


Differential accumulation mechanisms of purine alkaloids and catechins in *Camellia ptilophylla*, a natural theobromine-rich tea

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Abstract

Tea is consumed worldwide due to its charming flavor and the refreshing effects conferred by caffeine. Caffeine however has undesirable side effects, such as sleep disturbance. *Camellia ptilophylla* is known for its low caffeine content, and the biosynthesis of purine alkaloids in this species has become a hot topic. In this study, the accumulation of purine alkaloids in a natural *C. ptilophylla* population (32 plants) was analyzed, and the results showed that 81.25% of this population were caffeine-free, containing only theobromine (TB), while six plants contained both theobromine and caffeine (CAF). RNA-seq analysis of two *C. ptilophylla* plants with contrasting purine alkaloid contents (TB and CAF) revealed that xanthosine synthesis genes of the SAM cycle and AMP pathway were significantly related to the differential accumulation of purine alkaloids between TB and CAF. The high theobromine content in TB was attributed to the significantly higher expression of *TCS-2*, *TCS-3* and *MXMTs* and downregulation of the xanthosine degradation pathway in comparison to CAF. Additionally, *CsMYB184* was significantly upregulated in TB, opposing the expression pattern of *TCS1*, but consistent with that of other *TCSs* and *MXMTs*. Furthermore, the upregulated expression of catechin biosynthesis genes, *F3'H*, *F3'5'H* and *SCPLs* in TB corresponded to a higher gallicocatechin gallate (GCG) content. Overall, these findings provide new insights into the accumulation of theobromine and GCG, which may facilitate the development of tea plant cultivars with low-caffeine or high GCG to meet the diverse demands of consumers.

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Introduction

The widespread consumption of tea, which is derived from the tender shoots of *Camellia sinensis* and related species, can be attributed to its captivating flavors^[1]. Furthermore, tea is rich in various biologically active metabolites, such as flavonoids, theanine, and caffeine, which are known to confer a range of health benefits^[2–4]. Among these metabolites, caffeine (1,3,7-trimethylxanthine) is the dominant purine alkaloid in most teas and is globally recognized for its ability to stimulate the central nervous system^[5]. In addition to caffeine, theobromine (3,7-dimethylxanthine) and theacrine (1,3,7,9-tetramethyluric acid) are also typical purine alkaloids found in tea plants^[6,7]. The content of these purine alkaloids varies considerably among different tea accessions, with most containing caffeine at concentrations of 25.0–45.0 mg/g and theobromine at 2.4–4.3 mg/g^[5,8].

The excessive consumption of caffeine can have adverse effects, such as insomnia, migraines, and changes in intraocular pressure^[9,10]. This has led to a growing demand in the market for naturally low-caffeine or decaffeinated tea products. In response to this demand, researchers have turned their attention to identifying unique natural variations within tea plant species that accumulate low levels of caffeine or none at all^[11]. One such species, *Camellia ptilophylla*, possesses a natural variant that accumulates theobromine only^[12]. This variant represents an efficient and unique raw material for the development of low-caffeine tea products through breeding programs.

The biosynthesis pathway of caffeine has been extensively studied^[13,14]. Caffeine is synthesized from xanthosine^[15] in three methylation steps, by methyltransferase (XMT), 7-methylxanthine methyltransferase (MXMT) and tea caffeine synthase (TCS), and one nucleoside removal step^[16,17]. *TCS1*, which catalyzes 1-N and 3-N methylation of 7-methylxanthine, is essential to catalyze conversion of theobromine to caffeine. Prior studies have revealed that *TCS1* in most tea cultivars exhibits higher enzymatic activity and expression levels compared to *C. ptilophylla*, and furthermore, the *TCS1* enzyme in *C. ptilophylla* solely possesses 3-N methyltransferase activity^[18–20]. Thus, the low-caffeine levels in *C. ptilophylla* could be related to the reduced capacity of *TCS1*. Recent studies have documented that transcription factors (TFs) also play a critical role in caffeine biosynthesis^[21–24], such as *CsMYB184* and *CsNAC7*^[16,25]. Li et al. have proven that *CsMYB184* was significantly downregulated in *C. ptilophylla* compared to other tea cultivars^[16]. Therefore, the mechanism underlying caffeine deficiency in *C. ptilophylla* still remains elusive and requires further investigation.

Catechins, which confer bitter and astringent tastes, are also important indicators of tea quality^[26,27]. EGCG is the primary catechin monomer found in *C. sinensis*, with an average content of 94.6 ± 12.6 mg/g, whereas GCG is typically found in much smaller amounts (0.9 ± 2.2 mg/g)^[28,29]. However, in *C. ptilophylla*, GCG is the main catechin and can reach 93.85 ± 0.96 mg/g^[30]. Despite the extensive literature on tea catechins^[31], there remains a knowledge gap regarding the molecular

biology of different catechin monomers that accumulate in *C. ptilophylla*.

In this study, the content of purine alkaloids in a *C. ptilophylla* population (32 plants) were quantified by HPLC. Two *C. ptilophylla* plants, one was caffeine-free containing only theobromine (TB), and the other contained both theobromine and caffeine (CAF) were used to analyze content of catechins and theanine. RNA-seq was used to investigate the different mechanisms of purine alkaloid and catechins accumulation in TB and CAF.

Materials and methods

Plant materials

Samples containing one bud and two leaves each were collected from 32 individual *C. ptilophylla* plants in June 2022 in the Conghua tea garden (Guangzhou, Guangdong province, China), and the samples were stored at -80°C for chemical composition analysis and RNA extraction.

Chemicals

Caffeine, theobromine, theacrine and theophylline standards were bought from Beijing Weiye Research Institute of Metrology and Technology (Beijing, China). Magnesium oxide was purchased from FUCHEN Chemical Reagents LTD (Tianjin, China).

Theanine, catechin (C), gallic catechin (GC), epicatechin (EC), epigallocatechin (EGC), catechin gallate (CG), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and gallic catechin gallate (GCG) standards were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China).

Acetonitrile and methanol were obtained from BCR International Trading, Inc. (Florida, USA). Formic acid was purchased from Shanghai EN Chemical Technology Co., Ltd. (Shanghai, China)

Quantification of purine alkaloids

Freeze-dried powder (0.1 g) was used to extract four kinds of purine alkaloids in 30 mL ultrapure water containing 1.5% magnesium oxide (w/v) by ultrasonic vibration at 100°C for 30 min and then filtered through a 0.22 mm Millipore membrane. High-performance liquid chromatography (HPLC) was used to determine the purine alkaloids content according to a previous method with minimal changes^[32]. Briefly, 10 μL filtered extract was injected into an XSelect HSS C18 SB column (4.6×250 mm, 5 mm, Waters Technologies, Milford, MA, USA) at a flow rate of 0.9 mL/min, with the column maintained at a constant temperature of $35 \pm 1^{\circ}\text{C}$. The detection of caffeine, theobromine, theophylline and theacrine was performed on a Waters Alliance E2695 equipped with a 2489 UV/Vis detector (Waters Technologies) at a wavelength of 280 nm. The compounds were isocratically eluted with 30% methanol/70% ultrapure water (v/v).

Determination of theanine and catechins

Theanine was quantified according to a previous method^[2]. C, GC, EC, EGC, CG, GCG, ECG and EGCG content were detected with the instruments mentioned above at 280 nm. In brief, 0.2 g fine freeze-dried tea powders were subjected to extraction with 8 mL 70% methanol (v/v). One mL of the resulting supernatant was then filtered through a 0.22 mm Millipore membrane before being injected into an XSelect HSS C18 SB column (4.6×250 mm, 5 mm, Waters Technologies). Thereafter, catechin monomers were separated via gradient elution chromatogra-

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phy using a mobile phase consisting of 0.1% aqueous formic acid (v/v; A) and 100% acetonitrile (B). For the first 5 min, the mobile phase was 8% B; then from 5–14 min, B was increased from 8%–25%; finally, B was decreased from 25%–8% from 14–30 min.

RNA extraction and sequencing

Total RNA was isolated and purified from each sample using an R4151B-HiPure Plant RNA Kit B (Magen Biotechnology, Guangdong, China). The RNA concentration was quantified with a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). cDNA libraries were constructed according to the method in a previous study^[15]. Samples were designated into two groups, TB (TB-1, TB-2, TB-3) and CAF (CAF-1, CAF-2, CAF-3). Raw reads in FASTQ format were first processed through in-house Perl scripts^[33]. Paired-end clean reads were aligned to the reference genome obtained from the TPIA database (<http://tpdb.shengxin.ren/>) using Hisat2^[34]. Finally, the reads mapped to each gene were counted by feature-Counts^[35].

Functional annotation and analysis of differentially expressed genes

For the functional annotation of the proteins, the gene sequences of samples were compared against the Kyoto Encyclopedia of Genes and Genomes (KEGG), and the Gene Ontology (GO) database. GO and KEGG enrichment analyses of differentially expressed gene sets were implemented using the KOBAS package and topG (www.bioconductor.org/packages/release/bioc/html/topGO.html), respectively^[36]. Differential expression analysis was performed using EdgeR^[37]. False discovery rate control was implemented by adjusting the obtained P-values via the Benjamini and Hochberg's approach. Genes fulfilling the criteria of $|\log_2(\text{Fold Change})| > 1$ and p value < 0.05 were considered differentially expressed. In addition, differentially expressed candidate transcription factor (TF) genes were predicted with iTAK for plants^[38].

qRT-PCR

Premier 3 was used to design the primers for qRT-PCR, and the sequence is given in Supplemental Table S1. qRT-PCR was conducted by a BioRad CFX384TM Real-Time System (Bio-Rad, Hercules, CA, USA) according to our previously published research^[2]. *CsActin1* was used as the internal reference, and the relative expression levels were calculated based on the $2^{-\Delta\Delta\text{CT}}$ method^[39].

Statistical analysis

All data are presented as the mean \pm SD ($n = 3$). The double coordinate figure was produced with GraphPad Prism 9 software and correlation analysis was made in Origin 2022b R1. Heat maps were constructed with TBtools^[40]. IBM SPSS Statistics 25 was used to run a Duncan's multiple-range test at p value < 0.05 to reveal significant differences. Corrplot package in R (version 4.1.0) was used to determine the correlation coefficient between two genes, and the Co-expression network was visualized by Cytoscape (version 3.9.1).

Results

Analysis of the main secondary metabolites and morphology of *C. ptilophylla*

Concentrations of the main purine alkaloids in a *C. ptilophylla* population comprising 32 single plants were quantified

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(Fig. 1a). In 26 of the single plants, theobromine was the sole alkaloid, and its concentration ranged from 27.33–61.36 mg/g. The other six *C. ptilophylla* plants exhibited high levels of caffeine (37.43–54.86 mg/g) and low levels of theobromine (0.58–7.46 mg/g). As for theacrine and theophylline, they were not detected in the population (Fig. 1a).

To understand the differential pathways for accumulation of purine alkaloids, two *C. ptilophylla* plants with contrasting purine alkaloid contents (i.e., one of the theobromine-only plants (Cp3, TB) and one of the theobromine and caffeine plants (Cp4, CAF) were subjected to further analysis. The two had significantly different morphologies, especially in the size of the bud and first two leaves, which were significantly larger in TB than in CAF (Fig. 1c). The levels of the major flavor substances, theanine and catechins, varied considerably between TB and CAF. For example, theanine content was significantly higher in TB (3.60 mg/g) in comparison to CAF (0.55 mg/g; Fig. 1e). Notably, the catechins contents were greatly different between TB and CAF (Fig. 1d). GCG was the dominant catechin monomer in TB, with a content of 82.76 mg/g, while in CAF it was only 41.9 mg/g. In contrast, EGCG was the dominant catechin monomer in CAF, at 58.36 mg/g, while it was 10.05 mg/g in TB (Fig. 1d).

RNA-Seq and reference genome alignment

To investigate the mechanism of differential accumulation of purine alkaloids, TB and CAF were subjected to RNA-seq analysis. A substantial amount of high-quality RNA sequencing data was generated, amounting to 44.85 Gb of clean reads and an average of 7.47 Gb clean reads per sample. The base-calling accuracy of the sequencing data were assessed by Q20 (base-calling error probability of 99%) and Q30 (base-calling error probability of 99.90%) values, which were both above 90.00% (Supplemental Table S2). Moreover, the Pearson correlation

coefficients between the three replicates were all above 0.99, indicating a high level of consistency and reproducibility among the samples (Supplemental Fig. S1). The proportion of mapped reads, ranging from 85.54% to 86.33%, further indicated the high quality of the RNA-Seq data. These results collectively suggested that the RNA-Seq data in this study met the requirements for subsequent analyses.

Analysis of differentially expressed genes (DEGs) and functional enrichment

Analysis of the transcriptome data revealed 6,125 DEGs between TB and CAF, where DEGs differed by at least 2-fold, according to the transcript per kilobase million (TPM) mapped reads model. Among them, 2,514 were upregulated and 3,611 were downregulated (Fig. 2a). KEGG enrichment predicted that 127 metabolic pathways were associated with the annotated DEGs, including carbon metabolism, phenylpropanoid biosynthesis, photosynthesis, and glutathione metabolism (Supplemental Table S3). Particularly noteworthy was the enrichment of a considerable number of DEGs in the phenylpropanoid and flavonoid biosynthesis pathways, which are responsible for the biosynthesis of catechins. Purine metabolism, which was our primary focus, was also enriched as expected (Fig. 2c). In light of these findings, we focused on purine metabolism, phenylpropanoid biosynthesis, and flavonoid biosynthesis to delve into the underlying mechanisms responsible for the observed differences in the caffeine, theobromine, and catechins content at the transcript level. In addition, biological process enrichment of GO was shown in Supplemental Fig. S2. To validate the RNA-Seq data, 14 genes were chosen for RT-qPCR analysis, and the RT-qPCR analysis results were largely consistent with the RNA-seq results ($R^2 = 0.8012$; Fig. 2b), indicating the reliability of the RNA-seq data.

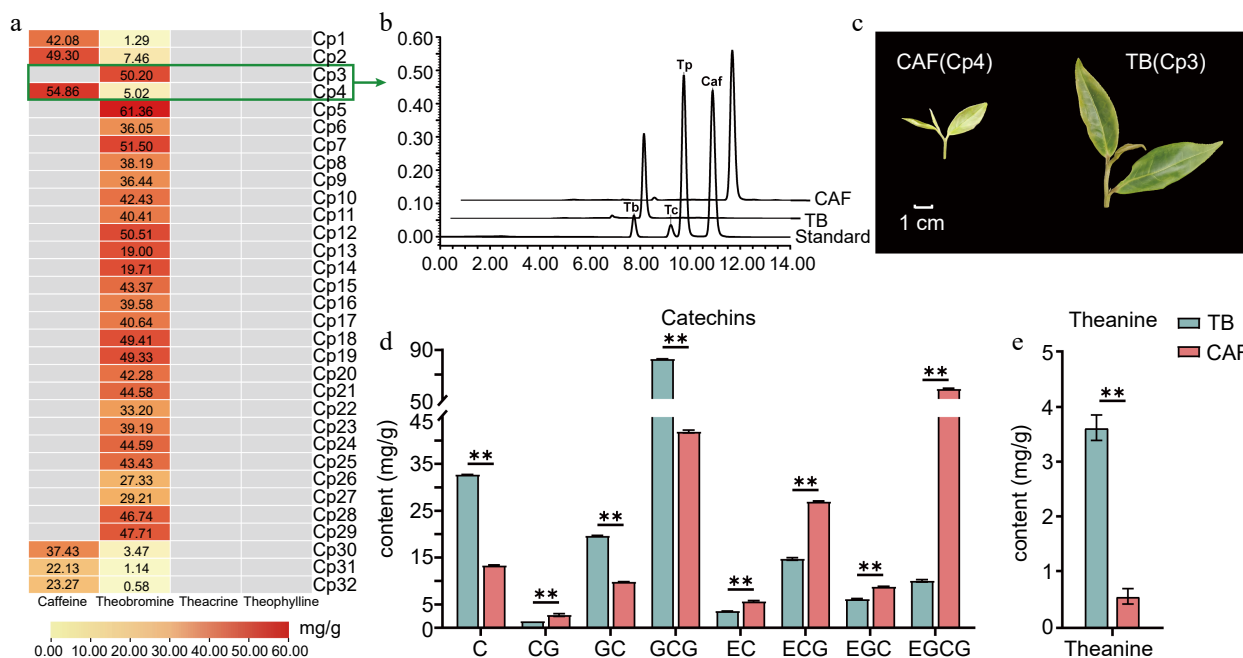


Fig. 1 The main secondary metabolites of *Camellia ptilophylla*. (a) Content of purine alkaloids in the tender leaves of 32 *C. ptilophylla*, where gray rectangles indicate the compound was not detected; (b) tender leaves of CAF and TB; (c) HPLC chromatogram of purine alkaloids (caffeine, theobromine, theacrine and theophylline) in TB and CAF; concentrations of (d) catechins and (e) theanine in TB and CAF. Catechin (C), gallic catechin (GC), epicatechin (EC), epigallocatechin (EGC), catechin gallate (CG), epicatechin gallate (ECG), gallic catechin gallate (GCG), and epigallocatechin gallate (EGCG). ** Significant difference between samples at the 0.01 level ($P < 0.01$) as determined by Student's t-test.

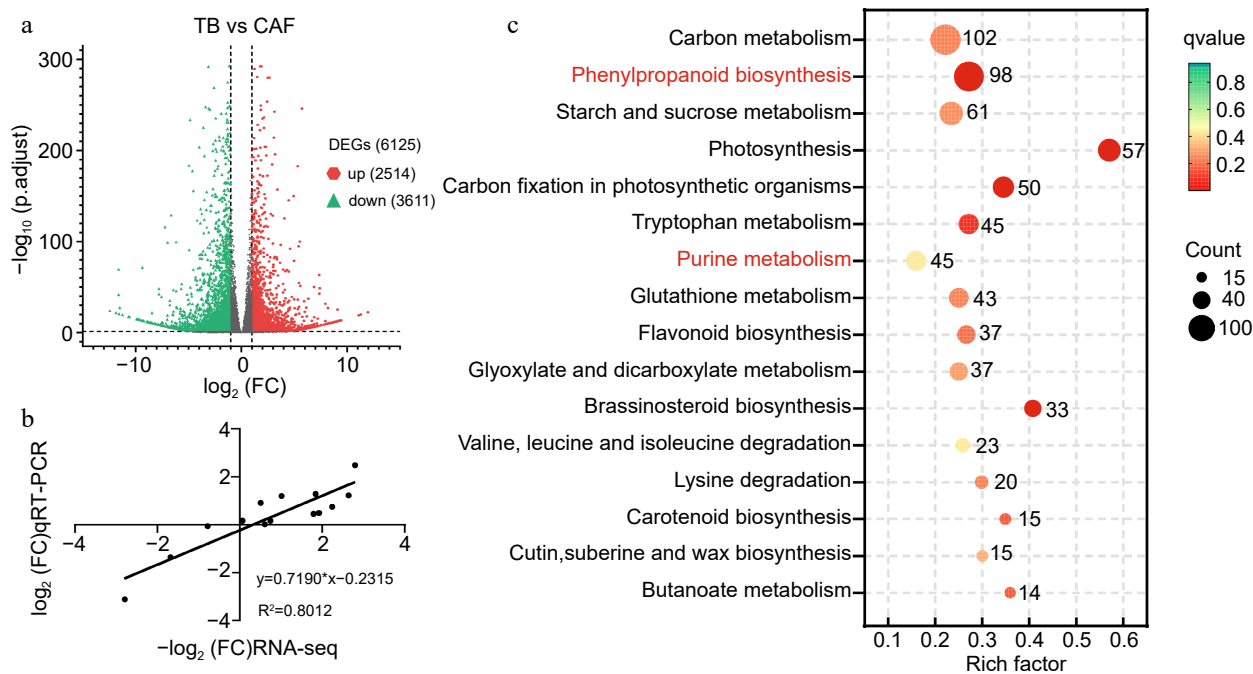


Fig. 2 (a) Volcano map showing differentially expressed genes (DEGs) between TB and CAF plants; (b) scatterplot of RNA-seq transcript abundance fold change values RNA-Seq (on the x-axis) plotted against RT-PCR expression values (on the y-axis). Actin (TEA019484) was used as the internal reference gene; and (c) KEGG pathways for the DEGs between TB and CAF. Red indicates the two pathways that were subsequently analyzed.

DEGs in purine alkaloid metabolism pathway

Purine alkaloid metabolism includes both synthesis and degradation pathways (Fig. 3a & b). Xanthosine, the main precursor of caffeine and theobromine, can be synthesized in five different pathways, including the 1) S-adenosylmethionine (SAM) cycle, 2) adenosine monophosphate (AMP) pathway, 3) guanine monophosphate (GMP) pathway, 4) *de novo* route, and 5) nucleic acids degradation (NAD) route (Fig. 3a). Heatmaps of the genes involved in purine alkaloid metabolism were generated, including xanthosine synthesis genes (Fig. 3c), methyltransferase genes (Fig. 3d), and degradation genes (Fig. 3e). The expression data is available in Supplemental Table S4. SAM synthetases (*SAMS-1*, *SAMS-3* and *SAMS-5*), S-adenosyl-L-homocysteine hydrolases (*SAHH-1* and *SAHH-2*), adenosine kinases (*ADK-1* and *ADK-5*) and IMP dehydrogenase-2 (*IMPDH-2*) in xanthosine biosynthesis pathway were all significantly upregulated in TB.

Xanthosine to theobromine and caffeine involves one nucleoside transfer, plus two and three methylations, respectively. The methyltransferase genes involved, *MXMT-4*, *MXMT-5*, and *MXMT-7*, were significantly upregulated in TB, where and *MXMT-5* exhibited over four-fold higher expression in TB than in CAF (Fig. 3d). *TCS-1*, which catalyzes the last methylation to produce caffeine, was downregulated in TB as anticipated. However, *TCS-2* and *TCS-3* were upregulated in TB, which indicated that these genes may have roles in theobromine biosynthesis.

Another aspect affecting alkaloid content is the degradation pathway (Fig. 3b). In general, degradation of purine alkaloids was less active in TB than in CAF. Two xanthine dehydrogenase genes (*XDH-4*, *XDH-5*), and urate oxidase-2 (*UOX-2*) were downregulated in TB, while *allantoinase-2* (*ALN-2*) and *urease-1* (*URE-1*) increased. In addition, six out of seven cytochrome P450

family 1 subfamily A polypeptide 2 genes (*CYP1A2*), which sequentially demethylate caffeine and the resulting metabolites, were downregulated in TB, possibly because there was no caffeine to degrade in TB.

Expression level of catechin biosynthesis genes

The biosynthesis pathway of catechins, encompassing phenylpropanoid and flavonoid biosynthesis, is shown in Fig. 4a. In the phenylpropanoid biosynthesis pathway, *phenylalanine ammonia-lyase* (*PAL*) and *cinnamate 4-hydroxylase* (*C4H*) were upregulated in TB, while *chalcone synthase* (*CHS*) and *cinnamate 3-hydroxylase* (*C3H*) exhibited no noticeable differences in expression (Fig. 4b).

Regarding the flavonoid pathway, two chalcone isomerase genes (*CHI-6*, *CHI-7*), *flavonoid 3'-hydroxylase-1* (*F3'H-1*) and *anthocyanidin reductase-2* (*ANR-2*) were significantly downregulated in TB. Conversely, *dihydroflavonol reductase-4* (*DFR-4*), *anthocyanidin synthase-2* (*ANS-2*), *flavonoid 3-hydroxylase* (*F3H*) and *flavonoid 3'5'-hydroxylase* (*F3'5'H-2*) were upregulated in TB, which was consistent with the contents of C, GC and GCG, while *leucoanthocyanidin reductases* (*LARs*) didn't have significant differences between samples. *Glucosyltransferases* and *serine carboxypeptidase-like acyltransferases 1 subfamily A* (*SCPL1A*), a kind of epigallocatechin acyltransferase, participates in the synthesis of ECG and EGCG^[41,42]. Among the four upregulated *SCPL1A* genes (*SCPL11-1*, *SCPL13-1*, *SCPL14-1*, *SCPL11-1*) in TB, *SCPL11-1* showed the highest expression, with a 3-fold increase in TB, which might be the primary reason for the accumulation of GCG in TB. The expression data is available in Supplemental Table S5.

Co-expression network

A total of 327 transcription factors (TFs) were identified by RNA-seq, with the AP2/ERF-ERF family being the most

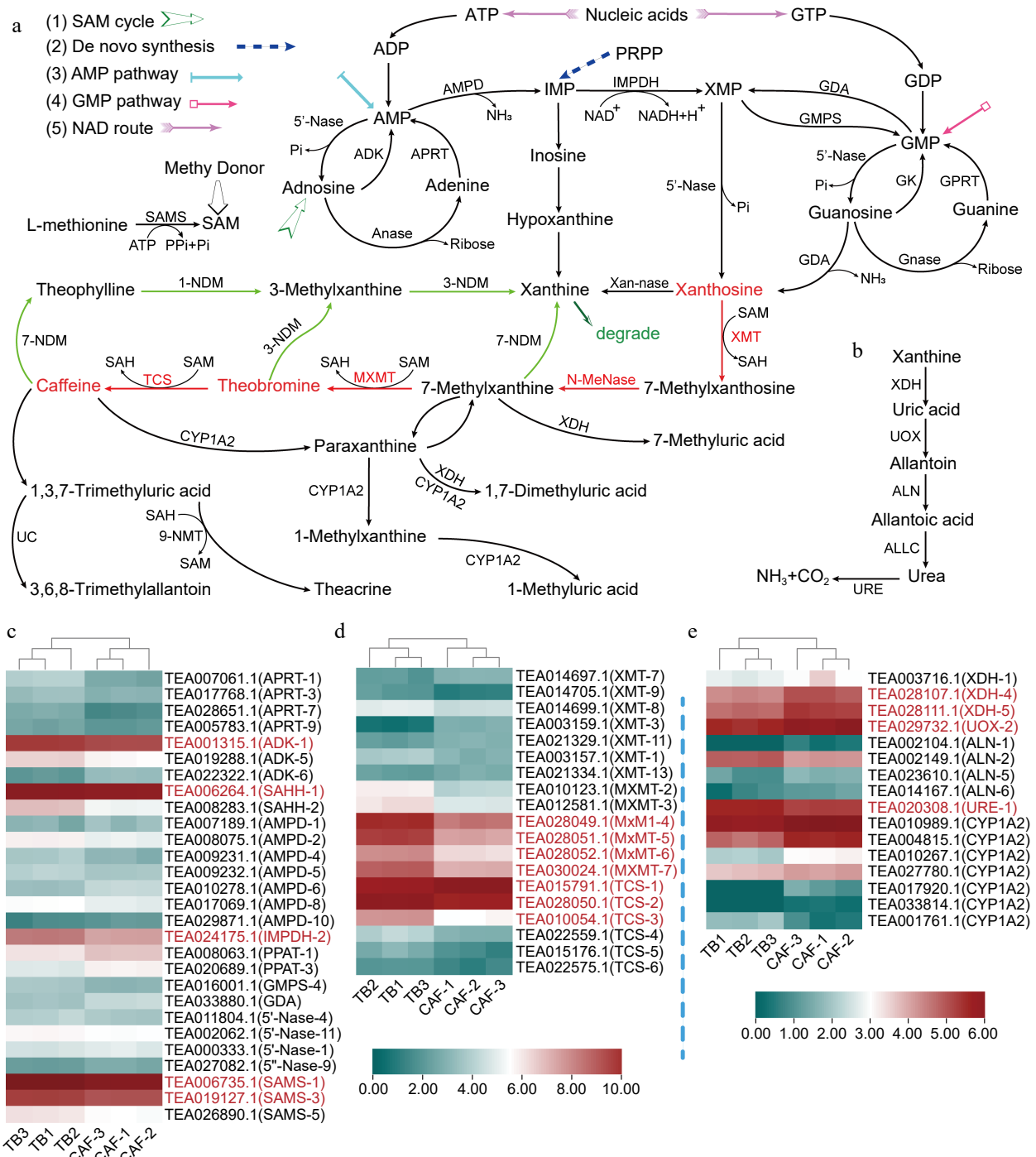


Fig. 3 Differential expression of genes involved in purine alkaloid biosynthesis and degradation; (a) xanthosine synthesis pathways: 1) SAM cycle, 2) *de novo* synthesis, 3) AMP pathway, 4) GMP pathway, and 5) the nucleic acid degradation (NAD) pathway, where red lines represent the main caffeine synthesis pathway, starting from xanthosine, and green lines represent the main pathway for caffeine degradation; (b) the xanthine degradation pathway; and heatmaps of the (c) genes in xanthosine biosynthesis; (d) methyltransferase genes involved in transforming xanthosine to caffeine; and (e) genes involved in xanthine degradation. Abbreviations: AMP deaminase (AMPD), 5'-nucleotidase (5'-Nase), IMP dehydrogenase (IMPDH), S-adenosyl-L-methionine synthase (SAMS), S-adenosyl-L-homocysteine hydrolase (SAHH), adenosine kinase (ADK), adenosine nucleosidase (Anase), adenosine phosphoribosyl transferase (APRT), GMP synthase (GMPS); guanosine deaminase (GDA), xanthosine methyltransferase/7-methylxanthosine synthase (XMT), N-methyl nucleosidase (N-MeNase), methylxanthine methyltransferase/theobromine synthase (MXMT), 1,3-dimethylxanthine methyltransferase/tea caffeine synthase (TCS), 5-phosphoribosyl-1-pyrophosphate (PRPP), N-demethylase (NDM), cytochrome P450 family 1 subfamily A polypeptide 2 (CYP1A2), xanthine dehydrogenase (XDH), urate oxidase (UOX), allantoinase (ALN), allantoinase (ALLC), urease (URE), and 5-phosphoribosyl-1-pyrophosphate (PRPP) amidotransferase (PPAT). Genes written in red had significant differences in gene expression.

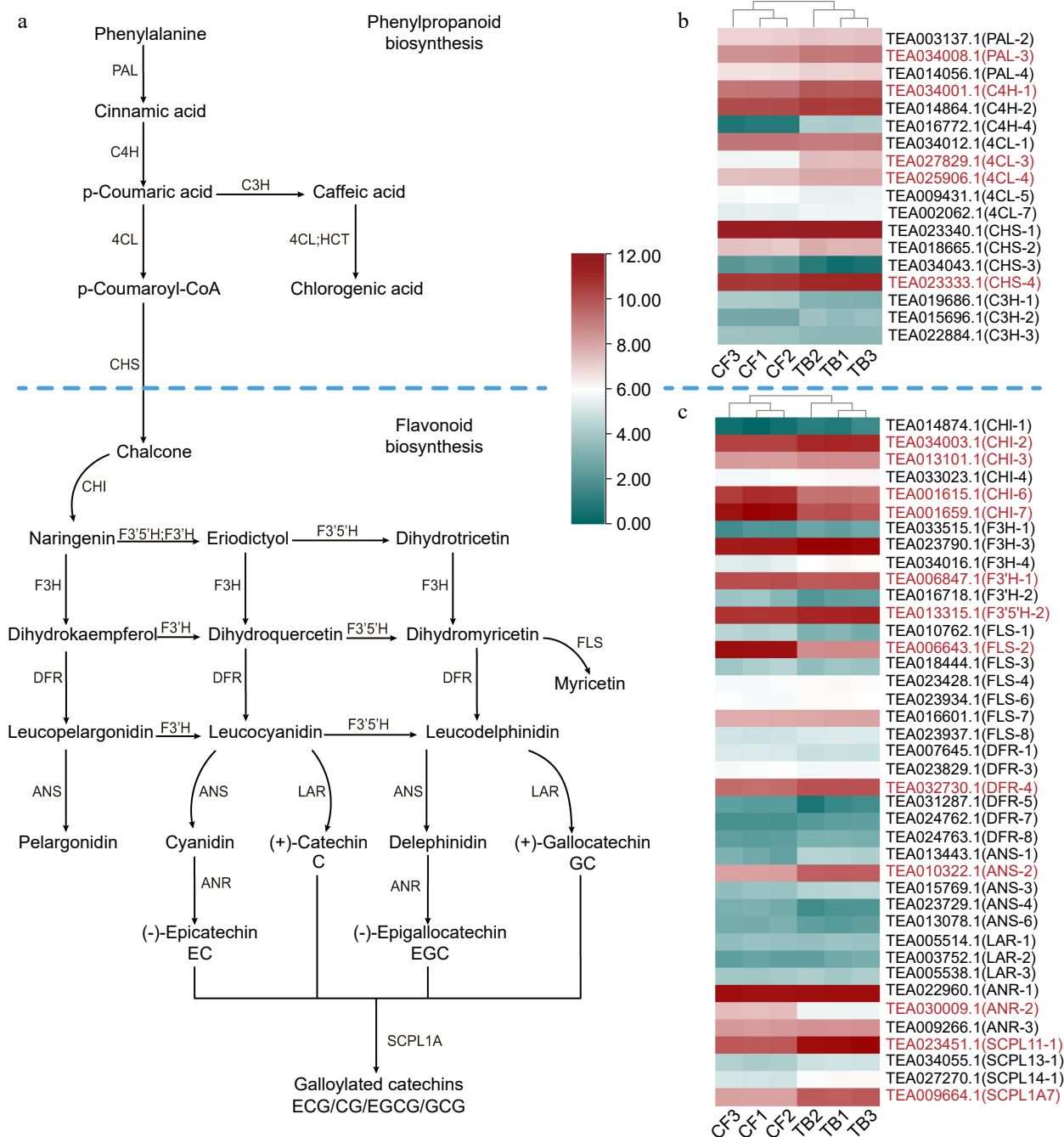


Fig. 4 (a) Phenylpropanoid, flavonoid, and catechin biosynthesis pathways and heatmaps of the expression level of (b) phenylpropanoid biosynthesis genes and (c) flavonoid biosynthesis genes. Phenylalanine ammonia-lyase (PAL), 4-coumarate-CoA ligase (4CL), cinnamate 4-hydroxylase (C4H), chalcone synthase (CHS), cinnamate 3-hydroxylase (C3H), chalcone isomerase (CHI), flavanone 3'-hydroxylase (F3'H), flavanone 3-hydroxylase (F3H), flavonoid 3'5'-hydroxylase (F3'5'H), flavonol synthase (FLS), dihydroflavonol reductase (DFR), leucoanthocyanidin reductase (LAR), anthocyanidin synthase (ANS), and serine carboxypeptidase-like acyltransferases (SCPL). Red represents a significant difference in gene expression.

prominent (48 TFs, 14.68%), followed by the MYB (33 TFs, 10.09%) and NAC families (22 TFs, 6.73%) (Supplemental Table S6). To explore the potential regulatory mechanisms underlying differential gene expression in the purine alkaloid and catechin biosynthesis pathways, co-expression networks were constructed (Fig. 5). The key gene of theobromine synthesis *MXMT-4*^[15] was significantly correlated with two *AP2/ERF-ERFs* (TEA013405.1, TEA028649.1) and two *MYBs* (TEA028555.1 and TEA024836.1). Interestingly, our studies showed that there was

no positive correlation between the expression level of *CsMYB184* and *CsTCS1*. In addition, *B3* (TEA015867.1), *GRF* (TEA028352.1) and *AP2/ERF-ERF* (TEA001056.1) were significantly related to *CsSCPL11-1* expression, indicating that those TFs might regulate GCG biosynthesis.

Data availability statement

The raw sequencing data in the study are publicly available in the NCBI BioProject: PRJNA972319.

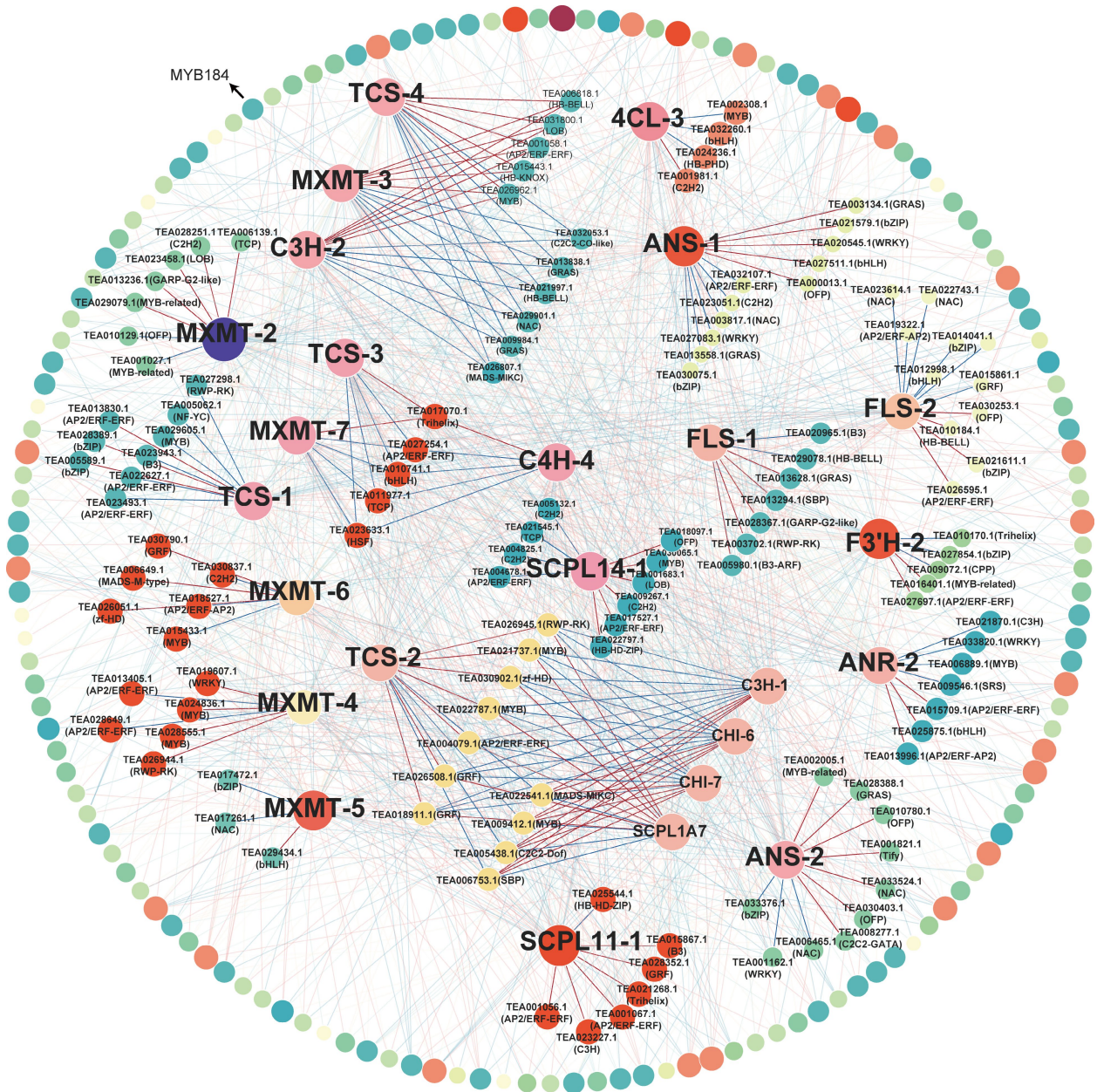


Fig. 5 Coexpression network between TFs and purine alkaloid and catechin biosynthesis genes. Pairs with $P < 0.05$ and $|\text{Spearman's rank correlation coefficient}| > 0.8$ were included in the analysis. Red lines and blue lines represent positive and negative correlations, respectively. The names of TFs with a high degree of co-expression are given. The size and color of nodes depend on the degree of co-expression. The outer ring with grey lines represents TFs with low correlation.

Discussion

C. ptilophylla was thought to contain theobromine only instead of caffeine, but the current evidence does not support this view^[16]. The random amplified polymorphic DNA (RAPD) technique was used to analyze *C. ptilophylla* that differed in purine base composition, and it revealed that *C. ptilophylla* containing only theobromine was more closely related to other *C. ptilophylla* plants containing both theobromine and caffeine but was more distantly related to cultivated species of tea plants^[43]. In our studies, *C. ptilophylla* showed intra-species diversity, i.e., a small number of single plants contained caffeine, which was consistent with the results of a recent

published study^[30]. In addition, we found that GCG was much more abundant than EGCG in TB, while EGCG was the major catechin in CAF, which means that not only purine alkaloids differ among *C. ptilophylla* populations, but that catechin compositions also have intraspecies diversity in this species. Whether there is any connection between the high GCG content and defective caffeine biosynthesis needs to be further confirmed. Our research has provided new insights into the metabolites of *C. ptilophylla* population.

RNA sequencing was used to discover the differential accumulation of purine alkaloids and catechins between TB and CAF. In agreement with previous studies^[16,44–47], we found that *TCS1* expression was downregulated in TB compared to CAF

(Fig. 3d), which might be the cause of defective caffeine biosynthesis in TB. Interestingly, *TCS-2*, *TCS-3* and xanthosine biosynthesis genes (*SAMS-1*, *SAHH-1*, and *ADK-1*) were upregulated in TB, which might be related to the high content of theobromine in TB. As expected, the expression level of *CYP1A2s*, which catalyze the demethylation of caffeine, were all downregulated in TB. In addition, *XDH-4*, *XDH-5*, and *UOX-2*, which participate in the degradation of purine alkaloids, were less active in TB (Fig. 3e). *CsMYB184*, a crucial transcription factor responsible for regulating caffeine biosynthesis in tea plants, has a proven link to *TCS1*, where lower transcription of this TF coincides with reduced *TCS1* expression in *C. ptilophylla*^[16]. Different from previous studies, our results showed that the expression level of *CsMYB184* in TB was significantly higher than that in CAF, and there was no positive correlation to the expression level of *CsMYB184* and *TCS1*. Based on the co-expression network, two *AP2/ERF-ERFs* (TEA013405.1, TEA028649.1) and two *MYBs* (TEA028555.1 and TEA024836.1) appeared to be highly related to *MXMT-4*, a key gene regulating the biosynthesis of theobromine, which implied that those TFs might regulate theobromine biosynthesis.

Regarding catechin biosynthesis, *F3'H-1* and *F3'5'H-2* were significantly upregulated in *C. ptilophylla*, which was consistent with the elevated GCG contents. Furthermore, *CsSCPL11-1*, thought to be related to the biosynthesis of EGCG^[26,41,42], was significantly upregulated in TB, which was consistent with the observed GCG content, but was contrary to the amount of EGCG in TB. Meanwhile, GC is the precursor to synthesize GCG^[28]. The significant increase of GC content in TB might be the reason for the high amount of GCG. These results suggest that the upregulation of *F3'H*, *F3'5'H* and *SCPLs* may played an important role in the synthesis of GCG in *C. ptilophylla*. Additionally, the co-expression network showed that transcription factors *B3* (TEA015867.1), *GRF* (TEA028352.1) and *AP2/ERF-ERF* (TEA001056.1) were significantly related to *CsSCPL11-1*.

Conclusions

C. ptilophylla is typically thought of as a naturally decaffeinated tea. However, there is intraspecies variation. In the population studied here, ~19% of the plants contained high levels of caffeine (CAF), while the majority were caffeine-free (TB). Xanthosine synthesis pathways were more active in TB than that in CAF, especially in the SAM cycle and AMP pathway, and the high theobromine content in TB may have been caused by significantly high expression of both *TCS* and *MXMT* and a lower level of alkaloid degradation compared to CAF. Additionally, *CsMYB184* (TEA029017.1) appeared to be involved in the biosynthesis of both caffeine and theobromine, which requires further verification. Furthermore, an increase in GCG content was observed in TB, which corresponded to the upregulated expression of *F3'H*, *F3'5'H* and *SCPL*, suggesting that these genes may play a crucial role in the accumulation of GC and GCG. This study lays out fundamental metabolite accumulation patterns in a *C. ptilophylla* population and provides possible regulation mechanisms of purine alkaloid and catechin biosynthesis. The understanding of purine alkaloid and catechins metabolism will facilitate the breeding of low-caffeine or high GCG tea plant cultivars for meeting diverse demands of consumers.

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Conflict of interest

The authors declare that they have no conflict of interest.

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References

- Li P, Fu J, Xu Y, Shen Y, Zhang Y, et al. 2022. *CsMYB1* integrates the regulation of trichome development and catechins biosynthesis in tea plant domestication. *New Phytologist* 234:902–17
- Li J, Liu S, Chen P, Cai J, Tang S, et al. 2022. Systematic analysis of the R2R3-MYB family in *Camellia sinensis*: evidence for galloylated catechins biosynthesis regulation. *Frontiers in Plant Science* 12:782220
- Jin J, Zhou C, Ma C, Yao M, Ma J, et al. 2014. Identification on purine alkaloids of representative tea germplasm in China. *Journal of Plant Genetic Resources* 2:279–85
- Zhong H, Wang Y, Qu F-R, Wei M-Y, Zhang C-Y, et al. 2022. A novel *TCS* allele conferring the high-theacrine and low-caffeine traits and having potential use in tea plant breeding. *Horticulture Research* 9:uhac191
- Jin J, Chai Y, Liu Y, Zhang J, Yao M, et al. 2018. Hongyacha, a naturally caffeine-free tea plant from Fujian, China. *Journal of Agricultural and Food Chemistry* 66:11311–19
- Ashihara H, Sano H, Crozier A. 2008. Caffeine and related purine alkaloids: biosynthesis, catabolism, function and genetic engineering. *Phytochemistry* 69:841–56
- Juliano LM, Griffiths RR. 2004. A critical review of caffeine withdrawal: empirical validation of symptoms and signs, incidence, severity, and associated features. *Psychopharmacology* 176:1–29
- Tritsch N, Steger MC, Segatz V, Blumenthal P, Rigling M, et al. 2022. Risk assessment of caffeine and epigallocatechin gallate in coffee leaf tea. *Foods* 11:263
- Kato M, Mizuno K, Crozier A, Fujimura T, Ashihara H. 2000. Caffeine synthase gene from tea leaves. *Nature* 406:956–57
- Kato M, Kanehara T, Shimizu H, Suzuki T, Gillies FM, et al. 1996. Caffeine biosynthesis in young leaves of *Camellia sinensis*: In vitro studies on N-methyltransferase activity involved in the conversion of xanthosine to caffeine. *Physiologia Plantarum* 98:629–36
- Deng WW, Ashihara H. 2010. Profiles of purine metabolism in leaves and roots of *Camellia sinensis* seedlings. *Plant and Cell Physiology* 51:2105–18
- Jin J, Yao M, Ma C, Ma J, Chen L. 2016. Association mapping of caffeine content with *TCS1* in tea plant and its related species. *Plant Physiology and Biochemistry* 105:251–9
- Negishi O, Ozawa T, Imagawa H. 1992. Biosynthesis of caffeine from purine nucleotides in tea plant. *Bioscience, Biotechnology, and Biochemistry* 56:499–503
- Ashihara H, Kato M, Ye CX. 1998. Biosynthesis and metabolism of purine alkaloids in leaves of cocoa tea (*Camellia ptilophylla*). *Journal of Plant Research* 111:599–604

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15. Deng C, Ku X, Cheng L, Pan S, Fan L, et al. 2020. Metabolite and transcriptome profiling on xanthine alkaloids-fed tea plant (*Camellia sinensis*) shoot tips and roots reveal the complex metabolic network for caffeine biosynthesis and degradation. *Frontiers in Plant Science* 11:551288
16. Li P, Ye Z, Fu J, Xu Y, Shen Y, et al. 2022. CsMYB184 regulates caffeine biosynthesis in tea plants. *Plant Biotechnology Journal* 20:1012–14
17. Yoneyama N, Morimoto H, Ye CX, Ashihara H, Mizuno K, Kato M. 2006. Substrate specificity of *N*-methyltransferase involved in purine alkaloids synthesis is dependent upon one amino acid residue of the enzyme. *Molecular Genetics and Genomics* 275:125–35
18. Teng J, Yan C, Zeng W, Zhang Y, Zeng Z, Huang Y. 2020. Purification and characterization of theobromine synthase in a Theobromine-Enriched wild tea plant (*Camellia gymnogyna* Chang) from Dayao Mountain, China. *Food Chemistry* 311:125875
19. Ma J, Jin J, Yao M, Ma C, Xu Y, et al. 2018. Quantitative trait loci mapping for theobromine and caffeine contents in tea plant (*Camellia sinensis*). *Journal of Agricultural and Food Chemistry* 66:13321–27
20. Ogino A, Taniguchi F, Yoshida K, Matsumoto S, Fukuoka H, et al. 2019. A new DNA marker CafLess-TCS1 for selection of caffeineless tea plants. *Breeding Science* 69:393–400
21. Yamada Y, Sato F. 2013. Transcription factors in alkaloid biosynthesis. In *International Review of Cell and Molecular Biology*, ed. Jeon KW. vol. 305. USA: Academic Press, Elsevier. pp. 339–82. <https://doi.org/10.1016/B978-0-12-407695-2.00008-1>
22. Wu Z, Li X, Liu Z, Li H, Wang Y, et al. 2015. Transcriptome-based discovery of AP2/ERF transcription factors related to temperature stress in tea plant (*Camellia sinensis*). *Functional & Integrative Genomics* 15:741–52
23. Zhao X, Zeng X, Lin N, Yu S, Fernie AR, et al. 2021. CsbZIP1-CsMYB12 mediates the production of bitter-tasting flavonols in tea plants (*Camellia sinensis*) through a coordinated activator–repressor network. *Horticulture Research* 8:110
24. Zhang Y, Li P, She G, Xu Y, Peng A, et al. 2021. Molecular basis of the distinct metabolic features in shoot tips and roots of tea plants (*Camellia sinensis*): Characterization of MYB regulator for root theanine synthesis. *Journal of Agricultural and Food Chemistry* 69:3415–29
25. Ma W, Kang X, Liu P, She K, Zhang Y, et al. 2022. The NAC-like transcription factor CsNAC7 positively regulates the caffeine biosynthesis-related gene *yhNMT1* in *Camellia sinensis*. *Horticulture Research* 9:uhab046
26. Wang Y, Zheng P, Liu P, Song X, Guo F, et al. 2019. Novel insight into the role of withering process in characteristic flavor formation of teas using transcriptome analysis and metabolite profiling. *Food Chemistry* 272:313–22
27. Shi J, Yang G, You Q, Sun S, Chen R, et al. 2021. Updates on the chemistry, processing characteristics, and utilization of tea flavonoids in last two decades (2001–2021). *Critical Reviews in Food Science and Nutrition*
28. Jin J, Ma J, Ma C, Yao M, Chen L. 2014. Determination of catechin content in representative Chinese Tea Germplasms. *Journal of Agricultural and Food Chemistry* 62:9436–41
29. Li K, Liu C, Tam JC, Kwok H, Lau C, et al. 2014. *In vitro* and *in vivo* mechanistic study of a novel proanthocyanidin, GC-(4→8)-GCG from cocoa tea (*Camellia ptilophylla*) in antiangiogenesis. *The Journal of Nutritional Biochemistry* 25:319–28
30. Ou C, Ou Y, Kong Q, Sun H, Luo Y, et al. 2022. Variation of major quality-related chemical components in leaves of seed-propagated offspring in 'Cocoa Tea'. *Journal of Tea* 3:169–72
31. Liu W, Feng Y, Yu S, Fan Z, Li X, et al. 2021. The Flavonoid Biosynthesis Network in Plants. *International Journal of Molecular Sciences* 22:12824
32. Mei S, Yu Z, Chen J, Zheng P, Sun B, et al. 2022. The physiology of postharvest tea (*Camellia sinensis*) leaves, according to metabolic phenotypes and gene expression analysis. *Molecules* 27:1708
33. Chen S, Zhou Y, Chen Y, Gu J. 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34:i884–i890
34. Kim D, Langmead B, Salzberg SL. 2015. HISAT: a fast spliced aligner with low memory requirements. *Nature Methods* 12:357–60
35. Liao Y, Smyth GK, Shi W. 2014. FeatureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30:923–30
36. Bu D, Luo H, Huo P, Wang Z, Zhang S, et al. 2021. KOBAS-i: intelligent prioritization and exploratory visualization of biological functions for gene enrichment analysis. *Nucleic Acids Research* 49:W317–W325
37. Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26:139–40
38. Zheng Y, Jiao C, Sun H, Rosli HG, Pombo MA, et al. 2016. iTAK: A program for genome-wide prediction and classification of plant transcription factors, transcriptional regulators, and protein kinases. *Molecular Plant* 9:1667–70
39. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* 25:402–8
40. Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, et al. 2020. TBtools: an integrative toolkit developed for interactive analyses of big biological data. *Molecular Plant* 13:1194–202
41. Wei C, Yang H, Wang S, Zhao J, Liu C, et al. 2018. Draft genome sequence of *Camellia sinensis* var. *sinensis* provides insights into the evolution of the tea genome and tea quality. *Proceedings of the National Academy of Sciences of the United States of America* 115:E4151–E4158
42. Ahmad MZ, Li P, She G, Xia E, Benedito VA, et al. 2020. Genome-wide analysis of serine carboxypeptidase-like acyltransferase gene family for evolution and characterization of enzymes involved in the biosynthesis of galloylated catechins in the tea plant (*Camellia sinensis*). *Frontiers in Plant Science* 11:848
43. Yuan C, Shi S, Ye C. 1999. Phylogenetic relationship of *Camellia ptilophylla* Chang with its allied species and disintegration of its population. *Acta Scientiarum Naturalium Universitatis Sunyatseni* 38:72–76
44. Jin J, Yao M, Ma C, Ma J, Chen L. 2016. Natural allelic variations of *TCS1* play a crucial role in caffeine biosynthesis of tea plant and its related species. *Plant Physiology and Biochemistry* 100:18–26
45. Li Mm, Xue Jy, Wen Yl, Guo Hs, Sun Xq, et al. 2015. Transcriptomic analysis of *Camellia ptilophylla* and identification of genes associated with flavonoid and caffeine biosynthesis. *Genetics and Molecular Research* 14:18731–42
46. Zhu B, Chen L, Lu M, Zhang J, Han J, et al. 2019. Caffeine content and related gene expression: novel insight into caffeine metabolism in *Camellia* plants containing low, normal, and high caffeine concentrations. *Journal of Agricultural and Food Chemistry* 67:3400–11
47. Zhang S, Jin J, Chen J, Ercisli S, Chen L. 2022. Purine alkaloids in tea plants: component, biosynthetic mechanism and genetic variation. *Beverage Plant Research* 2:13



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