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Transcription factor CcbHLH92 mediates ethephon-induced degreening in mandarin fruit by coordinately regulating chlorophyll and carotenoid metabolism

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Abstract

Early-ripening mandarin fruits exhibit asynchronous ripening between the peel and the flesh, making ethylene treatment essential in commercial production to promote peel coloration. This study sought to clarify the molecular basis of ethephon-induced degreening. We identified a basic helix-loop-helix transcription factor, CcbHLH92, whose expression was upregulated during ethephon-induced degreening in early-ripening mandarin fruit. CcbHLH92 exhibits transcriptional activation activity and is localized to the nucleus. Transient overexpression of *CcbHLH92* in tobacco leaves induced leaf yellowing and reduced chlorophyll accumulation. In mandarin fruit peel, *CcbHLH92* overexpression promoted coloration by upregulating chlorophyll degradation genes and enhancing carotenoid biosynthesis. Dual-luciferase reporter assays demonstrated that *CcbHLH92* directly activates key chlorophyll degradation genes (including *CcNYC1*, *CcChlase1*, *CcPAO*, and *CcRCCR*) and carotenoid biosynthesis and metabolism genes (such as *CcPSY1*, *CcLCYb*, *CcBCH2*, and *CcNCED5*). These regulatory effects accelerate chlorophyll degradation and promote the conversion of α-carotene and β-carotene into lutein and cryptoxanthin, ultimately enhancing fruit coloration in mandarins. Furthermore, BiFC and Co-IP assays provided complementary evidence supporting the physical interaction between CcbHLH92 and CcbHLH35, suggesting their potential involvement in the regulation of mandarin fruit coloration. Collectively, this study reveals the regulatory framework by which CcbHLH92 promotes mandarin fruit coloration, providing molecular insights into ethylene-mediated peel degreening.

Keywords: CcbHLH92, Mandarin fruit, Ethylene, Chlorophyll, Carotenoids, Degreening

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Introduction

The color of citrus peel is a crucial quality attribute and the first visual cue consumers perceive when selecting fresh citrus fruit[1]. As citrus fruits are non-climacteric, peel color is commonly used as an indicator of ripeness[2]. However, in some early-ripening mandarin varieties, such as certain types of mandarins, the external coloration often fails to accurately represent the internal ripeness of the fruit[3]. Even when the fruit has reached internal maturity—characterized by the development of distinctive flavor compounds and optimal levels of soluble solids, amino acids, and other components—its peel color may not align with the internal quality due to varietal traits and environmental influences. As a result, the presence of citrus fruits with green peels in the market often conveys an impression of unripeness to consumers, thereby misleading their judgment regarding the actual maturity of the fruit.

The color of citrus peel is primarily determined by the pigments present in the peel. The main color-forming pigments in citrus fruits are carotenoids and chlorophyll, while anthocyanidins are found exclusively in blood oranges. Carotenoids accumulate mainly in the membranes of chloroplasts and chromoplasts. Their biosynthesis starts from the five-carbon compounds isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are then converted into lycopene through the action of phytoene synthase (PSY). With the activities of phytoene desaturase (PDS), ζ-carotene isomerase (ZISO), ζ-carotene desaturase (ZDS), and carotene isomerase (CRTISO), phytoene is further transformed into lycopene. Lycopene then serves as a precursor for lutein via the ε-β pathway and for xanthophyll via the β-β pathway. In addition, 9-*cis*-neoxanthin is produced from neoxanthin through the catalytic function of neoxanthin synthase (NSY). It is subsequently transformed into the plant hormone abscisic acid by 9-*cis*-epoxycarotenoid dioxygenase (NCED), thereby contributing importantly to regulation of the abiotic stress signaling pathway[4].

Chlorophyll metabolism comprises three major processes: synthesis, cycling, and degradation. Chlorophyll synthesis can be divided into three stages. First, δ-aminolevulinic acid (ALA) is synthesized from glutamic acid. This step serves as a key regulatory node for chlorophyll precursor production and is influenced by both internal metabolic signals and external environmental factors. The second stage involves the conversion of δ-amino-ketoglutarate into protoporphyrin IX, which represents a common biosynthetic pathway shared by both chlorophyll and heme. Third, the magnesium branch proceeds specifically toward the formation of chlorophyll a[5].

During the chlorophyll cycle, chlorophyll a is converted to chlorophyll b through the catalytic action of chlorophyllide a oxygenase (CAO). Chlorophyll b can be reconverted to chlorophyll a via a two-step reduction. First, chlorophyll b reductase (CBR) reduces chlorophyll b to the intermediate 7-hydroxymethyl chlorophyll a (7-HMChl a), comprising two isozymes, Non-Yellow Coloring 1 (NYC1) and NYC1-Like (NOL). Then, 7-hydroxymethyl chlorophyll a reductase (HCAR) further reduces 7-HMChl a to regenerate chlorophyll a. In the chlorophyll degradation pathway, chlorophyll a is first dechelated by Mg-dechelatase, encoded by the Stay-Green (SGR) protein, to

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remove the central magnesium ion and yield pheophytin a (Phein a); then Phein a is dephytylated by pheophytin pheophorbide hydrolase (PPH) to produce pheophorbide a (Pheide a)[6].

Many factors impact the synthesis of pigments in citrus fruits. Under natural conditions, the breakdown and synthesis of pigments occur both while the fruit is on the tree and during the post-harvest period, leading to visible color changes. Variations in pigment ratios can even alter the direction of fruit coloration. Light exposure significantly influences color development in citrus fruits. Studies indicate that red light accelerates chlorophyll degradation and stimulates carotenoid accumulation, thereby promoting color change[7]. Similarly, blue light positively regulates the accumulation of total carotenoids in the fruit peel[8]. Temperature is another critical environmental factor regulating peel degreening and pigment transformation in citrus fruit; moderately low temperature can accelerate chlorophyll degradation and peel coloration independent of endogenous ethylene synthesis and signaling[9].

The application of exogenous ethylene significantly enhances peel color change in citrus fruits by stimulating chlorophyll breakdown and carotenoid synthesis[10–12]. Due to its cost-effectiveness and operational simplicity, ethephon has been widely adopted in the citrus industry for decades to regulate fruit coloration[1,13]. Recent research has shifted from studying single-factor effects to elucidating the mechanisms underlying multi-factor synergistic regulation of ethylene signaling sensitivity. For instance, blue light irradiation has been shown to enhance citrus sensitivity to ethephon, concurrent with alterations in plastid ultrastructure and fluctuations in chlorophyll and carotenoid levels. It also significantly upregulates key genes involved in the ethephon-induced ethylene signaling pathway, such as *CitACS1*, *CitACO*, *CitETR1*, *CitEIN2*, *CitEIL1*, and *CitERF2*[8]. These findings not only expand the scope of research on ethylene-mediated color regulation but also inspire further exploration into its underlying molecular mechanisms. During citrus color development, signaling molecules such as ethylene initiate a cascade of signal transduction events within the fruit, ultimately modulating the expression of downstream target genes and the activity of corresponding functional proteins, thereby influencing pigment synthesis and metabolic networks. This regulatory process operates across five levels: genetic, transcriptional, post-transcriptional, translational, and post-translational. Among these, transcriptional regulation is a key control point mediated through the interplay between *cis*-regulatory elements and trans-acting factors—fundamentally governed by transcription factors.

Transcription factors are specialized proteins that bind specific DNA sequences to regulate transcription, thereby modulating gene expression. In the regulatory network controlling fruit coloration in citrus, a range of transcription factors has been identified. These factors act either independently or in coordination with external signals to collectively mediate pigment biosynthesis. Based on conserved domains, transcription factors are grouped into families such as ARF, bZIP, bHLH, DOF, NAC, and WRKY. Among them, bHLH (basic helix-loop-helix) transcription factors form a large superfamily present across plants, animals, and fungi. They contain a conserved bHLH domain composed of a basic region, two α-helices, and a loop region, enabling their involvement in diverse signal transduction and anabolic pathways. Recent evidence indicates that bHLH transcription factors can modulate pigment biosynthesis and may contribute to fruit coloration regulation. For example, in *Arabidopsis*, SPL negatively regulates the R2R3-MYB-bHLH-WD40 complex, thereby repressing anthocyanin biosynthesis gene expression[14]. In tomato, *SlPRE2* is involved in chloroplast development and chlorophyll accumulation, and it also negatively regulates carotenoid accumulation during fruit ripening by inhibiting carotenoid synthesis[15]. In citrus, *CsbHLH6* promotes fruit coloration by inducing the expression of carotenoid metabolic genes in an abscisic acid-dependent manner[16]. These findings indicate that bHLH transcription factors can regulate, either directly or indirectly, the biosynthesis of major fruit pigments, including anthocyanins, carotenoids, and chlorophyll.

Furthermore, analysis of *cis*-regulatory elements in bHLH family genes has revealed an abundance of hormone- and light-responsive elements, which align with conventional color regulation treatments such as light exposure or hormone application. This suggests a molecular basis for the responsiveness of bHLH factors to external stimuli and provides a screening framework for identifying optimal conditions for their functional expression. Consequently, among various transcription factor families, bHLH-mediated regulatory mechanisms appear to play particularly prominent roles in fruit pigmentation.

In this study, we identified CcbHLH92, an ethylene-responsive transcription factor, as a candidate regulator from ethephon-induced degreened mandarin fruit. We employed a multifaceted approach, including transient overexpression, RNA-seq, quantitative reverse transcription PCR (qRT-PCR), EMSA, dual-luciferase reporter assay, BiFC, and Co-IP, to investigate the role and molecular mechanism of CcbHLH92 in regulating peel coloration. Specifically, we examined its effects on chlorophyll degradation and carotenoid metabolism, identified its downstream target genes, validated its direct binding to target promoters, and confirmed its interaction with CcbHLH35. The purpose of this study is to elucidate the regulatory mechanism by which ethylene-responsive CcbHLH92 coordinates chlorophyll degradation and carotenoid metabolism to promote peel coloration in early-ripening mandarin fruit, thereby providing new insights into the ethylene-mediated regulatory network of fruit coloration.

Materials and methods

Plant materials and experimental treatments

Mandarin fruit

Early-ripening mandarin fruits (*Citrus unshiu* Marc.) were harvested from an orchard located in Beibei District, Chongqing, China. Upon harvest, samples were promptly transferred to the laboratory, where they remained at ambient temperature overnight to eliminate field heat. Experimental materials were chosen based on uniformity in both color and size, while excluding any specimens with mechanical injuries. Selected fruits were rinsed under running tap water and allowed to dry naturally.

The initial harvest of fruits at the mature green stage was conducted in September 2017. These fruits were subsequently divided into two batches: one was immersed in a 1,000 mg/L ethephon solution for 1 min before air-drying, while the other served as a control and was treated identically using distilled water instead. All fruits were individually wrapped and kept in an incubator at 25 °C. Peel samples, approximately 1 cm in width, were collected from the equatorial region of the fruits after 0, 3, 6, and 9 d of storage. The collected samples were snap-frozen in liquid nitrogen and maintained at −80 °C until subsequent analysis.

Following the initial sampling, a subsequent harvest of mature green fruits was conducted from the same site in September 2021. All collected fruits were promptly transported to the lab for analysis. Employing identical procedures as outlined previously, these fruits underwent rigorous screening and processing. They were utilized as biological replicates for qRT-PCR validation to ensure reliability and reproducibility.

A third batch was harvested under identical conditions in September 2022. After the same selection and pretreatment procedures, these fruits were used for *Agrobacterium*-mediated transient overexpression experiments.

A fourth batch was harvested in October 2023 from the same orchard in Beibei District, Chongqing. Following the same selection and pretreatment procedures as described above, these fruits were used to validate the consistency of CcbHLH92-mediated enhancement of peel coloration across multiple batches of early-ripening mandarin fruits.

'Qingxiang' mandarin fruit

Post-harvest 'Qingxiang' mandarin fruit was purchased from a commercial orchard in Guangxi, China, in December 2022. Upon arrival at the laboratory, fruits were selected based on uniform color and size, with any specimens showing mechanical damage excluded. Selected fruits were rinsed under running tap water and allowed to dry naturally before use in transient overexpression experiments. Peel samples were collected and processed following the same procedures as described for early-ripening mandarin fruits.

Tobacco

The experimental plants were *Nicotiana benthamiana* grown under controlled conditions (25 °C; 16/8 h light/dark cycle). Seeds were sown and, at the two-true-leaf stage, seedlings were transferred to new pots. The plants were then cultivated for an additional four to five weeks before being used for experimental procedures.

RNA extraction and qRT-PCR

Following the protocol of the SteadyPure Plant RNA Extraction Kit (Accurate Biology, China), total RNA was extracted. Its integrity was verified on a 1% agarose gel. Prior to reverse transcription, genomic DNA was removed. cDNA was then generated using the PrimeScript RT Reagent Kit (Takara, China) and stored at −80 °C. qRT-PCR primers, designed by NCBI Primer-BLAST, were obtained from Sangon Biotech (Shanghai, China). The $2^{-\Delta\Delta C_T}$ method was used to calculate gene expression levels; each sample was analyzed in three technical replicates, and the entire experiment was independently repeated three times (n = 3 biological replicates)[17]. The specific primer sequences are shown in Supplementary Table S1.

Gene cloning and sequence analysis

The coding sequence (CDS) of *CcbHLH92* was obtained from the NCBI database, and specific primers for amplifying the full-length CDS were designed and synthesized by Sangon Biotech. Using cDNA derived from mandarin fruit peel as a template, PCR amplification was carried out in a 50 μL reaction volume. The detailed composition of the reaction mixture is provided in Table 1.

The PCR amplification protocol was set as follows: initial denaturation at 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min; and a final extension at 72 °C for 2 min. The resulting PCR products were separated via agarose gel electrophoresis, and bands of the expected size were excised. Gel slices containing the target DNA were purified with the SanPrep Columnar DNA Gel Recovery Kit (Sangon Biotech, Shanghai, China) following the manufacturer's instructions.

Table 1. PCR reaction mixture composition and volumes.

Component	Volume (μL)
Nuclease-free water	10
Phanta Max Super-Fidelity DNA Polymerase	1
2× Phanta Max Buffer	25
2× PrimeSTAR Max Premix	10
cDNA	2
Forward primer	1
Reverse primer	1

Bioinformatics analysis of CcbHLH92

Phylogenetic tree and amino acid sequence analysis

The CDS of *CcbHLH92* was obtained from the NCBI database, and its basic molecular characteristics were examined using DNAMAN. To investigate evolutionary relationships, a phylogenetic tree was constructed with MEGA10. Furthermore, potential *cis*-acting elements within the promoter region of *CcbHLH92* were predicted and characterized via the PlantCARE online tool.

Transcriptional activation activity assay in tobacco

The transcriptional activation potential of CcbHLH92 was assessed using a dual-luciferase reporter assay system in tobacco. The CDS of *CcbHLH92* was inserted into the pEAQ-BD vector (pBD) via the *Stu*I restriction site to generate an effector plasmid, in which expression is driven by the CaMV35S promoter. This effector plasmid was co-transformed with a reporter plasmid (containing the GAL4-responsive firefly luciferase (GAL4-LUC) gene and a constitutively expressed Renilla luciferase (REN) gene) into tobacco leaves at a 9:1 ratio. Following a 48-h incubation period, leaf discs were uniformly collected using a perforator. The dual-luciferase activity was then quantified to evaluate transcriptional activation, with six biological replicates performed per treatment.

Subcellular localization

The CDS of *CcbHLH92* was inserted into the pCAMBIA2300-GFP vector using *Kpn*I and *Pst*I restriction sites, and the resulting construct was introduced into *Agrobacterium tumefaciens* strain EHA105. Following subculture, bacterial cells were harvested by centrifugation and resuspended in an infiltration buffer composed of 10 mmol/L $MgCl_2$, 10 mmol/L MES monohydrate, and 0.1 mmol/L acetosyringone. The bacterial suspension was equilibrated by incubation in the dark at 28 °C for 2–3 h, after which it was infiltrated into the abaxial epidermis of tobacco leaves using a needleless syringe. GFP fluorescence signals in epidermal cells were observed 48–72 h post-infiltration under a Zeiss LSM780 laser scanning confocal microscope.

Transient heterologous expression of *CcbHLH92* in tobacco

The CDS of *CcbHLH92* was inserted into the pCAMBIA2300 vector to generate a recombinant plasmid, which can express the target gene, using the *Kpn*I and *Pst*I restriction sites. Following confirmation of the recombinant plasmid's sequence, it was transformed into *Agrobacterium tumefaciens* EHA105. The successful transformants were subsequently cryopreserved at −80 °C.

Tobacco leaves were infiltrated on their abaxial surfaces with *Agrobacterium* cultures harboring either the pCAMBIA2300-*CcbHLH92* construct or the empty vector control. Following infiltration, plants were kept under constant conditions at 25 °C. At 7 d post-infiltration (dpi), the leaves were photographed, after which the infiltrated areas were harvested. These tissue samples were immediately frozen in liquid nitrogen to preserve their integrity and then cryopreserved at −80 °C for downstream analysis.

Analysis of the function of CcbHLH92 in regulating color change in mandarin fruit

Transient overexpression of CcbHLH92 in mandarin fruit peels

The transient overexpression procedure was performed according to the protocol established by Yin et al.[18], with minor modifications, using the pCAMBIA2300 overexpression vector. Prior to *Agrobacterium* infiltration, two perforations (each 1 mm in both depth and diameter) were made in the equatorial region of the fruit using a sterile pipette tip. A total of 0.4 mL of bacterial suspension was injected into each hole via a needle-free syringe. After the suspension was fully absorbed, the fruits were individually wrapped and incubated at 25 °C. Peel tissue samples, approximately 1 cm wide and centered on the infiltration point, were harvested at predetermined intervals (0, 1, 2, 3, and 4 d post-infiltration). The samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C for subsequent analysis.

Measurement of peel color difference

A handheld CR-400 chroma meter was employed to determine the color of the fruit. In accordance with the instrument's guidelines, the L*, a*, and b* color coordinates were documented along the equatorial belt of the fruits. Each experimental group consisted of fifteen fruits, and three readings were taken at equidistant points around the equator of each individual fruit. The mean of these measurements was subsequently computed.

Quantification of chlorophyll a and b

Chlorophyll a and b were determined spectrophotometrically[19]. Briefly, fresh peel tissue was ground in liquid nitrogen and extracted with acetone in the dark. The absorbance of the extract was measured at 649 and 665 nm, and pigment concentrations were calculated using established formulas.

Quantification of individual carotenoid metabolites and ABA

Individual carotenoid metabolites were separated and quantified using the protocol described by Yuan et al.[8]. Specifically, carotenoid-related metabolites were separated and quantified using high-performance liquid chromatography (HPLC) following saponification and a C30 column gradient elution system[20]. The extraction and HPLC-based quantification of abscisic acid (ABA) were carried out based on the protocol established by Lado et al.[21], with minor modifications.

RNA sequencing

RNA-seq analysis was performed on peel samples collected under two experimental conditions. The samples obtained on day 3 of ethephon-induced degreening in 2017 were processed by Beijing Novogene Technology. In a separate experiment, peels harvested 48 h after the transient overexpression of *CcbHLH92* in 2022 were analyzed by Guangzhou GENE DENOVO Biotechnology. Differentially expressed genes (DEGs) were screened by applying the thresholds of fold change (FC) and false discovery rate (FDR). The screening thresholds were $|\log_2 FC| > 0$ and FDR < 0.05 to ensure strict statistical and biological significance.

Dual-luciferase reporter assay

Dual-luciferase reporter assay was performed to evaluate the transcriptional activation activity of CcbHLH92 toward target gene promoters. Briefly, the CDS of *CcbHLH92* was inserted into the pEAQ vector, while the promoter fragment of the target gene was ligated into the pGreenII 0800-LUC vector. After osmotic suspension for 2.5 h, the recombinant plasmids were co-infiltrated into tobacco leaves. The luminescence signal was detected 48 h after infiltration and normalized by calculating the ratio of LUC to REN activity. Six independent biological replicates per treatment were conducted to guarantee reliable results.

Electrophoretic mobility shift assay (EMSA)

Prokaryotic expression and purification of CcbHLH92 protein

The full-length CDS of *CcbHLH92* was amplified and inserted into the GST fusion expression vector via *Pst*I and *Bam*HI restriction sites. The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) competent cells. Positive clones were selected on LB plates containing ampicillin and cultured at 37 °C. Protein expression was induced, and the GST-CcbHLH92 fusion protein was purified using glutathione Sepharose beads. The purified protein was aliquoted and stored at −80 °C. Protein purity and molecular weight were verified by SDS-PAGE.

Probe design and labeling

Oligonucleotide probes containing the G-box core motif (with 17 bp flanking sequences on each side) were synthesized and labeled with biotin using a terminal deoxynucleotidyl transferase (TdT) kit according to the manufacturer's instructions. Complementary oligonucleotides were annealed to form double-stranded probes. The probe sequences are listed in Supplementary Table S1.

EMSA procedure

EMSA was performed using a chemiluminescent EMSA kit (Beyotime, China) following the manufacturer's protocol. Briefly, purified GST-CcbHLH92 protein was incubated with biotin-labeled probes in binding buffer for 20 min at room temperature. The reaction mixtures were separated on a 6% native polyacrylamide gel in 0.5× TBE buffer at 100 V for 60 min, then transferred onto a nylon membrane at 100 V for 40 min at 4 °C. After UV cross-linking, the membrane was incubated with streptavidin-HRP conjugate, and the signals were detected using a chemiluminescence imaging system.

Bimolecular fluorescence complementation (BiFC)

The BiFC assay was employed to investigate the potential interaction between CcbHLH35 and CcbHLH92 in tobacco. Full-length CDSs of *CcbHLH35* and *CcbHLH92* were individually fused to the N- and C-terminal fragments of YFP, using *Bam*HI and *Sal*I restriction sites, and both reciprocal combinations were tested. After sequence verification, the recombinant constructs were transformed into *Agrobacterium tumefaciens* strain EHA105. After cultivation, positive bacterial colonies were harvested and resuspended in infiltration buffer. *Agrobacterium* suspensions carrying the YNE and YCE constructs were mixed in equal proportions and co-infiltrated into tobacco leaves. Following a 48 h incubation at 25 °C, reconstituted yellow fluorescent protein signals were observed under a Zeiss LSM800 confocal laser scanning microscope.

Co-Immunoprecipitation (Co-IP) assay

The Co-IP assay was performed to verify the *in vivo* interaction between CcbHLH92 and CcbHLH35 in tobacco. The full-length CDSs of *CcbHLH92* and *CcbHLH35* were cloned into the plant expression vectors to generate HA-*CcbHLH92*-GFP and MYC-*CcbHLH35*-FLAG fusion constructs, respectively. The recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101, and the bacterial suspensions were adjusted to an OD_{600} of 0.6–0.8 with infiltration buffer (10 mmol/L $MgCl_2$, 120 μM acetosyringone). Equal

volumes of *Agrobacterium* suspensions carrying the indicated constructs were mixed and co-infiltrated into tobacco leaves. After 48–72 h of incubation, total proteins were extracted and incubated with anti-MYC agarose beads overnight at 4 °C. The immunoprecipitated proteins were detected by immunoblotting using anti-HA and anti-MYC antibodies.

Statistical analysis

All statistical analyses were conducted using SPSS 27.0, while figures were generated with GraphPad Prism 8.0. For comparisons involving two groups, a two-tailed Student's *t*-test was employed, with statistical significance set at * $p < 0.05$ and ** $p < 0.01$. When comparing three or more groups, a one-way ANOVA was performed, followed by Duncan's *post hoc* test using the same significance thresholds.

Results

Ethephon treatment elevates *CcbHLH92* expression in mandarin peel

Transcriptomic profiling of mandarin fruit subjected to ethephon-mediated degreening revealed that several bHLH transcription factor genes exhibited responsiveness to ethylene signaling, implicating their potential involvement in the regulation of fruit pigmentation[3]. Among these candidates, *CcbHLH92* exhibited pronounced upregulation and was therefore selected for further functional characterization. To validate its responsiveness to ethylene, qRT-PCR was performed to examine the expression pattern of *CcbHLH92* following ethephon treatment. The results showed that *CcbHLH92* expression was significantly induced in the peels of ethephon-treated mandarin fruit compared with the control, reaching approximately 2.4-fold and 9.0-fold of the control level at 3 and 6 d after treatment (Fig. 1), respectively ($p < 0.05$). These results confirmed that *CcbHLH92* is strongly and progressively responsive to ethephon treatment, supporting its selection as a candidate regulator for further functional analysis.

Bioinformatics analysis of *CcbHLH92*

CcbHLH92 sequence analysis

A query of the NCBI database revealed that the CDS of *CcbHLH92* comprises 705 bp and encodes a protein consisting of 234 amino acids. Alignment with representative bHLH transcription factors from *Arabidopsis* and mango demonstrated a high level of sequence conservation between *CcbHLH92* and its homologs in these species. It contains typical bHLH functional domains, confirming its classification within the bHLH family, and exhibits high evolutionary conservation—consistent with the structural hallmarks of bHLH transcription factors (Fig. 2a). A phylogenetic tree constructed using MEGA10 revealed that *CcbHLH92* is most closely related to *AtbHLH92* from *Arabidopsis* (Fig. 2b). Previous research has demonstrated that overexpression of *AtbHLH92* enhances plant tolerance to both salt and osmotic stress conditions[22].

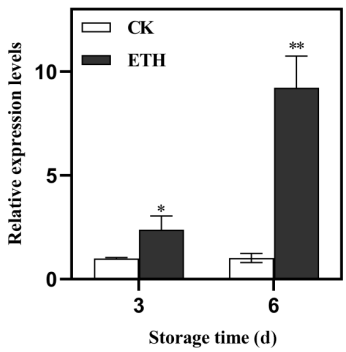


Fig. 1 Ethephon degreening treatment increases *CcbHLH92* expression in mandarin peel. The data represent the means ± SD of three biological replicates. CK: untreated group; ETH: ethephon-degreened treatment group; * $p < 0.05$; ** $p < 0.01$.

To investigate the evolutionary relationships of *CcbHLH92*, its sequence was aligned with those of various *Arabidopsis* bHLH genes, and a phylogenetic tree was constructed. The results (Fig. 2) indicate that *CcbHLH92* exhibits high homology with *AT5G43650.1*.

CcbHLH92 localizes to the nucleus and exhibits transcriptional activation activity

The nuclear localization of CcbHLH92 was initially predicted using the online tool (www.softberry.com). To experimentally confirm this prediction, the pCAMBIA2300-GFP vector was constructed and transiently expressed in tobacco leaves, with the successful transfection verified by detecting GFP fluorescence. As illustrated in Fig. 3a, the green fluorescent signals produced by the pCAMBIA2300-*CcbHLH92*-GFP fusion protein were exclusively observed in the nucleus. These findings provide strong evidence that CcbHLH92 is a nucleus-localized protein.

The transcriptional activation capability of CcbHLH92 was assessed using a dual-luciferase reporter (DLR) system. As shown in Fig. 3b, the LUC/REN ratio in the pBD-*CcbHLH92* group was significantly higher than that in the negative control pBD group ($p < 0.01$), indicating strong transcriptional activation activity and confirming its role as a functional transcriptional activator in tobacco.

Transient overexpression of *CcbHLH92* mediated by *Agrobacterium* in tobacco leaves

To rapidly and efficiently assess the function of CcbHLH92, transient overexpression was carried out in tobacco leaves via *Agrobacterium tumefaciens*-mediated transformation (strain EHA105). After agroinfiltration, the plants were kept at 25 °C for further incubation. As illustrated in Fig. 4a, leaf sectors expressing *CcbHLH92* exhibited a slight yellowing phenotype 7 d after infiltration, whereas those expressing the empty vector remained predominantly green. To quantify this phenotype, chlorophyll a and b contents were measured. In tobacco leaves transiently expressing *CcbHLH92*, a significant decrease was observed in the levels of both chlorophyll a and chlorophyll b relative to the control ($p < 0.05$). This reduction in chlorophyll content was consistent with the observed yellowing phenotype. These findings strongly suggest that *CcbHLH92* overexpression promotes chlorophyll degradation, leading to the visible loss of green pigmentation in tobacco leaves.

Mechanistic basis of *CcbHLH92*-mediated coloration in mandarin fruit

CcbHLH92 overexpression enhances mandarin peel coloration

CcbHLH92 was transiently overexpressed in mandarin fruit using an *Agrobacterium tumefaciens*-mediated method with strain EHA105. Three days after injection with the bacterial suspension and subsequent storage at 23 ± 1 °C, a distinct color change was observed around the injection sites on fruit peels transiently overexpressing *CcbHLH92*. In contrast, peels of the empty group remained predominantly green (Fig. 5a).

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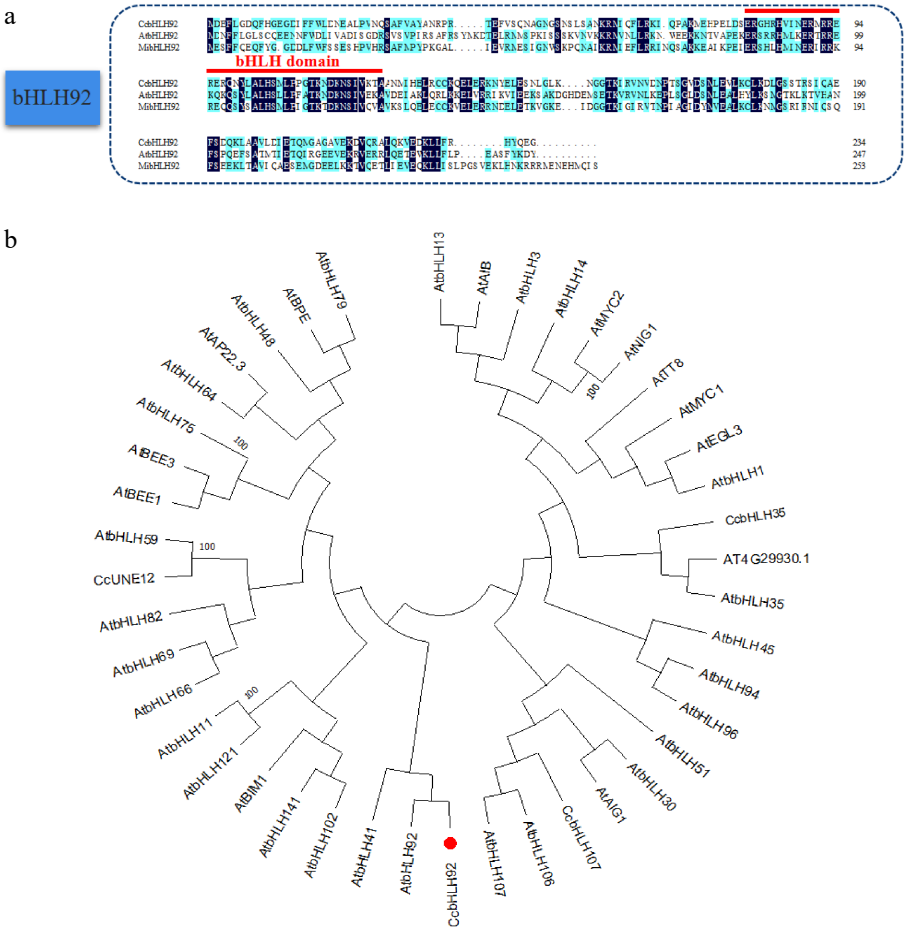


Fig. 2 Sequence comparison and phylogenetic placement of *CcbHLH92*. (a) Multiple alignment of *CcbHLH92* and its closest *Arabidopsis* homolog. Shading indicates the degree of amino acid sequence conservation, with dark blue representing identical residues and progressively lighter shades indicating decreasing similarity; unshaded residues are non-conserved. Red bars mark the conserved bHLH domain. (b) Phylogenetic analysis of *CcbHLH92* together with *Arabidopsis* bHLH family members.

To assess the temporal consistency of CcbHLH92 function, we conducted additional transient overexpression experiments in post-harvest early-ripening mandarin fruits harvested at a different time point (October 2023, Beibei, Chongqing, China). The peel color changes are documented in Supplementary Fig. S1, confirming that CcbHLH92-mediated enhancement of peel coloration is consistent across different batches of early-ripening mandarin fruits.

To further validate the effect of *CcbHLH92* overexpression on fruit peel coloration, we conducted parallel experiments in 2022 using 'Qingxiang' mandarin fruit, another commercially important mandarin cultivar. Consistent with the observations in mandarin fruit, transient overexpression of *CcbHLH92* in 'Qingxiang' mandarin fruit peel also resulted in similar color changes at the injection sites, as shown in Supplementary Fig. S2. These results demonstrate that the CcbHLH92-mediated enhancement of peel coloration is not cultivar-specific and may represent a conserved mechanism across mandarin varieties.

Verification of CcbHLH92 overexpression in mandarin peels

The expression level of *CcbHLH92* was markedly increased in mandarin fruit peel upon transient transformation ($p < 0.05$; Fig. 5b). These results confirmed the efficient overexpression of the target gene.

Effects of CcbHLH92 overexpression on chlorophyll metabolism-related genes and pigment contents

The green coloration of mandarin fruit peel is mainly attributed to chlorophyll, which obscures the visibility of underlying carotenoids. Therefore, chlorophyll degradation is a critical step in the peel degreening process. To assess the role of *CcbHLH92* overexpression in regulating the expression of chlorophyll catabolism genes, key genes involved in chlorophyll catabolism—including *CcChlase1*, *CcNYC1*, *CcPAO*, and *CcRCCR*—were selected based on RNA-seq data from mandarin peel 2 d after transient transformation (Fig. 6a). These genes were further verified using qRT-PCR. A significant upregulation of their expression was observed 2 d after *CcbHLH92* overexpression compared to the control ($p < 0.05$, Fig. 6b). Furthermore, the expression trends of these genes were consistent with the transcriptomic data.

In citrus peel, the green coloration is primarily maintained by chlorophylls, and their coordinated degradation is the fundamental process underlying peel degreening[11]. To investigate the role of CcbHLH92 in chlorophyll accumulation, we quantified chlorophyll a and b contents in peel samples following transient overexpression using spectrophotometry. A pronounced decrease was observed in the concentrations of both chlorophyll a and chlorophyll b throughout the storage period (Fig. 6c). Consistently, the accumulation of these pigments was substantially lower in the *CcbHLH92*-overexpressing fruit than in the control ($p < 0.05$).

In conclusion, our results establish that *CcbHLH92* overexpression promotes chlorophyll degradation and reduces chlorophyll content by modulating the expression of key pathway genes, namely *CcChlase1*, *CcNYC1*, *CcPAO*, and *CcRCCR*.

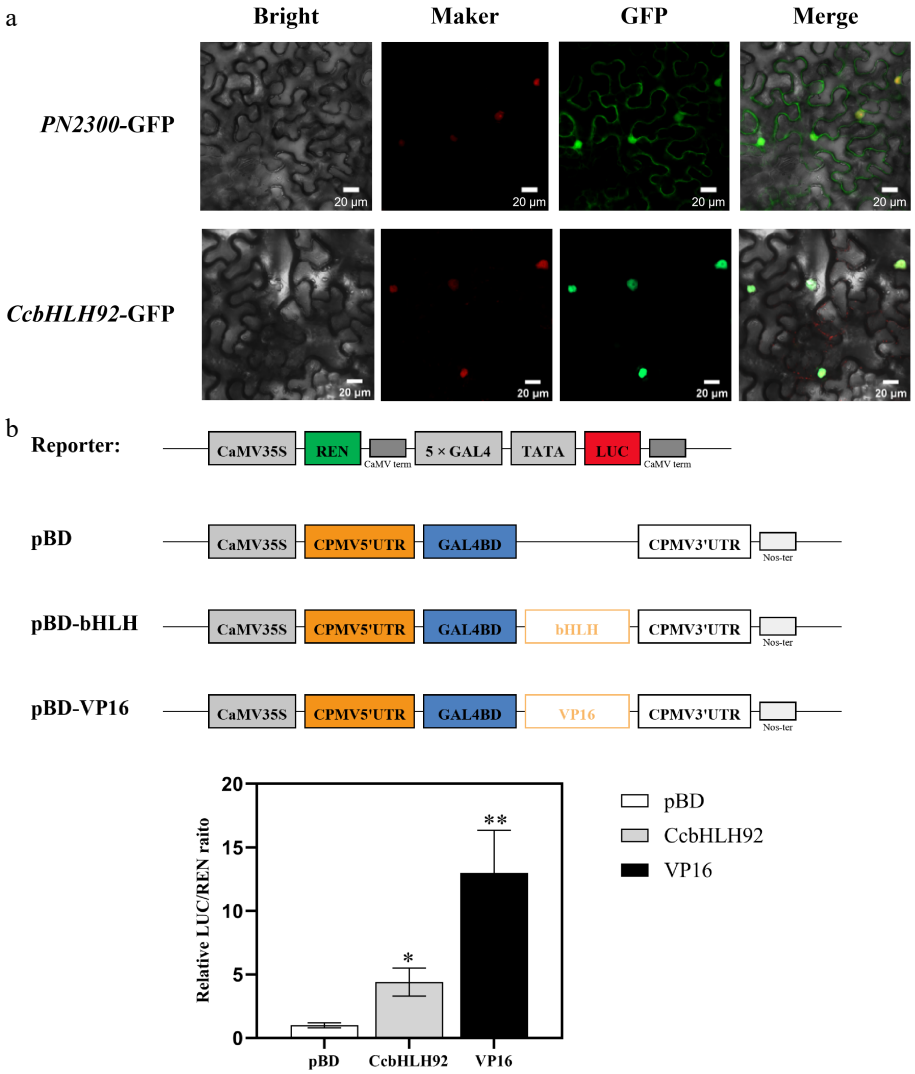


Fig. 3 Nuclear localization and transactivation ability of CcbHLH92. (a) Subcellular localization of CcbHLH92 in tobacco leaves. (b) Transcriptional activation activity of CcbHLH92. * $p < 0.05$; ** $p < 0.01$.

Effects of CcbHLH92 overexpression on carotenoid pathway genes and metabolite profiles

Carotenoid composition and their relative proportions, together with downstream derivatives, contribute to the diverse coloration of citrus peels. To decipher how *CcbHLH92* overexpression modulates carotenoid profiles, a transcriptome analysis was carried out. This led to the identification of critical carotenogenic genes, such as *CcBCH2*, *CcPSY1*, *CcLCYb*, and *CcNCED5* (Fig. 7a). These results were confirmed by qRT-PCR (Fig. 7b), which indicated a consistent upregulation in the expression levels of these genes, in full concordance with the transcriptome sequencing results.

After transient transformation and extended storage, the abundances of α-carotene, β-carotene, and violaxanthin progressively decreased. Conversely, lutein, cryptoxanthin, and zeaxanthin accumulated continuously. Notably, peels overexpressing *CcbHLH92* exhibited substantially lower concentrations of β-carotene, α-carotene, and violaxanthin than the control. In contrast, the contents of lutein and cryptoxanthin—compounds linked to the characteristic orange-yellow coloration of the peel—were significantly higher in the overexpression group ($p < 0.05$) (Fig. 7c).

As a downstream metabolite of the carotenoid pathway, ABA levels can be influenced by increased metabolic flux through this biosynthetic route. In this study, mandarin fruit peels overexpressing *CcbHLH92* accumulated significantly more ABA than the control ($p < 0.05$), indicating that CcbHLH92 potentially enhances ABA biosynthesis by activating carotenoid metabolism. The overexpression of *CcbHLH92* also upregulated critical carotenogenic genes (*CcPSY1*, *CcBCH2*, *CcLCYb*, and *CcNCED5*), which was associated with a reduction in β-carotene, α-carotene, and violaxanthin, and an accumulation of lutein and cryptoxanthin. Collectively, these findings suggest that CcbHLH92 modulates carotenoid composition and concentration by regulating key biosynthetic genes, ultimately promoting peel coloration.

CcbHLH92 interacts with and transactivates the promoters of its downstream target genes

Transcript analysis showed that *CcNYC1*, *CcChlase1*, *CcRCCR*, *CcPAO*, *CcLCYb*, *CcBCH2*, *CcPSY1*, and *CcNCED5* were significantly upregulated in mandarin fruit peels overexpressing *CcbHLH92*. Since the promoters of these genes contain G-box *cis*-elements, we hypothesize that CcbHLH92 may directly bind to and activate their promoters, thereby regulating their transcription.

EMSAs were performed to investigate whether CcbHLH92 directly binds to the target promoter sequences. The results (Fig. 8a) demonstrated that the GST-CcbHLH92 protein specifically bound to labeled probes corresponding to all eight genes, leading to a

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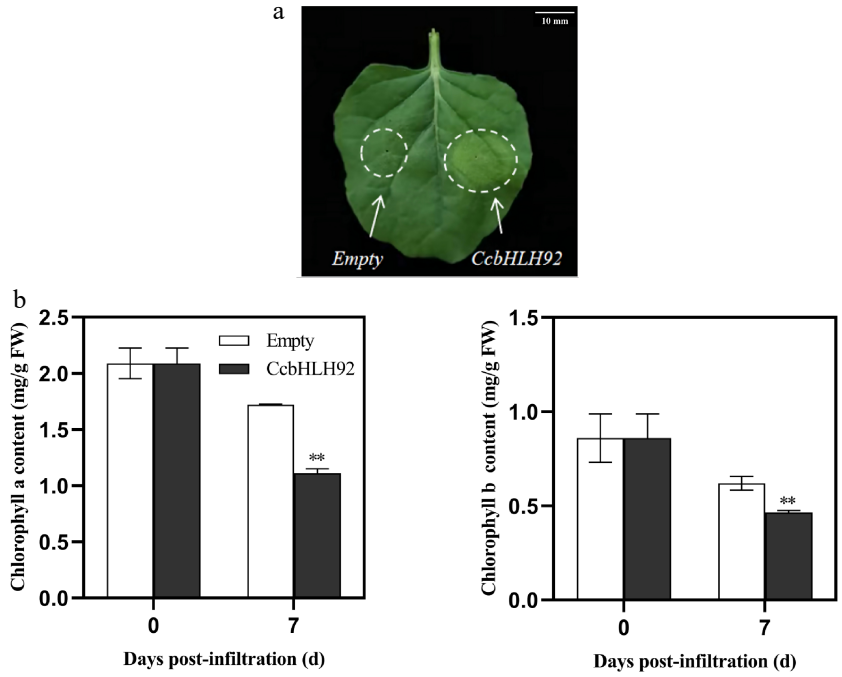


Fig. 4 Phenotypic and pigment changes following transient overexpression of *CcbHLH92* in tobacco. (a) Leaf appearance. (b) Chlorophyll a and b contents. 'Empty', empty vector; 'CcbHLH92', *CcbHLH92*-overexpression group. * $p < 0.05$; ** $p < 0.01$.

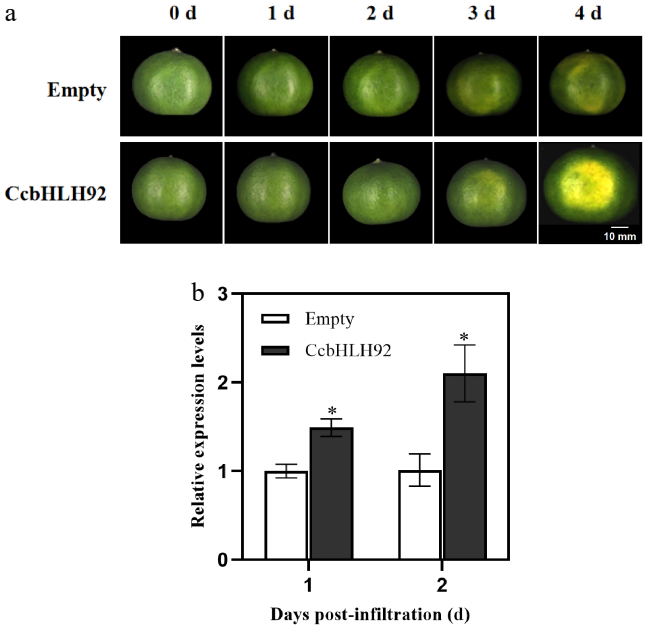


Fig. 5 Transient overexpression of *CcbHLH92* in mandarin fruit. (a) Peel phenotypes after *CcbHLH92* overexpression. (b) Relative *CcbHLH92* expression in peel following transient transformation. Data are shown as mean ± SD ($n = 3$). * $p < 0.05$; ** $p < 0.01$.

detectable mobility shift. These observations confirm that CcbHLH92 recognizes and binds to the G-box motifs located in the promoters of *CcChlase1*, *CcNYC1*, *CcPAO*, *CcRCCR*, *CcPSY1*, *CcLCYb*, *CcBCH2*, and *CcNCED5*, thereby promoting their transcriptional activation. These findings demonstrate that CcbHLH92 binds specifically to G-box *cis*-elements in the promoters of these eight genes and enhances their transcription.

To assess the transcriptional activation ability of CcbHLH92 toward its putative targets, a DLR assay was conducted. As shown in Fig. 8b, the co-expression of pEAQ-CcbHLH92 with promoter-LUC reporters of these eight genes in tobacco leaves markedly elevated the LUC/REN ratio. This indicates that CcbHLH92 functions as a transcriptional activator for these genes. Together with the EMSA results, these data confirm that CcbHLH92 directly binds G-box elements in their promoters to induce transcription.

Protein interaction between CcbHLH92 and CcbHLH35 in tobacco

In plants, bHLH transcription factors commonly dimerize among themselves or with other regulatory counterparts to attain diverse biochemical functions and participate in various physiological processes. Consequently, the functional characterization of individual bHLH proteins offers only partial understanding of their biological activities. Previous work has shown that both CcbHLH92 and CcbHLH35 regulate a common set of target genes—including *CcNYC1*, *CcChlase1*, *CcRCCR*, *CcPAO*, *CcBCH2*, and *CcNCED5*—which are key regulators of chlorophyll and carotenoid metabolism during mandarin fruit development[3]. Given the well-documented propensity of bHLH proteins to interact, we proposed that CcbHLH92 and CcbHLH35 might physically associate to coordinately control fruit coloration. To test this hypothesis, we employed bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (Co-IP) assays to detect potential protein–protein interactions.

For the BiFC assay, CcbHLH35 and CcbHLH92 were constructed into YNE and YCE vectors, respectively; the resulting constructs were co-introduced into tobacco leaf epidermis through *Agrobacterium tumefaciens* (strain EHA105)-mediated transient transformation. Following 48 h of incubation, reconstituted yellow fluorescent protein (YFP) signals were detected in the transformed cells using laser scanning confocal microscopy. As shown in Fig. 9, under YFP excitation light, yellow fluorescence was detected in tobacco leaf cells co-transformed with CcbHLH35-YNE + CcbHLH92-YCE and CcbHLH92-YNE + CcbHLH35-YCE, and the fluorescence signals were localized in the nuclei. Under the same YFP excitation light, no yellow fluorescence signals were detected in any other groups.

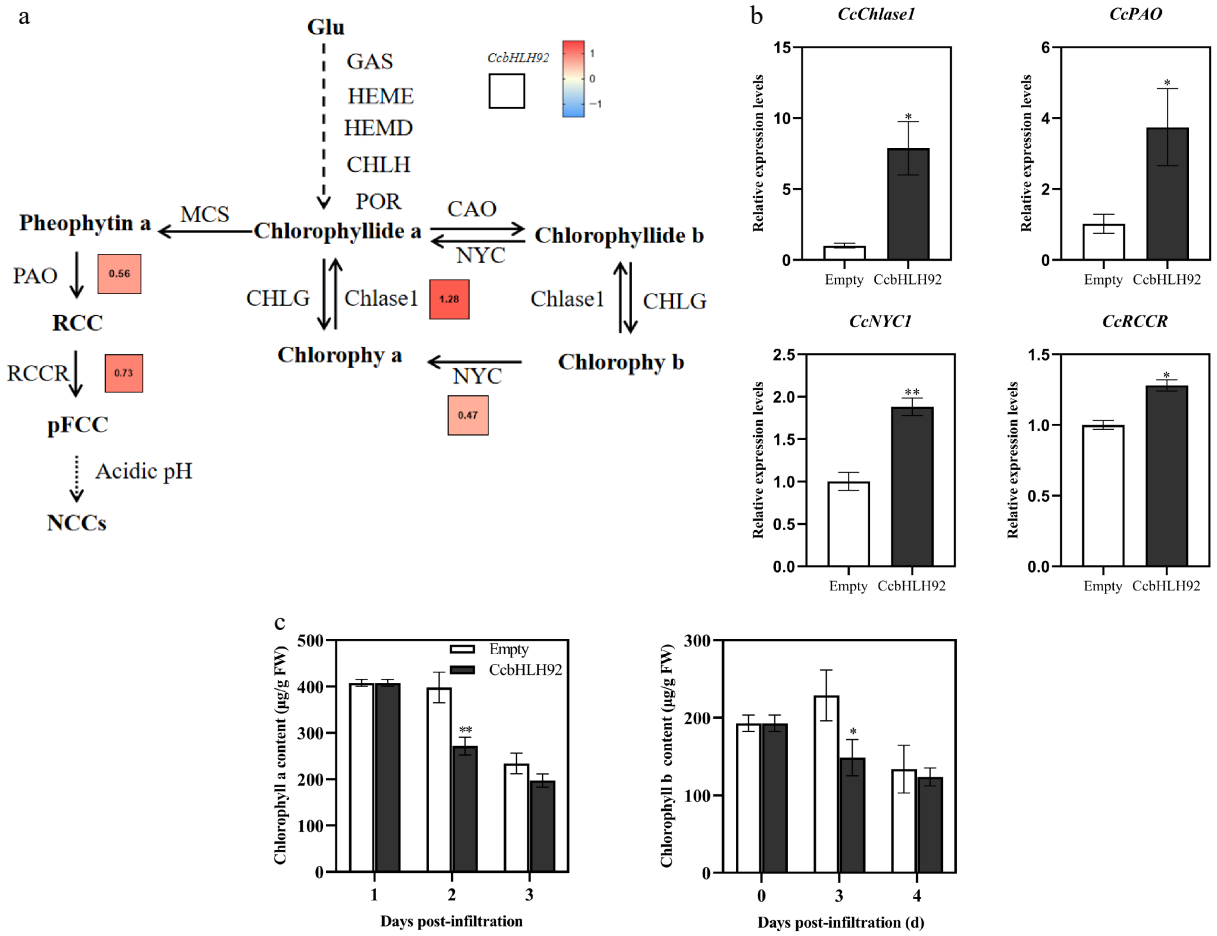


Fig. 6 Changes in chlorophyll catabolic genes and chlorophyll content after transient overexpression of *CcbHLH92*. (a) Abundance (fragments per kilobase of transcript per million mapped reads [FPKM]) of transcripts related to chlorophyll degradation. (b) qRT-PCR validation of differentially expressed genes identified by transcriptome analysis. Data are shown as mean ± SD ($n = 3$). (c) Contents of chlorophyll a and b. * $p < 0.05$; ** $p < 0.01$.

These results indicate that CcbHLH35 and CcbHLH92 interact at the protein level in tobacco leaf cells, and the protein complex formed by their interaction functions within the nucleus.

Although the BiFC assay demonstrated the interaction between CcbHLH92 and CcbHLH35 in plant cells, it is necessary to further confirm this protein–protein interaction using an independent biochemical approach. Co-immunoprecipitation (Co-IP) is a classic and widely accepted method for verifying physical protein interactions under near-physiological conditions, as it preserves the native conformation and post-translational modifications of proteins. Therefore, we performed a Co-IP assay to validate the *in vivo* interaction between CcbHLH92 and CcbHLH35.

The HA-CcbHLH92-GFP and MYC-CcbHLH35-FLAG fusion constructs were co-expressed in tobacco leaves via *Agrobacterium tumefaciens*-mediated transient transformation, with the GFP-MYC empty vector as the negative control. Total proteins were extracted from infiltrated leaves and immunoprecipitated with anti-MYC agarose beads, followed by immunoblot analysis with anti-HA and anti-MYC antibodies. As shown in Fig. 10, in the input samples, both CcbHLH92 and CcbHLH35 fusion proteins were successfully expressed at their expected molecular weights. In the anti-MYC immunoprecipitates, a clear band corresponding to CcbHLH92 was detected in the group co-expressed with MYC-CcbHLH35-FLAG, whereas no signal was observed in the negative control group co-expressed with GFP-MYC. These results provide independent biochemical evidence that CcbHLH92 and CcbHLH35 physically interact with each other in plant cells. The consistent results obtained from the BiFC and Co-IP assays provide complementary evidence supporting the physical interaction between CcbHLH92 and CcbHLH35 in plant cells. This interaction may contribute to their coordinated regulation of chlorophyll and carotenoid metabolism during mandarin fruit coloration.

Discussion

CcbHLH92 enhances mandarin fruit coloration by modulating chlorophyll and carotenoid metabolism

Light, temperature, and hormones are crucial environmental factors known to regulate chlorophyll and carotenoid biosynthesis, thereby influencing peel pigmentation in citrus fruits[7,9,10]. Exogenous ethylene treatment is a widely used postharvest degreening method. Citrus fruits perceive low-dose exogenous ethylene through ethylene receptors ETR1 and ETR2, and receptor-deficient mutants exhibit delayed peel coloration and late fruit ripening[10]. Ethylene signaling modulates target gene expression via transcription factors in the coloration network, affecting both chlorophyll breakdown and carotenoid buildup.

Multiple transcription factors mediate ethylene-induced coloration. In Satsuma mandarin flavedo, the ethylene-responsive factor CitERF061 directly binds the promoter of the C2H2-type zinc

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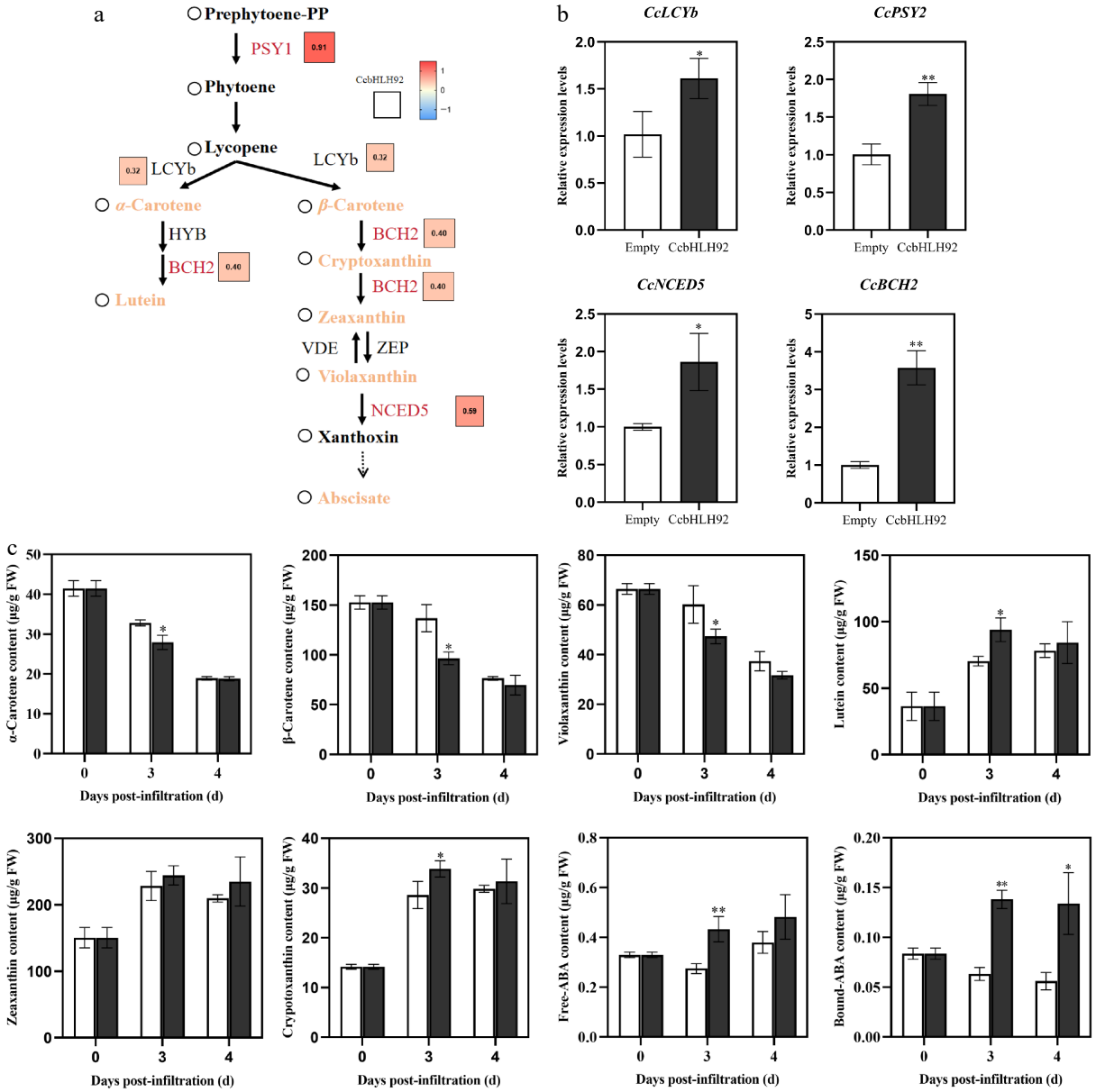


Fig. 7 Impact of *CcbHLH92* overexpression on transcriptional activity and individual metabolite accumulation in the carotenoid pathway. (a) FPKM expression levels of carotenoid biosynthetic and downstream genes in citrus peel. (b) Validation of transcript abundance using qRT-PCR. Data are shown as mean ± SD (n = 3). (c) Concentrations of individual carotenoid metabolites and ABA. * $p < 0.05$; ** $p < 0.01$.

finger gene *CitZAT4* and activates its transcription, and CitZAT4 in turn directly binds and activates *CitPSY*, increasing the supply of phytoene, the entry substrate of the carotenoid pathway, and ultimately contributing to orange-yellow pigmentation[23]. Beyond such transcriptional cascades, postharvest ethylene treatment has been shown to reproduce and accelerate the changes in carotenoid composition and carotenogenic gene expression that occur during natural maturation, including the induction of *PSY* and *ZDS* and the repression of *ε-LCY*[11]. Transcriptional control of carotenoid genes also operates in other non-climacteric fruits such as pepper, where a MADS-RIN–DIVARICATA1 module positively regulates carotenoid accumulation during largely ethylene-independent ripening[24].

Among the transcription factors studied, members of the bHLH family have emerged as important regulators of fruit pigmentation, and several have been functionally characterized in citrus and other fruit crops. For instance, Noemi, a citrus bHLH transcription factor, modulates flavonoid pigment biosynthesis and fruit acidity, illustrating the functional diversity of bHLH proteins in citrus[25]. In lemon, the expression of ClbHLH25 was induced by both ethylene and low temperature, and was correlated with the transcription of chlorophyll catabolic genes including *NYC1*, *PAO*, and *RCCR*[9]. In papaya, CpbHLH1 and CpbHLH2 bind the promoters of lycopene *β*-cyclase genes (*CpCYC-B*/*CpLCY-B*) and modulate their transcription, with CpbHLH2 acting as an activator and CpbHLH1 as a repressor, thereby affecting carotenoid accumulation during fruit ripening[26]. Collectively, these findings underscore the importance of bHLH factors in fruit coloration and provide a basis for assembling a more systematic regulatory network.

This study demonstrates that CcbHLH92 acts as a positive regulator of citrus fruit coloration by enhancing chlorophyll degradation. Its activity is mediated through the upregulation of genes involved in chlorophyll catabolism and the carotenoid biosynthetic pathway. Furthermore, CcbHLH92 promotes the conversion of *α*- and *β*-carotene and stimulates the production of lutein and cryptoxanthin,

thereby altering carotenoid composition and content to improve peel color. These results expand the known functional spectrum of the bHLH family in regulating pigment metabolism in citrus.

Intriguingly, our prior research revealed that CcbHLH35 triggers the expression of key genes involved in both chlorophyll degradation (e.g., *CcChlase1*, *CcNYC1*, *CcPAO*, *CcRCCR*) and carotenoid

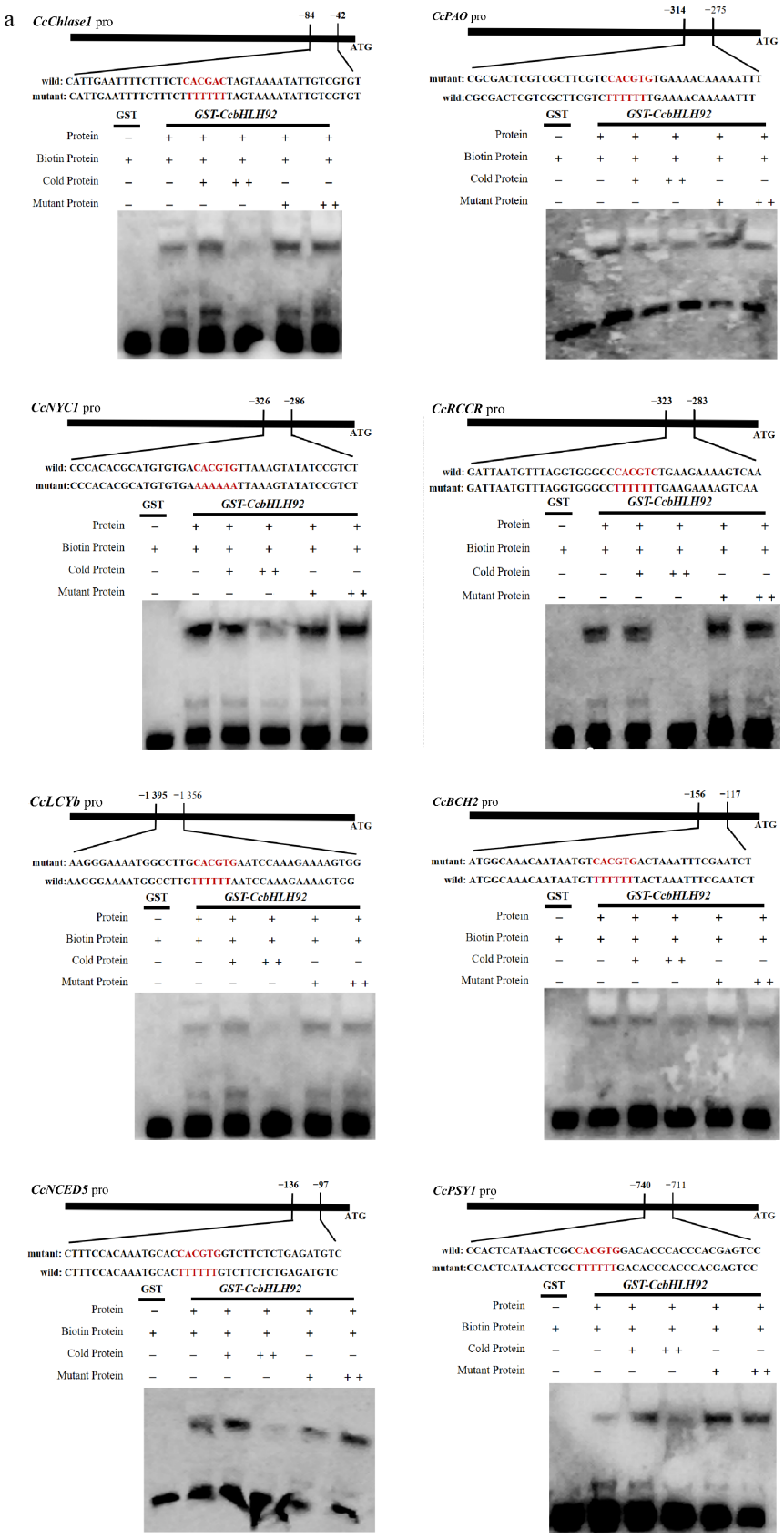


Fig. 8 to be continued.

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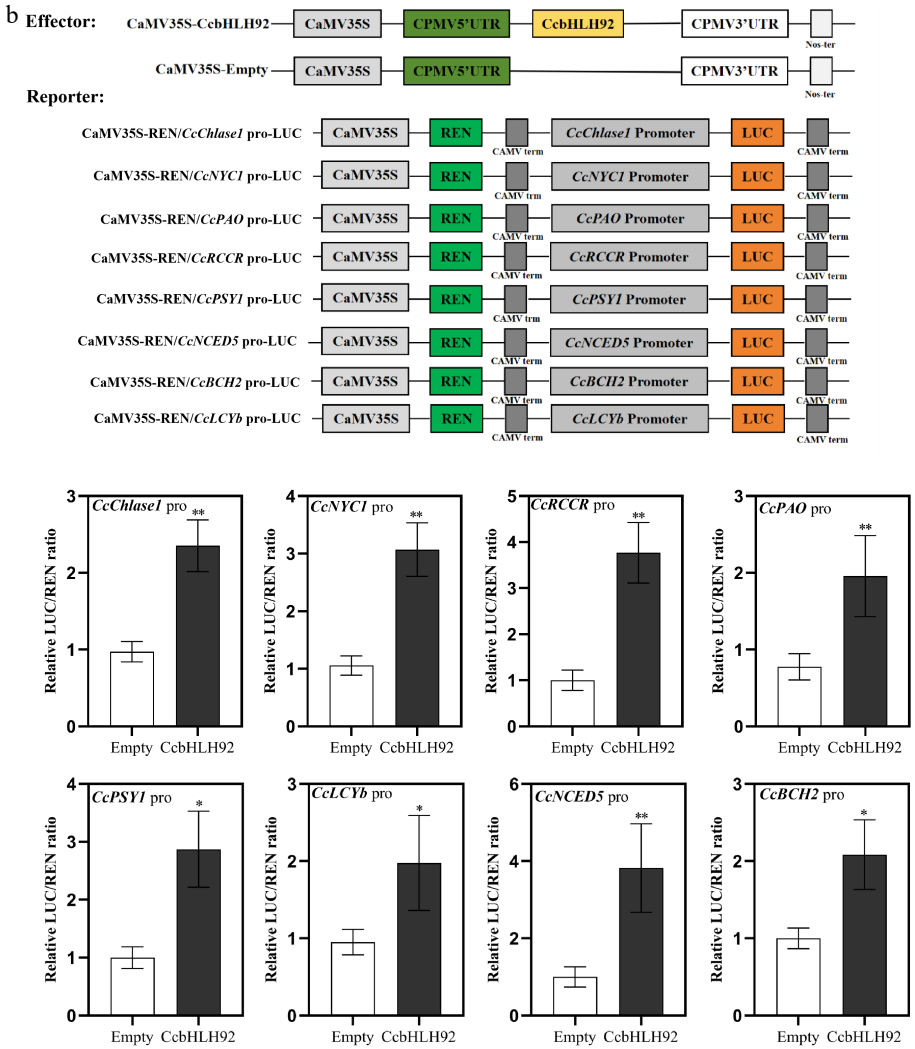


Fig. 8 CcbHLH92 binds and transactivates the promoters of downstream genes. (a) CcbHLH92 binds specifically to G-box *cis*-elements in promoters associated with metabolic pathways of chlorophyll and carotenoids. (b) Transcriptional activation of metabolic pathways of chlorophyll and carotenoid gene promoters by CcbHLH92. In both panels, 'pro' denotes the promoter region of the preceding gene. Data represent mean ± SD (n = 6). * $p < 0.05$; ** $p < 0.01$.

biosynthesis (e.g., *CcPSY1*, *CcBCH2*, *CcNCED5*), thereby accelerating pigment metabolism[3]. The target genes of CcbHLH92 show considerable overlap with those of CcbHLH35, suggesting that CcbHLH35 and CcbHLH92 may form a complex to co-regulate fruit coloration. Our BiFC and Co-IP assays have confirmed the physical interaction between CcbHLH35 and CcbHLH92 in tobacco leaf cells. However, further validation is required to fully elucidate the functional significance of this interaction. A summary model of this regulatory network is proposed in Fig. 11.

Dual roles of abscisic acid in carotenoid accumulation

In this study, we found that CcbHLH92, a mediator of ethylene-induced degreening, effectively regulates fruit coloration. This conclusion is consistent with the observed phenotypic changes, including decreased chlorophyll a and b contents and a distinct shift in carotenoid composition. Notably, the overexpression of CcbHLH92 led to a decline in β-carotene alongside an accumulation of lutein and cryptoxanthin. This specific pattern prompted us to explore the potential involvement of ABA biosynthesis in redirecting carotenoid metabolic flux.

ABA is a key hormone regulating fruit ripening in non-climacteric fruits such as citrus. However, studies examining exogenous ABA treatments across different citrus cultivars have reported inconsistent effects on carotenoid accumulation. Although most research supports a promotive role of ABA in citrus fruit coloration, studies on 'Cara Cara' navel orange, mandarin fruit, and Lisbon lemon juice sacs have demonstrated that ABA suppresses carotenoid accumulation[27]. Although exogenous ABA was not applied in the present study, the involvement of ABA in carotenoid metabolism may provide a potential explanation for the divergent patterns of carotenoid accumulation observed.

ABA exerts dual effects on carotenoid content through opposing mechanisms. In terms of carotenoid consumption, ABA biosynthesis utilizes carotenoids as precursors. Specifically, within the β-branch pathway, β-carotene is catalyzed by zeaxanthin epoxidase (ZEP) to yield zeaxanthin, which is subsequently oxidized by NCED enzymes to produce xanthoxin. Xanthoxin is then transported to the cytoplasm and converted into ABA via the actions of short-chain alcohol dehydrogenases and abscisic aldehyde oxidase (AAO3), the latter being activated by molybdenum cofactor sulfurase (MoCo). As NCED serves as the key rate-limiting enzyme in ABA biosynthesis, its

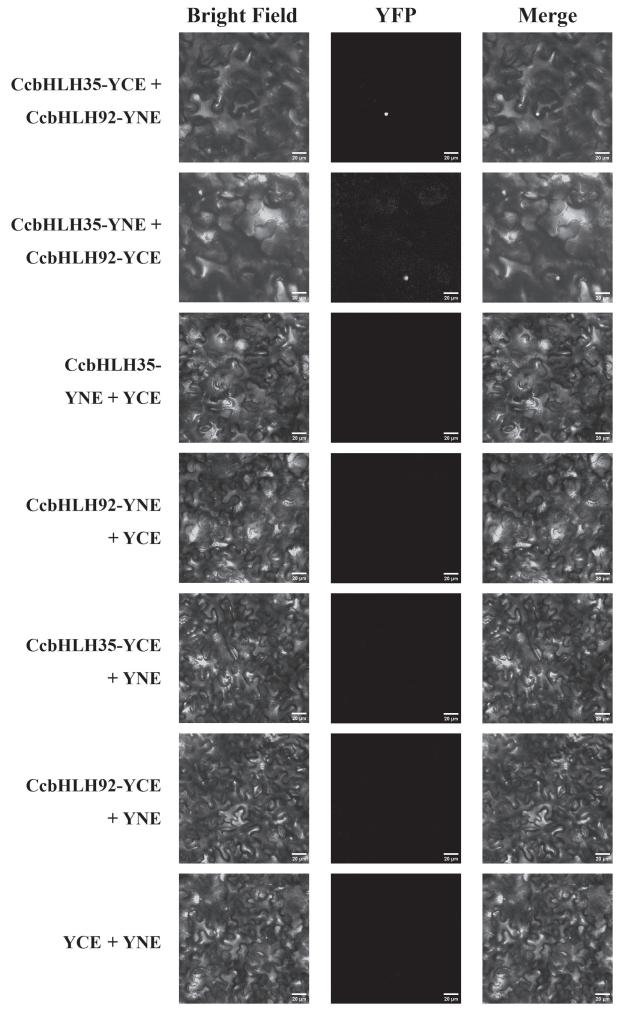


Fig. 9 Protein interactions between CcbHLH35 and CcbHLH92 in tobacco. (CcbHLH35 and CcbHLH92 were constructed into YNE and YCE vectors, respectively, and co-transferred into tobacco leaf cells through the mediation of *Agrobacterium*, and yellow fluorescent signals were observed in the cells).

elevated expression promotes ABA accumulation while concurrently leading to a reduction in carotenoid content—particularly that of β-carotene.

In contrast, in terms of carotenoid accumulation, ABA functions similarly to ethylene by accelerating carotenoid accumulation. As the primary hormone regulating the ripening of non-climacteric fruits, citrus fruits possess a well-established ABA signal transduction pathway. The signal is transmitted through the 'ABA-PYR1-PP2C-SnRK2' core signaling components to regulate fruit ripening[28]. Key receptor proteins of this pathway, including PP2C, PYR/PYL, and SnRK2, have been identified and shown to interact in citrus[29]. This conclusion is consistent with a previous study reporting that the ABA-responsive transcription factor CsbZIP44 enhances carotenoid accumulation by directly binding to the promoters of key biosynthetic genes (e.g., *CsDXR*, *CsGGPPs*, *CsBCH1*, *CsNCED2*), further supporting the critical role of transcriptional regulation in citrus pigmentation[30].

Based on these dual roles, we propose that the alteration in carotenoid composition—particularly the reduction in β-carotene and enrichment of lutein and cryptoxanthin—is determined by the interplay between ABA-driven precursor consumption and signaling-mediated biosynthetic activation. When ABA biosynthesis excessively consumes carotenoid precursors, the accumulation of carotenoids in other branches may be compromised, reshaping the carotenoid profile to promote the characteristic orange-yellow peel coloration.

Conclusions

This study identifies CcbHLH92 as a nuclear-localized transcription factor with transcriptional activation capability. Transient overexpression in tobacco leaves confirmed its role in accelerating chlorophyll degradation. Mechanistically, CcbHLH92 directly binds to and activates key genes in both chlorophyll and carotenoid metabolic pathways, thereby coordinating pigment metabolism in citrus peel. These findings establish a crucial role for CcbHLH92 in ethylene-mediated citrus coloration. Furthermore, BiFC assays and Co-IP provided complementary evidence supporting the physical interaction between CcbHLH92 and CcbHLH35, suggesting their potential involvement in the regulation of mandarin fruit coloration, although the functional significance of this interaction requires further investigation. Collectively, our work significantly advances the understanding of the bHLH-mediated regulatory network controlling citrus fruit coloration.

Ethical statements

Not applicable.

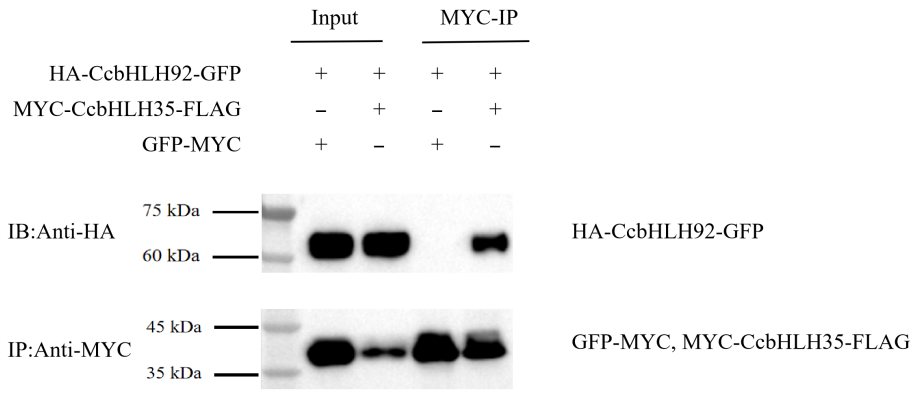


Fig. 10 Co-IP validation of the interaction between CcbHLH92 and CcbHLH35 in tobacco leaves. (Total proteins were extracted from *N. benthamiana* leaves co-expressing the indicated constructs and immunoprecipitated with anti-MYC agarose beads. The immunoprecipitated proteins were detected by immunoblotting with anti-HA and anti-MYC antibodies. GFP-MYC was used as the negative control).

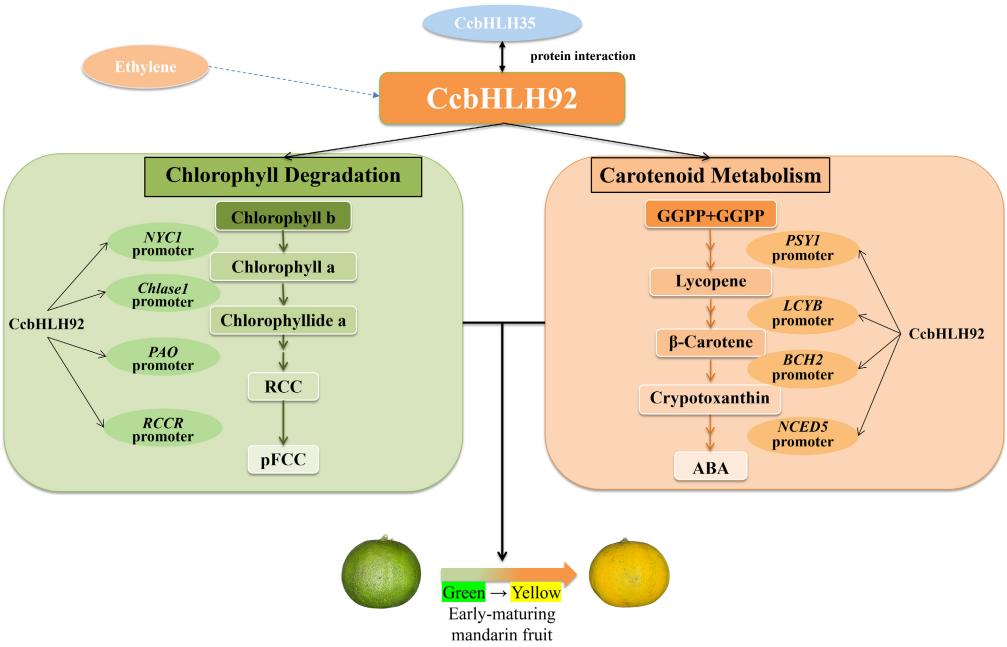


Fig. 11 Proposed transcriptional model for CcbHLH92-mediated chlorophyll degradation and carotenoid metabolism in ethylene-degreened mandarin peels. (CcbHLH92 is induced by ethephon treatment and may interact with CcbHLH35 during the regulation of mandarin fruit coloration. CcbHLH92 directly binds to and activates the promoters of chlorophyll degradation-related genes (*CcNYC1*, *CcChlase1*, *CcPAO*, and *CcRCCR*), thereby accelerating chlorophyll degradation and promoting peel degreening. Meanwhile, CcbHLH92 activates the promoters of carotenoid biosynthesis and metabolism-related genes (*CcPSY1*, *CcLCYb*, *CcBCH2*, and *CcNCED5*), contributing to carotenoid accumulation and metabolic adjustment during fruit ripening. Through the coordinated regulation of chlorophyll degradation and carotenoid metabolism, CcbHLH92 promotes the transition of peel color from green to yellow-orange in early-ripening mandarin fruit. Solid arrows indicate direct regulatory relationships confirmed by experimental evidence).

Author contributions

The authors confirm their contributions to the paper as follows: study conception and design: Deng L, Zeng K; data collection: Liu Q, Deng S; analysis and interpretation of results: Liu Q, Deng S, Liu L; draft manuscript preparation: Liu Q, Peng L, Xie Y. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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References

[1] Morales J, Tárrega A, Salvador A, Navarro P, Besada C. 2020. Impact of ethylene degreening treatment on sensory properties and consumer response to citrus fruits. *Food Research International* 127:108641

[2] Gupta AK, Pathak U, Tongbram T, Medhi M, Terdwongworakul A, et al. 2022. Emerging approaches to determine maturity of citrus fruit. *Critical Reviews in Food Science and Nutrition* 62:5245–5266

[3] Liu Q, Deng S, Liu L, Wang H, Yuan L, et al. 2024. The chlorophyll and carotenoid metabolism in postharvest mandarin fruit peels is co-regulated by transcription factor CcbHLH35. *Postharvest Biology and Technology* 216:113030

[4] Hadjipieri M, Georgiadou EC, Marin A, Diaz-Mula HM, Goulas V, et al. 2017. Metabolic and transcriptional elucidation of the carotenoid biosynthesis pathway in peel and flesh tissue of loquat fruit during on-tree development. *BMC Plant Biology* 17:102

[5] Solymosi K, Mysliwa-Kurdziel B. 2021. The role of membranes and lipid-protein interactions in the Mg-branch of tetrapyrrole biosynthesis. *Frontiers in Plant Science* 12:663309

[6] Tanaka A, Ito H. 2025. Chlorophyll degradation and its physiological function. *Plant and Cell Physiology* 66:139–152

[7] Gong J, Yang H, Xu Y, Zeng Y, Liu P, et al. 2023. Differential regulation of red light- and ethephon-induced degreening in postharvest kumquat fruit. *Postharvest Biology and Technology* 198:112264

[8] Yuan Z, Deng L, Yin B, Yao S, Zeng K. 2017. Effects of blue LED light irradiation on pigment metabolism of ethephon-degreened mandarin fruit. *Postharvest Biology and Technology* 134:45–54

[9] Mitalo OW, Otsuki T, Okada R, Obitsu S, Masuda K, et al. 2020. Low temperature modulates natural peel degreening in lemon fruit independently of endogenous ethylene. *Journal of Experimental Botany* 71:4778–4796

[10] Alós E, Distefano G, Rodrigo MJ, Gentile A, Zacarías L. 2014. Altered sensitivity to ethylene in 'Tardivo', a late-ripening mutant of Clementine mandarin. *Physiologia Plantarum* 151:507–521

[11] Rodrigo MJ, Zacarías L. 2007. Effect of postharvest ethylene treatment on carotenoid accumulation and the expression of carotenoid biosynthetic genes in the flavedo of orange (*Citrus sinensis* L. Osbeck) fruit. *Postharvest Biology and Technology* 43:14–22
[12] Stewart I, Wheaton TA. 1972. Carotenoids in citrus. Their accumulation induced by ethylene. *Journal of Agricultural and Food Chemistry* 20:448–449
[13] Lu Y, Li D, Li L, Belwal T, Xu Y, et al. 2020. Effects of elevated CO_2 on pigment metabolism of postharvest mandarin fruit for degreening. *Food Chemistry* 318:126462
[14] Zhao L, Gao L, Wang H, Chen X, Wang Y, et al. 2013. The R2R3-MYB, bHLH, WD40, and related transcription factors in flavonoid biosynthesis. *Functional & Integrative Genomics* 13:75–98
[15] Zhu Z, Liang H, Chen G, Li F, Wang Y, et al. 2019. The bHLH transcription factor *SlPRE2* regulates tomato fruit development and modulates plant response to gibberellin. *Plant Cell Reports* 38:1053–1064
[16] Sun Q, He Z, Zhang Y, Feng D, Wei R, et al. 2025. CsbHLH6 positively regulates abscisic acid-mediated citrus fruit coloration during postharvest storage. *Postharvest Biology and Technology* 219:113229
[17] Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 25:402–408
[18] Yin XR, Xie XL, Xia XJ, Yu JQ, Ferguson IB, et al. 2016. Involvement of an ethylene response factor in chlorophyll degradation during citrus fruit degreening. *The Plant Journal* 86:403–412
[19] Lichtenthaler HK, Wellburn AR. 1983. Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. *Biochemical Society Transactions* 11:591–592
[20] Noga G, Lenz F. 1983. Separation of citrus carotenoids by reversed-phase high-performance liquid chromatography. *Chromatographia* 17:139–142
[21] Lado J, Alós E, Manzi M, Cronje PJR, Gómez-Cadenas A, et al. 2019. Light regulation of carotenoid biosynthesis in the peel of mandarin and sweet orange fruits. *Frontiers in Plant Science* 10:1288
[22] Jiang Y, Yang B, Deyholos MK. 2009. Functional characterization of the Arabidopsis *bHLH92* transcription factor in abiotic stress. *Molecular Genetics and Genomics* 282:503–516
[23] Sun Q, He Z, Ye J, Wei R, Feng D, et al. 2025. A novel C2H2-type zinc-finger transcription factor, CitZAT4, regulates ethylene-induced orange coloration in Satsuma mandarin flavedo (*Citrus unshiu* Marc.). *Journal of Integrative Plant Biology* 67:294–310
[24] Pereira L, Teng C. 2025. Turn up the red: MADS-RIN-DIVARICATA1 module positively regulates carotenoid biosynthesis in nonclimacteric pepper fruits. *Plant Physiology* 197:kiaf104
[25] Butelli E, Licciardello C, Ramadugu C, Durand-Hulak M, Celant A, et al. 2019. *Noemi* controls production of flavonoid pigments and fruit acidity and illustrates the domestication routes of modern citrus varieties. *Current Biology* 29:158–164.e2
[26] Zhou D, Shen Y, Zhou P, Fatima M, Lin J, et al. 2019. Papaya *CpbHLH1/2* regulate carotenoid biosynthesis-related genes during papaya fruit ripening. *Horticulture Research* 6:80
[27] Liu C, Zhang H, He M, Liu X, Chen S, et al. 2021. Lycopene accumulation in cara cara red-flesh navel orange is correlated with weak abscisic acid catabolism. *Journal of Agricultural and Food Chemistry* 69:8236–8246
[28] Hou BZ, Chen XH, Shen YY. 2021. Interactions between strawberry ABA receptor PYR/PYLs and protein phosphatase PP2Cs on basis of transcriptome and yeast two-hybrid analyses. *Journal of Plant Growth Regulation* 40:594–602
[29] Romero P, Lafuente MT, Rodrigo MJ. 2012. The *Citrus* ABA signalosome: identification and transcriptional regulation during sweet orange fruit ripening and leaf dehydration. *Journal of Experimental Botany* 63:4931–4945
[30] Sun Q, He Z, Wei R, Zhang Y, Ye J, et al. 2024. The transcriptional regulatory module CsHB5-CsbZIP44 positively regulates abscisic acid-mediated carotenoid biosynthesis in citrus (*Citrus* spp.). *Plant Biotechnology Journal* 22:722–737

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