

CsMIEL1-CsMYB113 regulatory module mediates temperature-dependent anthocyanin accumulation in blood orange

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Research Article

Keywords: Blood orange, Anthocyanin biosynthesis, CsMYB113, High temperature, Ubiquitination

Posted Date: October 29th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11121895/v1>

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Additional Declarations: No competing interests reported.

Abstract

Background

The accumulation and pigmentation of anthocyanins in blood oranges are highly temperature-dependent. However, the molecular mechanism by which high temperatures suppress anthocyanin biosynthesis and metabolism remains unclear.

Results

In this study, blood oranges at different developmental stages were subjected to gradient temperature treatments, followed by comprehensive metabolic profiling analysis. The results revealed that high temperature specifically blocks the synthesis and metabolism of cyanidin-3-O-glucoside and cyanidin-3-O-(6-O-malonyl-beta-D-glucoside), which are responsible for the pigmentation of blood oranges. Transcriptional analysis revealed that the expression levels of *CsF3H*, *CsDFR*, *CsANS*, and *CsBZ1*, which are responsible for the biosynthesis of cyanidin-3-O-glucoside and cyanidin-3-O-(6-O-malonyl-beta-D-glucoside), were significantly and positively correlated with *CsMYB113* and were negatively regulated by high temperature. The transient transformation assay of tobacco leaves and the expression profile analysis revealed that the ubiquitinated protein *CsMIEL1*, activated by high temperature, inhibited the anthocyanin accumulation induced by *CsMYB113* in tobacco leaves. Protein-protein interaction assays further confirmed the interaction between *CsMIEL1* and *CsMYB113*, emphasizing the ubiquitination process of *CsMYB113* mediated by *CsMIEL1*.

Conclusion

In summary, this study identified the key anthocyanin subtypes that determine the pigmentation of blood oranges and the corresponding biosynthetic pathways. A hypothesis was constructed that *CsMIEL1* induced by high temperature ubiquitinated and degraded *CsMYB113*, blocking anthocyanin biosynthesis and resulting in the loss of pigment in blood oranges. These findings provide novel insights into the molecular mechanism by which high temperature inhibits anthocyanin biosynthesis in blood oranges.

1. Background

Anthocyanins are water-soluble flavonoid pigments, contributing to the coloration of fruits and flowers. The biosynthesis of anthocyanins is governed by a highly conserved yet species-specific regulatory network, modulated by developmental programs and environmental factors (Liu et al., 2021). Among abiotic stressors, high temperature represents a primary factor driving anthocyanin degradation, compromising both chromatic quality and nutritional integrity of horticultural produce during cultivation, postharvest storage, and thermal processing.

Extensive research has documented temperature-dependent modulation of anthocyanin accumulation across diverse plant species. In rose anthocyanin extracts subjected to spray drying, high-temperature treatment significantly reduced total anthocyanin content (Nguyen et al., 2022). In the study of grapevines, high temperature altered both the quantity and compositional profile of accumulation in cultivars including 'Malbec', 'Merlot', and 'Pinot Noir', and a response shown to be genotype-dependent (de Rosas et al., 2022). Integrated metabolomic and transcriptomic analysis of 'Summer Black' and 'Flame Seedless' grapes suggested that high-temperature stress during maturation suppresses anthocyanin biosynthesis at multiple molecular levels, providing mechanistic insights into thermally induced color loss (Dou et al., 2024). Transcription factor *FHY3* integrates auxin signaling and endoplasmic reticulum stress pathways under high-temperature conditions, directly activates core biosynthetic genes via promoter binding, and its activity is post-translationally modulated by endoplasmic reticulum stress, thereby highlighting a sophisticated, multi-layered control mechanism (Sun et al., 2024). Transcriptome analysis across different species further corroborates the complexity of thermal responses. The color changes in flowers under high-temperature stress indicated that the anthocyanin biosynthesis pathway has changed (Shi et al., 2022). Similarly, high temperature inhibits anthocyanin accumulation in sweet cherry fruits by promoting the decomposition of abscisic acid (ABA), and there is a significant correlation between hormone levels and anthocyanin content, suggesting that hormone regulation is a key component of the temperature response (Tan et al., 2023).

The MYB family of transcription factors plays a pivotal role in regulating anthocyanin biosynthesis across diverse plant species (Zhou et al., 2025; Lan et al., 2026). Extensive studies have elucidated the functional diversity and regulatory mechanisms of MYB proteins, particularly within the R2R3-MYB subgroup, highlighting their critical involvement in pigment accumulation and adaptive response to environmental cues. In pomegranate, an R2R3-MYB transcription factor was isolated and functionally characterized, revealing its essential role in anthocyanin accumulation during fruit color development (Khaksar et al., 2015). In blueberry, *MYBA* as a subgroup 6 R2R3-MYB activates anthocyanin biosynthesis. Functional assays demonstrated that *MYBA* can complement mutant phenotypes, in cooperation with bHLH co-factors, and robustly enhance pigment production, highlighting the conserved role of subgroup 6 MYBs as transcriptional activators (Plunkett et al., 2018). Similarly, *VrMYB90*, a subgroup 6 activator, forms a regulatory module with *VrMYB3* to coordinately control anthocyanin biosynthesis in mung bean. Their interaction suggests a finely tuned balance between activation and repression to precisely control pigment levels (Chen et al., 2023). Recent research has further expanded our understanding by integrating signaling pathways and environmental modulation. Niu et al. (2023) demonstrated that sugar signaling, rather than oxidative stress, predominantly regulates anthocyanin accumulation via R2R3-MYBs such as *RsMYB39* and *RsMYB82* in radish. These MYBs regulate genes involved in anthocyanin transport and biosynthesis, linking metabolic signals to pigment regulation. Conversely, *SlMYB7*, as an AtMYB4-like subgroup 4 MYB in tomato, functions as a transcriptional repressor that suppresses anthocyanin accumulation by downregulating key structural genes in the pathway (Zhang et al., 2023). Likewise, *VvMYB44-1*, a transcriptional repressor in grapevine, is strongly induced by high temperature in developing fruits. Its overexpression significantly inhibits anthocyanin

biosynthesis by directly repressing the expression of core biosynthetic genes, thereby achieving a mechanistic link between thermal stress and reduced pigmentation (Leng et al., 2025). This underscores the dual regulatory functions of MYB proteins in either promoting or suppressing pigment biosynthesis. Collectively, these studies illustrate that MYB transcription factors serve as central regulators of anthocyanin biosynthesis, exhibiting functional versatility ranging from activation to repression. Their interactions with partner proteins, responsiveness to environmental signals, and evolutionary diversification collectively highlight the intricate regulatory network governing pigment accumulation in plants.

Blood orange (*Citrus sinensis*) is a distinctive citrus cultivar of accumulating substantial levels of anthocyanins in its flesh, conferring a characteristic red color and enhanced commercial value. Anthocyanin accumulation in blood oranges is governed by a complex interplay among genetic determinants, environmental conditions, and physiological status. Flavonol 3-hydroxylase (F3H), a key enzyme in the flavonoid pathway, whose products serve as direct precursors for anthocyanin biosynthesis, represents a promising molecular target for enhancing anthocyanin content (Ma et al., 2023). Regulatory genes such as *CsTT8* and MYB transcription factors play pivotal roles in orchestrating anthocyanin biosynthesis. Specifically, *CsTT8* functions as a critical regulatory hub. Through alternative splicing, it promotes both anthocyanins and citric acid production and forms a transcriptional activation complex with MYB proteins to induce expression of biosynthetic genes (Wang et al., 2023). Similarly, the R2R3 MYB factor Ruby1 is regulated by ethylene-response factors that are induced under cold stress, thereby positively regulating anthocyanin biosynthesis (Wang et al., 2024). Light exposure exerts a significant impact on anthocyanin accumulation. Ultraviolet radiation promotes anthocyanin biosynthesis in blood orange peel by activating downstream glycosylation pathways (Yang et al., 2025). This finding is consistent with earlier research results, which indicate that ultraviolet radiation, as part of the spectrum, can stimulate anthocyanin synthesis. Additionally, coordinated regulation across multiple metabolic and signaling pathways is required for the temporal and spatial control of anthocyanin biosynthesis. *CsBBX24* modulates carotenoid and anthocyanin biosynthesis during fruit ripening, indicating coordinated regulation of the pigment biosynthetic pathway, which in turn influences both fruit coloration and nutritional quality (Fu et al., 2025).

Although the biosynthesis and metabolism of anthocyanins have been extensively and comprehensively elucidated, heat-induced anthocyanin degradation in blood oranges remains a prevalent and persistent challenge in production cultivation, and the underlying molecular mechanisms are still poorly understood. In this study, we employed blood orange samples subjected to gradient temperature treatments as experimental material. Based on integrating transcriptomics and metabolomics analyses with transient transformation and protein-protein interaction assays, it was confirmed that one of the anthocyanin subclasses, cyanidin, is the key factor responsible for the coloring of blood orange. Transcription factor *CsMYB113* was further identified to promote anthocyanin accumulation by inducing the expression of anthocyanin synthesis genes. A mechanistic hypothesis was proposed that high temperature triggers the ubiquitination and subsequent proteasomal degradation of *CsMYB113* mediated by *CsMIEL1*, thereby inhibiting anthocyanin biosynthesis.

2. Methods

2.1 Plant materials and processing

The blood oranges were sourced from the resource garden of Chongqing Academy of Agricultural Sciences. Four temperature gradients of 2.3°C (CK), 8°C (LT), 12°C (MT) and 16°C (HT) were employed using dedicated temperature facilities at 205 days after flowering (DAF). Treated samples were collected at 240, 255, 270 and 285 DAF, and labeled according to the corresponding four developmental stages.

2.2 Anthocyanin Metabolism Analysis

The sample was freeze-dried, ground into powder and extracted with 0.5 mL methanol/water/hydrochloric acid (500:500:1, v/v/v). Then the extract was vortexed for 5 min, sonicated for 5 min and centrifuged at 12,000 g under 4°C for 3 min. The residue was re-extracted by repeating the above steps under the same conditions. The supernatants were collected and filtered through a membrane filter (0.22 µm, Anpel) before LC-MS/MS analysis.

2.3 RNA-seq

Total RNA was extracted using Trizol reagent, and high-quality RNA samples with RIN value greater than 7.0 were selected to construct the sequencing library. Dual-end sequencing (PE150) was performed on an Illumina Novaseq™ 6000, and clean data were obtained after filtering. All the sample read sequences were aligned to the sweet orange reference genome using the HISAT2 software package. Use the StringTie software to assemble according to the default parameters. Subsequently, combine all the transcripts of all samples and use the gffcompare software to reconstruct a comprehensive transcriptome. After generating the final transcriptome, use StringTie and ballgown to estimate the expression levels of all transcripts and determine the expression abundance of mRNA by calculating the FPKM (fragments per kilobase of transcript per million mapped reads) value.

2.4 STEM

Using gradient temperature treatment as the horizontal axis, a gene spatiotemporal expression trend graph was generated by using STEM to normalize the data on a logarithmic scale. In the filtering module, the significance level of the model graph was 0.05. Spectra with significant differences were rendered in different background colors. The minimum correlation of the clustering graph was 0.7, and spectra with the same trend were represented by the same background color.

2.5 WGCNA

Among the 16 samples, a gene was retained if its FPKM value was greater than 0.3 in any sample. The co-expression network was constructed using the R software "WGCNA" package (version 1.7.3). The power estimation value was 12; the minimum module size was set to 30; and the merge cut-off was set to 0.25. The anthocyanin content and *CsMYB113* expression level were used to construct the module

correlation graph. The TOMsimilarityFromExpr function built into the WGCNA package was used to construct the co-expression network, and the selected threshold was 0.2.

2.6 RT-qPCR

The expression of *CsMYB113* and *CsMIEL1* was analyzed using the QuantStudio™ 6 Flex real-time PCR instrument (Amersham Company, USA), and three replicate samples were set up. The PCR mixture was prepared using the Power SYBR Green PCR master mix.

2.7 Tobacco injection assays

The constructed recombinant vector was transferred into GV3101 *Agrobacterium*. After activation, the OD₆₀₀ was adjusted to 0.6 with a buffer solution. It was injected into the leaves of *Nicotiana benthamiana* that had been growing for 6 weeks. After dark incubation for 2 days, the samples were observed and collected.

2.8 Total anthocyanin extraction

Place the target tissue in the pigment extraction buffer (containing 1% hydrochloric acid in methanol) and perform ultrasonic extraction at 4°C in the dark for 30 minutes. Centrifuge at 4000 rpm for 10 minutes, then aspirate the supernatant and measure the absorbance values at A530 and A657. Calculate the total anthocyanin content based on the formula $A530 - 0.25 \times A657$.

2.9 Dual luciferase assays

The transcription factor CDS and the gene promoter sequence were respectively ligated to the pGreen[®] 62-SK and pGreen[®] 0800-LUC vectors. After verification by Sanger sequencing and confirmation, they were transferred into GV3101 *Agrobacterium*. A bacterial solution with a ratio of transcription factor to promoter of 4:1 was prepared. The solution was injected into tobacco leaves and cultured in the dark for 2 days. For the morphological observation, the leaves were coated with D-luciferin fluorescent solution and then observed using laser confocal microscopy. The Promega kit was used to lyse and extract the target tissue, and the LUC and REN values were detected using a microplate reader.

2.10 Bimolecular fluorescence complementation (BiFC)

The *CsMYB113* gene was cloned into the pSPYNE vector, and the *CsMIEL1* gene was cloned into the pSPYCE vector. Both were then transferred into the GV3101 *Agrobacterium* strain. After streaking for activation, the OD₆₀₀ was adjusted to 0.6. The recombinant plasmid and the control plasmid were mixed with the nuclear marker and P19 at a ratio of 3:3:2:1. After being left to stand for 2 hours, they were injected into tobacco leaves. After dark cultivation for 2 days, they were observed using a laser confocal microscope.

2.11 Yeast two-hybrid assay

The CDS of *CsMIEL1* and *CsMYB113* were respectively cloned into the pGADT7 and pGBKT7 plasmids. The pGBKT7-*CsMYB113* + empty pGADT7 and pGBKT7-*CsMYB113* + pGADT7-*CsMIEL1* two combinations

were respectively co-transformed into Y2HGold, and then plated on two-hole and four-hole plates. The cultures were incubated at 30°C for 3–5 days, and the colony growth was observed to determine the interaction relationship.

3. Results

3.1 Changes in anthocyanin content of blood orange under gradient temperature treatments

To investigate the molecular mechanisms underlying natural pigmentation and the temperature-dependent suppression of pigmentation in blood oranges, fruit samples were collected at four distinct developmental stages (Fig. 1A). The pigmentation of blood orange did not commence at the early development stage (S1, 240 DAF), after which pigmentation progressively intensified throughout subsequent stages. Under controlled temperature treatments, coloration at each developmental stage exhibited a clear gradient response. Relative to low-temperature treatment, both moderate- and high-temperature treatments markedly suppressed pigmentation, with this inhibitory effect becoming more pronounced during later developmental stages (S3-S4, 270–285 DAF) (Fig. 1A). Given that anthocyanins are the primary determinants of blood orange coloration, we quantified individual anthocyanin compounds across developmental stages and temperature regimes (Fig. 1B, Table S1). Among the detected anthocyanin subclasses, cyanidin-3-O-galactoside and cyanidin-3-O-xyloside remained consistently low in abundance throughout development. Peonidin-3-O-(6-O-p-coumaroyl)-glucoside and rutin showed no significant variation across stages or temperature treatments. Notably, the contents of cyanidin-3-O-(6-O-malonyl-beta-D-glucoside) and cyanidin-3-O-glucoside were extremely low in S1, but increased significantly from S2 through S4. Moreover, at any given developmental stage, these two subclass anthocyanin content was inversely correlated with treatment temperature (Fig. 1B). A close correlation between the content changes of anthocyanin-3-O-(6-O-maleoyl- beta-D-glucoside) and anthocyanin-3-O-glucoside and color change of blood oranges, suggesting that cyanidin-type anthocyanins are the main substances responsible for blood orange pigmentation, and are negatively regulated by temperature within a certain range.

3.2 Transcriptome sequencing and expression profiling identification of blood oranges under gradient temperature conditions

To gain insight into the regulatory mechanisms underlying anthocyanin biosynthesis and metabolism, RNA-seq was performed on a total of 48 samples representing dynamic developmental stages and gradient temperature treatments. The three biological replicates exhibited tight clustering in multivariate space (Figure S1A), confirming high reproducibility in both sample collection and processing. Principal component analysis (PCA) revealed clear, stage-specific clustering corresponding to the four developmental stages. The first principal component (PC1) and second principal component (PC2)

accounted for 28.7% and 20.8% of the total transcriptional variation, respectively (Figure S1B). Notably, samples were ordered strictly along the PC1 axis according to their chronological developmental sequence, reflecting a coherent temporal progression in global gene expression patterns. Moreover, relative to control, low-temperature (LT) and moderate-temperature (MT) treatments, high-temperature (HT)-treated samples displayed greater dispersion in PCA space, indicating that HT stress induced substantial and heterogeneous transcriptional reprogramming.

The Short Time-series Expression Miner (STEM) was employed to construct co-expression modules under gradient temperature treatments to identify genes closely related to anthocyanin metabolism (Fig. 2A). Among the four developmental stages, several expression profiles were shared, whereas others were stage-specific. For instance, profile 37 and profile 47 were uniquely enriched in S1, suggesting potential roles in early-stage responses to temperature fluctuations together. Profile 26 was significantly enriched in S4 and included genes implicated in temperature-responsive regulation during the late phase of blood orange development. Profiles 9 and 42 exhibited linear downward and upward trends, respectively, across the low-to-high temperature gradient, implying that the genes within these modules are positively and negatively correlated with anthocyanin metabolism. Gene Ontology (GO) enrichment analysis was subsequently conducted for genes in these profiles (Fig. 2A). Nine biological processes potentially associated with anthocyanin metabolism were significantly enriched (Fig. 2B). Among them, the flavonoid biosynthesis process, the flavonoid metabolic process, the phenylpropanoid biosynthesis process and the phenylpropanoid metabolic process were markedly enriched in both S3_Profile 9 and S4_Profile 9, suggesting that the expression patterns of the corresponding genes are tightly correlated with anthocyanin accumulation.

3.3 Identification of anthocyanin synthesis genes and upstream regulatory factors

Integrated metabolomic and transcriptomic analyses indicated that the anthocyanin accumulation in blood oranges is cyanidin-type, and their biosynthesis proceeds via the flavonoid metabolic pathway. The analysis of the gene expression along the specific biosynthesis pathways of anthocyanins revealed that the expression levels of *CsANS*, *CsBZ1* and *Cs3MaT1*, which catalyze the synthesis of cyanidin, cyanidin-3-O-glucoside and cyanidin-3-O-(6-O-malonyl-beta-D-glucoside), exhibited strong temporal and quantitative concordance with anthocyanin accumulation (Fig. 3A). Notably, cyanidin itself was undetectable in blood orange, suggesting that *CsBZ1* may rapidly and efficiently catalyze the conversion of cyanidin into cyanidin-3-O-glucoside, or that the form of cyanidin is unstable. MYB is a key transcription factor family regulating anthocyanin synthesis, and 27 MYB transcription factors with distinct expressions were identified based on the differential expression analysis of comparison groups (Fig. 3B, Table S2). Expression correlation analysis between these MYB transcription factors and anthocyanin biosynthesis genes revealed that the expression of *CsMYB113* was downregulated by 2 to 17-fold under high temperature compared with CK. Additionally, particularly high correlations between the *CsMYB113* and *CsBZ1* (0.95), *CsANS* (0.93) and *Cs3MaT1* (0.62) (Fig. 3C) collectively supported that

CsMYB113 is negatively regulated by high temperature and serves as a potential candidate regulator of cyanidin-3-O-glucoside and cyanidin-3-O-(6-O-malonyl-beta-D-glucoside) biosynthesis.

3.4 Weighted gene co-expression network analysis

A total of 18,537 genes were subjected to weighted gene co-expression network analysis (WGCNA) to construct a co-expression network (Figure S2A, Table S3). This analysis identified 17 statistically significant modules, along with a gray module comprising genes that could not be confidently assigned to any specific module (Figure S2B). Notably, the eigengene expression profiles of several modules, such as the brown and lightcyan module, exhibited strong positive correlations with the temporal dynamics of anthocyanin accumulation across developmental stages (Figure S2C), suggesting functional relevance to anthocyanin biosynthesis. The purple and turquoise modules show a gradually decreasing expression trend across four developmental stages, but were negatively and positively correlated with the gradient temperature, respectively. The red module was not induced by the gradient temperature, but a significant upward trend is present at the stage S4. These genes may be responsible for the ripening of blood oranges. Building upon prior evidence implicating *CsMYB113* in anthocyanin regulation, we systematically evaluated its module membership and intramodular connectivity (Fig. 4A). *CsMYB113* showed the strongest association with the green module (correlation = 0.92), whose module eigengene displayed a consistent linear decline in expression from low to high temperature within each developmental stage (Fig. 4B). Intriguingly, peak expression of the green module eigengene occurred at stage S3 rather than S4, indicating that *CsMYB113* transcriptional activation precedes maximal anthocyanin accumulation by one developmental stage. This temporal offset is biologically plausible, as *CsMYB113* likely functions upstream in the regulatory genes involved in anthocyanin biosynthesis. GO enrichment analysis of green module genes revealed significant overrepresentation of terms related to flavonoid biosynthesis, in addition to core metabolic processes such as biosynthesis of amino acids and carbon metabolism (Fig. 4C). This result highlighted the central role of *CsMYB113* in flavonoid metabolism and anthocyanin synthesis. A refined coexpression network was subsequently extracted from the green module, yielding 20 transcription factors that exhibit highly correlated expression patterns (Fig. 4D). These co-regulated factors represent a functionally coordinated transcriptional module potentially governing the spatiotemporal regulation of anthocyanin production.

3.5 Regulatory module analysis of anthocyanin degradation induced by high temperature

To clarify the biological function of *CsMYB113* and the molecular mechanism underlying heat-induced anthocyanin degradation in blood oranges, we identified a ubiquitination protein-coding gene, *CsMIEL1*. The expression profiling revealed an inverse correlation between *CsMYB113* and *CsMIEL1* across developmental stages (Fig. 5A). *CsMYB113* expression shows a negative correlation with the gradient temperature treatment, especially during the first three periods of anthocyanin biosynthesis. Conversely, *CsMIEL1* expression was markedly elevated under MT and HT treatments relative to CK and LT

conditions, indicating that its expression is induced by a relative range of high temperatures rather than being completely linear (Fig. 5A). The instantaneous injection of tobacco (*N. benthamiana*) was used to conduct the primary verification of the functions of *CsMYB113* and *CsMIEL1*. The results showed that anthocyanin accumulation occurred only upon *CsMYB113* expression, either alone or in combination with *CsMIEL1*, whereas no accumulation was observed with empty vector or *CsMIEL1*-only controls (Fig. 5B). Notably, anthocyanin levels were reduced in the *CsMYB113*-*CsMIEL1* co-expression group compared to *CsMYB113* alone, suggesting that *CsMIEL1* likely mediates *CsMYB113* protein degradation via ubiquitination, thereby attenuating anthocyanin biosynthesis (Fig. 5B, C). Quantitative PCR revealed that co-transfection of *CsMYB113* and *CsMIEL1* suppressed the transcript levels of both genes. The expression inhibition originated from the reduction of *CsMYB113* protein level mediated by *CsMIEL1*, as the protein can activate the promoter of *CsMYB113* (Fig. 5D).

Bimolecular fluorescence complementation assay was employed to validate direct physical interaction. A robust yellow fluorescence signal was detected exclusively in co-infiltrated leaves expressing *CsMYB113*-YNE and *CsMIEL1*-YCE fusions, whereas no fluorescence was observed in any negative controls, including YNE/YCE pairwise combinations and either protein paired with its respective empty vector (Fig. 6A). Consistent with these findings, yeast two-hybrid and pull-down assays confirmed a specific, direct protein-protein interaction between *CsMYB113* and *CsMIEL1* (Fig. 6B-C). These results emphasize that the heat-induced *CsMIEL1*, through ubiquitination-mediated degradation of *CsMYB113*, inhibits the anthocyanin biosynthesis pathway, ultimately leading to the failure of the blood orange coloration.

4. Discussion

Anthocyanins are water-soluble pigments that give fruits their rich colors and enhance their commercial value. To date, more than 20 types of anthocyanins have been identified, among which anthocyanin (cyanidin), pelargonidin, delphinidin, peonidin, malvidin, and petunidin are widely present in fruit trees (Liu et al., 2018). The types and modification structures of anthocyanin substances jointly determine the diversity of anthocyanins, thereby presenting various colors. In strawberries, the p-cyanidin-3-glucoside accounts for more than 70% of the total anthocyanin content (Kosar et al., 2004). The main component of anthocyanins is anthocyanin-3-galactoside in the skins of apples and pears (Pomar et al., 2005; Zhai et al., 2014). For blood orange, several studies suggested cyanidin 3-glucoside and cyanidin 3-(6-malonyl-glucoside) were the dominant pigments (Fabroni et al., 2016). In our study, only the content changes of cyanidin 3-O-glucoside and cyanidin 3-O-(6-O-malonyl-beta-D-glucoside) were consistent with the appearance changes of blood oranges under dynamic development and gradient temperature treatment (Fig. 1A-E), which is in line with the previous research results.

The anthocyanin synthesis pathway is highly conserved in plants, with structural genes such as PAL, 4CL, CHS, F3H, DFR, and ANS responsible for anthocyanin synthesis (Saito et al., 2013). The transcription of these structural genes is dependent on the MYB-bHLH-WD40 complex, of which the MYB transcription factor is the core component. Multiple transcription factors and small RNAs have been

reported to interact with the MBW complex or MYB genes, affecting the transcriptional regulation of anthocyanin synthesis genes to cause changes in anthocyanin content (Liu et al., 2016; Verweij et al., 2016; Zhang et al., 2025). The latest research indicates that the stability of the MBW complex components is a key target of temperature influence on anthocyanin synthesis. For example, the heat-induced transcription factor *PpHSFB2A* inhibits the expression of *PpHY5L*, thereby inhibiting anthocyanin synthesis in pear (Wang et al., 2025). In apples, the heat-induced GATA transcription factor *MdGATA15* inhibits the expression of anthocyanin synthesis-related genes *MdMYB11* and *MdANS*, while activating the expression of anthocyanin synthesis inhibitor *MdMYB308*, resulting in a dual inhibition of anthocyanin synthesis (Fang et al., 2025). In this study, the expression levels of *CsF3H*, *CsDFR*, *CsANS*, and *CsBZ1* in the anthocyanin glycoside synthesis pathway all show negative regulation by high temperature (Fig. 3A). At the same time, MYB transcription factors, especially *CsMYB113*, exhibit similar expression patterns and high expression correlations to these genes (Fig. 3B, C). The transient injection experiment in tobacco leaves confirmed that overexpression of *CsMYB113* promotes anthocyanin accumulation, but which key target gene this phenomenon mainly depends on still needs further clarification (Fig. 5B, C). Interestingly, *CsMYB113* can bind to its own promoter to form a feedback pathway (Figure S3), enhancing the dynamic regulation of anthocyanin synthesis, which is similar to *MdGATA15* induced by heat expression (Fang et al., 2025).

Low temperatures commonly promote anthocyanin biosynthesis as a photoprotective response (Zhang et al., 2026), whereas high temperatures consistently suppress pigment accumulation. This thermosensitive inhibition has historically been attributed to transcriptional downregulation of core anthocyanin biosynthetic genes, including *F3H*, *DFR*, *ANS*, and *BZ1* (Leng et al., 2025). Consistent with this, our study demonstrates that high-temperature stress significantly represses the expression of these four structural genes, and their transcript levels correlate strongly with temporal dynamics of anthocyanin content (Fig. 1A, 3A). However, emerging proteomic and molecular evidence indicates that heat-induced anthocyanin loss is an active, regulated process mediated by the ubiquitin–26S proteasome system (UPS). As a highly selective post-translational regulatory machinery, UPS targets specific proteins for degradation via polyubiquitination. Notably, recent studies have shown that UPS directly destabilizes the MYB-bHLH-WD40 (MBW) transcriptional activation complex, particularly through ubiquitin-dependent degradation of R2R3-MYB factors, which rapidly dismantles the transcriptional framework essential for sustained anthocyanin production (Hong et al., 2023). Conversely, transcription factor stability can be preserved through counter-regulatory deubiquitination. The deubiquitinating enzyme MdUBP15 stabilizes MdGLK1 by antagonizing the E3 ligase MdPUB17 in apple (An et al., 2026). Similarly, heat stress upregulates the expression of *VvMYB44*, thereby inhibiting anthocyanin accumulation in grapevine (Leng et al., 2025). In pear, the E3 ligase PpPUB59 mediates ubiquitination and degradation of PpBBX24, leading to diminished anthocyanin synthesis (Li et al., 2025). Collectively, these findings underscore a conserved adaptive strategy, which post-translational regulation enables plants to rapidly terminate the energetically costly anthocyanin biosynthetic pathway upon thermal disruption of cellular homeostasis.

In our study, *CsMYB113* was identified as a central transcriptional regulator of anthocyanin biosynthesis in blood orange. The direct physical interaction between *CsMYB113* and the ubiquitinated protein *CsMIEL1* was verified (Fig. 6). In previous studies, it has been demonstrated that *CsMIEL1* mediates the ubiquitination and degradation of MYB transcription factors (An et al., 2020), which is consistent with our research results. Expression profiling reveals that *CsMIEL1* is markedly upregulated under high-temperature treatment relative to the control, whereas *CsMYB113* exhibits a reciprocal downregulation pattern (Fig. 5A). Intriguingly, *CsMYB113* transcript abundance peaks at developmental stage S3, distinct from the peak of anthocyanin accumulation, indicating a temporal lag between transcriptional activation and metabolic output. Furthermore, across all four sampled stages (control and three gradient temperature treatments), *CsMIEL1* and *CsMYB113* display inverse expression trends at stage S4. *CsMIEL1* is consistently upregulated while *CsMYB113* is downregulated, supporting a model wherein *CsMIEL1*-mediated regulation actively attenuates the *CsMYB113*-driven anthocyanin synthesis program (Fig. 5A). These studies have emphasized that high temperatures activate ubiquitination, promoting the degradation of the *CsMYB113* protein and ultimately inhibiting the expression of genes involved in anthocyanin synthesis. Additional regulators, including *CsBBX24*, *CsMYB3*, and *CsMYB10*, exhibit expression dynamics congruent with *CsMYB113* (Figure S4). Notably, *BBX24* has been shown to interact with *HY5* and modulate *MYB10* expression in pear, thereby forming a hierarchical regulatory module for anthocyanin biosynthesis (Li et al., 2025). These observations imply that *CsMYB113*, *CsBBX24*, *CsMYB3*, and *CsMYB10* likely function within an integrated, interdependent regulatory network in blood orange, which requires further investigation and verification.

5. Conclusions

In summary, an integrated transcriptomic and metabolomic analysis was conducted to identify the key anthocyanin subtypes responsible for the characteristic red pigmentation and fading under high-temperature conditions of blood oranges. Furthermore, the study revealed that the high-temperature-induced ubiquitin ligase gene *CsMIEL1* physically interacts with the transcription factor *CsMYB113*, thereby repressing the expression of core anthocyanin biosynthetic genes and anthocyanin biosynthesis (Fig. 7). Collectively, these findings elucidate a molecular mechanism underlying both the accumulation and heat-induced breakdown of anthocyanins in blood oranges, providing a mechanistic foundation and actionable targets for improving the heat stability of anthocyanins in blood oranges.

Declarations

Ethics approval and consent to participate

Not applicable.

Clinical trial

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The original RNA-seq data of this study can be accessed with PRJNA1541567 in NCBI.

Competing interests

The authors declare that they have no conflict of interest.

Funding Declaration

This work was supported by the National Modern Citrus Industry System (CARS-26); the Hubei Province Technology Innovation Plan Project (2025BBB009); the Chongqing City Germplasm Resource Collection, Utilization, and Variety Testing Consortium Project of Research Institution and Enterprise (KYLX20250400027, KYLX20240500120, KYLX20231000025).

Authors' contributions

YH: Writing–original draft, Funding acquisition, Validation. WL: Validation, Visualization. CH: Resources. WJ: Conceptualization, Funding acquisition. LL: Visualization, Writing–original draft, Writing–review and editing.

Acknowledgements

Not applicable.

References

1. An JP, Wang XF, Zhang XW, et al. An apple MYB transcription factor regulates cold tolerance and anthocyanin accumulation and undergoes MIEL1-mediated degradation. *Plant Biotechnol J*. 2020;18:337–53. <https://doi.org/10.1111/pbi.13201>.
2. An JP, Zhao L, Liao L, et al. The deubiquitinase UBP15 and the E3 ubiquitin ligase PUB17 coordinate GLK1 stability in apple anthocyanin deposition. *Plant Cell*. 2026;38:koag029. <https://doi.org/10.1093/plcell/koag029>.
3. Chen J, Zhan J, Wang H, et al. VrMYB90 Functions Synergistically with VrbHLHA and VrMYB3 to Regulate Anthocyanin Biosynthesis in Mung Bean. *Plant Cell Physiol*. 2023;64:221–33. <https://doi.org/10.1093/pcp/pcac160>.
4. de Rosas I, Deis L, Baldo Y, et al. High Temperature Alters Anthocyanin Concentration and Composition in Grape Berries of Malbec, Merlot, and Pinot Noir in a Cultivar-Dependent Manner. *Plants (Basel)*. 2022;11:926. <https://doi.org/10.3390/plants11070926>.

5. Dou F, Phillip FO, Liu H. Combined Metabolome and Transcriptome Analysis Revealed the Accumulation of Anthocyanins in Grape Berry (*Vitis vinifera* L.) under High-Temperature Stress. *Plants* (Basel). 2024;13:2394. <https://doi.org/10.3390/plants13172394>.
6. Fabroni S, Ballistreri G, Amenta M, et al. Anthocyanins in different Citrus species: an UHPLC-PDA-ESI/MSn -assisted qualitative and quantitative investigation. *J Sci Food Agric*. 2016;96:4797–808. <https://doi.org/10.1002/jsfa.7916>.
7. Fang Y, Ma HY, Wu ZM, et al. High temperature induces MdGATA15 to suppress anthocyanin accumulation in apple peels. *Plant J*. 2025;123:e70381. <https://doi.org/10.1111/tpj.70381>.
8. Fu J, Liao L, Sun J, et al. CsBBX24 regulates both carotenoid and anthocyanin biosynthesis in citrus. *Plant Physiol*. 2025;199:kiaf539. <https://doi.org/10.1093/plphys/kiaf539>.
9. Hong Y, Lv Y, Zhang J, et al. The safflower MBW complex regulates HYSA accumulation through degradation by the E3 ligase CtBB1. *J Integr Plant Biol*. 2023;65:1277–96. <https://doi.org/10.1111/jipb.13444>.
10. Khaksar G, Sayed Tabatabaei BE, Arzani A, et al. Functional Analysis of a Pomegranate (*Punica granatum* L.) MYB Transcription Factor Involved in the Regulation of Anthocyanin Biosynthesis. *Iran J Biotechnol*. 2015;13:17–25. <https://doi.org/10.15171/ijb.1045>.
11. Kosar M, Kafkas E, Paydas S, et al. Phenolic composition of strawberry genotypes at different maturation stages. *J Agric Food Chem*. 2004;52:1586–9. <https://doi.org/10.1021/jf035093t>.
12. Lan M, Kou M, Li C, et al. Sweetpotato solute carrier family 35 member F1 (IbSLC35F1), a nucleotide sugar transporter transcriptionally activated by IbMYB1, modulates anthocyanin accumulation in storage roots. *Int J Biol Macromol*. 2026;339:150076. <https://doi.org/10.1016/j.ijbiomac.2026.150076>.
13. Leng X, Li C, Wang P, et al. The transcription factor VvMYB44-1 plays a role in reducing grapevine anthocyanin biosynthesis at high temperature. *Plant Physiol*. 2025;197:kiae657. <https://doi.org/10.1093/plphys/kiae657>.
14. Li S, Ou C, Liu X, et al. Plant U-box E3 ligase PpPUB59 regulates anthocyanin accumulation by ubiquitinating PpBBX24 in 'Zaosu' pear and its red bud mutation. *Plant Physiol Biochem*. 2025;219:109354. <https://doi.org/10.1016/j.plaphy.2024.109354>.
15. Liu H, Liu Z, Wu Y, et al. Regulatory Mechanisms of Anthocyanin Biosynthesis in Apple and Pear. *Int J Mol Sci*. 2021;22:8441. <https://doi.org/10.3390/ijms22168441>.
16. Liu R, Lai B, Hu B et al. Identification of MicroRNAs and Their Target Genes Related to the Accumulation of Anthocyanins in Litchi chinensis by High-Throughput Sequencing and Degradome Analysis. *Front Plant Sci*. (2016). 7, 2059. <https://doi.org/10.3389/fpls.2016.02059>
17. Liu Y, Tikunov Y, Schouten R, et al. Anthocyanin Biosynthesis and Degradation Mechanisms in Solanaceous Vegetables: A Review. *Front Chem*. 2018;6:52. <https://doi.org/10.3389/fchem.2018.00052>.
18. Ma G, Zhang L, Yamamoto R, et al. Molecular characterization of a flavanone 3-hydroxylase gene from citrus fruit reveals its crucial roles in anthocyanin accumulation. *BMC Plant Biol*. 2023;23:233.

<https://doi.org/10.1186/s12870-023-04173-3>.

19. Nguyen QD, Dang TT, Nguyen TV, et al. Microencapsulation of roselle (*Hibiscus sabdariffa* L.) anthocyanins: Effects of drying conditions on some physicochemical properties and antioxidant activities of spray-dried powder. *Food Sci Nutr*. 2022;10:191–203. <https://doi.org/10.1002/fsn3.2659>.
20. Niu M, Chen X, Guo Y, et al. Sugar Signals and R2R3-MYBs Participate in Potassium-Repressed Anthocyanin Accumulation in Radish. *Plant Cell Physiol*. 2023;64:1601–16. <https://doi.org/10.1093/pcp/pcad111>.
21. Plunkett BJ, Espley RV, Dare AP, et al. From Blueberry (*Vaccinium* Section *Cyanococcus*) Is a Subgroup 6 Type R2R3MYB Transcription Factor That Activates Anthocyanin Production. *Front Plant Sci*. 2018;9:1300. <https://doi.org/10.3389/fpls.2018.01300>.
22. Pomar F, Novo M, Masa A. Varietal differences among the anthocyanin profiles of 50 red table grape cultivars studied by high performance liquid chromatography. *J Chromatogra A*. 2005;1094:34–41. <https://doi.org/10.1016/j.chroma.2005.07.096>.
23. Saito K, Yonekura-Sakakibara K, Nakabayashi R, et al. The flavonoid biosynthetic pathway in *Arabidopsis*: structural and genetic diversity. *Plant Physiol Biochem*. 2013;72:21–34. <https://doi.org/doi:10.1016/j.plaphy.2013.02.001>.
24. Shi Z, Han X, Wang G, et al. Transcriptome analysis reveals chrysanthemum flower discoloration under high-temperature stress. *Front Plant Sci*. 2022;13:1003635. <https://doi.org/10.3389/fpls.2022.1003635>.
25. Sun Y, Zheng Y, Wang W, et al. VvFHY3 links auxin and endoplasmic reticulum stress to regulate grape anthocyanin biosynthesis at high temperatures. *Plant Cell*. 2024;37. <https://doi.org/10.1093/plcell/koae303>.
26. Tan Y, Wen B, Xu L, et al. High temperature inhibited the accumulation of anthocyanin by promoting ABA catabolism in sweet cherry fruits. *Front Plant Sci*. 2023;14:1079292. <https://doi.org/10.3389/fpls.2023.1079292>.
27. Verweij W, Spelt CE, Bliet M, et al. Functionally Similar WRKY Proteins Regulate Vacuolar Acidification in *Petunia* and Hair Development in *Arabidopsis*. *Plant Cell*. 2016;28:786–803. <https://doi.org/10.1105/tpc.15.00608>.
28. Wang J, Xu R, Qiu S, et al. CsTT8 regulates anthocyanin accumulation in blood orange through alternative splicing transcription. *Hortic Res*. 2023;10:uhad190. <https://doi.org/10.1093/hr/uhad190>.
29. Wang L, Gao YH, Zhang L, et al. Reduced proteasome degradation of HsfB2a at higher temperatures is responsible for the inhibition of anthocyanin synthesis in pear. *Plant Cell*. 2025;37. <https://doi.org/10.1093/plcell/koaf102>.
30. Wang Y, Li S, Shi Y, et al. The R2R3 MYB Ruby1 is activated by two cold responsive ethylene response factors, via the retrotransposon in its promoter, to positively regulate anthocyanin biosynthesis in citrus. *Plant J*. 2024;119:1433–48. <https://doi.org/10.1111/tpj.16866>.

31. Yang H, Chen H, Wang W, et al. UV radiation promotes anthocyanins biosynthesis in the fruit peel of blood oranges (*Citrus sinensis*). *Front Plant Sci.* 2025;16:1679102. <https://doi.org/10.3389/fpls.2025.1679102>.

32. Zhai R, Liu XT, Feng WT, et al. Different biosynthesis patterns among flavonoid 3-glycosides with distinct effects on accumulation of other flavonoid metabolites in pears (*Pyrus bretschneideri* Rehd). *PLoS ONE.* 2014;9:e91945. <https://doi.org/10.1371/journal.pone.0091945>.

33. Zhang L, Duan Z, Ma S, et al. SIMYB7, an AtMYB4-Like R2R3-MYB Transcription Factor, Inhibits Anthocyanin Accumulation in *Solanum lycopersicum* Fruits. *J Agric Food Chem.* 2023;71:18758–68. <https://doi.org/10.1021/acs.jafc.3c05185>.

34. Zhang MJ, Zhou XQ, Wang ZY, et al. MdSPL6 regulates the accumulation of anthocyanins in apple leaves induced by low temperature by activating the promoters of anthocyanin-related genes. *Hortic Plant J.* 2026. <https://doi.org/10.1016/j.hpj.2025.12.018>.

35. Zhang Y, Wang SS, Wang XS, et al. FaMADS6 negatively regulates anthocyanin accumulation in strawberry by inhibiting FaMYB10 expression and ABA biosynthesis. *Hortic Plant J.* 2025;12:300–16. <https://doi.org/10.1016/j.hpj.2025.02.013>.

36. Zhou H, Deng Q, Li M, et al. R2R3-MYB transcription factor CaMYB5 regulates anthocyanin biosynthesis in pepper fruits. *Int J Biol Macromol.* 2025;308:142450. <https://doi.org/10.1016/j.ijbiomac.2025.142450>.

Figures

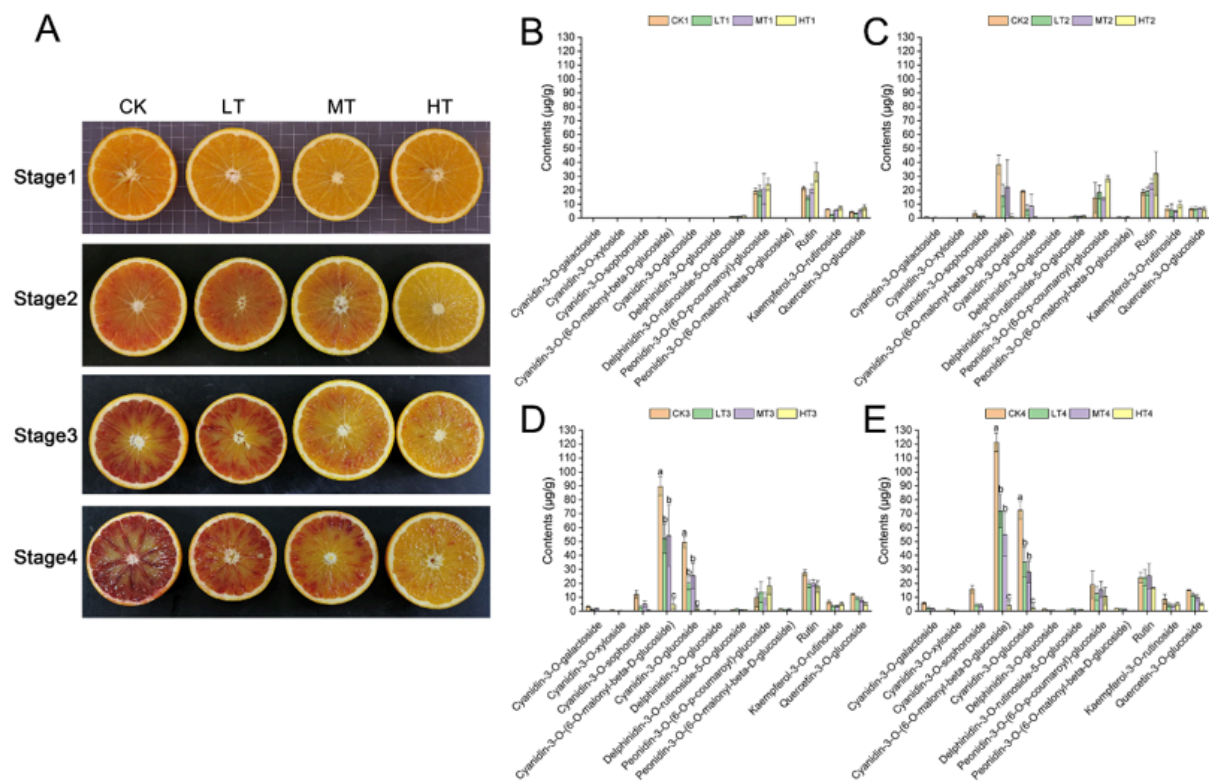


Figure 1

Changes in anthocyanin content of blood oranges under low-temperature treatment across four developmental stages

A: The color changes of blood oranges across four developmental stages under CK, LT, MT and HT. B-E: Quantitative anthocyanin contents at each developmental stage under gradient temperature treatment. The data are presented as mean $\pm$ SD. Statistical significance among multiple groups were conducted by one-way analysis of variance (One-Way ANOVA), and post hoc multiple comparisons were performed using the Duncan method (Post Hoc Test). The significance of differences between groups was marked using the letter marking method. The significance level was set at $P < 0.05$.

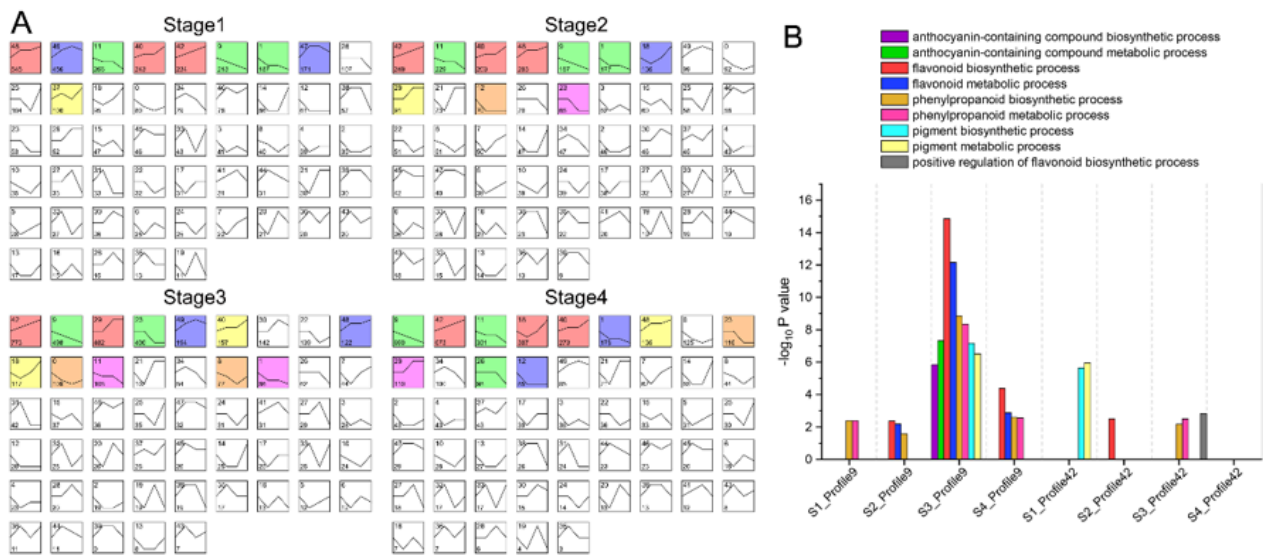


Figure 2

Identification of gene expression profiles under gradient temperature treatment and related GO enrichment analysis

A: The expression profile generated by the STEM analysis across four stages. The line graph in the profile represents the gene expression trends of CK, LT, MT and HT. Profiles with the same background color indicate similar expression trends. The number in the lower left corner represents the gene number of the profile. B: GO functional enrichment (biological process) of genes in Profile 9 and Profile 42 across four stages.

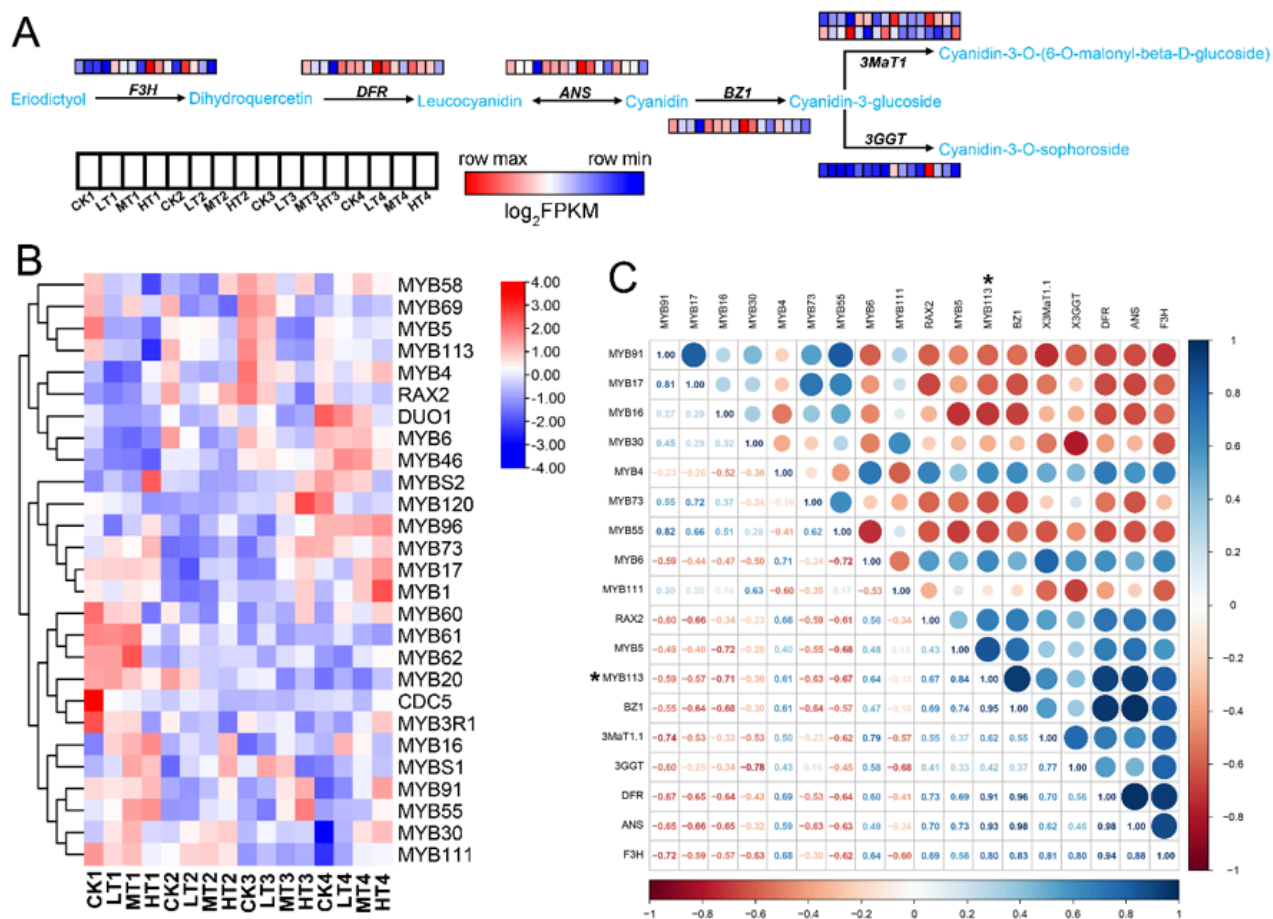


Figure 3

Expression patterns of anthocyanin synthesis genes and MYB transcription factors

A: Expression patterns of key genes in the cyanidin-3-glucoside and cyanidin-3-O-(6-O-malonyl-beta-D-glucoside) biosynthesis pathway. B: Heatmap of MYB transcription factors that are different expression among different comparison groups. C: Expression correlation analysis between anthocyanin biosynthesis genes and MYB transcription factors using the Pearson correlation coefficient.

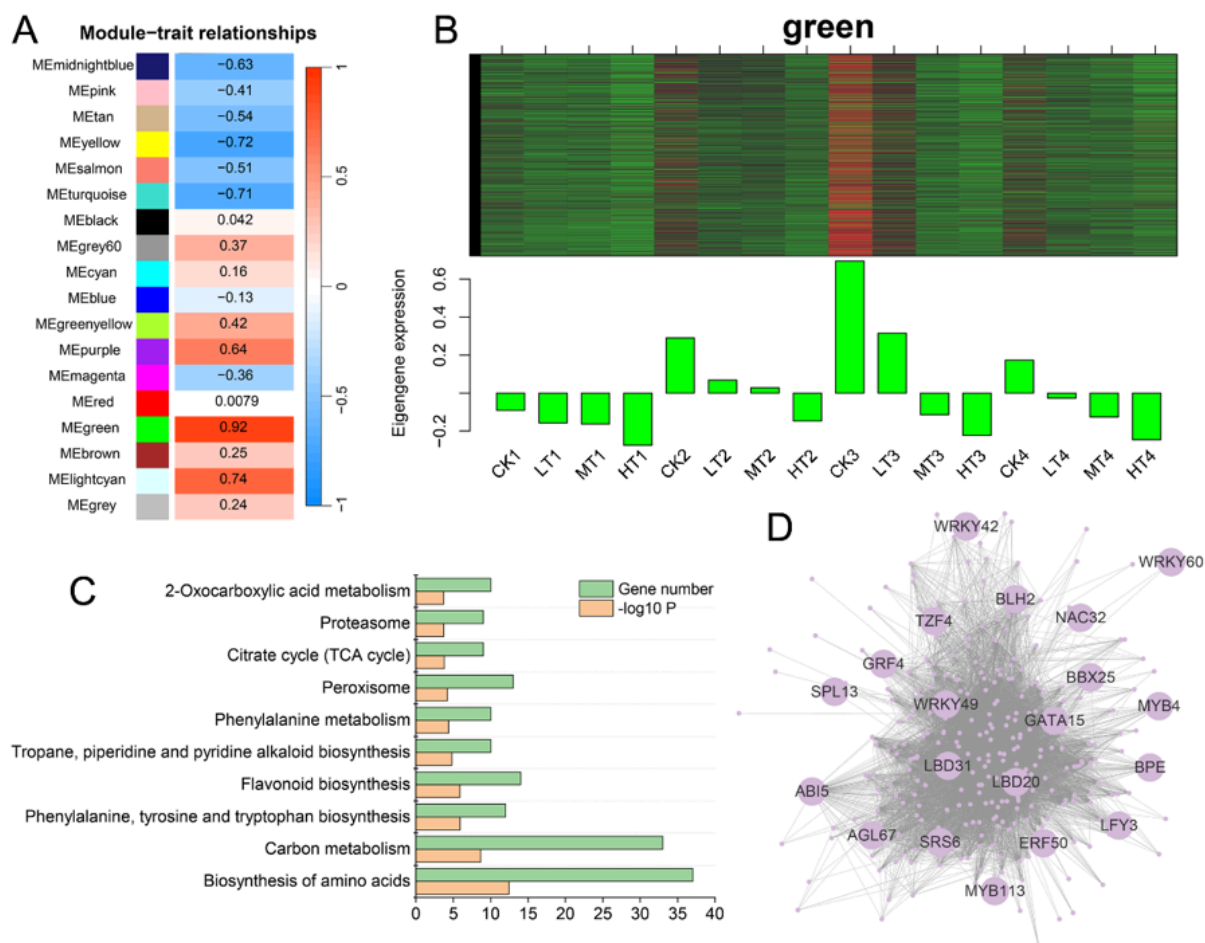


Figure 4

Co-expression network analysis of *CsMYB113*

A: The correlation between *CsMYB113* and the different modules generated by WGCNA. B: Expression trend of the green module. C: GO functional enrichment (biological process) of the genes in the green module. D: Co-expression network of *CsMYB113* produced by WGCNA. The pink circles represent transcription factors.

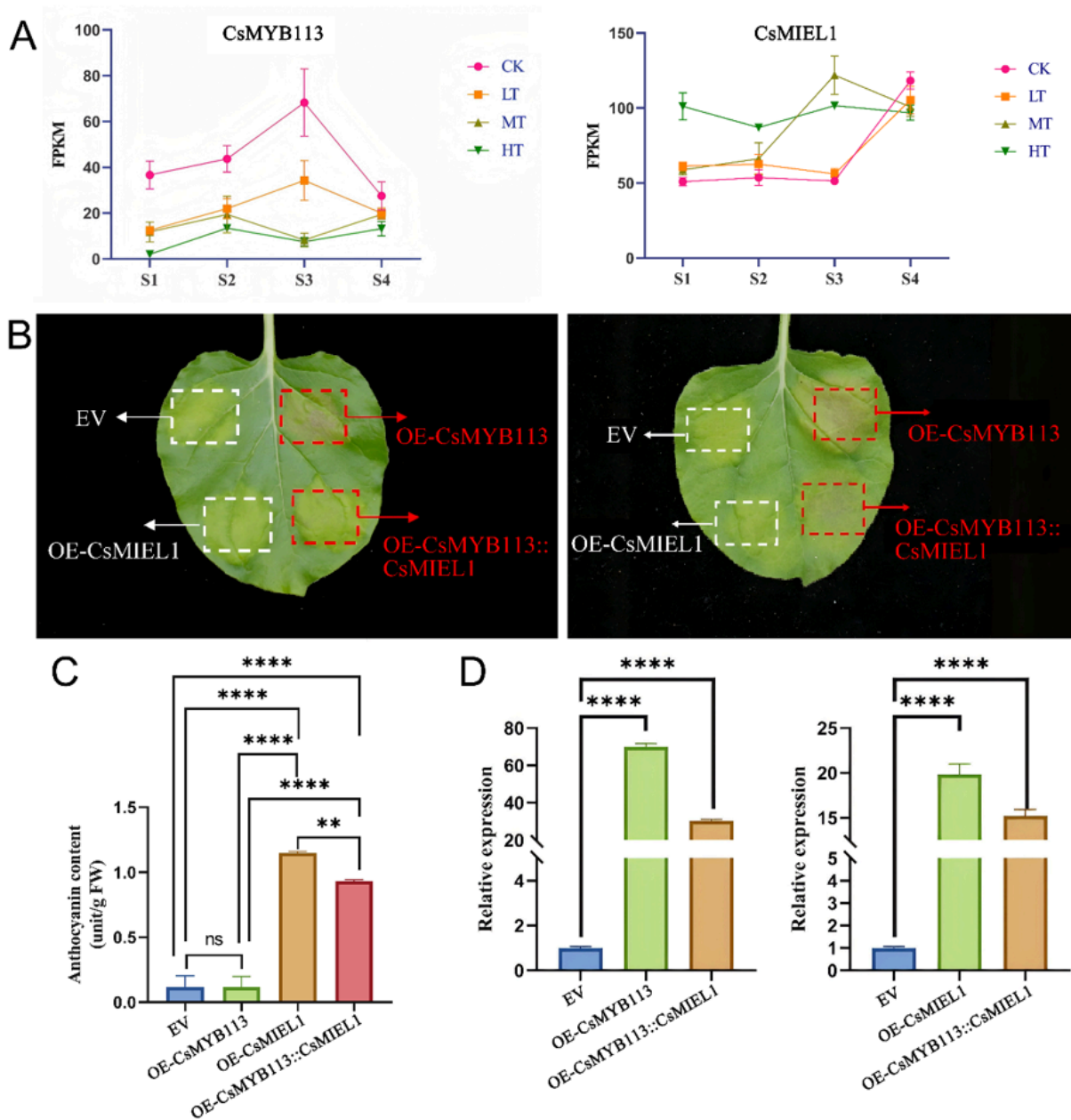


Figure 5

Identification of upstream regulatory factors and biological functions of *CsMYB113*

A: Expression patterns of *CsMYB113* and *CsMIEL1*. B: Separate and together injection assays of *CsMYB113* and *CsMIEL1* in tobacco leaves. The left and right columns represent two independent biological replicates. C: The anthocyanin content of tobacco leaves transformed by the separate and together injections of *CsMYB113* and *CsMIEL1*. The independent samples t-test was used for the significance analysis. **, $P < 0.01$, ****, $P < 0.0001$. D: The relative expression of *CsMYB113* and *CsMIEL1* in tobacco leaves transformed by the separate and together injections of *CsMYB113* and *CsMIEL1*. The independent samples t-test was used for the significance analysis. **, $P < 0.01$, ****, $P < 0.0001$. EV: empty vector, pK7WG2D.

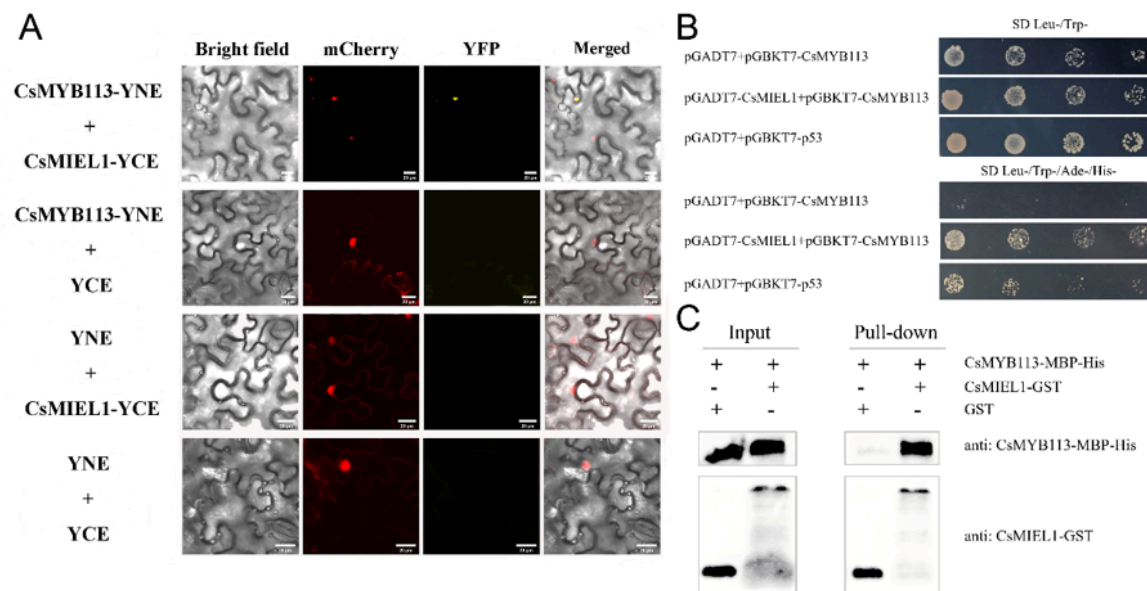


Figure 6

The protein-protein interaction assays between CsMYB113 and CsMIEL1

A: The fluorescence manifestations of the different domain combinations of CsMYB113 and CsMIEL1 based on BiFC. B: The yeast strains with different domain combinations of CsMYB113 and CsMIEL1 were grown in nutrient-deficient media. C: The pull-down assay between CsMYB113 and CsMIEL1 using GST as the protein tag.

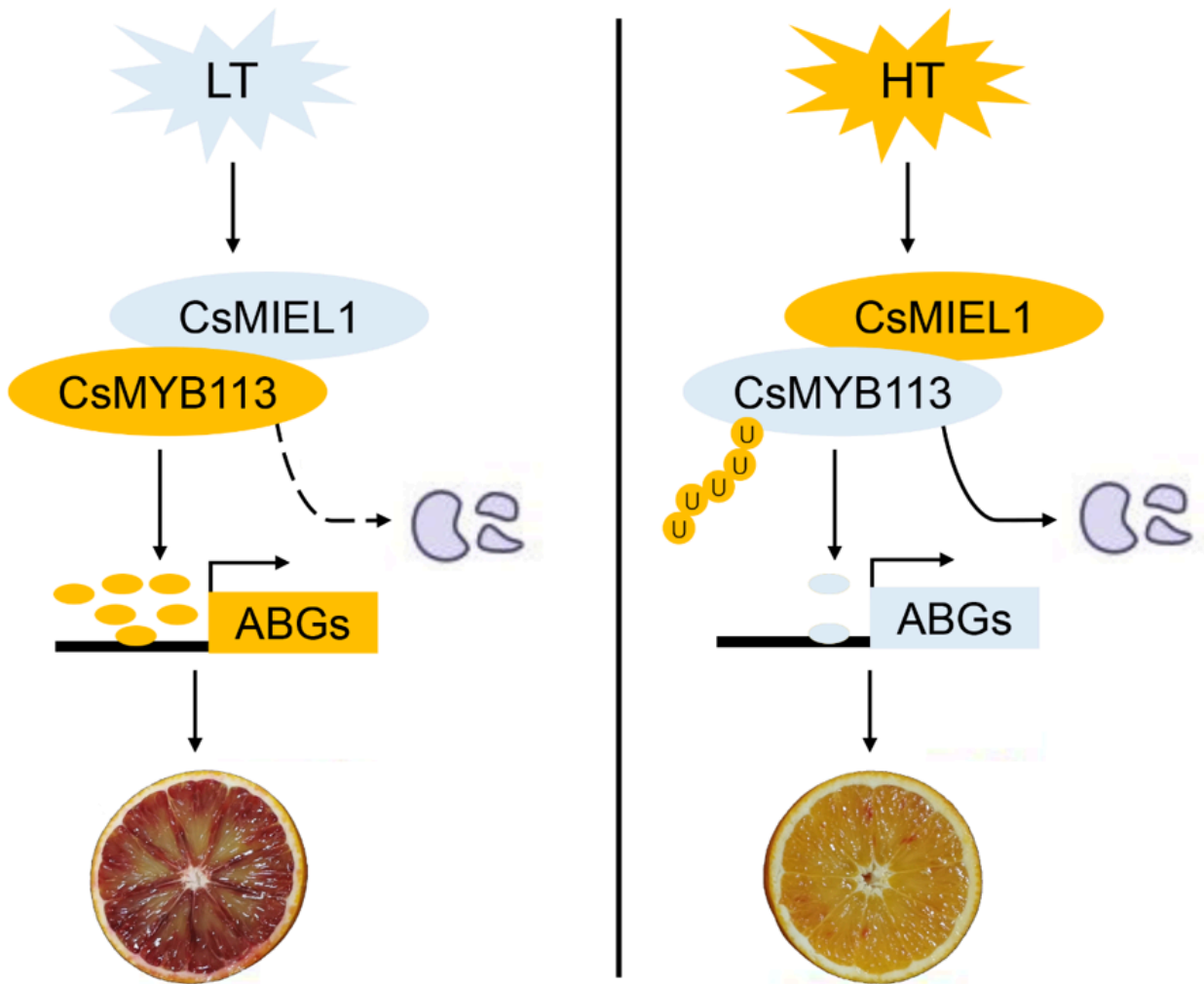


Figure 7

A hypothesis diagram of high temperature blocking the coloring of blood oranges

At low temperatures, the relatively low expression level of *CsMIEL1* is insufficient to fully mediate the ubiquitination of the *CsMYB113* protein. *CsMYB113* functions as a transcriptional activator that promotes the expression of anthocyanin biosynthesis genes, thereby facilitating anthocyanin accumulation and the characteristic red pigmentation of blood oranges. In contrast, high temperatures induce robust upregulation of *CsMIEL1* expression. The resulting high abundance of *CsMIEL1* enhances ubiquitin-mediated proteasomal degradation of *CsMYB113*, leading to a marked reduction in *CsMYB113* protein levels. Since the *CsMYB113* protein autoactivates its own promoter, the transcriptional level was decreased stem from a reduction in protein levels of *CsMYB113*, establishing a feedback loop between transcription and translation. Consequently, transcriptional activation of anthocyanin biosynthetic genes is blocked and anthocyanin synthesis is suppressed, resulting in the failure of blood orange pigmentation. LT: low temperature; HT: high temperature; U: ubiquitination; ABGs: anthocyanin biosynthesis genes. The light blue background color indicates down-regulated expression, while the orange background color indicates up-regulated expression.

Supplementary Files

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