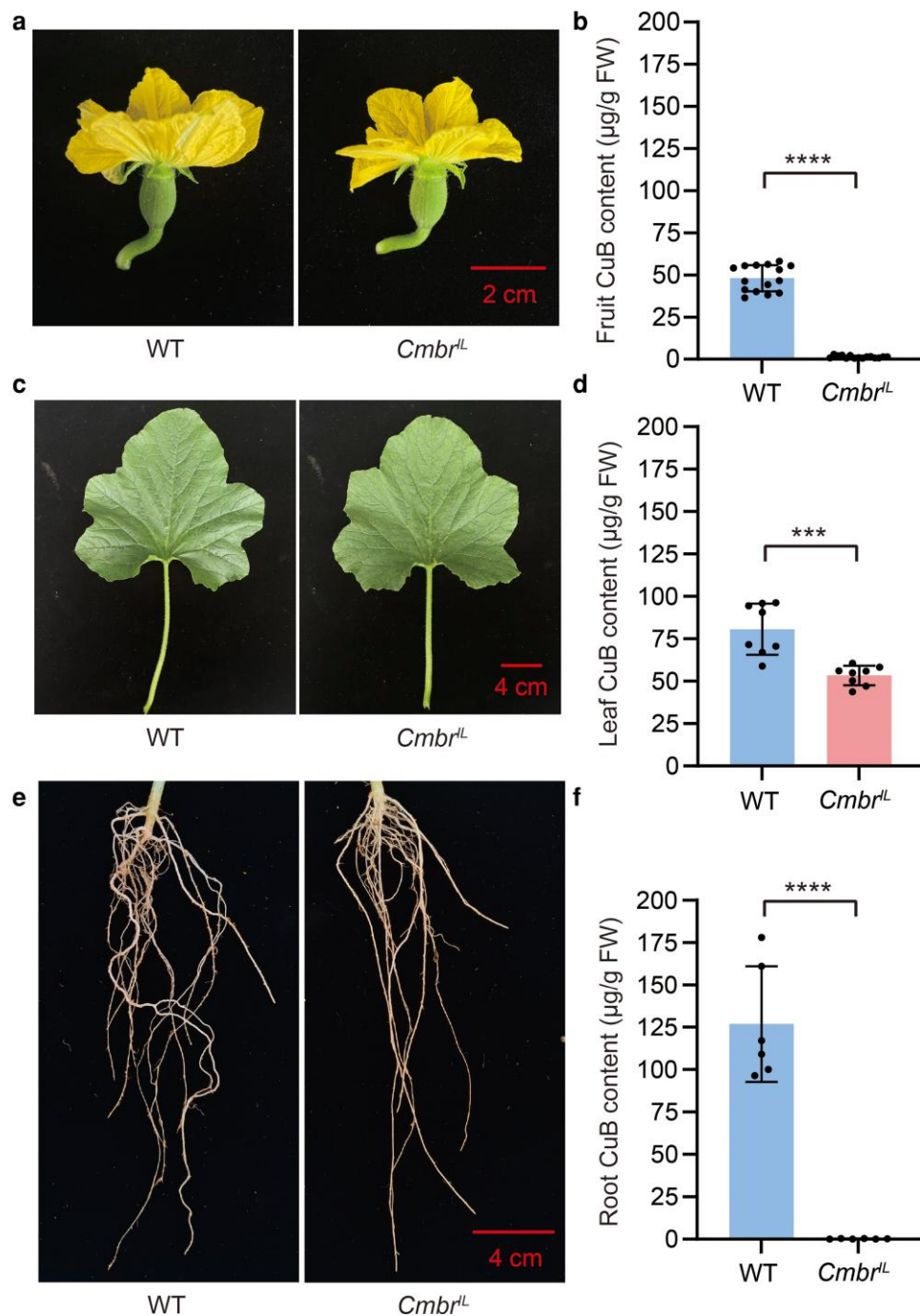


Figure 2 CuB levels in fruits, leaves, and roots of *Cmbr^L* mutant. **a)** Representative fruits of WT and *Cmbr^L* mutant at 0 DAA. Scale bar = 2 cm for all images. **b)** CuB content in young fruits (0 DAA) of *Cmbr^L* mutant and WT measured by LC-MS and expressed in μg/g fresh weight (FW). Data are given as mean ± SEM (n = 15 biological replicates). Statistical significance by 2-tailed *t* test: *****P* < 0.0001. **c)** Representative leaves of WT and *Cmbr^L* mutant. Scale bar = 4 cm for all images. **d)** CuB levels in leaves of *Cmbr^L* mutant and WT measured by LC-MS and expressed in μg/g FW. Data are given as mean ± SEM (n = 8 biological replicates). Statistical significance by 2-tailed *t* test: ****P* < 0.001. **e)** Representative roots of WT and *Cmbr^L* mutants. Scale bar = 4 cm for all images. **f)** CuB levels in roots of *Cmbr^L* mutant and WT measured by LC-MS and expressed in μg/g FW. Data are given as mean ± SEM (n = 6 biological replicates). Statistical significance by 2-tailed *t* test: *****P* < 0.0001. NOTE: WT data shared with Fig. 1, derived from the same LC-MS measurements.

transcriptional level, nor does *CmBr* regulate *CmBt*. Both *CmBt* and *CmBr* belong to the Ib₃ subgroup of bHLH transcription factors, a family that is widely recognized to function by forming heterodimeric complexes (Lei et al. 2020; Xu et al. 2022). We therefore hypothesized that *CmBt* might physically interact with *CmBr*. Yeast 2-hybrid (Y2H)

assay showed that coexpression of *CmBt* and *CmBr* enabled AH109 yeast cells to grow on selective medium, demonstrating a physical interaction between the *CmBt* and *CmBr* proteins (Fig. 3c). Tobacco-based firefly luciferase complementation assay showed detectable luciferase (LUC) signals when NLuc-*CmBr* and *CmBt*-CLuc

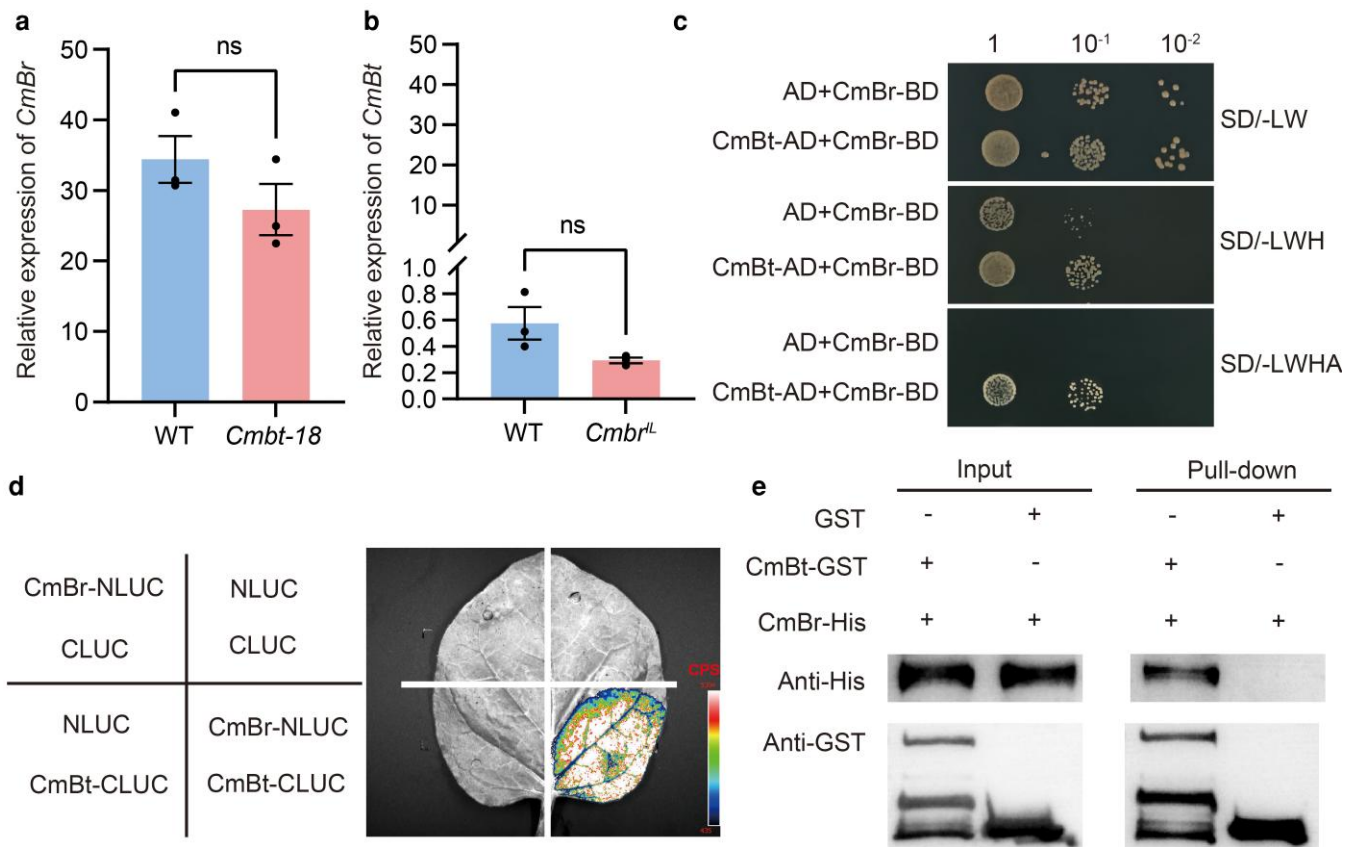


Figure 3 *Cmbt* physically interacts with *CmBr*. **a**) Relative expression of *CmBr* in WT and *Cmbt-18* mutant determined by RT-qPCR. Data are given as mean $\pm$ SEM ($n = 3$ biological replicates). ns, not significant ($P > 0.05$, 2-tailed t test). **b**) Relative expression of *CmBt* in WT and *Cmbt-18* mutant determined by RT-qPCR. Data are given as mean $\pm$ SEM ($n = 3$ biological replicates); ns, not significant ($P > 0.05$, 2-tailed t test). **c**) Y2H assay showing coexpression of CmBt and CmBr enabled growth of AH109 yeast on simple dropout medium. -LW, -Trp-Leu; -LWH, -Trp-Leu-His; -LWHA, -Trp-Leu-His-Ade. AD, pGADT7 vector; BD, pGBKT7 vector. Spots from left to right in each row indicate the gradient dilution (1 , 10^{-1} , 10^{-2}) of yeast clones. **d**) Luciferase complementation imaging (LCI) assay validating the interaction between CmBt and CmBr. Luciferase (LUC) signals were detected in tobacco leaves when NLuc-CmBr and CLuc-CmBt were coexpressed. The luminescence intensity range is shown by the pseudocolor scale bar, with units of CPS (counts per second). **e**) Pull-down assay showing the interaction between CmBt-GST and CmBr-His. Recombinant GST, CmBt-GST, and CmBr-His proteins expressed in *Escherichia coli* were incubated with glutathione-agarose beads. The total proteins (input) and proteins eluted from the beads (pull-down) were analyzed by immunoblotting with anti-His and anti-GST antibodies.

were coexpressed, while no obvious LUC signals was observed for the negative control (Fig. 3d). Pull-down assay in vitro provided further support that CmBt-GST pulled down CmBr-His, whereas GST alone did not (Fig. 3e). Together, these biochemical data demonstrate that CmBt physically interacts with CmBr.

CmBt and CmBr module might cooperatively regulate transcription of CuB biosynthetic genes

To investigate the mechanism by which CmBt and CmBr module cooperatively regulate CuB accumulation, we performed transcriptome sequencing (RNA-seq) on fruits at 0 DAA from WT, *Cmbt-18* mutant, and *Cmbt-18* mutant (Table S1). A total of 886 differentially expressed genes (DEGs) were identified in the *Cmbt-18* vs WT group (Table S2), while 850 DEGs were detected in the *Cmbt-18* mutant versus the WT group [false discovery rate < 0.05 , $|\log_2(\text{fold change})| \geq 0.7$] (Fig. 4a, Table S3). We focused on the 443 DEGs overlapping between the *Cmbt-18* mutant and *Cmbt-18* mutant (Table S4), as these genes are likely regulated by CmBt and CmBr module. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and Gene Ontology (GO) analysis were subsequently conducted on these overlapping

DEGs. KEGG pathway analysis revealed high enrichment in “sesquiterpenoid and triterpenoid biosynthesis” and “biosynthesis of various plant secondary metabolites” among the DEGs (Fig. 4b, Table S5). Furthermore, GO analysis also showed significant enrichment of biological processes related to triterpenoid biosynthetic process (Fig. 4c, Table S6). Notably, the expression levels of CuB biosynthetic genes were significantly downregulated in both the *Cmbt-18* mutant and *Cmbt-18* mutant compared with WT, including the cyclase gene *CmBi*; the cytochrome oxidase P450 genes *Cm160*, *Cm170*, *Cm180*, *Cm710*, and *Cm490*; and the acetyltransferase gene *CmACT* (Fig. 4d). These results indicate that CmBt and CmBr cooperatively regulate the expression of CuB biosynthetic genes and thereby positively regulate CuB accumulation in melon.

CmBt and CmBr synergistically activate the expression of *CmBi* and promote CuB biosynthesis

We aimed to verify that CmBt and CmBr cooperatively regulate CuB biosynthetic genes and thereby promote CuB accumulation. Firstly, we generated a *CmbtCmbr* double mutant by editing *CmBr* in the *Cmbt-18* lines. In the *CmbtCmbr* double mutant, CuB content in fruits

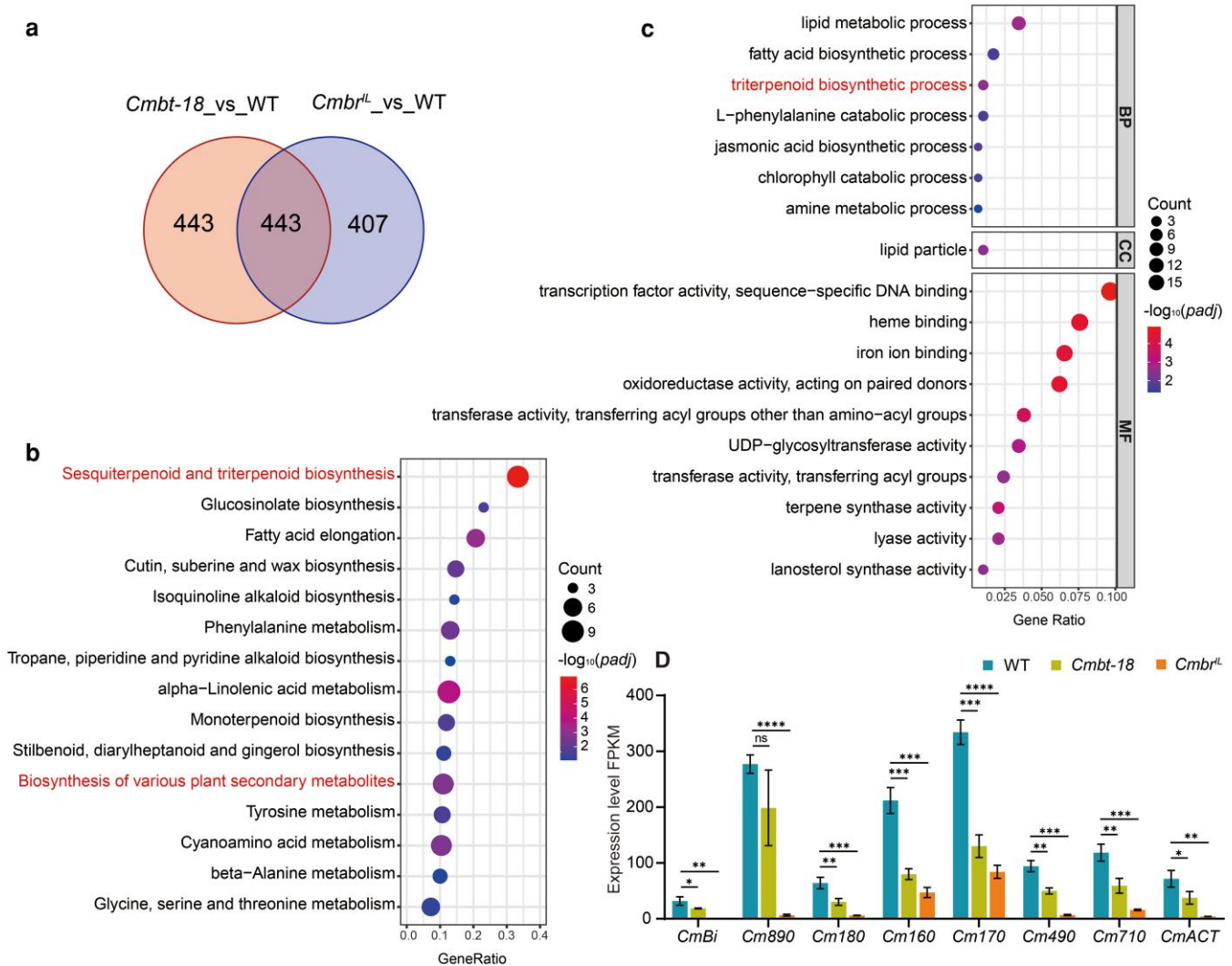


Figure 4 Analysis of DEGs in young fruits of *Cmbt-18* mutant and *Cmbr^L* mutant relative to WT. **a**) Venn diagram showing overlapping DEGs between *Cmbt-18* mutant vs WT and *Cmbr^L* mutant vs WT. Red circle: DEGs in *Cmbt-18* vs WT; blue circle: DEGs in *Cmbr^L* vs WT. **b**) Significantly enriched KEGG pathways among overlapping DEGs. **c**) Significantly enriched GO terms among overlapping DEGs. **d**) Expression levels of CuB biosynthetic pathway genes in young fruits of WT, *Cmbt-18* mutant, and *Cmbr^L* mutant. Data are given as mean $\pm$ SEM ($n=3$ biological replicates). Statistical significance by 2-tailed t test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. ns, not significant ($P > 0.05$, 2-tailed t test).

was undetectable and lower than in either the *Cmbt-18* single mutant or the *Cmbr^L* single mutant (Fig. 5a, b). Similarly, CuB levels in leaves and roots were lowest in the double mutant compared with the single mutants (Fig. 5c–f). These results indicate that *CmBt* and *CmBr* synergistically promote CuB biosynthesis across tissues.

Notably, CuB content in fruits and roots showed a larger reduction in the *Cmbr^L* mutant than in the *Cmbt-18* mutant (Fig. 5b, f), consistent with higher *CmBr* expression than *CmBt* in fruits and roots (Fig. S1c). Conversely, CuB content in leaves exhibited a greater reduction in the *Cmbt-18* than in the *Cmbr^L* (Fig. 5d), consistent with the higher expression of *CmBt* than *CmBr* in leaves (Fig. S1c). These results further confirm that *CmBt* is leaf predominant and *CmBr* is fruit- and root-predominant, which depends on their expression levels in different tissues.

To further verify that *CmBt* and *CmBr* cooperatively regulate CuB biosynthetic genes, we qualified the transcriptional levels of *CmBi* (a key CuB biosynthetic gene) in the *Cmbt-18* single mutant, *Cmbr^L* single mutant, and the *CmbtCmbr* double mutant using RT-qPCR. The

expression of *CmBi* was reduced in *Cmbt-18* and *Cmbr^L* single mutants and further decreased in the *CmbtCmbr* double mutants (Fig. 5g). To verify whether *CmBt* and *CmBr* synergistically regulate *CmBi* expression, we coexpressed 35S:*CmBt*, 35S:*CmBr*, and a *proCmBi-LUC* reporter in tobacco leaves (Fig. 5h). Cotransformation of 35S:*CmBt* and 35S:*CmBr* with *proCmBi-LUC* produced significantly higher LUC activity than either 35S:*CmBt* + *proCmBi-LUC* or 35S:*CmBr* + *proCmBi-LUC* alone (Fig. 5i). These results confirm that *CmBt* and *CmBr* cooperatively activate the expression of the CuB biosynthetic gene *CmBi*, thereby promoting CuB biosynthesis.

The *CmbtCmbr* double mutant produces non-bitter fruits under CPPU treatment during melon production

In melon production, the cytokinin-like regulator CPPU is commonly used to improve fruit set, boost yield, and reduce artificial pollination labor (Hayata et al. 2000; Huang et al. 2017; Cheng et al. 2021; Li et al. 2021; Liu et al. 2023). However, CPPU often induces fruit bitterness,

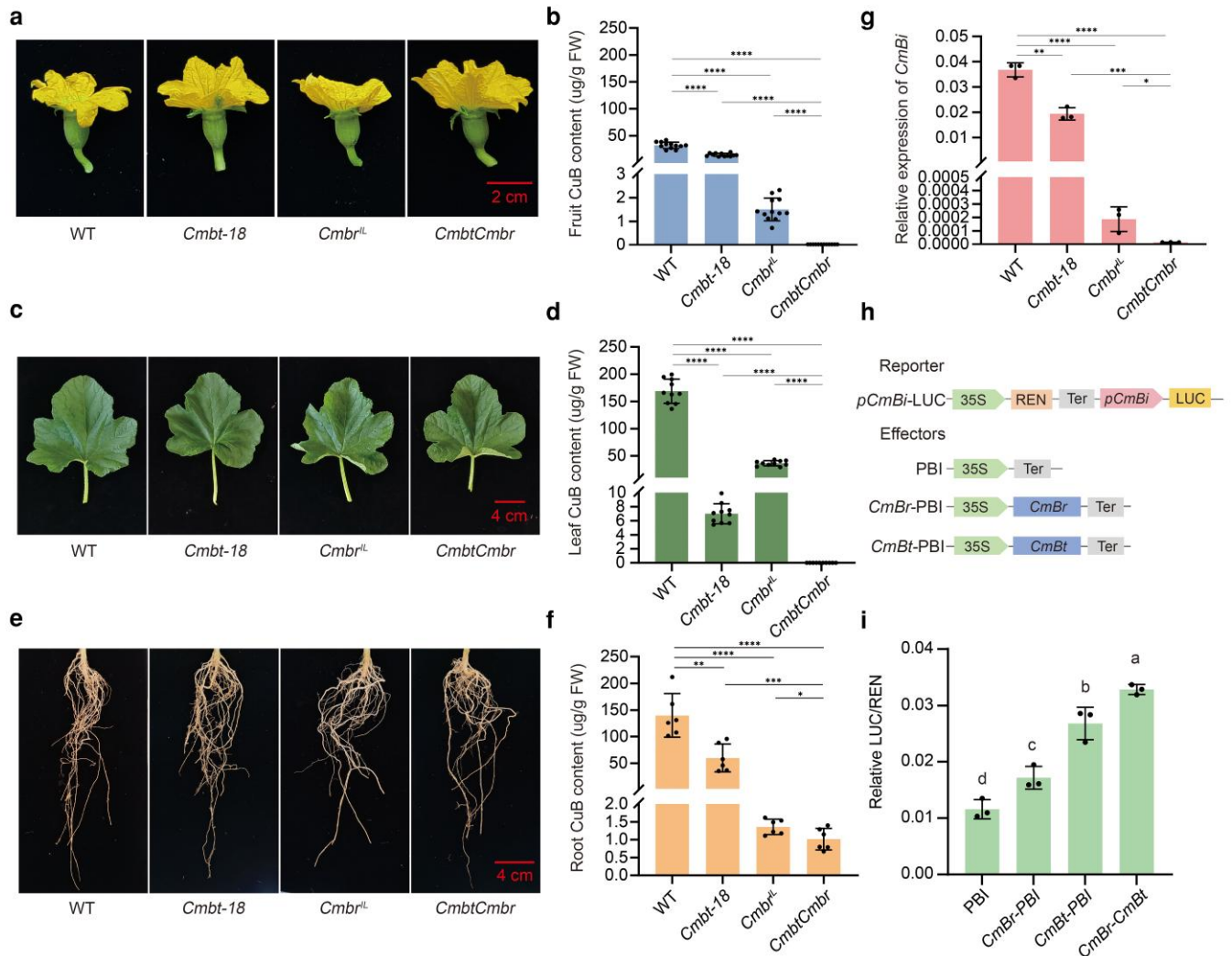


Figure 5 *CmBt* and *CmBr* synergistically activate *CmBi* expression and promote CuB biosynthesis. **a**) Representative young fruits of WT, *Cmbt-18* mutant, *Cmbt-18* mutant, and the *CmbtCmbr* double mutant. Scale bar = 2 cm for all images. **b**) CuB levels in young fruits of WT, *Cmbt-18* mutant, *Cmbt-18* mutant, and the *CmbtCmbr* double mutant measured by LC-MS and expressed in $\mu\text{g/g}$ fresh weight (FW). Data are given as mean $\pm$ SEM ($n = 12$ biological replicates). Statistical significance by 2-tailed t test: **** $P < 0.0001$. **c**) Representative young leaves of WT, *Cmbt-18* mutant, *Cmbt-18* mutant, and the *CmbtCmbr* double mutant. Scale bar = 4 cm for all images. **d**) CuB levels in young leaves of WT, *Cmbt-18* mutant, *Cmbt-18* mutant, and the *CmbtCmbr* double mutant measured by LC-MS and expressed in $\mu\text{g/g}$ FW. Data are given as mean $\pm$ SEM ($n = 10$ biological replicates). Statistical significance by 2-tailed t test: **** $P < 0.0001$. **e**) Representative roots of WT, *Cmbt-18* mutant, *Cmbt-18* mutant, and the *CmbtCmbr* double mutant. Scale bar = 4 cm for all images. **f**) CuB levels in roots of WT, *Cmbt-18* mutant, *Cmbt-18* mutant, and the *CmbtCmbr* double mutant measured by LC-MS and expressed in $\mu\text{g/g}$ FW. Data are given as mean $\pm$ SEM ($n = 6$ biological replicates). Statistical significance by 2-tailed t test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. **g**) Relative expression of *CmBi* in young fruits (0 DAA) of WT, *Cmbt-18* mutant, *Cmbt-18* mutant, and *CmbtCmbr* double mutant measured by RT-qPCR. Data are given as mean $\pm$ SEM ($n = 3$ biological replicates). Statistical significance by 2-tailed t test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. **h**) Schematic of constructs used for dual-luciferase (LUC) assays in a tobacco transient expression system. Effectors contained the coding sequences of *CmBr*, *CmBt*. Reporters contained *CmBi* promoter fragments upstream of the translation start site fused to the firefly LUC gene. LUC, firefly luciferase; REN, Renilla luciferase; 35S, CaMV 35S promoter; Ter, terminator. **i**) Synergistic activation of the *CmBi* promoter by *CmBt* and *CmBr* in tobacco leaves. Data are given as mean $\pm$ SEM ($n = 3$ biological replicates). Different letters indicate significant differences ($P < 0.05$, 1-way ANOVA).

impairing melon quality. Given that *CmBt* and *CmBr* are reported to respond to CPPU (Luo et al. 2020; Wang et al. 2024), we therefore evaluated the fruit bitterness in the *Cmbt* mutant and *CmbtCmbr* double mutant following CPPU treatment. Measurement of CuB content in young fruit at 3 DAA after CPPU treatment showed a 37.75% increase in the WT line and a smaller 32.95% increase in the *Cmbt-18* mutant, whereas the *CmbtCmbr* double mutant showed no significant change (Fig. 6a, b). Specifically, CuB content reached approximately 37.72 $\mu\text{g/g}$ in the WT lines and 21.43 $\mu\text{g/g}$ in the *Cmbt-18* lines but was nearly

undetectable in the *CmbtCmbr* double mutant lines, resulting in a non-bitter phenotype (Fig. 6a, b).

To evaluate the breeding potential for producing non-bitter fruit under CPPU treatment, we scored the incidence of bitter ripe fruit at 35 DAA in field trials following application of a high CPPU concentration (Fig. 6c). In the ivf05 WT line, 58.57% of fruits developed CPPU-induced bitterness. This incidence decreased to 27.27% in the *Cmbt-18* single mutant (Fig. 6d) and to 4.17% in the *Cmbt-18* introgression lines under CPPU treatment (Wang et al. 2024). Importantly, fruit

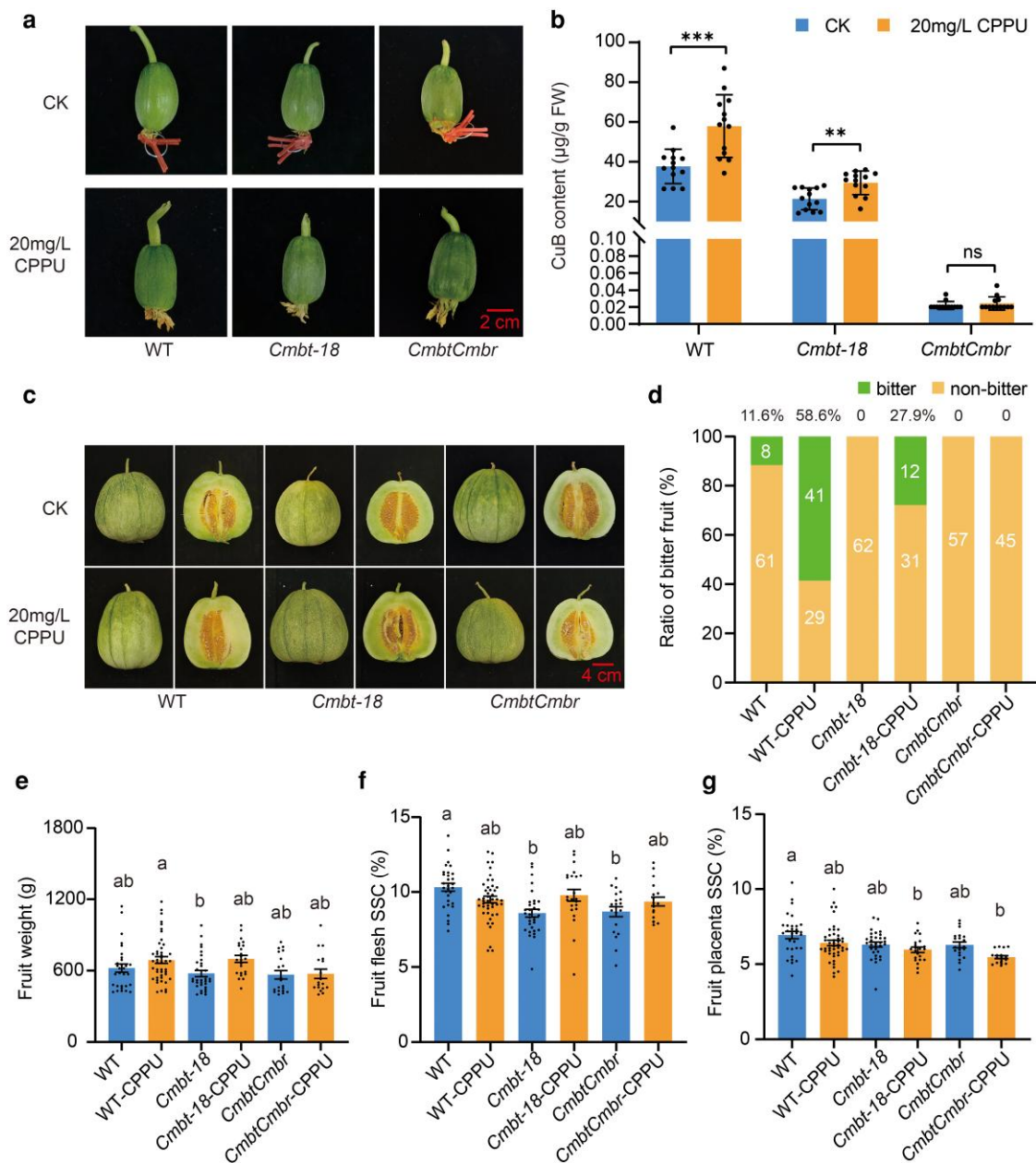


Figure 6 The *CmbtCmbr* double mutant produces non-bitter fruits under CPPU treatment. **a**) Representative fruits of WT, *Cmbt-18* mutant, and *CmbtCmbr* double mutant at 3 DAA under hand-pollination control (CK) and CPPU treatment (20mg/L CPPU). Scale bar = 2 cm for all images. **b**) CuB content in fruits of WT, *Cmbt-18*, and *CmbtCmbr* at 3 DAA under CK and CPPU treatment. Data are given as mean $\pm$ SEM ($n \geq 12$). Statistical significance by 2-tailed t test: *** $P < 0.01$; **** $P < 0.001$; ns, not significant ($P > 0.05$, 2-tailed t test). **c**) Representative fruits of WT, *Cmbt-18*, and *CmbtCmbr* at ripening (25 to 28 DAA) under CK and CPPU treatment. Scale bar = 4 cm for all images. **d**) Ratio of bitter fruits in WT, *Cmbt-18*, and *CmbtCmbr* at ripening (25 to 28 DAA) under CK and CPPU treatment. Numbers indicate total number of fruits per group ($n \geq 45$). **e-g**) Fruit weight (e), fruit flesh soluble solids content (SSC) (f), and placenta SSC (g) for WT, *Cmbt-18*, and *CmbtCmbr* in response to CPPU at 25 to 28 DAA. Data are given as mean $\pm$ SEM ($n \geq 18$). Different letters indicate significant differences ($P < 0.05$, 1-way ANOVA).

bitterness was effectively abolished in the *CmbtCmbr* double mutant (Fig. 6d). These results indicate that the nonfunctional *CmbtCmbr* allele reliably prevents CPPU-induced fruit bitterness and therefore has strong potential for application in melon breeding.

Given that melon fruits are primarily grown for commercial purposes, we also evaluated agronomic and quality traits of WT, *Cmbt-18*, and the *CmbtCmbr* double mutant. Important fruit traits, including fruit weight, fruit length, fruit width, and soluble solids

content (SSC), were measured. There were no significant differences in fruit weight, length, and width among WT, *Cmbt-18*, and the *CmbtCmbr* double mutant after CPPU treatment (Fig. 6e and Fig. S4a, b). Similarly, SSC in both flesh and placenta did not differ among these genotypes after CPPU treatment (Fig. 6f, g). Therefore, the nonfunctional *CmbtCmbr* allele prevents CPPU-induced bitterness without detectable negative effects on measured fruit weight or quality traits when CPPU is used during melon production.

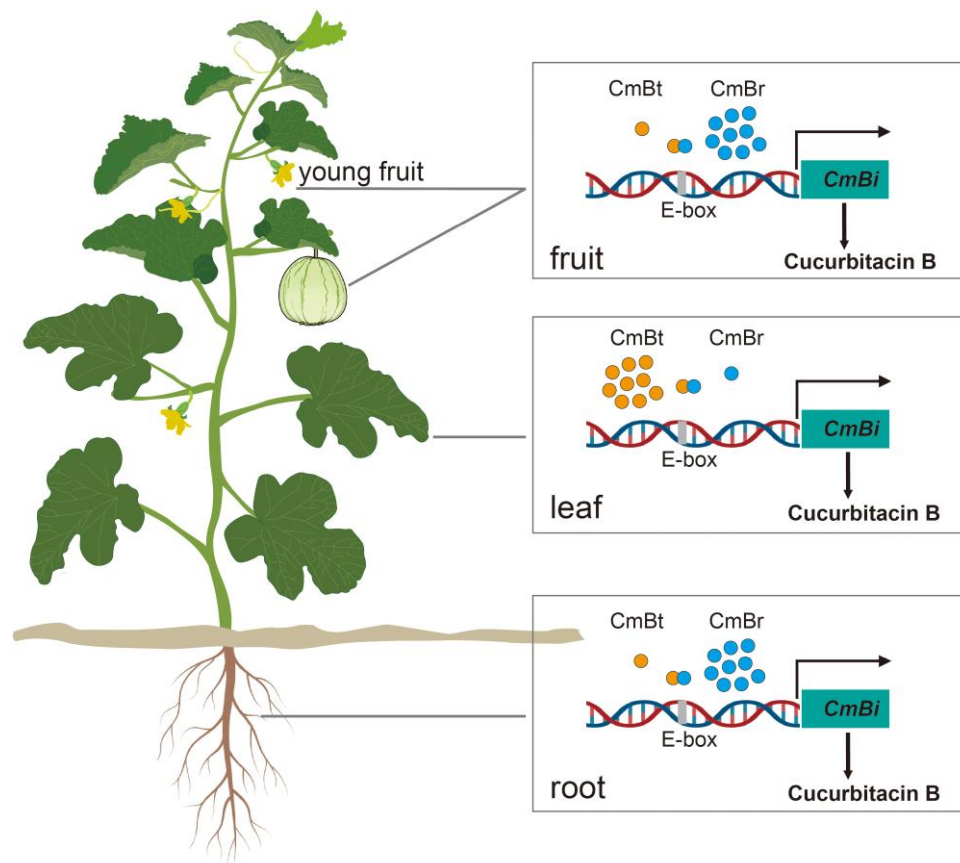


Figure 7 A regulatory model for cucurbitacin B biosynthesis by *CmBt* and *CmBr* in melon. *CmBt* (*Bitter fruit*) predominantly regulates CuB accumulation in leaves, whereas its paralog *CmBr* (*Bitter root*) plays a more prominent role in fruits and roots. *CmBt* physically interacts with *CmBr*, and the *CmBt*-*CmBr* module synergistically activates the CuB biosynthetic gene *CmBi* to promote CuB accumulation. In summary, *CmBt* and *CmBr* exert tissue-specific, partially redundant and synergistic roles in activating *CmBi* and promoting CuB biosynthesis across tissues.

Discussion

CmBt and *CmBr* have tissue-specific roles of CuB biosynthesis in melon

Previous studies characterized *CmBt* as a fruit-specific regulator and *CmBr* as a root-specific regulator of cucurbitacin B (CuB) in melon (Zhou et al. 2016). However, the previously proposed regulatory mechanisms underlying CuB biosynthesis across different tissues are not fully accurate. RNA-seq showed that *CmBt* is highly expressed in young leaves and young fruits, whereas *CmBr* is highly expressed in young fruits (0 to 4 DAF) and roots (Fig. S1c). Consistent with these expression patterns, *Cmbt-18* and *Cmbt-19* mutant lines exhibited significantly reduced CuB content in fruits, leaves, and roots compared with WT, producing non-bitter leaves (Fig. 1b–g). Meanwhile, CuB content in leaves exhibited a greater reduction in *Cmbt-18* than in *Cmbr^{IL}* (Fig. 5d). These results indicate that *CmBt* predominantly controls CuB accumulation in leaves and also contributes to CuB accumulation in fruits and roots. By contrast, *Cmbr^{IL}* mutant exhibited significantly reduced CuB content in fruits, leaves, and roots compared with WT, resulting in a non-bitter fruits and roots phenotype (Fig. 2a–f). Moreover, the *Cmbr^{IL}* mutant exhibit a larger reduction of CuB than the *Cmbt* mutant relative to WT in fruits and roots (Fig. 5b, f). These results confirmed that *CmBr* plays a predominant role in regulating CuB accumulation in fruits and roots while also contributing to CuB

accumulation in leaves. Previous study reported that *CmBt* and *CmBr* directly bind to the E-box motifs of the promoters of CuB biosynthetic genes (*CmBi*, *Cm160*, *Cm170*, *Cm180*, *Cm490*, *Cm710*, *Cm890*, and *CmACT*) and activate their expression (Zhou et al. 2016). In summary, *CmBt* and *CmBr* have tissue-specific roles in CuB biosynthesis that *CmBt* predominantly controls CuB accumulation in leaves, whereas *CmBr* plays a more prominent role in fruits and roots (Fig. 7). Both *CmBt* and *CmBr* belongs to the bHLH Clade Ib subfamily (Zhang et al. 2018; Gao and Dubos 2024). *MELO6W008960* and *MELO8W026330* show relatively high expression in roots, leaves, or fruits, suggesting their potential roles in regulating CuB biosynthesis (Fig. S1c). Among these, *MELO6W008960* was highly expressed in roots and has been reported to regulate cucurbitacin B biosynthesis in roots (Xu et al. 2022).

CmBt is functionally conserved with cucumber leaf regulator *CsBI*

In the Cucurbitaceae family, cucurbitacin biosynthesis is controlled by tissue-specific bHLH transcription factors. Comparative genomic and phylogenetic analyses revealed that the *CmBt*/*CmBr* gene cluster is collinearly conserved across Cucurbitaceae species. However, the copy number of genes within this cluster varies among species, with 3 genes in cucumber, 4 in melon, and 5 in watermelon (Fig. S5a).

Phylogenetic analysis further classified these genes into 3 distinct clades (Fig. S5b).

In cucumber, *CsBl* controls cucurbitacin C (CuC) in leaves while *CsBt* functions in fruit (Shang et al. 2014). The cucumber *CsBt* ortholog *ClBr* in watermelon has been shown to regulate cucurbitacin E biosynthesis in roots. In melon, *CmBr* regulates cucurbitacin B biosynthesis in both fruits and roots (Zhou et al. 2016; Wang et al. 2024); however, its orthologs in cucumber (Cs220) and watermelon (*Cl509*) have not yet been experimentally characterized. The cucumber *CsBl* ortholog in melon is *CmBt*. In this study, The *Cmbt* loss-of-function mutants (*Cmbt-18* and *Cmbt-19*) show undetectable leaf CuB and non-bitter leaves (Fig. 1d, e), paralleling the role of *CsBl* in cucumber. In contrast, the cucumber *CsBl* ortholog *ClBt* in watermelon has been reported to control cucurbitacin E biosynthesis in fruits (Yuan et al. 2023).

In summary, the *CmBt/CmBr* gene cluster shows conserved synteny but functional diversification across the Cucurbitaceae family. Integrating synteny, orthology, and functional evidence indicates that *CmBt* and *CsBl* share a conserved role in regulating leaf cucurbitacin biosynthesis across species.

CmBt and CmBr physically interact and exhibit partial redundancy as well as synergistic functions across tissues in melon

Because *CmBt* and *CmBr* have tissue-specific roles in CuB biosynthesis in fruits, leaves, and roots, the CuB content in the *CmbtCmbr* double mutant was lower than in either the *Cmbt-18* single mutant or the *Cmbr^{IL}* single mutant (Fig. 5a–f), indicating that *CmBt* and *CmBr* have partially redundancy function across tissues.

Both *CmBt* and *CmBr* encode bHLH transcription factors, a family known to form heterodimers (Lei et al. 2020). Yeast 2-hybrid, tobacco luciferase complementation, and GST pull-down assays demonstrate a physical interaction between *CmBt* and *CmBr* (Fig. 3c–e). Together, our genetic and biochemical evidence supports a cooperative role for *CmBt* and *CmBr* in activating CuB biosynthesis.

Notably, both *CmBt* and *CmBr* genes are located in the bHLH cluster within a collinear genomic region (Fig. S5a). Analysis of overlapping DEGs both in *Cmbt-18* mutant and *Cmbr^{IL}* mutant showed the expression levels of CuB biosynthetic genes (the cyclase gene *CmBi*; the cytochrome oxidase P450 genes *Cm160*, *Cm170*, *Cm180*, *Cm710*, and *Cm490*; and the acetyltransferase gene *CmACT*) were significantly downregulated both in *Cmbt-18* mutant and *Cmbr^{IL}* mutant compared with WT (Fig. 4d). Specifically, *CmBi* is a member of the oxidosqualene cyclase (OSC) gene family and encodes cucurbitadienol synthase, which catalyzes the cyclization of 2,3-oxidosqualene into the tetracyclic cucurbitane skeleton, the most central and first committed step in CuB biosynthesis (Zhou et al. 2016). The *CmBi* expression levels in the *CmbtCmbr* double mutant were lower than in either the *Cmbt-18* single mutant or the *Cmbr^{IL}* single mutant (Fig. 5g). Tobacco dual-LUC assay showed coexpression of *CmBt* and *CmBr* has significantly higher LUC activity than either alone (Fig. 5h, i). Collectively, these results indicate that *CmBt-CmBr* module cooperatively upregulates the expression of biosynthetic gene *CmBi*, thereby promoting CuB accumulation in melon (Fig. 7). A previous study has reported that *CmBt* and *CmBr* directly bind to the E-box motifs in the promoters of CuB biosynthetic genes to activate their transcription (Zhou et al. 2016). Based on these findings, we hypothesize that *CmBt* and *CmBr* synergistically activate all CuB biosynthetic genes (*CmBi*, *Cm160*, *Cm170*, *Cm180*, *Cm710*, and *Cm490* and *CmACT*), and this hypothesis will be experimentally validated in

future studies. In summary, we propose a regulatory model wherein *CmBt* and *CmBr* exert tissue-specific and partially synergistic roles in promoting CuB biosynthesis, with this synergy largely dependent on their intrinsic spatiotemporal expression patterns (Fig. 7).

The *CmbtCmbr* double mutant produces non-bitter fruit under CPPU compared with single mutant

CPPU treatment can induce CuB accumulation and cause fruit bitterness. In our field assays, in the ivf05, 58.57% of fruits developed CPPU-induced bitterness, whereas the *Cmbt-18* mutant reduced the percentage of bitter fruit to 27.27% (Fig. 6c, d). *Cmbr^{IL}* introgression lines decreased CPPU-induced bitterness to 4.17% (Wang et al. 2024). Notably, the *CmbtCmbr* double mutant exhibited dramatically reduced CuB levels and a near-complete loss of bitterness (Fig. 6c, d). Thus, the *CmbtCmbr* double mutant reliably produces non-bitter fruit after CPPU treatment and is therefore a more attractive solution for eliminating CPPU-induced bitterness than disruption of either gene alone. The *CmbtCmbr* double mutant showed significantly lower CuB in leaves and roots than the WT or single mutant (Fig. 5c–f). Given the defensive role of cucurbitacins, it will be essential to evaluate resistance to herbivores and microbial pathogens in the double mutant background before large-scale deployment in breeding in the further field trials. As an alternative strategy to preserve foliar defenses while reducing fruit bitterness, targeted promoter engineering of *CmBt* could be used to maintain high CuB accumulation in leaves.

Materials and methods

Plant materials and growth conditions

The seeds of the inbred line ivf05 were kindly provided by Huaisong Wang (Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences). Ivf05 is a cultivated agrestic variety; its young fruits, leaves, and roots display a pronounced bitterness. The ivf05 line and the derived mutants (*Cmbt-18*, *Cmbt-19*, and the *CmbtCmbr* double mutant) and introgression lines of WT and *Cmbr^{IL}* were cultivated in a greenhouse at the Jimo Agricultural High-tech Industrial Development Zone, Qingdao, Shandong, China, during 2022–2025. RNA-seq data of *CmBt* in fruits, leaves, and roots were obtained from a public database Melonet-DB (<https://melonet-db.dna.naro.go.jp/ap/top>) from the Japanese muskmelon cultivar “Earls favorite Harukei-3” (Yano et al. 2020).

Sample preparation and CPPU treatment

In the ivf05 *agrestis* inbred line and a series of ivf05-derived mutants, more than 18 samples were collected from ovaries, leaves, and roots (6 biological replicates, 3 tissues per replicate). All tissues were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. In addition, CPPU (Yuanye Biotech, Cat. No. S18139) was prepared at 20 mg/L and sprayed onto the ovaries at the lateral branch nodes on the day of bio-sexual flower anthesis in both the ivf05 inbred line and the derived mutants (Luo et al. 2020; Wang et al. 2024). Hand-pollinated plants were used as untreated controls. Fruits from CPPU-treated and control plants were harvested at 3 d after anthesis (DAA) and at 25 to 28 DAA, then immediately frozen in liquid nitrogen and stored at -80°C for subsequent analyses.

Bitterness sensory evaluations and LC-MS/MS quantification of cucurbitacin B

To assess the bitterness phenotype, we conducted sensory evaluations with at least 3 panelists, who each described the taste sensation as either “bitter” or “non-bitter” when biting the plant tissues. Based on these sensory assessments, we defined the “bitter” and “non-bitter” phenotypes (Horie et al. 2007).

To quantify cucurbitacin B content, we first flash-froze the samples. Frozen samples were ground to a fine powder in liquid nitrogen using a grinder. A 100-mg aliquot of the powdered tissue was extracted with 1 mL methanol, vortexed thoroughly, and sonicated for 15 min. The extract was centrifuged at 12,000 rpm for 10 min and the supernatant collected. The supernatant was diluted 1:100 with methanol and filtered through a 0.22- μ m membrane prior to instrumental injection.

Chromatographic separation and mass spectrometric detection were performed using an Agilent 1290 HPLC system (Agilent Technologies, Santa Clara, CA) coupled to a QTRAP 6500 + triple quadrupole mass spectrometer (Sciex, Framingham, MA). Chromatographic separation was performed on an Acquityr Premier Peptide CSH C18 column (1.7 μ m, 2.1 $\times$ 100 mm; Waters, Milford, MA) using gradient elution. The mobile phase comprises methanol (solvent B) and 0.1% (v/v) formic acid in water (solvent A). The flow rate was maintained at 0.2 mL/min, and the injection volume was 1 μ L. The total run time was 18 min, and the column oven temperature was set at 50 °C. The electrospray ionization (ESI) source was operated in a positive mode. The mass spectrometer was conducted in the multiple reaction monitoring (MRM) mode with a dwell time of 300 ms per MRM channel. The MRM transition m/z 576.0 $\rightarrow$ 499.0 was used for quantification of CuB. The sheath gas temperature was 350 °C and the ion spray voltage was 5.5 kV. CuB concentrations were quantified with internal calibration.

CRISPR/Cas9 vector construction

The single guide RNA (sgRNA) targeting *CmBt* was designed using the CRISPR-Genome Editing (GE) web tool (<http://skl.scau.edu.cn/targetdesign/>). The annealed sgRNA oligonucleotides were cloned into pBSE402 by Golden Gate assembly to generate pBSE402-sgRNA-*CmBt* (Xin et al. 2022). The resulting construct was verified by Sanger sequencing and introduced into *Agrobacterium tumefaciens* GV3101 for subsequent melon transformation. Primer sequences are provided in Table S7.

Melon transformation and generation of gene-edited plants

Melon transformation was performed following established protocols with minor modifications (Hu et al. 2017; Xin et al. 2022; Li et al. 2025). *Agrobacterium tumefaciens* GV3101 carrying pBSE402-sgRNA-*CmBt* was cultured in LB medium containing 50 mg/L kanamycin and 25 mg/L rifampicin at 28 °C until OD₆₀₀ reached 0.4–0.8. Cells were pelleted and resuspended to OD₆₀₀ = 0.2 in inoculation medium composed of 4.43 g/L Murashige and Skoog (MS), 30 g/L sucrose, 0.5 g/L 2-morpholinoethanesulfonic acid (MES) monohydrate, 2 mg/L 6-benzylaminopurine (6-BA), 80 mg/L acetosyringone (AS), and 1 mg/L abscisic acid (ABA).

Seeds of the ivf05 inbred line were de-hulled, surface-sterilized and germinated in the dark at 28 °C for 1 to 2 d. Cotyledons were excised, the hypocotyl removed, and each cotyledon cut in half; the proximal halves with V-shaped ends were used as explants. Explants were submerged in the *Agrobacterium* suspension and sonicated briefly to improve infiltration. For vacuum infiltration, explants and

approximately 15 mL of suspension were placed into a 20-mL syringe, and the plunger was gently withdrawn and released to ensure complete penetration of the suspension into the tissue. After infection, explants were co-cultivated on moist filter paper placed over solid medium B (4.43 g/L MS, 30 g/L sucrose, 3.3 g/L Gelzan, 1 mg/L ABA, 0.5 g/L MES, 80 mg/L AS, 2 mg/L 6-BA, and 150 mg/L dithiothreitol) for 72 h in the dark. Explants were then rinsed with sterile water and transferred to selection medium C (4.43 g/L MS, 30 g/L sucrose, 1 mg/L ABA, 3.3 g/L Gelzan, 200 mg/L Timentin, 0.5 mg/L 6-BA, 2 mg/L Basta) for regeneration. Shoots typically emerged within 3 to 4 wk and putative transformants were identified by green fluorescent protein (GFP) fluorescence screening. Regenerated shoots were transferred to rooting medium (4.43 g/L MS, 15 g/L sucrose, 9.4 g/L agar, 1 mL/L (v/v) plant preservative mixture, 200 mg/L Timentin) to induce root formation. Successfully rooted plantlets were acclimated and transplanted into soil for subsequent growth.

We transformed a CRISPR vector harboring the Cas9 and GFP cassettes to generate T₀ plants carrying both cassettes, which were backcrossed with WT plants to produce F₁ progeny. From the F₁ generation, we screened *Btbt* heterozygous mutants free of the Cas9 and GFP cassettes; these mutants were self-pollinated to obtain homozygous *bttb* mutants (*Cmbt-18/19*) lacking the 2 cassettes in the F₂ generation. The successful removal of the Cas9 and GFP cassettes in F₁ and F₂ progeny was verified by PCR-based genotyping and GFP fluorescence detection. Off-target, phenotypic and transcriptome analyses were all performed using these F₂ homozygous *bttb* mutants (*Cmbt-18/19*). Primer sequences are provided in Table S7.

Transcriptome data analyses

To improve gene expression quantification, a subset of melon genes was manually re-annotated to optimize the genome annotation reference. High-quality total RNA was extracted from 0 DAA fruits of WT, *Cmbr^{FL}*, and *Cmbt-18* plants, respectively, with 3 biological replicates per genotype. cDNA libraries were constructed from the extracted RNA and sequenced on an Illumina NovaSeq 6000 platform (Annoroad Gene Technology, Beijing Co., Ltd.). After adapter trimming and quality filtering, clean reads from each sample were aligned to the reference genome (Chen et al. 2024) using HISAT2 v2.2.1 with default parameters (Kim et al. 2019). Alignments were converted to BAM format with SAMtools v1.22 and processed with StringTie v3.0.0 to calculate Fragments Per Kilobase of exon per Million mapped reads (FPKM) (Pertea et al. 2015). Raw read counts for downstream differential expression analysis were obtained with featureCounts (Liao et al. 2014).

Gene re-annotation

We manually reannotated a subset of genes in the P84 genome by refining their gene structure annotations. Specifically, RNA-seq datasets derived from young fruits of P84 and wild-type ivf05 were used as transcriptional evidence. RNA-seq reads were aligned to the P84 genome assembly and integrated into the GSAMAN software to correct gene boundaries, including exon–intron structures (Chen et al. 2021). The revised gene models were subsequently validated by sequence alignment against the annotated gene set of the DHL92 reference genome to ensure annotation accuracy. All gene structures that underwent manual inspection and validation were incorporated into the final P84 genome annotation file, which was then used as the reference for downstream gene expression quantification.

GO and KEGG enrichment of DEGs

Differential expression analysis (DEGs) was carried out with the DESeq2 R package using the raw read count matrix as input (Love et al. 2014). Significantly DEGs were defined by an adjusted *P*-value (*padj*) < 0.05 and an absolute log2 fold change ($|\log_2\text{FoldChange}|$) $\geq$ 0.7. Functional enrichment of the identified DEGs was conducted using the R package clusterProfiler (Yu et al. 2012). Enrichment analyses were performed against the Gene Ontology (GO) (Ashburner et al. 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa et al. 2023). GO terms and KEGG pathways with *padj* < 0.05 were considered significantly enriched.

Identification of the bHLH gene family in melon and phylogenetic analysis

The melon genome sequence was obtained from the P84 wild melon reference assembly (<https://ngdc.cncb.ac.cn/gwh/Assembly/67928/show>) (Chen et al. 2024). Watermelon and cucumber genome data were retrieved from the Cucurbit Genomics Database (<http://cucurbitgenomics.org/v2/>), and *Arabidopsis thaliana* sequences were obtained from TAIR (<https://www.arabidopsis.org>). Candidate bHLH proteins in melon, watermelon, and cucumber were identified by HMM-based searches of each species' proteome using HMMER v3.4 and the bHLH HMM profile (Pfam: PF00010) retrieved from InterPro (<https://www.ebi.ac.uk/interpro/>) (Blum et al. 2025). Candidate sequences were further validated by batch submission to SMART (<http://smart.embl-heidelberg.de/>) and by manual inspection to confirm the presence of a complete bHLH domain (Letunic et al. 2021).

Two phylogenetic analyses were conducted to resolve evolutionary relationships among *CmBt* homologs. For cross-species comparison, validated melon bHLH proteins were aligned with *Arabidopsis* bHLH sequences using MEGA 11. Based on the alignments of conserved bHLH domains, a neighbor-joining (NJ) tree was constructed with 1,000 bootstrap replicates under the Jones-Taylor-Thornton (JTT) model and 80% partial deletion for gap treatment (Tamura et al. 2021). To highlight clades relevant to *CmBt*, only the clade containing *Arabidopsis* bHLH members homologous to *CmBt* was retained in the final presentation, with other clades collapsed. For intra-cucurbit analysis, bHLH proteins from melon, watermelon, and cucumber were aligned with MEGA 11, and an NJ phylogenetic tree was constructed. The tree was pruned to the clade containing *CmBt* homologs.

Multiple sequence alignment of *CmBt* homologs was performed using MEGA11 with default parameters (Tamura et al. 2021). Alignments were visualized and edited in GENEDOC (Nicholas et al. 1997). The bHLH domain boundaries were annotated based on conserved motif positions reported for the *Arabidopsis* bHLH family (Toledo-Ortiz et al. 2003).

Reverse transcription quantitative PCR (RT-qPCR) analyses

Fruits transiently overexpressing *CmBt* were sampled 4 d after infiltration into 10 DAA fruits of the WT and *Cmbr*^{IL} introgression lines for RT-qPCR analysis. Total RNA was extracted using the Quick RNA Isolation Kit (Huayueyang, Cat. No. 0416GK) following the manufacturer's protocol. First-strand cDNA was synthesized from 1 μ g total RNA using the GoScript Reverse Transcription Mix (Promega, Cat. No. A2790). Quantitative PCR was performed on a CFX96TM Real-Time System (BIO-RAD) with SYBR Premix (Roche, Cat. No. 04707516001) according to the manufacturer's instructions. Three

independent biological replicates were analyzed; each biological replicate consisted of a pooled sample of 3 to 6 fruits. Relative expression levels were calculated using the comparative $2^{-\Delta\Delta C_t}$ method (Schmittgen and Livak 2008), with *CmActin2* (MELO3C008032.2.1) as the internal reference gene. Primers were designed with Primer Premier 5 software (Premier Biosoft, Palo Alto, CA, USA), and the primer sequences are listed in Table S7.

Yeast 2-hybrid (Y2H) assay

The full-length CDS of *CmBr* was cloned into the pGBKT7 vector to generate *CmBr*-pGBKT7 (bait), and the full-length CDS of *CmBt* was cloned into pGADT7 to generate *CmBt*-pGADT7 (prey). Empty pGADT7 was used as a negative control. For the Y2H assay, *CmBt* (GAL4 activation-domain fusion) and *CmBr* (GAL4 DNA-binding-domain fusion) were cotransformed into Y2HGold yeast cells. Protein-protein interactions were assessed on SD/-Trp-Leu-His-Ade selective medium. Primer sequences are listed in Table S7.

Luciferase complementation imaging (LCI) assay

The full-length CDS of *CmBr* and *CmBt* were cloned into the 35S-NLUC and 35S-CLUC vectors, respectively. The resulting constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 (pSoup-p19). Transformed cells were grown in Luria-Bertani (LB) liquid medium containing kanamycin and rifampicin to an OD₆₀₀ of 1.0. The cells were then resuspended in infiltration buffer (total volume 200 mL: 2 mL 1 M MES, 2 mL 1 M MgCl₂, and 400 μ L 40 mM acetosyringone) and incubated in the dark for 2 h. Equal volumes of the appropriate *Agrobacterium* suspensions were mixed and infiltrated into *Nicotiana benthamiana* leaves. The plants were maintained under a 16 h light/8 h dark photoperiod for 48 h. Leaves were then evenly coated with firefly luciferase substrate, incubated in the dark for 2 min, and imaged with a Tanon-5200 imaging system (23T12NP6500-12146). Primer sequences are listed in Table S7.

Dual-luciferase reporter assay

The full-length CDS of *CmBt* and *CmBr* were incorporated into pBI121 vector to generate effectors, respectively. The 1800-bp sequence upstream of the translation initiation start site of the *CmBi* gene was cloned into pGreenII 0800-Luc vector to generate the reporter constructs. Effector and reporter constructs (*CmBt*-pBI121, *CmBr*-pBI121, and *proCmBr*-LUC reporter) were transformed into *Agrobacterium tumefaciens* strain GV3101 (pSoup-p19). Empty pBI121 was used as the effector control. *Agrobacterium tumefaciens* containing effector and reporter constructs were mixed at a 9:1 ratio, infiltrated into *Nicotiana benthamiana* leaves, and maintained in the dark at room temperature for 8 h. Samples were collected within 60 h post-infiltration, and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Cat. No. E1910) on a GloMax 20/20 Luminometer (Promega) according to the manufacturer's instructions. The relative promoter activity was determined as the LUC/REN ratio. Each experiment was repeated at least 5 times. Primer sequences are listed in Table S7.

A Glutathione S-transferase (GST) pull-down assay

The full-length CDS of *CmBt* was inserted into the expression vector pGEX4T-2 (GST Tag) and *CmBr* into the expression vector pCold-TF (His Tag). The resulting constructs were transformed into Rosetta

(DE3) Chemically Competent Cell (weidi Cat. No. EC1010). Recombinant protein expression was induced and confirmed by western blot. CmBt-GST was used as the bait to test interaction with CmBr-His. HaloTag pull-down assays were performed according to the Magne HaloTag Beads Technical Manual (Promega, USA). Primer sequences are provided in Table S7.

Statistical analysis

The soluble solid content (SSC) of the placenta and flesh was determined using a Refractometer PAL-1 (ATAGO), with 3 replicate measurements taken per fruit. The length and width of the fruits were quantified using ImageJ software. All data were processed with GraphPad Prism 9.2 software, and the statistical differences among the data were tested by 1-way ANOVA.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *CmBt* (MELO9W045730), *CmBr* (MELO9W045720), and *CmBi* (MELO11W106450) in the melon (P84) v1 reference genome.

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Author contributions

Z.H.Z. and H.M.Z. designed the research and supervised the program. S.Q.W., X.Z., M.Y.W., and C.J.W. performed experiments, X.R.W. and Y.C.X. analyzed data. Z.H.Z., H.M.Z., S.Q.W., X.Z., and X.R.W. drafted the manuscript. All authors read and approved the final manuscript.

Supplementary material

Supplementary material is available at *Plant Physiology* online.

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Conflict of interest

The authors declare no conflict of interests.

Data availability

The RNA sequencing data generated in this study have been deposited in the NCBI under the accession number PRJNA1338512.

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