

CsMYB5 regulates anthocyanin accumulation and stress responses through responding to hormone signaling of *Camellia sinensis*

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Abstract

Background

Anthocyanins are major secondary metabolites that determine tea quality and contribute to plant stress adaptation. However, the transcriptional mechanisms coordinating their biosynthesis in response to environmental cues remain largely unclear.

Result

In this study, we characterized the R2R3-MYB transcription factor *CsMYB5* from tea plant (*Camellia sinensis*) and investigated its role in hormone-responsive secondary metabolism regulation. Expression analyses revealed that *CsMYB5* is preferentially expressed in anthocyanin-rich tea cultivars and leaf tissues and is strongly induced by SA, MeJA and multiple abiotic stresses. Genome-wide DNA affinity purification sequencing revealed that *CsMYB5* binding sites are enriched in promoter regions of genes associated with transcriptional regulation and secondary metabolism, including flavonoid and caffeine biosynthetic pathways. Notably, *CsMYB5* directly targets *CsTCP15*, a TCP transcription factor potential regulation of anthocyanin synthesis and accumulation, suggesting the existence of a *CsMYB5*–*CsTCP15* regulatory module. Heterologous overexpression of *CsMYB5* in *Arabidopsis thaliana* resulted in enhanced vegetative growth and pronounced anthocyanin accumulation.

Conclusions

Based on these findings, we propose that *CsMYB5* acts as a central transcriptional hub linking hormone signaling with coordinated regulation of anthocyanin accumulation biosynthesis. This study provides new insights into the transcriptional integration of flavonoid metabolism and offers potential targets and research direction for improving tea quality and stress resilience through molecular breeding.

1. Background

During growth and development, plants are frequently exposed to adverse environmental conditions, such as drought, low temperature, high salinity, and pathogen infection[1]. To cope with these complex and fluctuating environments, plants have evolved sophisticated gene regulatory networks to finely control the biosynthesis of secondary metabolites[2]. Anthocyanins and caffeine are two core secondary metabolites in tea plants (*Camellia sinensis*)[3]. Anthocyanins contribute to leaf coloration and antioxidant capacity[4], whereas caffeine influences tea flavor and physiological activity; Anthocyanins are also involved in stress responses in tea plants[5].

In plants, anthocyanins enhance tolerance to drought and salt stress through multiple mechanisms, including antioxidative defense, osmotic adjustment, photoprotection, and signal regulation[6]. Moreover, environmental stresses such as drought, low temperature, and pathogen infection can significantly affect the biosynthesis and accumulation of anthocyanins and caffeine[7]. However, the molecular mechanisms underlying the regulation of their biosynthetic pathways under stress conditions remain

largely unclear[8]. In recent years, the completion of genome sequencing for multiple tea plant varieties, together with the extensive application of transcriptomic technologies[9], has provided valuable resources for elucidating the molecular mechanisms governing anthocyanin biosynthesis and for improving our understanding of the regulatory networks controlling secondary metabolism in tea plants.

Anthocyanins generally accumulate at very low levels in most tea cultivars, typically below 0.1 mg/g. However, anthocyanin content can be markedly increased under specific conditions, such as in purple-leaf tea varieties or in response to environmental stresses[10], reaching moderate accumulation levels[11]. For example, in the tea cultivar 'Meizhan', which occasionally exhibits purple pigmentation in young shoots and leaves, anthocyanin content can reach 0.1–0.3 mg/g. Previous studies have also demonstrated that the stability of purple pigmentation and anthocyanin accumulation in tea plants is regulated at the transcriptional level[12], and that this process is closely associated with leaf color transition[13]. Transcription factors play central roles in signal transduction and gene regulatory networks by recognizing and binding to specific cis-regulatory elements in the promoter regions of target genes, thereby activating or repressing downstream gene transcription[14]. Among them, MYB transcription factors are particularly important regulators[15], as they integrate hormonal signaling pathways to coordinate plant responses to internal and external environmental cues, ultimately influencing diverse physiological traits[16].

The R2R3-MYB subfamily plays a central role in regulating plant secondary metabolism, growth and development, as well as responses to biotic and abiotic stresses[17]. Previous studies have shown that overexpression of *A. thaliana* *AtMYB12* significantly activates the expression of flavonoid biosynthetic genes, promotes flavonoid accumulation, and consequently enhances drought and salt tolerance in transgenic plants[18]. In *Brassica rapa*, *BcMYB111* positively regulates flavonoid biosynthesis by directly binding to the promoters of *BcF3H* and *BcFLS1*[19]. Over-expression of *MdMYB48* in apple and *P. notoginseng* enhanced the elongation of primary root and the formation of lateral roots normal and drought stress conditions, exhibiting better drought resistance[20]. Especially MYB transcription factors play a critical role in activating the expression of anthocyanin biosynthetic genes. The MYB-bHLH-WDR (MBW) ternary complex is a key regulatory component of anthocyanin biosynthesis[21]. Meanwhile, plant hormones are also involved in anthocyanin biosynthesis. Exogenous application of MeJA promotes anthocyanin accumulation, while GA negatively regulates anthocyanin biosynthesis in *Arabidopsis* by promoting DELLA degradation, thereby releasing suppressors of anthocyanin biosynthesis.

Previous studies have identified a total of 122 CsR2R3-MYB genes in the tea plant genome[22], some of which have been demonstrated to be involved in the regulation of catechin biosynthesis[23], phosphorylation modification[24], anthocyanin accumulation[25], low-temperature stress responses[26], and pathogen infection responses[27]. Although the functions of several CsMYB genes have been predicted and experimentally validated, the regulatory mechanisms of most R2R3-MYB genes in tea plants remain poorly understood, particularly with respect to their roles in responses to pathogen infection and environmental stresses. In our previous transcriptomic analysis of tea leaves, we identified a MYB family gene *CsMYB5* that was significantly upregulated following infection by the tea

gray blight pathogen. This gene contains a typical R2R3-MYB domain structure, and sequence analysis revealed a high degree of homology between *CsMYB5* and *A.thaliana AtMYB5*, suggesting a potential role for *CsMYB5* in integrating jasmonic acid (JA) and abscisic acid (ABA) stress signaling pathways to regulate anthocyanin biosynthesis in tea plants.

In this study, the *CsMYB5* gene was cloned from tea plants, and its sequence characteristics, evolutionary relationships, and physicochemical properties were systematically analyzed. Quantitative real-time PCR (qRT-PCR) was employed to investigate the expression dynamics of *CsMYB5* in different tissues and in response to SA, MeJA, and pathogen infection. Recombinant *CsMYB5* protein was subsequently obtained through prokaryotic expression, and potential downstream target genes

enabling a preliminary characterization of the molecular regulatory network mediated by *CsMYB5*. Furthermore, an overexpression construct was generated and introduced into *A.thaliana* to evaluate phenotypic changes in transgenic plants and to assess their responses to abiotic stresses, including salinity, drought, and low temperature. The results of this study aim to elucidate the potential role of *CsMYB5* in plant growth and stress responses, providing new insights into the functional mechanisms of MYB transcription factors in woody plants, and offering candidate gene resources for the genetic improvement of stress tolerance in tea plants.

2. Material and Method

2.1 Plant material and related treatment methods

Tea plants (*Camellia sinensis*), including the cultivar 'Shuixian' and an additional 17 cultivars (Table S1), were obtained from the Tea Germplasm Resource Garden of Wuyi University (27.731°N, 117.999°E). Seeds of *A. thaliana* (Col-0) were provided by the Fujian Provincial Key Laboratory of Green Technology for Ecological Industry. Plants were treated by foliar spraying with phytohormones, including methyl jasmonate (MeJA) at a concentration of 100 µM and salicylic acid (SA) at 5 mM. To assess the response of target genes to pathogen infection, plants were inoculated using a needle-pricking method. Two punctures were made on each side of the main leaf vein, followed by inoculation with pathogen suspensions ($OD_{600} = 0.2$) of CLBB1 and CYBB1. To evaluate the responses of transgenic *A. thaliana* plants to abiotic stresses, stress treatments were applied as described below. Salt stress was simulated using 100 mM NaCl, drought stress was induced with 200 mM mannitol, and cold stress was applied by incubating plants in a growth chamber at 10°C under light conditions. Samples were collected at multiple time points between 0 and 48 h after treatment. Total RNA was extracted and reverse-transcribed into cDNA for subsequent gene expression analysis.

2.2 Cloning and Protein Sequence Analysis of *CsMYB5*

The *CsMYB5* gene was cloned based on sequence information obtained from the tea plant genome database (Table S2). The cloned *CsMYB5* coding sequence was translated into an amino acid sequence, and the three-dimensional protein structure was predicted and visualized using AlphaFold 3.0[28].

Homologous protein sequences of CsMYB5 were identified using the BLAST program in the NCBI database(<https://www.ncbi.nlm.nih.gov/>,accessed 13 March 2023) [29], and multiple sequence alignment was performed using ESPript [30]. AtMYBs proteins were selected as references, and a phylogenetic tree was constructed using the Neighbor-Joining method in MEGA 11.0 to analyze the evolutionary relationship of CsMYB5[31]. The resulting phylogenetic tree was visualized using the Interactive Tree Of Life (iTOL, <https://itol.embl.de/>,accessed 24 May 2024) online tool [32].

2.3 Expression profiling of CsMYB5

To analyze the expression patterns of *CsMYB5* in different organs and under exogenous stress conditions, eight-year-old tea plants were used. Root, stem, flower, mature leaf, and young leaf tissues, as well as mature leaves treated as described in Section 2.1 (50 g per sample), were collected for RNA extraction and subsequent cDNA synthesis. Gene-specific primers for quantitative real-time PCR were designed based on the *CsMYB5* sequence (Table S3), and the tea plant 18S rRNA gene was used as an internal reference[33]. Quantitative real-time PCR was performed using a Bio-Rad CFX Connect 96 system with SYBR Green chemistry. The PCR cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 30 s. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Three biological replicates were performed for each sample, and each biological replicate contained three technical replicates.

2.4 Induced expression of CsMYB5 protein and Western blot analysis

The pGEX-4T vector was used for prokaryotic expression. Gene-specific primers were designed to amplify the open reading frame (ORF) of CsMYB5 (Table S3), and the amplified fragment was ligated into the pGEX-4T vector to generate the recombinant expression construct CsMYB5-pGEX-4T-1. The recombinant plasmid CsMYB5-pGEX-4T-1 was transformed into Escherichia coli BL21 (DE3) competent cells. Protein expression was induced with 0.2 mM IPTG at 16°C for 24 h, after which bacterial cells were harvested. Bacterial cells were lysed according to standard GST fusion protein purification protocols, and the supernatant was subjected to affinity chromatography to purify the fusion protein. Purified proteins were separated by SDS-PAGE, transferred onto membranes under ice-cooling conditions, blocked, and subsequently analyzed by Western blotting. After revision, this section meets Nature's standards for rigor and clarity in recombinant protein expression and immunoblot analysis.

2.5 DAP-seq analysis of CsMYB5

DNA fragments bound by CsMYB5 protein in tea plants were sequenced using the Illumina NovaSeq platform to generate raw DAP-seq data[34]. Raw DAP-seq sequencing data were subjected to quality control and preprocessing using fastp (v0.23.2) [35]. The cleaned reads were aligned to reference genome using BWA-MEM (v0.7.17)[35]. Peak calling was performed using MACS2 (v2.2.7.1) to identify CsMYB5-binding sites[36]. Technical replicates were merged using HOMER (v4.11) to improve the reliability of the results[37]. Peak annotation was performed using ChIPseeker (v1.36.0) [38] assigning peaks to genomic features including promoters, exons, introns, downstream regions, distal intergenic

regions, 5' untranslated regions (5' UTRs), and 3' untranslated regions (3' UTRs). Peaks enriched near transcription start sites (TSSs) were defined as candidate transcription factor binding sites. Genes associated with CsMYB5-binding peaks were subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses to elucidate their potential biological functions.

2.6 Phenotypic and gene expression analysis of CsMYB5-transgenic *A. thaliana*

The coding region of CsMYB5 together with its native promoter was cloned into the overexpression vector pBWA(V)HS-35S using the In-Fusion cloning method, generating the eukaryotic expression construct pBWA(V)HS-35S-CsMYB5, which was subsequently introduced into *Agrobacterium tumefaciens* strain GV3101. After bolting, wild-type *A. thaliana* Col-0 plants were transformed using the floral dip method with *Agrobacterium* suspensions harboring the pBWA(V)HS-35S-CsMYB5 construct. Plants were kept in darkness for 24 h and then grown under long-day conditions (15 h light, 10,000–12,000 lx, 50–60% relative humidity, and 21–24°C) until silique maturation. Homozygous T3 CsMYB5-transgenic *A. thaliana* lines were grown on half-strength MS medium. At the 7–8 true-leaf stage, qRT-PCR was performed to assess CsMYB5 expression levels in different lines, and lines with elevated expression were selected for subsequent experiments. Phenotypic differences between transgenic and wild-type plants were then evaluated, and *CsMYB5* expression patterns under various stress conditions were analyzed. The qRT-PCR reaction system and cycling conditions were the same as those described in Section 2.3.

3. Results

3.1 Phylogenetic relationship and structural characterization of CsMYB5

Phylogenetic analysis revealed that CsMYB5 (TEA027333) clustered closely with *A. thaliana* MYB5 (AtMYB5) and AtMYB111, forming a well-supported monophyletic clade (Fig. 1,a). All members within this clade were classified as typical R2R3-MYB transcription factors and were clearly separated from other MYB subgroups, indicating a conserved evolutionary origin and potential functional specialization. Previous studies have demonstrated that AtMYB5 and AtMYB111 play critical roles in regulating secondary metabolism, particularly flavonoid and anthocyanin biosynthesis. The close phylogenetic relationship between CsMYB5 and these functionally characterized MYB proteins suggests that CsMYB5 may perform analogous biological roles in tea plants, potentially participating in flavonoid-related metabolic regulation.

NCBI BLAST analysis showed that CsMYB5 shares high sequence similarity with MYB5-, MYB308-, and MYB111-type proteins from multiple plant species. Multiple sequence alignment of the conserved R2R3-MYB domains further confirmed the high degree of structural conservation among these proteins

(Fig. 1,b). Both R2 and R3 repeat regions contain several highly conserved α -helices, with key amino acid residues preserved across species, highlighting their essential roles in DNA binding and transcriptional regulation. In contrast, the C-terminal region of CsMYB5 exhibits greater sequence divergence, consistent with the known role of this region in transcriptional activation or regulatory specificity in MYB transcription factors. The predicted three-dimensional structure of CsMYB5, generated using AlphaFold, is shown in (Fig. 1,c). The structural model indicates that the R2 and R3 repeat regions adopt a stable helix–turn–helix conformation, forming the canonical MYB DNA-binding domain. This structural feature is highly consistent with previously reported R2R3-MYB transcription factors, further supporting the classification of CsMYB5 as a typical R2R3-MYB protein.

3.2 Expression patterns of CsMYB5 across tea cultivars, tissues and stress conditions

To investigate the transcriptional regulation and expression dynamics of *CsMYB5* (*TEA027333*), we first analyzed the promoter regions of it and its homologous gene cluster (*TEA012130*, *TEA012145*, and *TEA014311*). Promoter sequence analysis identified a diverse array of cis-acting regulatory elements, including elements responsive to phytohormones (abscisic acid, salicylic acid, and methyl jasmonate), environmental stresses (anaerobic conditions and circadian rhythms), and light, as well as multiple MYB-binding sites (Fig. 2,a). Further counting analysis revealed that all four promoters were enriched in core regulatory elements, such as CAAT-boxes and TATA-boxes (Fig. 2,b). Notably, compared with other CsMYB transcription factors, the *CsMYB5* promoter showed a pronounced enrichment of MeJA-responsive and stress-related cis-elements, including ARE, MYB, and MYC motifs, suggesting that *CsMYB5* expression is co-regulated by endogenous phytohormone signaling and external environmental cues.

Using the expression level of *CsMYB5* in leaves of the ‘SX’ cultivar as a reference, we systematically examined its expression profiles across different 16 tea cultivars. *CsMYB5* expression exhibited pronounced cultivar-specific variation among the 15 tea cultivars analyzed (Fig. 2,c). The highest expression levels were observed in the anthocyanin-rich cultivar ‘MZ’, followed by ‘FY6’ and ‘BYTZ’, whereas relatively low expression was detected in ‘RG’, ‘TGY’, and ‘HD’. This differential expression pattern suggests that *CsMYB5* may be associated with cultivar-specific traits, particularly flavonoid and anthocyanin accumulation. Tissue-specific expression analysis revealed that *CsMYB5* transcripts accumulated predominantly in old leaves, while expression in stems and flowers was barely detectable (Fig. 2,d). This expression pattern implies that *CsMYB5* primarily functions in leaf-associated metabolic processes, such as the biosynthesis of secondary metabolites and responses to environmental stimuli.

Under exogenous hormone treatments, *CsMYB5* exhibited a rapid and robust transcriptional response to SA, reaching an initial expression peak at 3 h followed by a second peak at 24 h (Fig. 2,e). MeJA treatment also significantly induced *CsMYB5* expression, with a maximum at 3 h and a subsequent gradual decline. These results indicate that *CsMYB5* transcription is regulated by defense-related phytohormones, implicating this gene in hormone-mediated stress-response pathways. Upon pathogen

inoculation, *CsMYB5* displayed distinct expression dynamics depending on the infecting strain. Inoculation with CLBB1 strain resulted in a marked upregulation of *CsMYB5* at 24 h, whereas infection with strain CCYB1 led to a delayed induction, with peak expression observed at 72 h (Fig. 2,f). This strain-dependent expression response suggests that *CsMYB5* may participate in pathogen-specific defense mechanisms in tea plants. Collectively, *CsMYB5* shows elevated expression in anthocyanin-rich tea cultivars and leaf tissues and is differentially induced in response to diverse environmental stress conditions. These transcriptional features suggest that *CsMYB5* is closely associated with leaf secondary metabolism and hormone- or stress-related responses, thereby warranting further investigation into its protein accumulation and molecular regulatory mechanisms underlying tea plant growth and metabolism.

3.3 Western Blotting and DAP-seq of *CsMYB5* protein

After preliminary optimization experiments, the GST–*CsMYB5* fusion protein was successfully expressed *in vitro* under induction conditions of 20°C with 0.2 mM IPTG for 20 h. The recombinant GST–*CsMYB5* protein was subsequently purified and subjected to Western blot analysis (Fig. 3,a). A distinct immunoreactive band corresponding to the GST–*CsMYB5* fusion protein was detected at approximately 60 kDa. Given that the predicted molecular weight of *CsMYB5* is 34.55 kDa and that of the GST tag is 26 kDa, the observed band size is consistent with the expected molecular weight of the fusion protein. These results confirm the successful expression and purification of the GST–*CsMYB5* protein and further validate the accuracy of the prior protein structure prediction. The purified *CsMYB5* protein was subjected to DAP-seq analysis to systematically characterize its genome-wide DNA-binding landscape. Chromosomal distribution analysis of DAP-seq peaks revealed that *CsMYB5* binding sites were widely distributed across all chromosomes (Fig. 3,b). Genomic annotation of *CsMYB5* binding peaks indicated a clear positional preference, with 37.81% of peaks located within promoter regions and 61.09% within intronic regions, whereas only a minor proportion was associated with untranslated regions (UTRs) (Fig. 3,c).

Notably, *CsMYB5* binding peaks were significantly enriched in the vicinity of transcription start sites (TSS), with a pronounced accumulation in upstream promoter regions (Fig. 3,d), supporting the role of *CsMYB5* as a transcriptional regulator. Gene Ontology (GO) enrichment analysis of promoter-associated *CsMYB5* target genes revealed significant enrichment in biological processes related to RNA metabolism, DNA biosynthesis, transcriptional regulation, and secondary metabolism (Fig. 3,e), including RNA phosphodiester bond hydrolysis, nucleotidyltransferase activity, and UDP-glycosyltransferase activity.

KEGG pathway analysis further demonstrated that *CsMYB5* target genes were predominantly enriched in secondary metabolism-related pathways, including terpenoid backbone biosynthesis, tyrosine metabolism, caffeine metabolism, and multiple alkaloid biosynthesis pathways (Fig. 3,f).

Network analysis revealed extensive interconnections among these pathways, suggesting that *CsMYB5* may function as a central regulator coordinating tea-specific secondary metabolic networks

(Fig. 3,g). Motif analysis of CsMYB5 binding peaks identified several significantly enriched conserved sequences, including a canonical MYB-binding motif (Fig. 3h). Furthermore, a prominent CsMYB5 binding peak (peak_6276) was detected within the promoter region of *CsTCP15* homology to *AtTCP15*, which has been reported to participate in transcriptional networks regulating plant development and secondary metabolism, indicating that *CsTCP15* is a potential direct target of CsMYB5 (Fig. 3,i). Integrated DAP-seq and functional enrichment analyses revealed that the direct targets of CsMYB5 are significantly enriched in transcriptional regulation and secondary metabolism-related pathways. Notably, these targets form regulatory modules that modulate the expression of downstream genes involved in the biosynthesis of tea-specific secondary metabolites, including anthocyanins and caffeine.

3.4 Phenotypic and transcriptional effects of CsMYB5 overexpression

To elucidate the biological function of *CsMYB5*, an overexpression construct containing the native promoter region of *CsMYB5* was generated and introduced into *A. thaliana*, resulting in stable transgenic lines. Gene expression analysis confirmed stable overexpression of *CsMYB5* in multiple independent T3 lines, with the highest transcript abundance detected in line T3-20, followed by T3-40 and T3-45 (Fig. 4,a). Comparative analysis of phenotypic characteristics between CsMYB5-overexpressing *A. thaliana* and wild-type plants revealed that during early growth stages, there were no significant differences between wild-type (Col-0) plants and CsMYB5-overexpressing lines T3-20, T3-40, and T3-45 (Fig. 4b). Two weeks after sowing, transgenic seedlings exhibited slightly larger rosette leaves and accelerated growth compared to wild-type plants, with all three lines (T3-20, T3-40, and T3-45) bolting (Fig. 4c). By week three, transgenic lines showed increased vegetative biomass and larger leaves, with distinct anthocyanin accumulation in rosette leaves of T3-40 and T3-45 lines, presenting a reddish-purple phenotype (Fig. 4d). At week five, all transgenic lines had produced siliques, while Col-0 plants were just beginning to bolt. Moreover, anthocyanin accumulation in *A. thaliana* leaves was more pronounced (Fig. 4e). Throughout the entire growth period, CsMYB5-overexpressing plants exhibited earlier bolting and significantly increased inflorescence height compared to wild-type plants. The transition to reproductive growth occurred 14–21 days earlier in transgenic plants than in wild-type Col-0 (Supplementary Fig. 1). Additionally, transgenic plants produced 5–6 more siliques per stem and had stems 5–8 cm taller than wild-type Col-0. These results demonstrate that overexpression of CsMYB5 accelerates the transition to reproductive growth in *A. thaliana* and promotes anthocyanin accumulation in leaves.

Consistent with the expression patterns observed in tea plants, CsMYB5-overexpressing *A. thaliana* lines exhibited pronounced transcriptional responses to exogenous hormone treatments. MeJA rapidly induced *CsMYB5* expression, reaching a maximum at 6 h, whereas SA treatment triggered a characteristic biphasic response with prominent peaks at 3 h and 24 h (Fig. 4,f), indicating that CsMYB5 retains strong responsiveness to defense-related hormone signaling in a heterologous system. Under abiotic stress conditions, CsMYB5-overexpressing lines also displayed distinct and time-dependent transcriptional responses. Cold treatment induced *CsMYB5* expression to peak levels at 48 h, while

drought and NaCl treatments caused rapid and significant upregulation at early time points (6 h and 12 h, respectively) (Fig. 4,g). Taken together, overexpression of *CsMYB5* not only markedly altered plant growth and development and enhanced anthocyanin accumulation in *A. thaliana*, but also conferred dynamic transcriptional responsiveness to hormonal and environmental cues. These findings suggest that *CsMYB5* exhibits a degree of functional conservation between *C. sinensis* and *A. thaliana* and support its potential role as a key regulatory factor linking plant development, secondary metabolism, and stress-responsive signaling pathways, thereby providing a solid foundation for further dissection of its underlying molecular regulatory network.

4. Discussion

In this study, we systematically characterized the R2R3-MYB transcription factor *CsMYB5* and demonstrated its pivotal role in integrating hormone signaling, secondary metabolism, and stress responses in tea plant (*Camellia sinensis*). By combining expression profiling, DAP-seq analysis, and functional validation in *A. thaliana*, we propose a regulatory model in which *CsMYB5* functions as a central transcriptional hub coordinating anthocyanin accumulation and caffeine biosynthesis in response to environmental cues (Fig. 5).

Anthocyanins are key flavonoid compounds that contribute not only to plant pigmentation but also to antioxidant capacity and stress tolerance[39]. R2R3-MYB transcription factors act as core regulators of anthocyanin biosynthesis across diverse plant species. For instance, the *A. thaliana* homolog of *CsMYB5*, *AtMYB5*, collaborates with *AtMYB2* to promote anthocyanin accumulation in plants[40]. Similarly, *FaMYB5* in strawberry performs an analogous function, enhancing anthocyanin biosynthesis[41].

In this study, we found that *CsMYB5* is highly expressed in anthocyanin-rich tea cultivars and leaf tissues, and its overexpression in *A. thaliana* significantly promotes anthocyanin accumulation, indicating that *CsMYB5* in *Camellia sinensis* has a conserved function in regulating anthocyanin biosynthesis. These findings strongly support a conserved role of *CsMYB5* in promoting flavonoid biosynthesis, particularly anthocyanin accumulation, in agreement with its close phylogenetic relationship to *A. thaliana* MYB5. Beyond flavonoid metabolism, DAP-seq and functional enrichment analyses uncovered an unexpected yet biologically meaningful association between *CsMYB5* and caffeine biosynthesis pathways. *CsMYB5* binding peaks were significantly enriched in promoter regions of genes involved in caffeine metabolism, including *CsXMTs*, which encode key N-methyltransferases catalyzing caffeine biosynthesis[42]. DAP-seq observation suggests that *CsMYB5* may possess a multiple regulatory capacity, coordinating the biosynthesis of polyphenols and alkaloids—two hallmark metabolic features that collectively determine tea quality.

Hormone signaling appears to act as a critical upstream regulator of *CsMYBs* activity[43]. The *CsMYB5* promoter is enriched in SA and MeJA, and *CsMYB5* transcription is rapidly induced by both hormones in *Camellia sinensis* and transgenic *A. thaliana*. Importantly, our DAP-seq analysis identified *CsTCP15* as a

potential direct downstream target of CsMYB5 protein. *CsTCP15* shares high sequence homology with *AtTCP15*, which has been implicated in the regulation of plant development[44] and secondary metabolism[45]. The proposed *CsMYB5* and *CsTCP15* regulatory module therefore offers a mechanistic framework through which *CsMYB5* may exert both direct and indirect control over secondary metabolism. Taken together, this study support a working model in which stress-induced SA and MeJA signaling activate *CsMYB5* expression, which in turn regulates transcriptional networks associated with anthocyanin accumulation and caffeine biosynthesis. Future plans include further metabolomic and transcriptomic analyses to dissect the hierarchical structure of the CsMYB5-centered transcriptional regulatory network, and multi-dimensional experimental validation of the direct regulatory relationship between *CsMYB5* and *CsTCP15* in modulating secondary metabolism. These efforts will provide new research directions and molecular resources for understanding tea quality improvement and the genetic breeding of elite tea cultivars.

5. Discussion

This study systematically characterized the R2R3-MYB transcription factor *CsMYB5* in *Camellia sinensis* and verified its pivotal role as a central transcriptional hub integrating hormone signaling, secondary metabolism and stress responses. *CsMYB5* shows a conserved function in promoting anthocyanin biosynthesis, with high expression in anthocyanin-rich tea tissues and enhanced anthocyanin accumulation in overexpressed *Arabidopsis thaliana*. Notably, it unexpectedly regulates caffeine biosynthesis by binding to promoters of key caffeine metabolic genes like *CsXMTs*, coordinating both polyphenol and alkaloid production that determines tea quality. Additionally, *CsMYB5* transcription is rapidly induced by SA and MeJA, and it targets *CsTCP15* to form a regulatory module for secondary metabolism. These findings elucidate a novel hormone-CsMYB5-mediated regulatory network, providing molecular insights for tea quality improvement and elite cultivar breeding.

Abbreviations

DAP-seq:DNA affinity purification sequencing. TSS:transcription start sites. UTRs:untranslated regions . KEGG:Kyoto Encyclopedia of Genes and Genomes.GO:Gene Ontology . ABA:abscisic acid.SA:Salicylic Acid. MeJA:Methyl Jasmonate

Declarations

Competing interests:

The authors declare no competing interests.

Ethics approval and consent to participate:

Not applicable.

Consent for publication:

Not applicable.

Availability of data and materials:Competing interests

The original sequence data of the tea plant genome in this project is derived from the Tea Plant Information Archive (TPIA) and is publicly accessible via <https://tpia.teaplants.cn/>.

Authors' contributions:

These Jinxian Liu and Yukun Peng contributed equally to this work.J.L. conceived the project, designed the experiments and drafted the initial manuscript. Y.P. and J.Z. performed the experiments, drafted the initial manuscript and prepared the figures. Z.F. assisted with figure visualization. L.L., X.F., F.L. and G.W. supervised the study, and provided experimental equipment and technical support. Z.L. refined the manuscript to enhance scientific rigor and handled correspondence and submission. All authors reviewed and approved the final manuscript.

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Figures

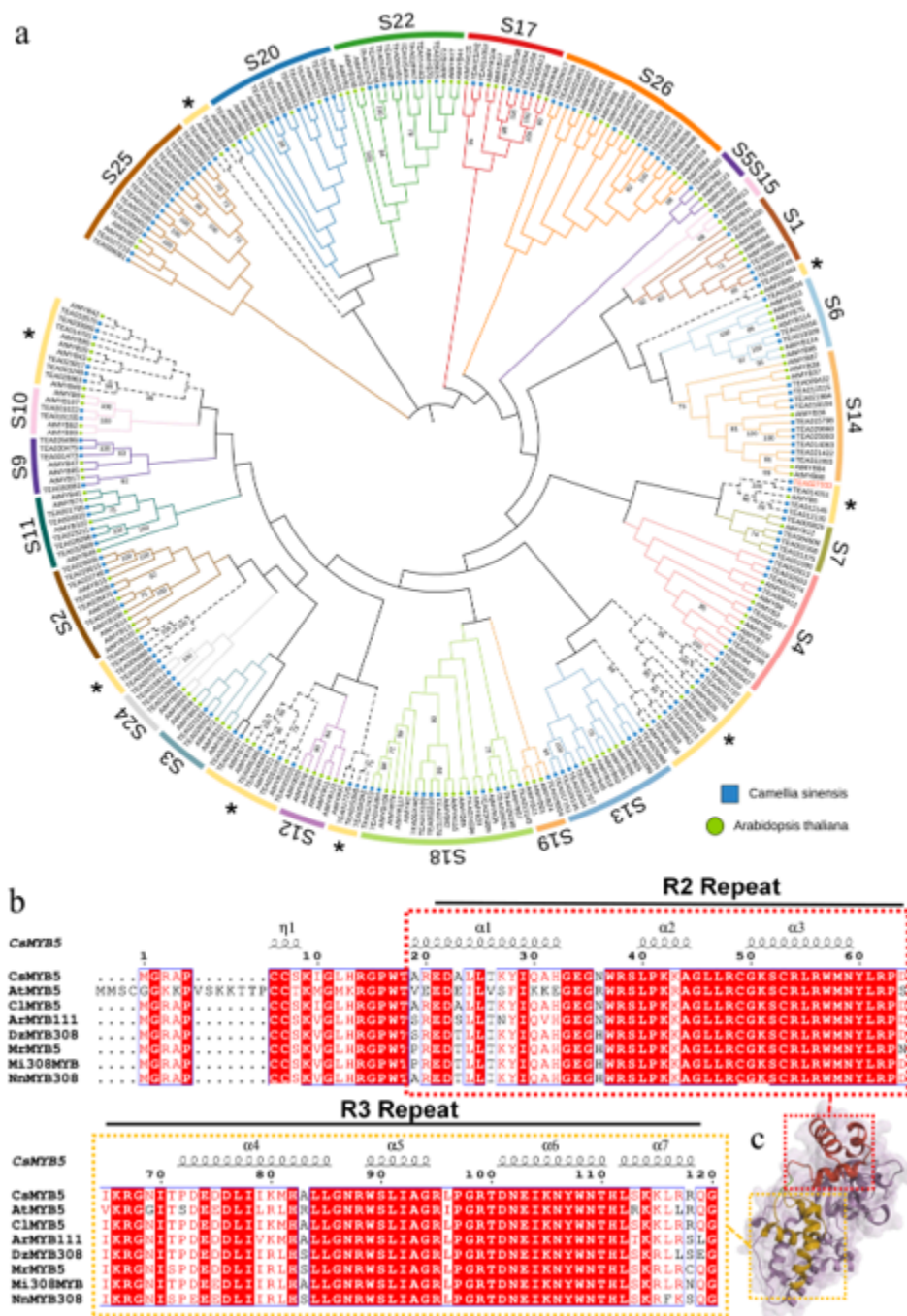


Figure 1

Phylogenetic analysis and protein structure of CsMYB5. (a) Phylogenetic tree of MYB proteins from *Camellia sinensis* and *Arabidopsis thaliana*. (b) Multiple sequence alignment of R2R3-MYB domains from CsMYB5 and its homologs. (c) Alpha Fold-predicted 3D structure of CsMYB5 of the R2 and R3 DNA-binding domains.

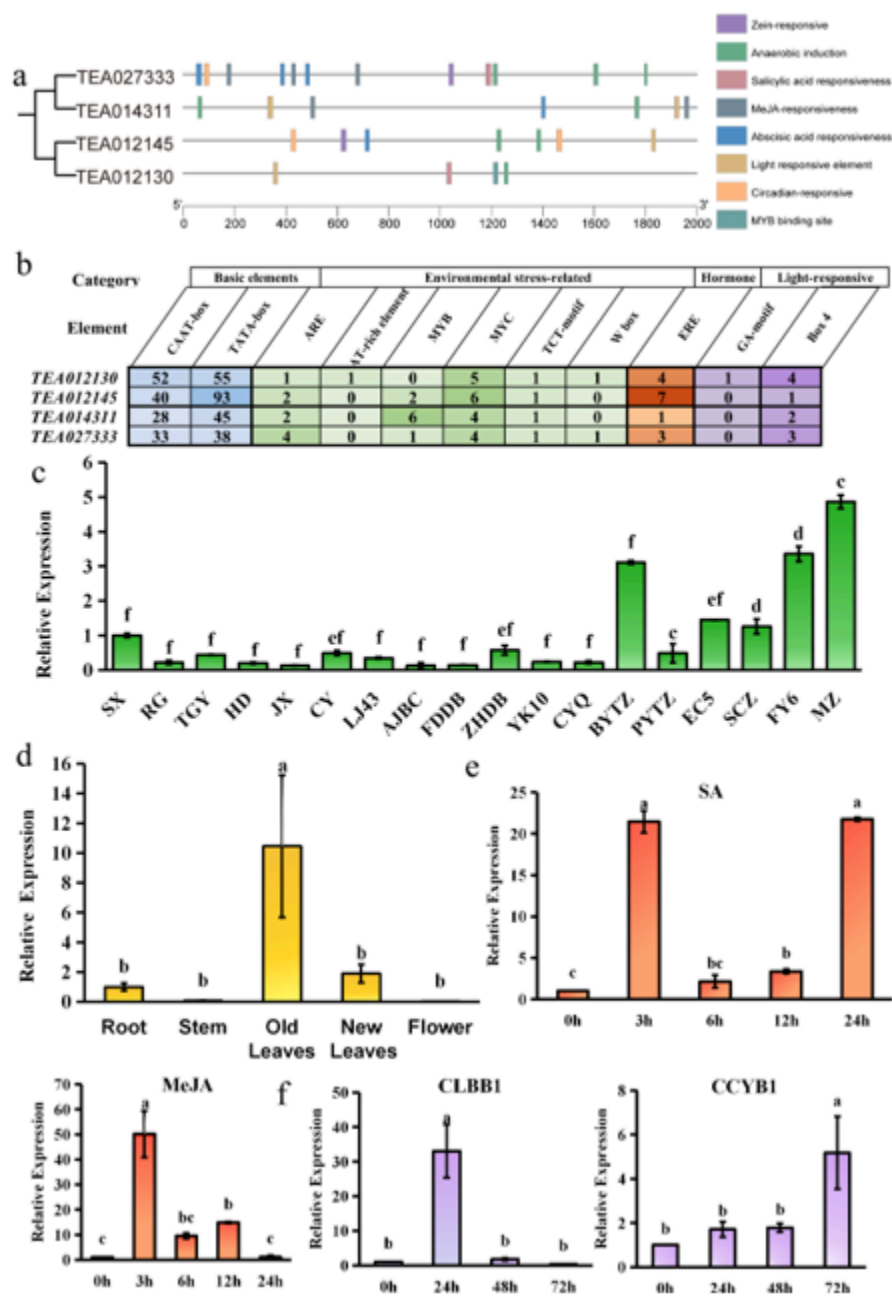


Figure 2

Expression characteristics and promoter analysis of *CsMYB5* in tea plants.

(a) Distribution of cis-acting regulatory elements in the promoters of *CsMYB5* and its homologs.

(b) Comparison of cis-acting core regulatory and stress-responsive elements number across the four promoters of *CsMYB5* and its homologs. (c) Relative *CsMYB5* expression in 15 tea cultivars.

(d) Tissue-specific *CsMYB5* expression, with maximal transcript accumulation in old leaves.

(e) *CsMYB5* expression dynamics under SA and MeJA treatments.(f) *CsMYB5* expression profiles after inoculation with CLBB1 and CCYB1.

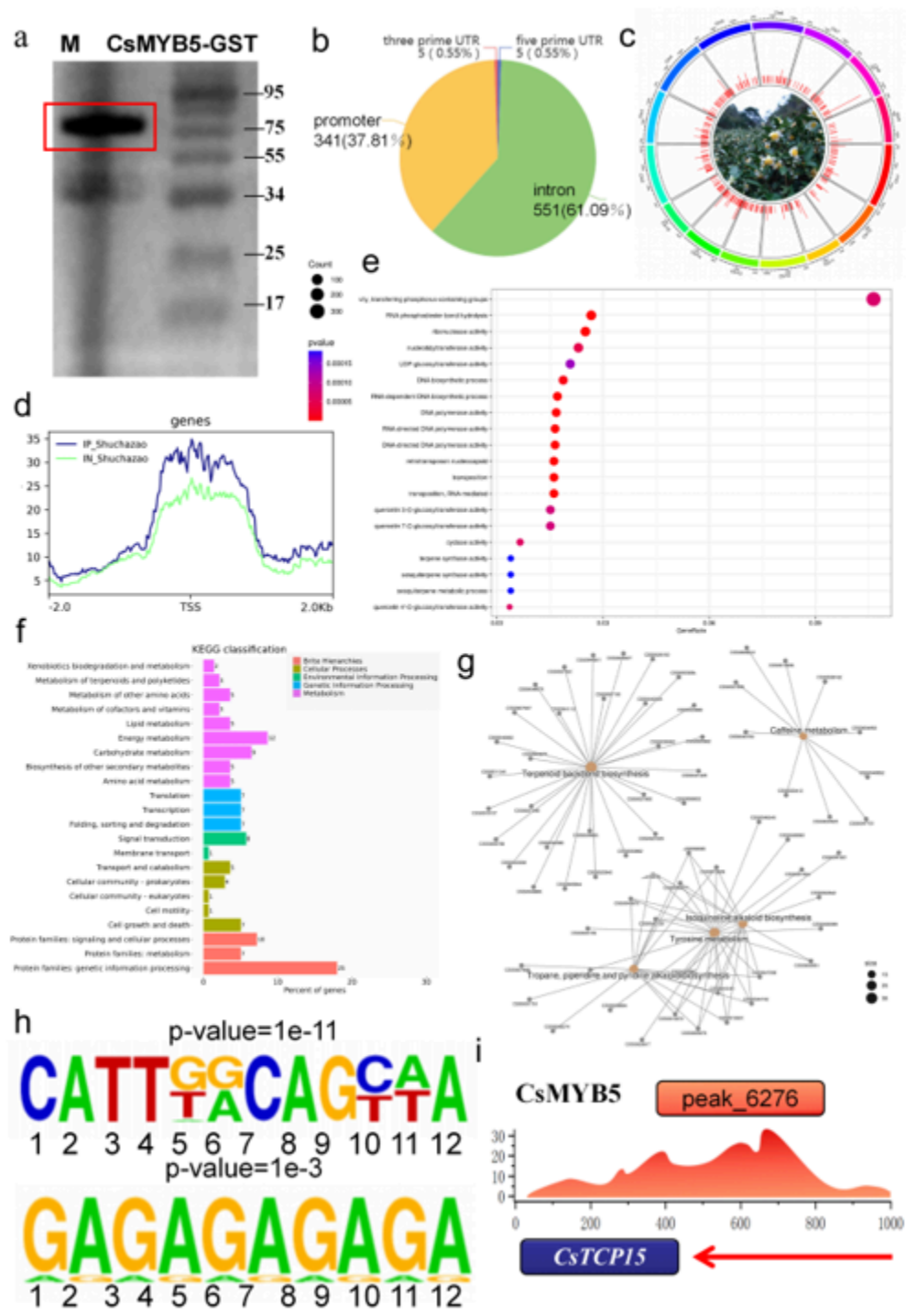


Figure 3

Western blot and genome-wide DNA-binding analysis of CsMYB5 protein.

(a) Western blot analysis of the purified GST-CsMYB5 fusion protein.(b) Chromosomal distribution of CsMYB5 DAP-seq binding peaks across the tea plant genome.(c) The genomic distribution of CsMYB5 binding peaks.(d) Binding signal enrichment profile of CsMYB5 around transcription start sites (TSS).(e) Gene Ontology (GO) enrichment analysis of CsMYB5 target genes.(f) KEGG pathway classification of

CsMYB5 target genes.(g) Network analysis of KEGG pathways regulated by CsMYB5.(h) Significantly enriched DNA-binding motifs identified from CsMYB5 DAP-seq peaks.(i) Visualization of CsMYB5 binding peak (peak_6276) in the promoter region of *CsTCP15*gene.

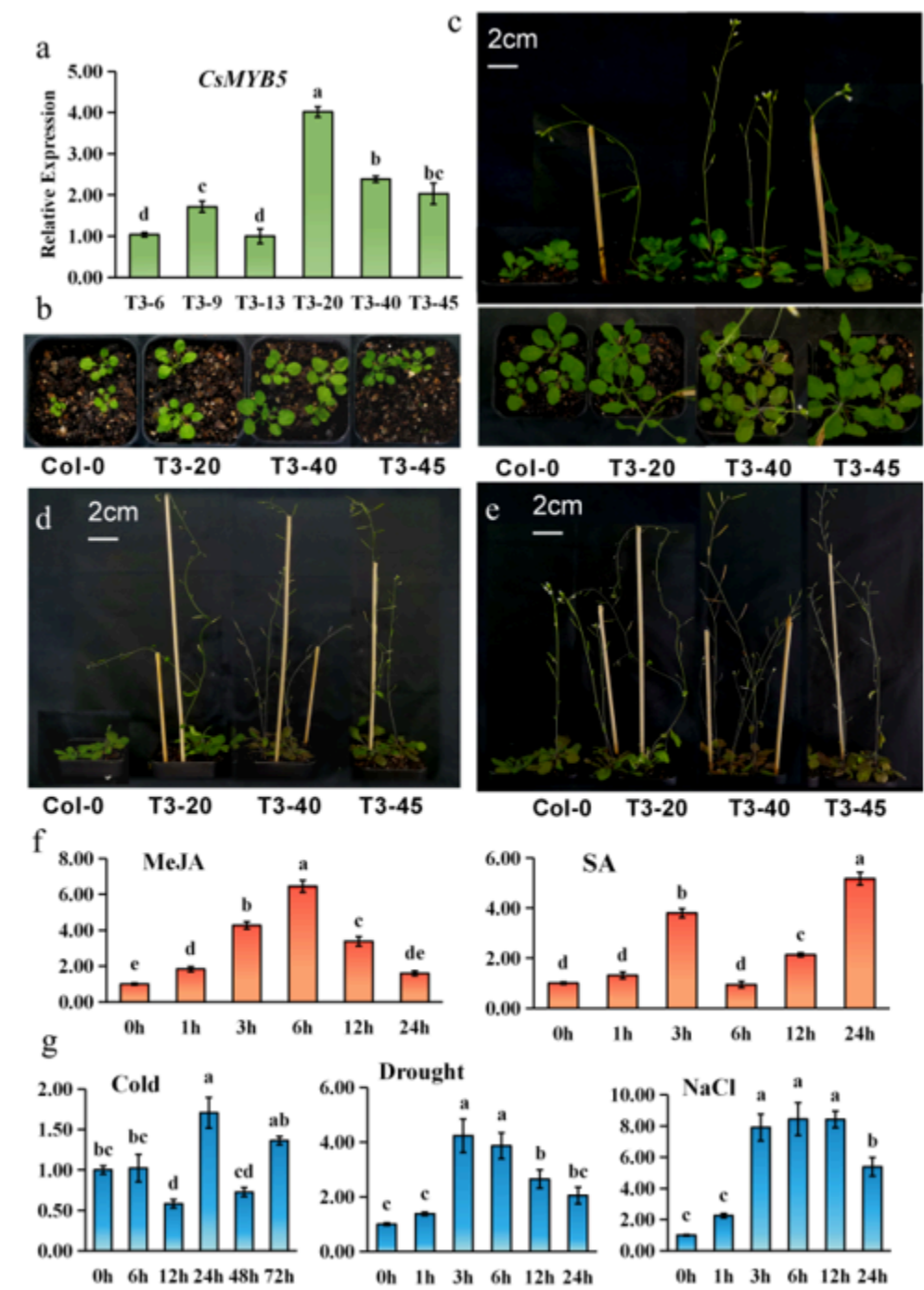


Figure 4

Phenotypic and transcriptional analysis of CsMYB5-overexpressing *Arabidopsis thaliana*.

(a) Relative expression of CsMYB5 in independent T3 transgenic lines. (b) Growth phenotype of one-week-old seedlings, showing accelerated early growth in transgenic lines compared to wild-type (Col-0). (c) Three-week-old plants, displaying increased leaf size and anthocyanin accumulation in transgenic

lines. (d) Five-week-old plants, showing intensified anthocyanin pigmentation in leaves of transgenic lines. (e) Mature plants, demonstrating earlier bolting and taller inflorescence stems in *CsMYB5*-overexpressing lines. (f) *CsMYB5* expression profiles under MeJA and SA treatments.(g) *CsMYB5* expression dynamics under cold, drought, and NaCl stresses.

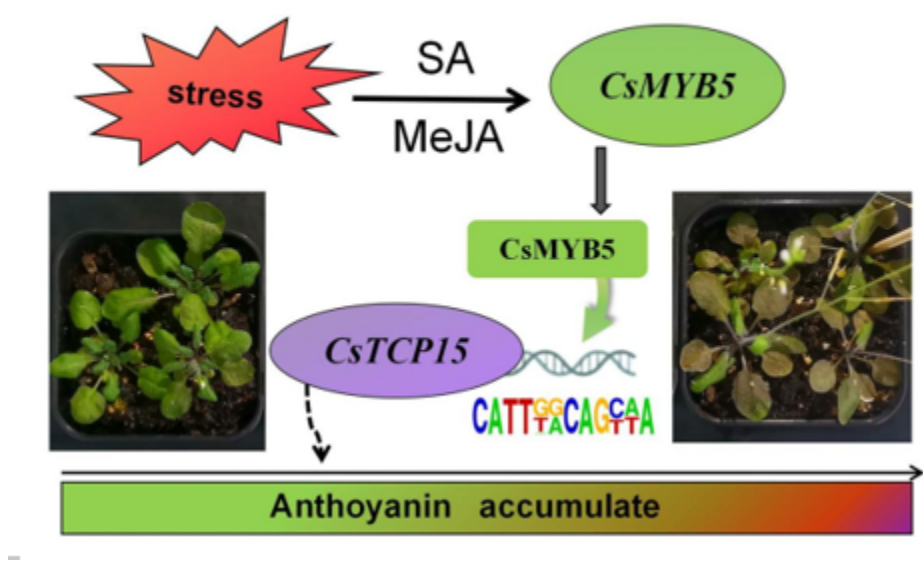


Figure 5

Proposed regulatory model of *CsMYB5* in *Camellia sinensis*.Figure illustrating how stress-induced hormonal signaling activates *CsMYB5* expression, which in turn regulates the transcription of downstream genes such as *CsTCP15*, thereby influencing the metabolic accumulation of anthocyanins and other secondary metabolites.

Supplementary Files

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