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miR828a-CsMYB114 Module Negatively Regulates the Biosynthesis of Theobromine in *Camellia sinensis*

Qifang Jin,[#] Zhong Wang,[#] Devinder Sandhu, Lan Chen, Chenyu Shao, Siyi Xie, Fanghuizi Shang, Shuai Wen, Ting Wu, Huiying Jin, Feiyi Huang, Guizhi Liu, Jinyu Hu, Qin Su, Mengdi Huang, Qian Zhu, Biao Zhou, Lihua Zhu, Lvwen Peng, Zhonghua Liu,* Jianan Huang,* Na Tian,* and Shuoqian Liu*

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ABSTRACT: Theobromine is an important quality component in tea plants (*Camellia sinensis*), which is produced from 7-methylxanthine by theobromine synthase (*CsTbS*), the key rate-limiting enzyme in theobromine biosynthetic pathway. Our transcriptomics and widely targeted metabolomics analyses suggested that *CsMYB114* acted as a potential hub gene involved in the regulation of theobromine biosynthesis. The inhibition of *CsMYB114* expression using antisense oligonucleotides (ASO) led to a 70.21% reduction of theobromine level in leaves of the tea plant, which verified the involvement of *CsMYB114* in theobromine biosynthesis. Furthermore, we found that CsMYB114 was located in the nucleus of the cells and showed the characteristic of a transcription factor. The dual luciferase analysis, a yeast one-hybrid assay, and an electrophoretic mobility shift assay (EMSA) showed that CsMYB114 activated the transcription of *CsTbS*, through binding to *CsTbS* promoter. In addition, a microRNA, miR828a, was identified that directly cleaved the mRNA of *CsMYB114*. Therefore, we conclude that *CsMYB114*, as a transcription factor of *CsTbS,* promotes the production of theobromine, which is inhibited by miR828a through cleaving the mRNA of *CsMYB114*.

KEYWORDS: *"Jianghua Kucha", theobromine, broadly targeted metabolomic analysis, gene suppression, tea plant*

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■ INTRODUCTION

Theobromine (3,7-dimethylxanthine), a purine alkaloid precursor for caffeine synthesis, differs from caffeine primarily due to the absence of a methyl group at the N−1 position,[1−4] and exhibits diuretic, cardiac excitatory, vasodilatory, and smooth muscle relaxing properties.[5] Theobromine is predominantly found in cocoa, coffee, and tea plants.[6−8] Young leaves contain the highest concentration of theobromine, and this concentration declines as the leaves age, while other parts of the tea plant, including the flowers, stems, seeds and roots, contain traces.[9]

In tea plants, the predominant biochemical pathway for the synthesis of caffeine follows these sequential steps: starting from xanthosine, it transforms into 7-methylxanthosine, then proceeds to become 7-methylxanthine, followed by conversion to theobromine, and finally culminates in the formation of caffeine.[10] In the pathway, *S*-adenosyl-L-methionine (SAM) functions as a methyl donor, facilitating the methylation of purine bases at specific stages of the process.[11] In most plants, the conserved structural domains A, B′, and C serve as binding sites for the methyl donor SAMa.[12] These domains play an important role in recognizing the purine ring *N*-methylation site and catalyzing the methylation reaction. 7-Methylxanthoside synthases are a class of substrate-specific enzymes involved in the first step of the methylation reaction catalyzing the formation of 7-methylxanthosine from xanthosine.[13] The first 7-methylxanthosine synthase gene (*CmXRS1*) was cloned from coffee leaves and has a 1307 base pair coding cDNA sequence that encodes 372 amino acids.[14] Theobromine synthase catalyzes the conversion of 7-methylxanthine to theobromine as the second methylation step.[13] The catalytic activity of theobromine synthase and caffeine synthase was first demonstrated in a crude extract of tea leaves.[15] Theobromine synthase is also substrate-specific, catalyzing only the 3-*N*-methylation of 7-methylxanthine and its conversion to theobromine. Coffee plant theobromine synthase genes include *CTS1*, *CaMXMT1*, *CTS2*, *CaMXMT2*, and *BTS1*, which share 80% amino acid sequence similarity and possess conserved structural domains such as the SAMs binding sites A, B′, C, and YFFF.[16] The *CgcTS* gene, which encodes theobromine synthase, was successfully cloned from the young leaves of *Camellia gymnogyna* Chang, a plant species native to Dayaoshan Mountain in Guangxi.[9] The *CgcTS* encodes a protease that most actively catalyzes 7-methylxanthine, followed by xanthosine, when presented with different purine derivatives as substrates.[9] However, the enzyme displayed no activity when tested with substrates, such as theobromine, caffeine, and theophylline. TCS1 is a caffeine synthase originally purified from leaves of tea plant (*C. sinensis*),[17] and it has a corresponding cDNA sequence spanning 1483 base

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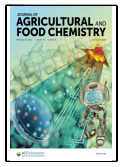

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pairs, which translates to 369 amino acids.[17] In vitro experiments showed that the TCS1 substrate specificity closely resembles that of the naturally occurring, purified enzyme, it promotes theobromine formation by catalyzing the 3-*N*-methylation using 7-methylxanthine and caffeine formation by catalyzing the 1-*N*-methylation with theobromine as the substrate.[17]

Related studies have shown that alkaloid synthesis is also regulated by transcription factors.[18] In vitro experiments, employing techniques such as dual luciferase, yeast one-hybridization, trans-activation analysis, and electrophoretic mobility shift assay (EMSA), confirmed the interaction of MYB184 to the MYB1AT-MYBPLANT motif, which is integral to regulating caffeine biosynthesis in tea plants.[19] A novel senescence-associated factor, CsS40, has been reported to negatively regulate *TCS1* and inhibit the accumulation of caffeine in the tea cultivar 'Fuding Dabai'.[20] In addition, CsNAC7 positively regulates the caffeine synthase gene *yhNMT1*, promoting the accumulation of caffeine within tea plants.[21] The purification and characterization of theobromine has been comprehensively understood, and have successfully isolated and cloned both the *N-methyltransferase* (*NMT*) gene and its promoter.[22−24] However, there is no report on the mechanism of transcription regulation of theobromine biosynthesis.

microRNA (miRNA) acts as a negative regulator of plant gene expression, primarily controlling target gene methylation at the transcriptional level and influencing mRNA translation at the post-transcriptional level to modulate target gene expression.[25] Current literature offers limited insight into the role of miRNA in theobromine biosynthesis in tea plants.[26,27] The post-transcriptional regulatory effects of miRNA on theobromine biosynthesis warrant further investigation.

Tea is one of the world's top three drinks, and the World Health Organization recognizes it as one of the top ten health foods.[27] Its global popularity stems from its health benefits, including antioxidant properties, cardiovascular disease prevention, and potential cancer-fighting ability.[27] At the same time, tea is a major cash crop, and its processing, cultivation, and trade hold substantial economic importance.[28] In this study, we identified a theobromine synthase gene, *CsTbS* (novel-G000134, Figure S1), and a MYB transcription factor, CsMYB114 (CSS0026328, Figure S2), by correlation analysis of extensively targeted metabolomic and transcriptomic data. The regulatory relationship between CsMYB114 and *CsTbS* was investigated by using a dual-luciferase reporter gene assay, electrophoretic mobility shift, and yeast one-hybridization techniques. The antisense oligonucleotide assay (ASO) was employed to validate the relationship between CsMYB114 and *CsTbS,* exploring its potential role in regulating theobromine biosynthesis in tea plant. In addition, the relationship between miR828a and CsMYB114 and its possibility to regulate theobromine biosynthesis were verified by a cleavage site validation assay. Our research uncovers a miR828a-CsMYB114-CsTbS pathway pivotal to theobromine production, offering potential applications in tea plant breeding to either elevate or diminish theobromine content.

■ MATERIALS AND METHODS

Tea Plant Materials. In this study, two cultivars of tea plants, "Xianghong 3" (XH3H) and "Yaoshan Xiulv" (YSXL), served as the experimental subjects. XH3H belongs to "Jianghua Kucha" and YSXL originated from Guangxi Province of Southwest China. The experimental methodology was adapted from the previously established protocols of the subject group.[29] The procedures for sample preparation and metabolite extraction were conducted in accordance with the methods described earlier.[30]

High Performance Liquid Chromatography (HPLC) Conditions. A liquid chromatography tandem mass spectrometry system (LC−ESI−MS/MS) was used to analyze the sample extracts. The analytical procedures were conducted following the conditions outlined in a previous study.[31] The effluent was connected alternatively to an electrospray ionization quadrupole linear ion trap tandem mass spectrometer (ESI-Q TRAP−MS/MS), which features a triple quadrupole-linear ion trap. HPLC conditions were based on the method of Lou et al.[32]

mRNA and miRNA Expression Profile Analyses. The data of mRNA and miRNA expression profiles were downloaded from our previous submission in GenBank (PRJNA986690, PRJNA1056246).[29]

Real-Time Quantitative RT-PCR Analysis. Total RNA from tea plant leaves was extracted using RNAprep Pure Plant Kit (Tiangen Biotech, Beijing, China), RNA integrity was detected by 1.1% agarose gel, and RNA samples were detected by ultramicro-UV spectrophotometer (Thermo Fisher Scientific). TransScript miRNA First-strand cDNA Synthesis Super Mix (Transgene) and the Prime-Script Reagent Kit (Takara) were used for cDNA synthesis. Primers required for q-RT-PCR were obtained by Primer Premier 5 design (Supporting Information Table S1). The expression levels of miR828a and target genes were detected according to the instructions of TransStart Tip Green qPCR SuperMix (Transgene) and using a Roche Light Cycler 480 real-time fluorescence quantitative PCR instrument with three replicate determinations. The 5.8S rRNA and U6 small nuclear RNA were used as the internal reference genes of miR828a expression analysis, and the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the internal reference gene of the target gene express analysis. The equation $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression level of the genes.

Binding Profiles Analysis of CsMYB114 and Motif Analysis of *CsTbs*. The binding profiles analysis of CsMYB114 and the motif analysis of *CsTbs* were performed on the JASPAR online software (https://jaspar.elixir.no/). The *Arabidopsis thaliana* core database was selected, and a threshold value of 90% was set.

Subcellular Localization Analysis. As an experimental group, tobacco leaves were injected with bacterial broth containing 35S::CsMYB114-GFP. Simultaneously, an empty pSuper-1300 GFP vector was injected into tobacco leaves as a control. The detailed subcellular localization procedure was described elsewhere.[33]

Dual Luciferase Assay. The cDNA of *CsMYB114* was cloned into the vector pGreenII62-SK, resulting in the creation of recombinant plasmid pGreenII62-SK-CsMYB114, which was then utilized as the effector in subsequent experiments. The *CsTbS* promoter sequence was simultaneously cloned in the pGreenII-0800-LUC dual reporter vector. These plasmids were subsequently introduced into *Agrobacterium tumefaciens* to facilitate transient expression studies.[34]

EMSA. The pMAI-C6T-CsMYB114 fusion construct was generated by incorporating the complete CsMYB114 sequence into the expression vector (pMAI-C6T). MBP-tagged protein is 44 kDa. The additional steps of EMSA were as described previously.[35]

A DNA segment of 50 base pairs, featuring the promoter region of a MYC element at the 5′ end of *CsTbS*, was utilized to create the universal probe. This was used for biosynthesis purposes. Probes designed for competition were structured in the same sequence as the universal probe. In the case of the mutated probe, the MYC site sequence (CAATTG) was altered to a single adenine base (A), while the rest of the sequence remained unchanged, matching the universal probe.[36] In the Electrophoretic Mobility Shift Assay (EMSA) setup, the probe was used at a concentration of 0.4 μM/μL.

Yeast One-Hybrid Assays. The entire *CsMYB114* sequence was integrated into yeast vector pGADT7, resulting in the formation of the pGADT7-CsMYB114 recombinant plasmid, which encodes a protein of 193 amino acids. Concurrently, the *CsTbS* promoter

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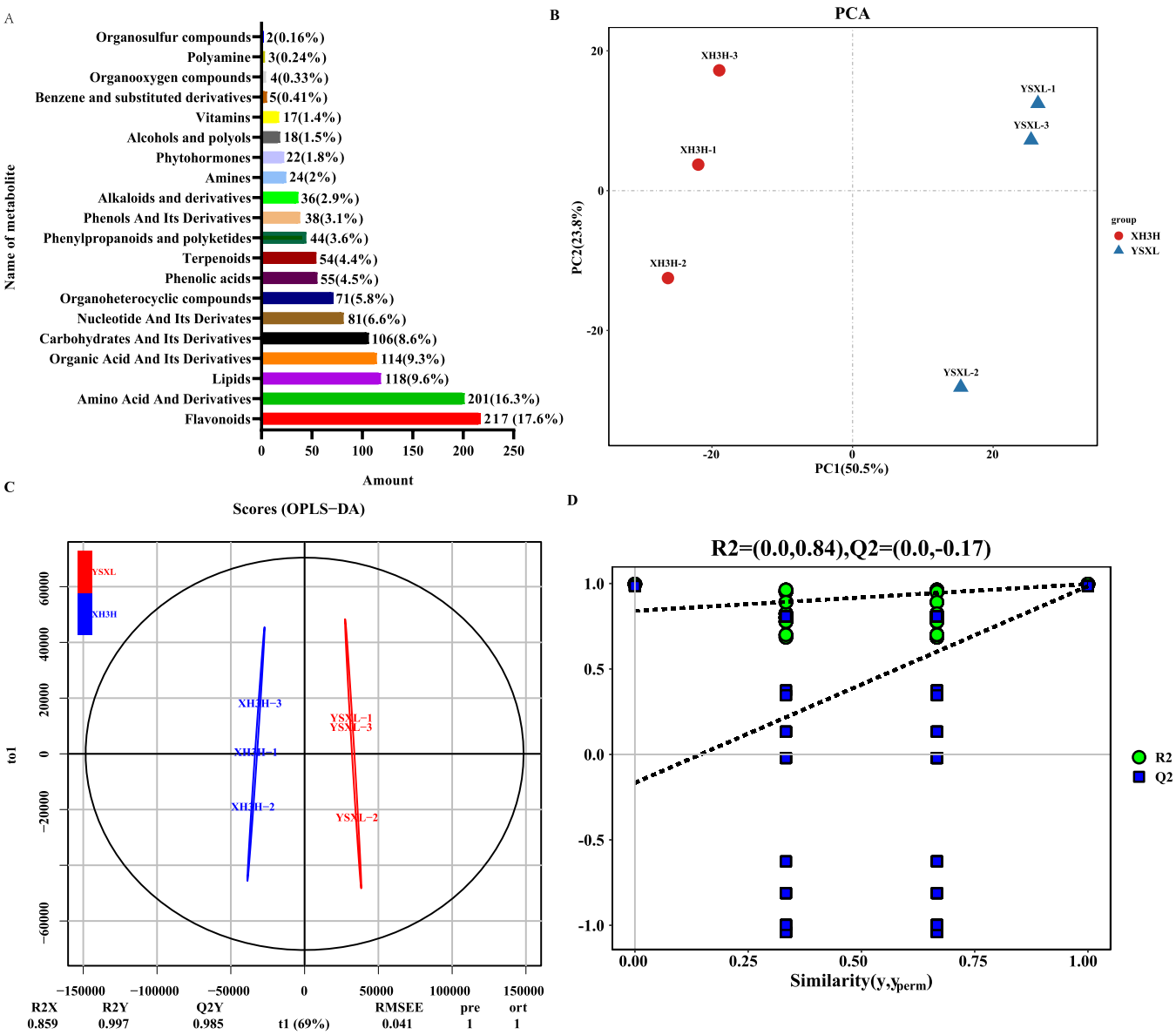

Figure 1. Analysis of widely targeted metabolites in YSXL and XH3H tea plant. (A) Classes of metabolites were identified in YSXL and XH3H. (B) Principal component analysis. (C) Orthogonal partial least-squares discriminant analysis (OPLS-DA). (D) Displacement test analysis.

region, spanning from −1071 to −1386 bp, was inserted into the pHis2 vector, yielding the pHis2-TbS recombinant plasmid. For the experimental setup, a mixture of the pHis2-CsTbS and pGADT7-CsMYB114 plasmids was prepared. In contrast, the control group used a combination of the pHis2-CsTbS and the empty pGADT7 vectors. These plasmid mixtures were then used to transform the yeast strain Y187. The transformed yeast cells were spread on SD/-Leu/-Trp plates and incubated at 28 °C for a period ranging from 2 to 5 days. Yeast cells were cultured on a triple-deficiency medium (SD/-His/-Leu/-Trp), both with and without the addition of 3-AT. These cultures were prepared in a series of dilutions, reducing the concentration by factors of 10, 100, and 1000 respectively, and then incubated at 28 °C for 3 days.[37] The presence of white colonies in the experimental group, in contrast with their absence in the control group (which utilized self-activation), would indicate effective trans-activation.

Antisense Oligonucleotide Assay (ASO). Based on the sequences of the tea plant gene *CsMYB114*, several optimal ASOs were designed and selected using the online soft Soligo[38] (http://sfold.wadsworth.org/cgi-bin/index.pl). Shoots from the XH3H tea plant were positioned in centrifuge tubes, each containing 20 μM CsMYB114-associated ASO. As a control, tubes were filled with water. To detect changes in *CsMYB114* gene expression and caffeine levels, samples were collected after 6, 24, and 48 h.

Validation of Cleavage Sites Using Modified RLM-RACE. The verification of cleavage sites was conducted in accordance with the methods outlined in an earlier study.[39] The primers for cleavage of the target gene were designed using DNAMAN software, and the nested PCR amplification was executed utilizing a combination of specific and universal primers (Table S1).

RESULTS

Widely Targeted Metabolites Analysis of YSXL and XH3H Tea Cultivars. The metabolites of the two tea plant species were analyzed by ultraperformance liquid chromatography tandem mass spectrometry (UPLC−MS/MS) in ESI positive and negative ion mode, respectively. First, the total ion chromatograms (TIC) of the quality control samples were overlaid for comparison (Figure S3). For the two species, the coefficient of variation (CV) was assessed. In XH3H, 86.6% of the total ESI positive ion intensities and 85.7% of the total ESI

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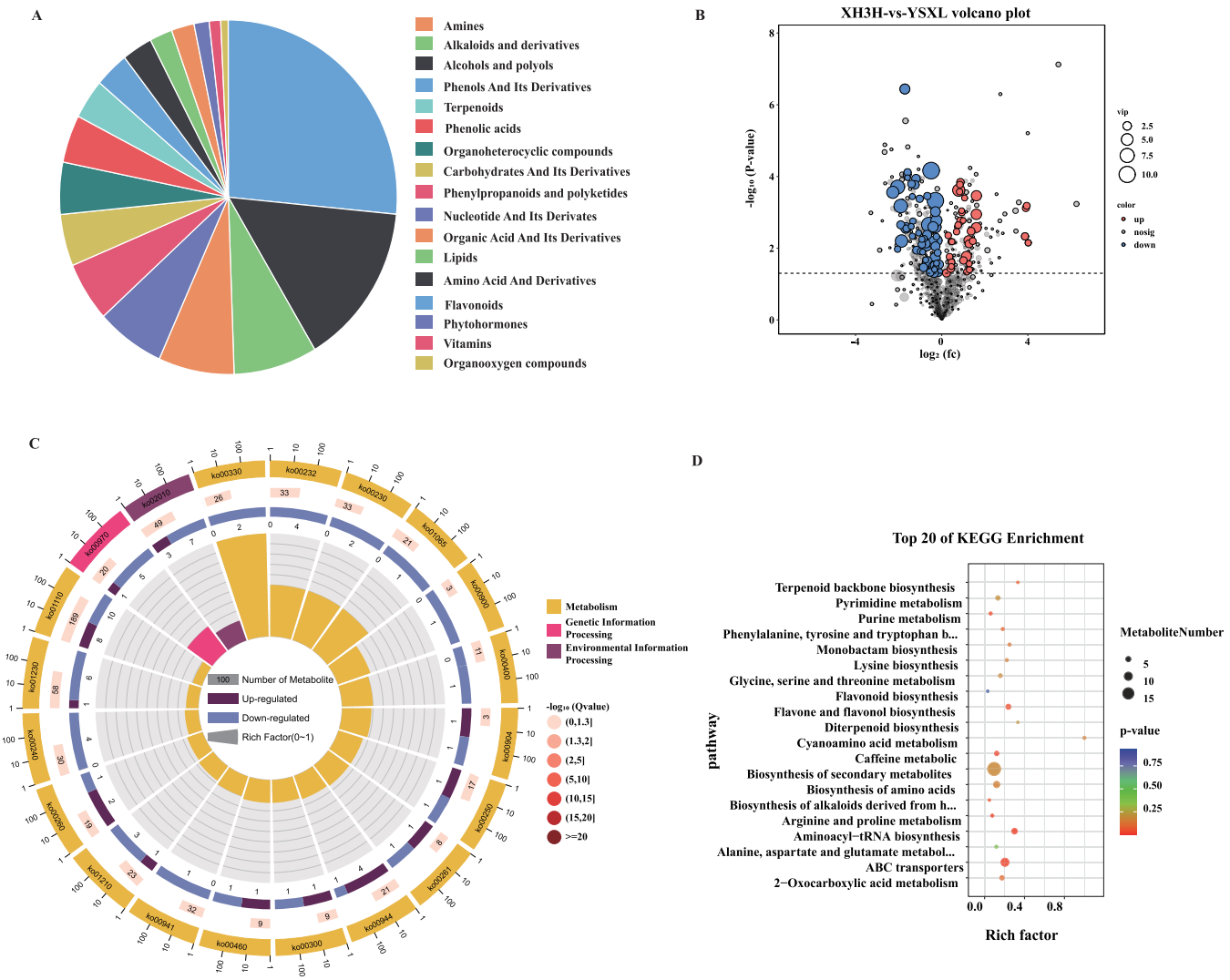

Figure 2. Differential metabolite analysis. (A) Pie chart of YSXL and XH3H significant differential metabolites. (B) Volcano chart of differential metabolites. (C) KEGG enrichment circles (first circle: top 20 enriched pathways; outside the circle is the coordinating scale for the number of differential metabolites. Different colors represent different A classes; the second circle: the number of this pathway in the differential metabolite background and the *Q* value. The length of the bar represents the number of differential metabolites in the background, and a more intense red color indicates a smaller *Q* value; third circle: the bar of the up and down differential metabolite ratios, dark purple represents up-regulated differential metabolite ratios, light purple represents down-regulated differential metabolite ratios; the specific values are shown below; fourth circle: the Rich Factor value of each pathway (the number of differential metabolites in that pathway divided by the pathway, background grid lines, each cell represents 0.1)). (D) KEGG enrichment bubble plot (using the top 20 pathways with the smallest *Q* values to make the plot, the vertical coordinate is the pathway, horizontal coordinate is the enrichment factor (the number of differential metabolites in that pathway divided by all quantities in that pathway), size indicates how many quantities, and a more intense red color indicates a smaller *Q* value).

negative ion intensities had relative standard deviations below 20%. Similarly, in YSXL, 87.5% of the ESI positive ion intensities and 83.5% of the ESI negative ion intensities displayed relative standard deviations under 20%. The above results indicate that the metabolomics method for tea plant samples has good signal stability, reliable data, and good reproducibility.

Using a broadly targeted metabolomics approach, 1230 metabolites spanning 20 classes were identified in XH3H and YSXL (Figure 1A). The predominant metabolites included flavonoids (like quercetin-*O*-glucoside, quercetin, and peonidin chloride), amino acids and derivatives (such as L-arginine, DL-leucine, and *S*-adenosylmethionine), lipids (examples are linoleic acid, sorbic acid, and methyl myristate), organic acids and derivatives (e.g., gallic acid, gallocatechin gallate, and 1,5-dicaffeoylquinic acid), and sugars and their derivatives (including ribulose-5-phosphate, lactose, D-glucopyranose). These classes respectively comprised 217, 201, 118, 114, and 106 species (Figure 1A). This was followed by nucleotides and their derivatives (s-adenosyl-methionine, 2,6-dihydroxypurine, adenosine, etc.), organic heterocyclic compounds (7-methyl-guanine, allantoin, purine, etc.), phenolic acids (2-*O*-caffeoylmalic acid, 3-hydroxycinnamic acid, cinnamic acid, etc.), and terpenoids (examples being alpha-ionone, lactucopicrin, geraniol, etc.). Other significant groups were Phenylpropanoid and polyketone compounds (including coumarin, caffeic aldehyde, and scopolin), phenols and their derivatives (pyrogallol, among others), and alkaloids and their derivatives (like lappaconitine, gramine, and aloperine). The rest of the groups had fewer than 35 species. Notably, the least diverse

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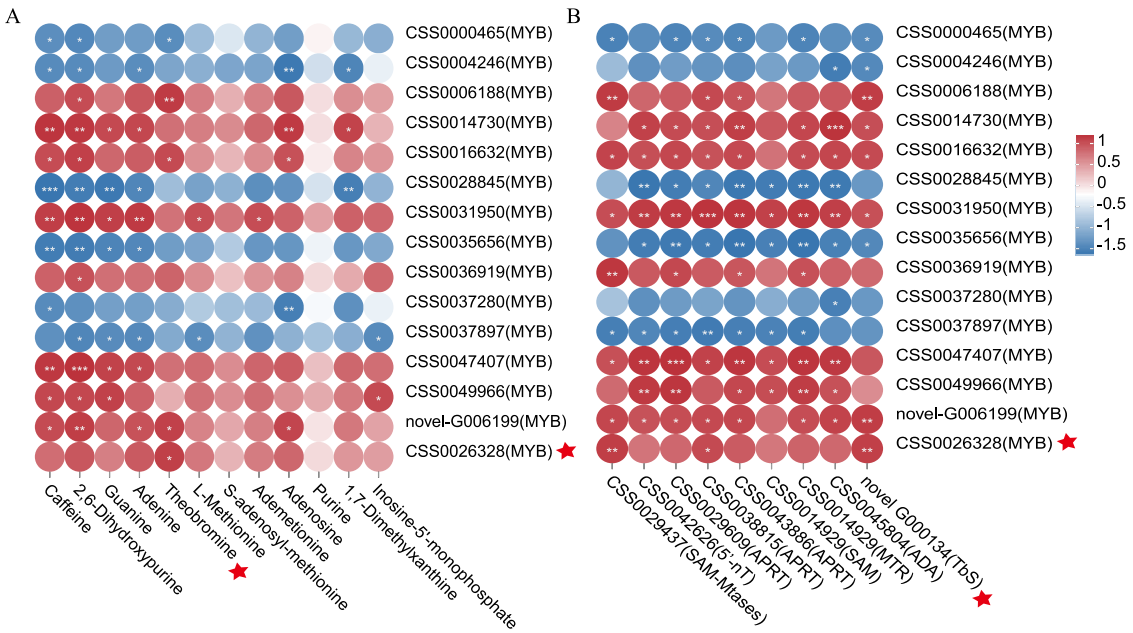

Figure 3. (A) Correlation between MYB TFs expression and the relative content of purine alkaloids; (B) correlation between MYB TFs expression and the expression of key genes for the biosynthesis of theobromine.

group of metabolites was organosulfur compounds, with only two detected species, representing a mere 0.9% of the flavonoids (Figure 1A). The metabolites of YSXL and XH3H were subjected to PCA and OPLS-DA analysis. The first principal component (PC1) accounted for 50.5% variance and the second principal component (PC2) for 23.8%, collectively contributing over 74.3% (Figure 1B, Table S2). The OPLS-DA model evaluation showed R2X = 0.859, R2Y = 0.997, and Q2 = 0.985, indicating that the model was stable and reliable with Q2 greater than 0.9. (Figure 1C). Cross-validation and permutation tests were also performed to verify the reliability of the OPLS-DA model (Figure 1D). The scores from the OPLS-DA and the outcomes of the permutation test suggest that the model is both a reliable predictor and an effective evaluation tool. Furthermore, this indicates that the differential metabolites identified through VIP analysis are accurately represented.

Differential Metabolite Identification in YSXL and XH3H. Based on the OPLS-DA model, the YSXL and XH3H were screened for significantly different metabolites according to the criteria of VIP $\geq$ 1, FC $\geq$ 2 or FC $\leq$ 0.5. A total of 276 significantly different metabolites were identified in the two tea cultivars (Table S3; Figure 2A), accounting for 22.36% of the total metabolites measured. The most prominent categories of differential metabolites were flavonoids, followed by amino acids and their derivatives, with lipids and organic acids and their derivatives also being notably present (Figure 2A). In XH3H compared to YSXL, 25 metabolites showed significantly increased levels, while 17 displayed significantly decreased levels (Figure 2B). The relative contents of 4 amino acids and their derivatives, 10 flavonoids, 3 nucleotides, and their derivatives, 3 organic heterocyclic compounds, 2 alcohols and polyols, 1 terpene, 1 phenol and its derivatives, and 1 phenolic acid in XH3H were higher. YSXL had a high relative content of nine flavonoids, four lipids, two organic acids and their derivatives, one alcohol and polyols, one phenol and its derivatives, and one sugar and its derivatives. The above results showed that the metabolite content in XH3H and YSXL is highly variable.

Differential Metabolite Pathway Analysis. Pathway enrichment analysis was performed on 276 differential metabolites through the KEGG database. Of these, 91 differential metabolites (61 up-regulated and 30 down-regulated in XH3H compared to YSXL) were successfully annotated into 91 metabolic pathways (Table S4). The top 20 most enriched pathways were (the closer the *p*-value to 0 the more significant the enrichment): arginine and proline metabolism, caffeine metabolism, purine metabolism, adenine ribonucleotide biosynthesis, terpenoid backbone biosynthesis, phenylalanine, tyrosine, and tryptophan biosynthetic pathways, diterpenoid biosynthesis, alanine, aspartate and glutamate metabolic pathways, flavonoid biosynthetic, flavonoid and flavonol biosynthetic, ABC transport, aminoacyl-tRNA biosynthesis, lysine biosynthesis, cyan amino acid metabolism, glycine, serine, and threonine metabolism, pyrimidine metabolism, 2-oxocarboxylic acid metabolism, biosynthesis of amino acids, biosynthesis of secondary metabolites, monobactam biosynthesis (Figure 2C,D). The KEGG pathway annotation analysis identified the arginine and proline metabolic pathways, the caffeine metabolic pathway, and the purine metabolic pathway as the main differential metabolic pathways in XH3H and YSXL (Figure 2D).

Analysis of the Main Differential Metabolite Components. To gain a clearer understanding of the differences in purine alkaloids between the two samples, the main differential metabolite caffeine metabolism and purine metabolic pathways of XH3H and YSXL were analyzed in this study. There are seven purine differential metabolites in XH3H and YSXL (Figure 2D), and six purine alkaloid substances (xanthine, guanine, adenine, *S*-adenosylmethionine, theobromine, and caffeine) were more abundant in XH3H than in YSXL. The KEGG results also showed that the purine metabolic pathway was one of the main metabolic pathways that differed between the two tea plants.

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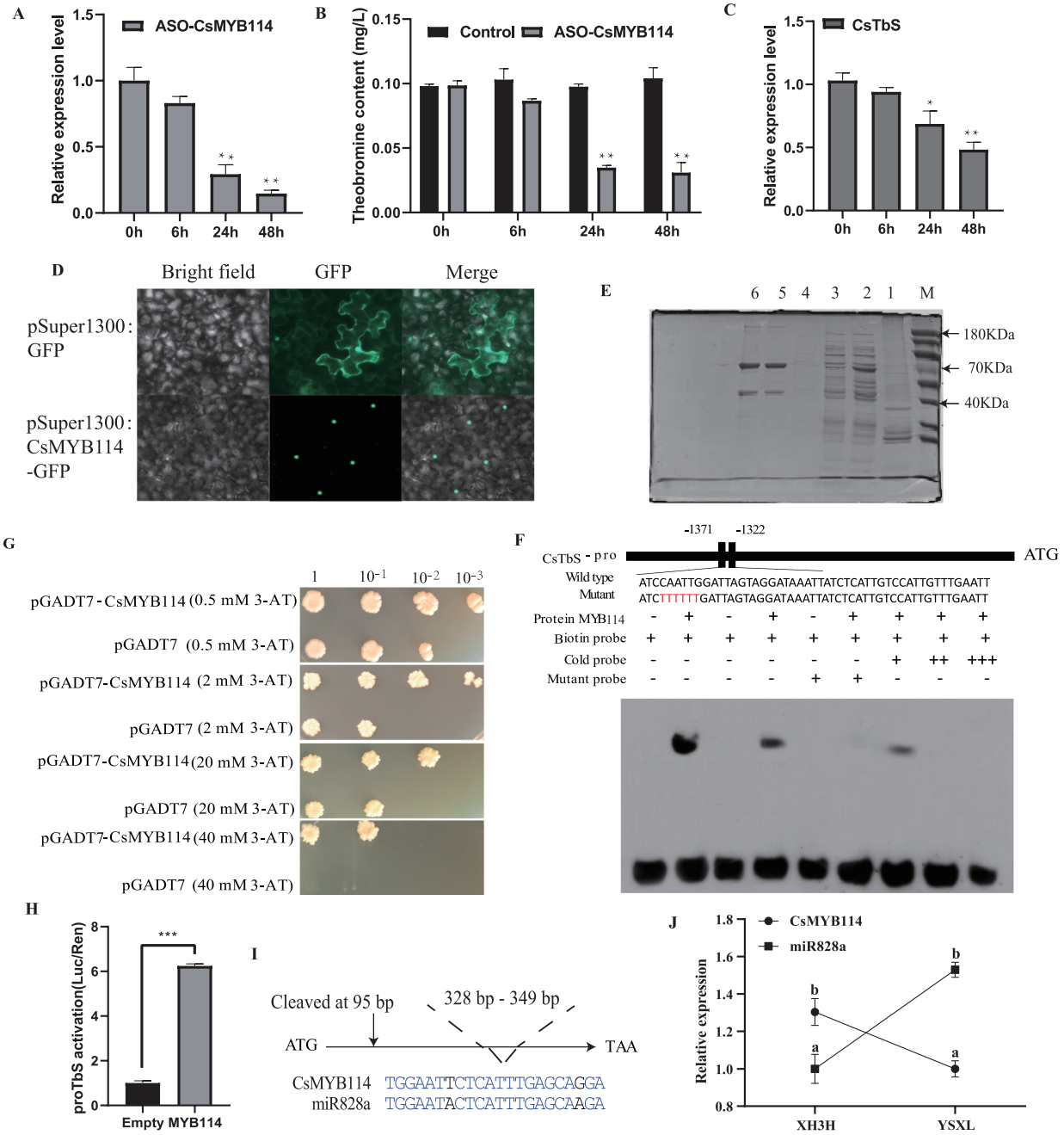

Figure 4. CsMYB114 is localized in the nucleus and has transactivational activity, CsMYB114 binds to the promoter of *CsTbS* and miR828a is able to cleave *CsMYB114* target genes. (A) Gene expression of CsMYB114 in leaves after ASO-CsMYB114 treatment expression. (B) Theobromine content in young shoots treated with water and ASO-CsMYB114. (C) Relative expression of *CsTbS* after the inhibition of CsMYB114. (D) Subcellular localization of CsMYB114 in tobacco leaves; scale bars = 10 μm. (E) Protein purification to obtain a fusion protein of MBP-CsMYB114. M: Protein Marker; 1: the expression of CsMYB114 protein was detected without induction; 2−3: Supernatant and precipitation of 1 mmoL/L IPTG-induced protein; 4: Washing solution; 5−6: Purified CsMYB114 recombinant protein washing solution. (F) EMSA, from left to right: Negative control; Positive control; CsTbS probe; The CsTbS probe binds to the CsMYB114 protein; CsTbS mutant probe; CsTbS mutant probe and CsMYB114 protein; CsMYB114, CsTbS mutant probe and 10× CsTbS competitive probe; CsTbS mutant probe and 50× CsTbS competitive probe; CsTbS mutant probe and 100× CsTbS competitive probe; The 10× CsTbS competitive probes can compete with part of the strip, and the 50× and 100× CsTbS competitive probes can compete with all the strip. (G) Transcriptional activity of *CsMYB114* in yeast cells. (H) Transcriptional transactivation of *CsMYB114* in tobacco. (I) The miR828a mature sequence binds to the target gene *CsMYB114*, prompting cleavage of the target gene at 95 bp. (J) Relative expression of the *CsMYB114* and *CsTbS* genes in XH3H and YSXL tea plant shoots. All experiments were performed in 3 biological replicates. Different letters and "*" indicate statistical differences in the expression or substance content of the corresponding genes ($p < 0.05$, Duncan's multiple range test).

Regulatory Transcription Factors Associated with Theobromine Synthesis. In order to screen the TFs involved in the theobromine biosynthetic pathway, the gene expression profiles of YSXL and XH3H were analyzed based on the data from our previous transcriptome (PRJNA986690), and the results indicated 88 TFs and 9 key genes in purine alkaloid biosynthesis pathway were differentially expressed between 2 tea cultivars (Figure S4). Furthermore, the

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correlation between the content of theobromine and the gene expression level of 88 TFs was investigated, and the results indicated that 3 MYBs (CSS0006188, CSS0026328, and novel-G006199) showed the highest correlation coefficient (r^2 > 0.9), which implied MYB might be the key regulator in the biosynthesis of theobromine (Figures S5 and 3A, and Table S5). Moreover, the bindings of MYB transcription factors to the *CsTbS* promoter were predicted using JASPAR online software. The results showed that a MYB (CSS0026328), which is annotated as MYB114 in the reference genome of shuchazao2 (http://tpia.teaplants.cn/), had the highest binding score of 11.33, which indicated that it had a strong affinity for the promoter of *CsTbS* (Table S6). Finally, analysis of the correlation between *CsMYB114* and *CsTbS* gene expression levels revealed a notably high correlation coefficient of 0.92 (Figure 3B), which further verified that CsMYB114 might be a TF of *CsTbS*.

Effect of *CsMYB114* Inhibition on Theobromine Biosynthesis. The designed antisense oligodeoxynucleotide, ASO-CsMYB114, aimed at targeting and reducing CsMYB114 expression, demonstrated considerable effectiveness in suppressing activity at both 24 and 48 h in the samples within the centrifuge tubes (Figure 4A). This result underscores the potent inhibitory action of ASO-CsMYB114. In this study, we compared tea plant shoots treated with ASO-CsMYB114 to a control group treated with water. After 48 h, we observed a significant decrease in both theobromine content (70.21%) and the *CsTbS* gene expression (58.18%) in the ASO-CsMYB114 treated group (Figures 4B,C and S6). These results suggest a direct association between the suppression of *CsMYB114* gene expression and the reduction of the theobromine content.

Subcellular Localization Analysis of CsMYB114. To ascertain the subcellular position of CsMYB114, construct pSuper1300-CsMYB114-GFP underwent transient expression in the leaves of tobacco plants. For comparison, pSuper-1300-GFP served as a control within the epidermal cells of tobacco leaves. Observations revealed that CsMYB114 predominantly resides in the nucleus (Figure 4D).

CsMYB114 Interacts with the Promoter Region of *CsTbS*, Enhancing Its Transcriptional Activity. The MBP-CsMYB114 fusion protein produced through IPTG induction and subsequent protein purification was used in the EMSA (Figure 4E). The *CsTbS* promoter sequence (−1322 ∼ −1371 bp) features a potential MYC binding site (CAATTG). EMSA results revealed that CsMYB114 protein binds specifically to the *CsTbS* promoter, with this binding activity diminishing upon the introduction of an unlabeled cold probe. (Figure 4F). The results demonstrated that CsMYB114 exhibited specific binding to the promoter of *CsTbS*.

The yeast one-hybrid (Y1H) method was employed to verify the binding affinity of CsMYB114 for the *CsTbS* promoter. The findings showed that introducing CsMYB114-pGADT7 (pHis2-pro-CsTbS and AD-CsMYB114) into yeast cells (Figure S7) facilitated their growth on SD/-His/-Leu/-Trp selection medium with 40 mM 3-AT (Figure 4G). This growth pattern substantiates the interaction between CsMYB114 and the *CsTbS* promoter within the yeast environment. To further corroborate the interaction between CsMYB114 and the *CsTbS* promoter, a dual luciferase assay was conducted. Findings revealed that the LUC/REN ratios of the reporter gene, when coinjected with pGreenII 62-SK-CsMYB114, were elevated by 6.24 times compared to the negative control, which used pGreenII 62-SK-Empty (Figure 4H). This indicates that the CsMYB114 protein can enhance the transcriptional activity of the *CsTbS* promoter.

Characterization of miR828a Target Gene. The prediction of CsMYB114 was made using psRNA Target software, utilizing miRNA data from tea leaves acquired through bioinformatics and high-throughput sequencing. Eighty miRNAs were identified as potential agents for cleaving CsMYB114 (Table S7). The validation of these cleavage sites was conducted by using a modified RLM-RACE technique. Our results conclusively show that miR828a targets and specifically cleaves the downstream transcription factor CsMYB114 at the 95 bp position (Figures 4I and S8). This process plays a crucial role in the regulation of theobromine biosynthesis. To further verify the correlation between miR828a and its target gene CsMYB114, quantitative reverse transcription-PCR (q-RT-PCR) analysis was performed (Figure 4J). The study revealed distinct expression levels of the *CsMYB114* gene in different tea cultivars: it was 1.53 times higher in the XH3H cultivar compared to YSXL. In contrast, the expression level of miR828a was greater in YSXL, being 1.3 times that of XH3H. Notably, miR828a and CsMYB114 exhibited a strong negative correlation with a coefficient of −0.96. This significant observation strongly supports the idea that miR828a plays an inhibitory role in controlling the expression of *CsMYB114*.

■ DISCUSSION

The tea made from "Jianghua Kucha" shows a special characteristic in taste and flavor. So far, there is less information about the chemical composition of "Jianghua Kucha". In this study, we determined the chemical composition of "Jianghua Kucha" (XH3H) using the ESI-Q TRAP−MS/MS technique (Table S3). In the theobromine synthesis pathway, XH3H contains higher levels of *S*-adenosylmethionine, caffeine, guanine, adenine, and theobromine compared to the common tea cultivar (YSXL). More importantly, we found that theobromine content in XH3H was 2.82 times higher than that in YSXL (Figure S5), which provided an excellent material for research on the regulation of theobromine biosynthesis.

Theobromine has received more and more attention as an important alkaloid in tea plant. Up to this point, only CsNAC7 and CsMYB184 have been functionally identified as regulators of caffeine biosynthesis, with each one specifically enhancing the expression of the critical biosynthetic genes *NMT1* and *TCS1*, respectively.[19,21] In addition, a novel senescence-associated transcription factor, CsS40, was found to regulate caffeine biosynthesis by downregulating the biosynthetic gene *TCS1*.[20] However, there is limited understanding regarding the regulatory mechanisms of theobromine biosynthesis. It has been reported that CsMYB184 (TEA029017.1) seemed to be involved in the biosynthesis of theobromine, but this needs to be further verified.[40] In this work, we analyzed the relationship between the metabolite concentrations and the gene expression levels in the theobromine synthesis pathway, utilizing both metabolomic and transcriptomic data. We found that the expression level of *CsMYB114* showed a high correlation with that of *CsTbS* and theobromine content (Figure 3 and Table S5), indicating that CsMYB114 may function as a TF for *CsTbS*. Interestingly, we found that the sequence similarity between *CsMYB184* and *CsMYB114* was 99.83% (Figure S9), implying that *CsMYB184* and *CsMYB114*

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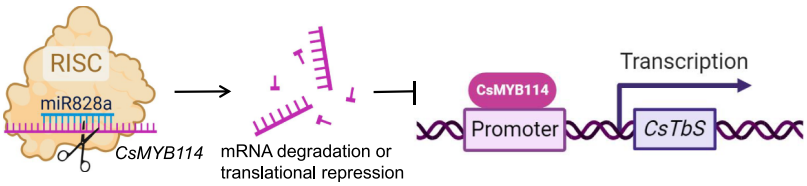

Figure 5. A possible model for the involvement of CsMYB114 and miR828a in the biosynthetic regulation of tea plant theobromine. *CsTbS*, theobromine synthases.

shared a similar function of regulating the biosynthesis of theobromine.[40]

To validate our hypothesis, we conducted studies on the functional role of CsMYB114 and its potential interaction with the *CsTbS* promoter. The expression pattern of CsMYB114 was identical to that of *CsTbS* and the content of theobromine, which also suggests that CsMYB114 might be regulating the content of theobromine. We found that the inhibition of *CsMYB114* expression in leaves of "XH3H" using ASO led to a significantly reduced theobromine content (Figure 4B), which further verified that *CsMYB114* was involved in the biosynthesis of theobromine. Furthermore, motif analysis identified the sequence CAATTG in the *CsTbS* promoter, recognized as a transcription factor (TF) binding site for CsMYB114. The binding of CsMYB114 to this CAATTG sequence was confirmed through yeast one-hybrid, EMSA, and dual luciferase assays (Figure 4E−H). These findings indicate that CsMYB114 acts as a positive TF for *CsTbS*, regulating the theobromine content in tea plants by specifically binding to and activating the expression of the *CsTbS* gene. Recently, CiMYB42 has been found to bind the TTGTTG motif (type II MYB core) in the CiOSC promoter.[41] Similarly, TcMYB29a can bind and activate the promoter of *TcT5OH* to regulate taxol biosynthesis in *Taxus chinensis*.[42] Our research offers an additional understanding of the importance of MYB transcription factors in the control of secondary metabolite biosynthesis in plants.

Moreover, the regulation of CsMYB114 expression was investigated in the present study. Our study uncovered an inverse relationship between the expression levels of CsMYB114 and miR828a (Figure 4J). This suggests that miR828a, acting as a negative regulator of theobromine biosynthesis, can target and cleave CsMYB114 (Figure 4I), leading to reduced theobromine accumulation. microRNAs a category of small noncoding RNAs, are universally present in living organisms and play regulatory roles. Current research focuses on the involvement of miRNAs in regulating biological activities such as the synthesis of secondary metabolites and stress response in plants.[43−47] In pitaya, HmTT2-like, a MYB TF, was found to be negatively correlated with betalain accumulation.[48] Hmo-miR828a targets HmTT2-like, suggesting a positive role in betalain accumulation in pitaya.[48] Similarly, in purple potato, miR828a targets a MYB gene (PGSC0003DMT400078477, MYB3-like) to regulate anthocyanin biosynthesis.[49] In tea plant leaves infected with *Colletotrichum gloeosporioides*, the expression profile of csn-miR828a showed a negative correlation with its target gene MYB75,[50] which is known as a positive regulator of abiotic stress responses. This implies that csn-miR828a might play a negative role in abiotic stress response.[50] These findings indicate that a single miRNA can target multiple genes and be involved in regulating the synthesis of various secondary metabolites as well as stress responses in plants. Nonetheless, our results indicate an unprecedented function of miR828a in controlling the production of theobromine in tea plants.

To summarize, our research demonstrates that *CsMYB114* acts as a transcription factor for *CsTbS*, engaging with the *CsTbS* promoter to stimulate its transcription. We also demonstrated that ASO-CsMYB114 effectively represses the expression of *CsMYB114*, leading to a reduced theobromine level. Furthermore, we found that miR828a could precisely target and cleave *CsMYB114*, thereby inhibiting its expression and consequently affecting theobromine biosynthesis in tea plant (Figure 5).

■ ASSOCIATED CONTENT

Data Availability Statement

The raw sequence data have been submitted to the NCBI Sequence Read Archive database under accession number PRJNA986690 and PRJNA1056246.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c07736.

List of primers used in this study, XH3H-vs-YSXL pc, 276 significantly different metabolites, XH3H-vs-YSXL differential metabolite pathways, correlation between gene expression and broad target metabolite content, prediction of the binding site of MYB transcription factor to the promoter of theobromine synthase, miRNA and target gene cleavage site prediction (XLSX)

Sequence information on *CsTbS* gene (novel-G000134) and qRT-PCR expression in the two cultivars (XH3H and YSXL), sequence information on *CsMYB114* gene (CSS0026328), TIC overlap map of quality control samples, 88 TFs and 9 key genes in purine alkaloid biosynthesis pathway were differentially expressed between two tea cultivars, Theobromine content of XH3H and YSXL, HPLC profiles of theobromine content in young shoots treated with water and ASO-CsMYB114, SD/Leu/Trp plate maps of pGADT7 and pGADT7-CsMYB114, miR828a targets the cleavage of the downstream transcription factor CsMYB114, sequence alignment of CsMYB184 (TEA029017.1) and CsMYB114 (CSS0026328) (DOCX)

■ AUTHOR INFORMATION

Corresponding Authors

Zhonghua Liu − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China;*

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Phone: +86 0731 84635304; Email: zhonghua-liu-ms@hunau.edu.cn; Fax: +86 0731 84635304

Jianan Huang − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China;* Phone: +86 0731 84635304; Email: Jian7513@hunau.edu.cn; Fax: +86 0731 84635304

Na Tian − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China;* Phone: 086(0)73184635304; Email: tianna5678@hunau.edu.cn; Fax: 086(0)73184635306

Shuoqian Liu − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China;* Phone: 086(0)73184635304; Email: shuoqianliu@hunau.edu.cn; Fax: 086(0)73184635306

Authors

Qifang Jin − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China;* orcid.org/0000-0002-6108-0628

Zhong Wang − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

Devinder Sandhu − *United States Salinity Laboratory, United States Department of Agriculture, Agricultural Research Service, Riverside, California 92507, United States;* orcid.org/0000-0003-4193-3408

Lan Chen − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

Chenyu Shao − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

Siyi Xie − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

Fanghuizi Shang − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

Shuai Wen − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

Ting Wu − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

Huiying Jin − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

Feiyi Huang − *Tea Research Institute, Hunan Academy of Agricultural Sciences/National Small and Medium Leaf Tea Plant Germplasm Resource Nursery, Changsha 410125, China*

Guizhi Liu − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

Jinyu Hu − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

Qin Su − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

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Mengdi Huang − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

Qian Zhu − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

Biao Zhou − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

Lihua Zhu − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

Lvwen Peng − *Key Laboratory of Tea Science of Ministry of Education, Hunan Agricultural University, Changsha 410127, China; National Research Center of Engineering and Technology for Utilization of Botanical Functional Ingredients and CoInnovation Center of Education Ministry for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410127, China*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acs.jafc.3c07736

Author Contributions

[#]Q.J. and Z.W. contributed equally. Q.J. designed and performed the experiments, analyzed the data, and wrote the manuscript. Z.W. performed the experiments, analyzed the data, and wrote the manuscript. D.S. performed data analysis, reviewed and revised the manuscript. L.C., C.S., S.X., F.S., S.W., T.W., H.J., F.H., G.L., J.H., Q.S., M.H., Q.Z., B.Z., L.Z., and L.P. analyzed the data. Z.L., J.H., N.T., and S.L. conceived the study, participated in the coordination, data analysis and interpretation, and drafted and reviewed the manuscript. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

CsTbS, theobromine synthase; ASO, antisense oligonucleotides; SAM, *S*-adenosyl-L-methionine; *CmXRS1*, 7-methylxanthosine synthase gene; *TCS1*, caffeine synthase; EMSA, Electrophoretic Mobility Shift Assay; LUC, firefly luciferase; REN, Renilla luciferase; TIC, the total ion chromatograms; LC-ESI-MS/MS, liquid chromatography tandem mass spectrometry; HPLC, High performance liquid chromatography; ESI-Q TRAP−MS/MS, electrospray ionization quadrupole linear ion trap tandem mass spectrometry; UPLC-MS/MS, ultraperformance liquid chromatography tandem mass spectrometry; CV, coefficient of variation; NMT, *N*-methyltransferase

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