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Multi-omics analysis of the mechanisms of abundant theacrine and EGCG3"Me in tea (*Camellia sinensis*)

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Abstract

Theacrine and epigallocatechin-3-*O*-(3-*O*-methyl) gallate (EGCG3"Me) are notable secondary metabolites in tea (*Camellia sinensis*), celebrated for their unique flavors and significant health effects. Theacrine has a mild effect on nerve stimulation, while EGCG3"Me exhibits better stability, higher oral bioavailability and stronger biological activity. However, tea plant varieties naturally rich in both theacrine and EGCG3"Me are rare. This study unveils a unique tea variety 'Anxi kucha', which is abundant in both theacrine and EGCG3"Me. Through integrated transcriptome-proteome-metabolome analysis, SAMS3, APRT1, IMPDH, and TCS1 were identified as critical enzymes for theacrine synthesis; while CHI1, CHI2, FLS2 and LAR1 were key for EGCG3"Me synthesis. Additionally, transcription factor analysis revealed that MYB4 and bHLH74 were positively correlated with the contents of theacrine and EGCG3"Me. This study provides valuable materials for further exploring theacrine and EGCG3"Me in tea plants, and establishes a theoretical basis for their biosynthesis.

Keywords *Camellia sinensis*, Theacrine, epigallocatechin-3-*O*-(3-*O*-methyl) gallate, Anxi Kucha, Metabolic pathways, Proteomics; transcriptomics

Introduction

Tea, derived from the young leaves and shoots of the evergreen perennial tea plant (*Camellia sinensis*), is the world's most popular beverage and enjoys global consumption. Its health-promoting properties have been widely explored. Its health-promoting properties have been widely explored [1]. The health benefits of tea are attributed to its phytochemical content, which includes approximately 4,000 bioactive compounds [2]. The secondary metabolites such as purine alkaloids (caffeine) and flavonoids (catechins) are particularly abundant [3]. These major secondary metabolites in tea not only enhance human health but also influence tea quality.

Theacrine significantly contributes to the bitterness of tea and has a mild stimulatory effect on the nervous system [4]. It has various physiological functions, including

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locomotor activation, improving sleep, inhibiting breast cancer cell metastasis, etc [5]. In certain tea plants, caffeine can be converted into non-irritating theacrine. Studies have shown that caffeine is oxidized at *C8* to produce the intermediate 1,3,7-trimethyluric acid, which is then methylated to *N9* to synthesize theacrine [6]. Caffeine is a central nervous system stimulant and its consumption may cause occasional adverse reactions [7]. Naturally reducing caffeine intake has significant economic and health implications. Tea plant resources containing theacrine have been found in many places in Fujian Province. The earliest discovery was in Anxi County, and its main biochemical components have been studied [8, 9].

Methylated (-)-Epigallocatechin gallate (EGCG), a polyphenolic compound and derivative of EGCG, is biosynthesized through the phenylpropanoid and flavonoid biosynthetic pathways. Japanese scholars first discovered EGCG3"Me in the 'Benifuuki' cultivar and observed that it has superior stability, fat solubility, higher oral bioavailability and stronger biological activity compared with EGCG [10]. It also possesses antiallergic, antioxidant and cytoprotective properties [11]. In addition, the methylation of phenolic hydroxyl groups enhances the bioavailability of polyphenols, which has garnered significant scholarly attention [12]. However, the tea plant resources containing EGCG3"Me that have been screened are very scarce, with only a few germplasms containing natural EGCG3"Me (> 10 mg/g) [13]. The tea plants abundant in EGCG3"Me are mainly found in Fujian, Guangdong and Yunnan provinces, in China [13, 14].

Related research has found that tea plants with high theacrine content are suitable for processing black tea, which has a mellow and fresh taste [4]. Theacrine impacts the astringency of EGCG by promoting the binding of salivary proteins to EGCG, thus enhancing its astringency [15]. EGCG and EGCG3"Me are positively correlated with bitterness [16]. Transcriptome technology has been used to compare the differences in gene expression among tea cultivars with varying theacrine levels [17]. It was found that CsWRKY31 and CsWRKY48 might negatively participate in EGCG3"Me biosynthesis by downregulating the expression of *CsLAR*, *CsDFR* and *CCoAOMT* [18]. Our research group has extensively studied tea plant resources containing theacrine and EGCG3"Me across various regions in Fujian. Based on the transcriptome analysis, we explored the genes related to theacrine synthesis in the leaves and flowers of 'Jiaocheng kucha' [8, 14, 19, 20].

'Anxi kucha' (AXKC) is a tea variety resource discovered by our research group in Wulang Mountain, Anxi. Through targeted metabolomics, we found that the concentrations of theacrine and EGCG3"Me in three varieties showed interesting changes. With the help of transcriptomics, proteomics and metabolomics, we revealed the reasons why AXKC is rich in theacrine and EGCG3"Me from multiple perspectives, providing data background for subsequent in-depth research such as genetic engineering and enzyme engineering. The aim is to provide a new tea plant experimental material for the study of theacrine and EGCG3"Me, and to provide a reference for the development and utilization of AXKC.

Materials and methods

Tea samples

In early April 2024, 'Anxi kucha' (AXKC) (also known as the *Camellia sinensis* 'Anxi Langshan Tea') and 'Tieguanyin' (TGY) were picked at the Wulang Mountain in Anxi County, Fujian Province. The control cultivar 'Fudingdabaicha' (FDDB) was picked in the tea plant cultivar resource garden of Fujian Agriculture and Forestry University. Previous studies indicated that theacrine content and EGCG3"Me increased initially and then decreased with leaves maturation [20, 21]. Consequently, the third leaf of the tender shoot with relatively consistent growth was selected as the experimental material. Three biological replicates were prepared for each sample and each omics experiment. The third leaf was promptly frozen in liquid nitrogen and stored at -80 ℃ refrigerator for subsequent experiments.

Targeted metabolite assays

Chemicals

The following 9 catechin standards were used: epigallocatechin-3-*O*-(3-*O*-methyl) gallate (EGCG3"Me), catechin (C), epicatechin (EC), epigallocatechin gallate (EGCG), epigallocatechin (EGC), gallocatechin (GC), epicatechin gallate (ECG), gallocatechin gallate (GCG), catechin gallate (CG). Purine alkaloid standards: theacrine (TC), caffeine (CAF), theobromine (TB), and theophylline (TP) were purchased from Yuanye Bio-technology Co., Ltd. (Shanghai, China). Methanol (chromatographic grade) and acetonitrile (chromatographic grade) were purchased from Shanghai Merck Chemical Technology Co., Ltd. Formic acid (chromatographic grade) was purchased from China National Pharmaceutical Group Chemical Reagent Co., Ltd., and ultrapure water was used.

Instruments and equipment

UltiMate 3000 high performance liquid chromatograph (Thermo Fisher Scientific, USA), diode array detector (DAD), C18 reversed-phase chromatographic columns (5 μm, 4.6 × 250 mm, 2.6 μm, 2.1 × 100 mm) (Phenom, Guangzhou, China), Nexera X2 LC-30 A HPLC system (Shimadzu, Kyoto, Japan), tandem Sciex 4500 Q-Trap mass spectrometer (Sciex, Massachusetts, USA); AB204-N analytical balance (Mettler, USA); ultrapure water

system (Medium Touch, Shanghai Hetai Instrument Co., Ltd., China). KQ-800E ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd., China); MS3 basic vortex shaker (Aika (Guangzhou) Instrument Co., Ltd., China).

Determination of catechins and purine alkaloid components

Catechins sample preparation

The catechin components were carried out using high-performance liquid chromatography (HPLC). The specific method was referred to this study [22]. A 200 mg portion of crushed freeze-dried sample was treated with 30 mL of methanol and vortexed. Ultrasonic extraction was performed at room temperature for 30 min, followed by refrigerated centrifugation at 4℃ and 10,000 r/min for 5 min. Then the supernatant was filtered through the 0.22 μm organic phase microporous membrane.

On-machine data collection and analysis

Catechins chromatographic conditions were as follows: C18 reverse phase column (5 μm, 4.6 × 250 mm) was used, with mobile phase A being 0.2% formic acid-water solution (v: v), and mobile phase B being methanol. The flow rate was set to: 1.00 mL/min, the column temperature to: 40 ℃, and the injection volume: to 10.0 μL. Gradient elution conditions were: 0–2 min, 88% A; 2–10 min, 88%-75% A; 10–15 min, 75%-73% A; 15–25 min, 73%-68% A; 25–30 min, 68% A; 30–32 min, 68-88% A. The detector wavelength was set at: 280 nm.

Preparation of purine alkaloid samples and on-machine data collection and analysis

The steps for obtaining the supernatant were the same as for catechins. The supernatant was diluted 10-fold and 1000-fold, respectively. The dilution was passed through a 0.22 μm organic phase microporous membrane for UPLC-MS/MS analysis. This was performed using a Nexera X2 LC-30 A HPLC system and a tandem Sciex 4500 Q-Trap mass spectrometer at a column temperature of 40 ℃, a wavelength of 231 nm, an injection volume of 5 μL, and a flow rate of 0.3 mL/min. A C18 column (2.6 μm, 2.1 × 100 mm) was used with solvent A (0.1% formic acid) and solvent B (acetonitrile) as the mobile phase. Gradient elution conditions: 0–0.2 min, 10% B; 0.2–2.5 min, 10-90% B; 2.5–4.0 min, 90% B; 4.0–4.2 min, 90%-10% B; 4.2–6.0 min, 10% B. Mass spectrometry conditions: the electrospray ionization source (ESI) was operated in positive ion mode with the following parameters: curtain gas (N_2) pressure at 30 psi, electrospray voltage at 4500 V, auxiliary gas (N_2) temperature at 550 °C, nebulizer gas (N_2) pressure at 55 psi, and heater gas (N_2) pressure at 55 psi [19]. All samples underwent three replicates.

RNA isolation and RNA-seq analysis

RNA extraction and sequencing

Total RNA was extracted from the test materials using the Omega Plant RNA kit (Omega Bio-Tek, Inc, Cat. No. R6827, USA) following the manufacturer's protocol. RNA sequencing was performed by Genedenovo Biotechnology Co., Ltd. (Guangzhou, China), for detailed methods, please refer to the study by Huang et al. [23]. Nine libraries (3 samples, 3 biological replicates) were sequenced on Hiseq™ 4000 platform.

Data quality control

Fastp was used to perform quality control on the raw data, filter low-quality data to obtain high-quality clean read length data. HISAT 2 was used to align the sequences obtained from the paired-end sequencing to reference tea genome of Tieguanyin, with the parameters set to default [24]. Based on the alignment results, transcripts were reconstructed using Stringtie, and the gene expression levels in each sample were calculated using RSEM software.

Identification and quantification of proteins

Proteome extraction and sequencing

To extract proteins from the samples, the DIA experimental method was employed. In brief, it involved protein denaturation, reduction, alkylation, enzymatic digestion and peptide purification [25]. These procedures were conducted by Genedenovo Biotechnology Co., Ltd. (Guangzhou, China). After preparing the protein samples, DIA protein detection was performed.

Protein qualitative and quantitative analysis

The UltiMate 3000 (Thermo Fisher Scientific, MA, USA) liquid chromatography system was connected to the tims TOF Pro2 Mass spectrometer (Bruker Daltonics), an ion-mobility spectrometry quadrupole time of flight mass spectrometer (Bruker Daltonics). DIA data were acquired in the diaPASEF mode. Raw DIA data were processed and analyzed using Spectronaut 18 (Biognosys AG, Switzerland) with default settings. To ensure the reliability of results, checked whether the qualitative analysis results of the protein meet the following identification criteria: precursor thresholds of 1.0% FDR and protein thresholds of 1.0% FDR at the peptide and protein levels, respectively. Normalization strategy was set to local normalization. Peptides which passed the 1% Qvalue cutoff were used to calculate the major group quantities with MaxLFQ method. After counting the reads for each protein, the set of genes expressed in each time period was counted for each variety, and differences between varieties were analyzed by Venn diagrams.

Quantitative real-time PCR (qRT-PCR) analysis

cDNA synthesis, qRT-PCR, and specific primer design were based on previously reported methods [26]. Primers were designed using an online website (https://www.primer3plus.com/index.html. September 10, 2024) (Table S2). The tea plant *GAPDH* (registration number GE651107) was selected as the internal reference gene [27]. qRT-PCR analysis was performed using a CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA), according to the instructions of the Hieff® qPCR SYBR Green Master Mix (No Rox) Mixing kit (Yisheng Biotechnology (Shanghai) Co., Ltd., Shanghai, China). All samples were analyzed in three biological replicates. Relative expression levels were calculated using the $2^{-\triangle\triangle Ct}$ method [28]. The correlation coefficients of the RNA-seq data and qRT-PCR was performed in Omicshare Tools (https://www.omicshare.com/tools).

Data analysis

Each experiment was repeated at least three times, and data presented as mean ± standard deviation. Differences between groups were assessed using one-way ANOVA followed by Duncan's test. Principal Component Analysis (PCA) was performed using R studio (4.0.5) [29]. Cluster analysis and correlation analysis were performed with the Mfuzz R package. The chemical structures were created using ChemDraw 22.0.0. The network diagram of transcription factors and genes was drawn using Cytoscape_v3.10.2 software. Differentially abundant proteins (DAPs) were analyzed using Student's t test, with selection criteria of a fold change > 1.2, $P < 0.05$ [30]. The edge R package was applied to identify differentially expressed genes (DEGs) across samples with a criterion of fold change > 2, FDR < 0.05 [31]. The gene functions of DEGs and the enriched metabolic pathways were annotated based on Gene Ontology (GO, http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/), respectively. Transcriptome data have been uploaded to the National Center for Biotechnology Information https://submit.ncbi.nlm.nih.gov/subs/sra/ via the SRA partner repository under the data set identifier PRJNA1179900. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD062468.

Results

Analysis of purine alkaloids and catechins content in 'Anxi Kucha'

We measured the purine alkaloid and catechin contents of the leaves of FDDB, TGY and AXKC. Among them, the contents of theacrine and EGCG3"Me were significantly different among the three varieties. The total purine alkaloid content of AXKC was significantly higher than that of the other two tested samples. The difference in the total content of purine alkaloids among the three tested samples was mainly reflected in the theacrine content. The theacrine content of AXKC was as high as 17.44 ± 0.40 mg/g, which was significantly higher than that of TGY and FDDB (not detected) (Fig. 1A).

The EGCG3"Me content of AXKC was 11.25 ± 0.17 mg/g. For TGY, the EGCG3"Me content was 5.32 ± 0.30 mg/g. FDDB had an EGCG3"Me content of 0.93 ± 0.01 mg/g (Table 1). Analysis of significant differences indicated that the differences in the EGCG3"Me content of AXKC, TGY and FDDB varied significantly ($p < 0.05$), and the contents showed a low, medium and high distribution in order (Fig. 1B).

In general, the contents of theacrine and EGCG3"Me were significantly different among the three tea varieties, with AXKC leaves exhibiting substantially higher levels of both metabolites compared to the other two varieties.

Multivariate proteomics and transcriptomics analysis of 'Anxi Kucha'

The content of theacrine and EGCG3"Me in AXKC was higher, and the content was significantly different from the other two varieties. To further explore the synthesis pathways and enzymes/genes of theacrine and EGCG3"Me, proteomic and transcriptomic studies were continued. A total of 10,035 proteins were identified from the proteomics dataset, and the samples were clustered into three groups in the PCA plot based on their protein abundance values. The proteome profiles of different samples were well differentiated (Fig. 2A). Further research is warranted. RNA sequencing identified 53,422 transcripts, with 7,521 were novel genes. Pearson analysis of TPMs (Transcripts Per Millions) distribution across samples (Fig. 2B) and the heatmap depicted dynamic trends of transcripts. Strong correlations between different biological replicates were observed, confirming analysis's reliability.

Using DIA and RNA-seq analysis to explore the enzymes and genes that synthesize theacrine and EGCG3"Me. Proteomic analysis identified 6049 DAPs (Fig. 2C), while transcriptome data revealed, 16,102 DEGs (Fig. 2D). In the FDDB vs TGY comparison, there were 1419 up-regulated and 1537 down-regulated DAPs. There were 4239 up-regulated and 5763 down-regulated DEGs. In the FDDB vs AXKC comparison, there were 2254 up-regulated and 2067 down-regulated DAPs. There were 4831 up-regulated and 5646 down-regulated DEGs. In the TGY vs AXKC comparison, there were 1894 up-regulated and 1689 down-regulated DAPs. There were 4413 up-regulated and 3713 down-regulated DEGs.

In organisms, different proteins coordinate to perform various functions. Pathway analysis aids in understanding

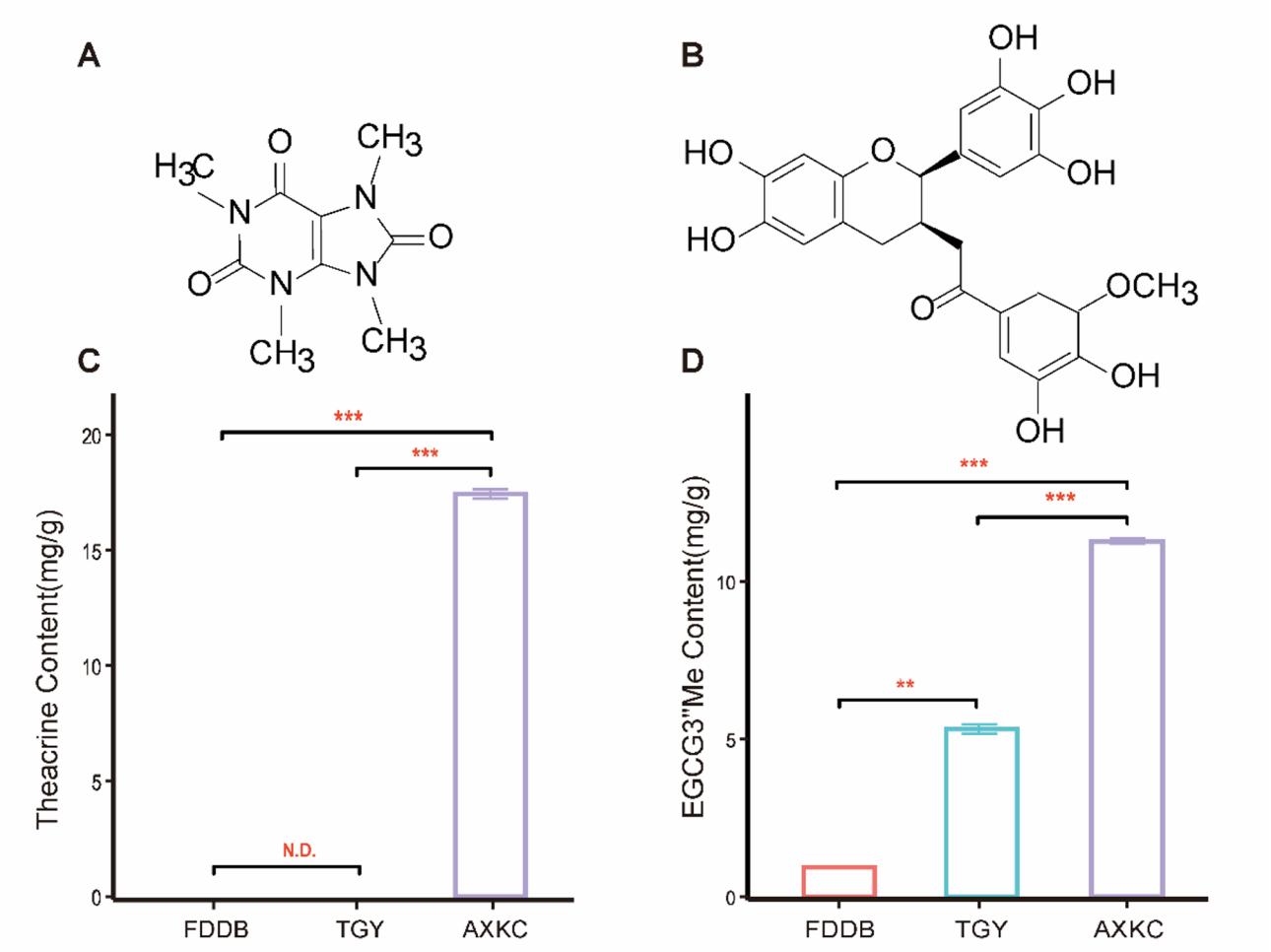

Fig. 1 Theacrine and EGCG3"Me content of FDDB, TGY and AXKC varieties. (**A/C**) The chemical structure and content of theacrine. (**B/D**) The chemical structure and content of EGCG3"Me. The significance of differential compounds between the two control groups is indicated as $^{**}P < 0.05$, $^{***}P < 0.01$. FDDB: 'Fudingdabaicha', TGY: 'Tieguanyin', AXKC: 'Anxi kucha'. N.D. means not detected

these biological functions of proteins. Pathway enrichment analysis identified pathways related to theacrine and EGCG3"Me synthesis. Key pathways, including purine metabolism and flavonoid biosynthesis, were highlighted in the KEGG enrichment results of the proteome (Fig. 2E). The abundance levels of DAPs enzymes in these two pathways were analyzed (Fig. 2F). High enzyme abundance levels of SAMS3, TCS1, TCS2, APRT3, IMPDH and ADK2 were found in the purine metabolism pathway, while CHI1, CHI2, FLS2 and ANR4 were prominent in the flavonoid biosynthesis pathway. These enzymes played a role in the synthesis of theacrine and EGCG3"Me, respectively. In the transcriptome data, the KEGG enrichment results of DEGs also enriched the purine metabolism and flavonoid biosynthesis pathways (Fig. 2G). The transcriptome data were verified by qRT-PCR. The strong correlation coefficient between qRT-PCR analysis and RNA-seq data indicated that the transcriptome data in this study accurately reflected transcript abundance (Fig. 2H).

The results of proteomic and transcriptomic analysis showed that the three tea varieties could be well distinguished from each other. The key pathways regulating theacrine and EGCG3"Me synthesis were successfully enriched.

Integrated transcriptome-proteome-metabolome analysis

Protein and mRNA in organisms represent gene expression at the protein level and transcription level. Protein-transcriptome association analysis can focus on enzymes expressed in both omics simultaneously. Venn diagram analysis screened the three comparison groups for DAPs and DEGs expressed in both omics, resulting in 808, 1167, and 759, respectively. The specific distribution and up-regulation and down-regulation of the commonly expressed enzymes in each comparison group were shown in Fig. 3A and B.

Table 1 Relative quantitative analysis of alkaloid and Catechin in the third leaf of different tea varieties

Cultivar	AXKC	TGY	FDDB
CAF	32.29 ± 0.48[a]	33.27 ± 1.10[a]	30.37 ± 0.30[b]
TC	17.44 ± 0.40[a]	ND	ND
TB	1.39 ± 0.01[a]	0.65 ± 0.01[b]	0.48 ± 0.01[c]
TP	ND	ND	ND
Total alkaloids	51.11 ± 0.75[a]	33.92 ± 1.10[b]	30.85 ± 0.30[c]
GC	1.99 ± 0.07[c]	3.61 ± 0.11[a]	2.46 ± 0.030[b]
EGC	16.83 ± 0.09[c]	27.69 ± 0.08[b]	48.01 ± 0.07[a]
C	0.37 ± 0.05[b]	0.55 ± 0.05[a]	0.17 ± 0.03[c]
EGCG	97.33 ± 0.12[a]	75.13 ± 0.32[b]	67.98 ± 0.26[c]
EC	3.02 ± 0.15[c]	7.19 ± 0.13[a]	6.26 ± 0.02[b]
EGCG3"Me	11.25 ± 0.17[a]	5.32 ± 0.30[b]	0.93 ± 0.01[c]
ECG	24.56 ± 0.04[a]	19.61 ± 0.17[b]	13.98 ± 0.03[c]
CG	0.29 ± 0.04[a]	0.16 ± 0.05[b]	0.28 ± 0.01[a]
Total catechins	155.64 ± 0.17[a]	139.26 ± 0.44[c]	140.08 ± 0.20[b]

All data are presented as mean ± standard statistical (SD) of three replicates. Values are expressed as mg/g. The letters (a, b, c) indicate statistical significance ($P < 0.05$). ND means not detected. FDDB: 'Fudingdabaicha', TGY: 'Tieguanyin', AXKC: 'Anxi kucha'. The contents of TC.

Nine quadrant graphs were drawn based on the log2 values of fold differences between the two omics (Fig. 3C, D, and E). In the purine metabolism pathway, common mRNAs and corresponding proteins were mostly concentrated in quadrant 6. Related proteins and mRNAs in the flavonoid biosynthesis pathway were relatively more common in quadrants 3, 6, and 7. Various enzymes in the proteome directly participate in metabolic processes and regulate metabolic reactions. Therefore, we focused on the DAPs in quadrants 6, 8, and 9. KEGG enrichment analysis (Fig. 3F) revealed that purine metabolism and flavonoid biosynthesis pathways related to theacrine and EGCG3"Me synthesis were enriched.

We further focused on the two enriched pathways and performed correlation analysis between their DAPs (quadrant 3) that were upregulated in both proteomic and transcriptomic data and the targeted metabolites, displayed as a correlation heat map (Fig. 3G). There was a highly significant correlation between the abundance of IMPDH, TCS1, SAMS3, APRT1 enzymes and theacrine content. This was partially consistent with the analysis results in Fig. 2F, indicating that SAMS3, APRT1, IMPDH and TCS1 enzymes abundance levels regulated theacrine synthesis. There was a highly significant correlation between EGCG3"Me content and the abundance of LAR1, CHI1 and FLS2 enzymes, as well as a significant correlation with CHI2. There was a significant correlation between LAR1, FLS2 and CHI1 enzymes abundance and EGCG content. This further illustrating that CHI1, CHI2, and FLS2 enzymes regulated EGCG3"Me synthesis.

The results of transcriptome-proteome-metabolome analysis showed that IMPDH, TCS1, SAMS3, and APRT1 enzymes were closely related to theacrine synthesis, and LAR1, CHI1, CHI2 and FLS2 enzymes were closely related to EGCG3"Me synthesis. Further analysis of these related enzymes in the regulation of theacrine and EGCG3"Me synthesis pathways was needed.

Analysis of DAPs and DEGs involved in purine metabolism and flavonoid biosynthesis pathways in 'Anxi kucha'

Purine metabolism pathway

At the protein level, SAMS3, ADK1, ADK2, APRT1, APRT2, APRT3, AMPD, and IMPDH (Fig. 4A) were expressed at higher levels in AXKC leaves, and all were upregulated. These enzymes are crucial in xanthine nucleoside synthesis. High-level expression promoted the accumulation of more xanthine nucleosides in AXKC leaves, providing more abundant initial substrates for purine alkaloids synthesis. Conversely, these enzymes were generally down-regulated in FDDB and TGY leaves. TCS1 and TCS2 enzymes were upregulated in AXKC, providing more precursors for the subsequent theacrine synthesis. *N*-methyltransferase plays a vital role in theacrine synthesis, with higher expression of the NMT2 enzyme in AXKC leaves. Previous studies have shown that most genes involved in caffeine metabolism are upregulated. URE is part of the caffeine metabolic pathway [32]. In this study, the expression level of URE1 enzyme was consistent with the changes in caffeine content in FDDB and AXKC leaves. URE1 enzyme was highly expressed in samples with low caffeine content, which is consistent with the results of previous studies [33]. Caffeine seems to be a precursor for theacrine synthesis, and the catalytic effect of URE1 enzyme on caffeine may affect the synthesis of theacrine by AXKC.

Gene expression changes related to theacrine synthesis regulation in the purine metabolism pathway were also analyzed. The *SAMSs* genes were mainly up-regulated in FDDB leaves, with *SAMS3* up-regulated in AXKC leaves. *ADK1* and *ADK3* genes were highly expressed and upregulated in TGY and AXKC leaves. *APRT* genes were all up-regulated in FDDB leaves, with *APRT1* solely up-regulated in AXKC leaves. The *IMPDH* gene was up-regulated in AXKC leaves. The expression levels of these genes in FDDB, TGY and AXKC leaves varied between up-regulated and down-regulated, facilitating xanthosine accumulation and laying the foundation for caffeine synthesis. *TCS1*, *TCS2*, *TCS3* and *TCS4* genes were all up-regulated in AXKC leaves. The *NMT1* gene was upregulated in AXKC and FDDB leaves, while *URE2* and *URE3* genes had higher expression in FDDB leaves. Transcriptomic data were slightly less consistent with the theacrine concentrations changes across the three varieties, aligning with the nine-quadrant diagram analysis, where genes and corresponding enzymes related to the purine metabolic pathway were mainly concentrated in quadrant 6, with higher protein expression abundance

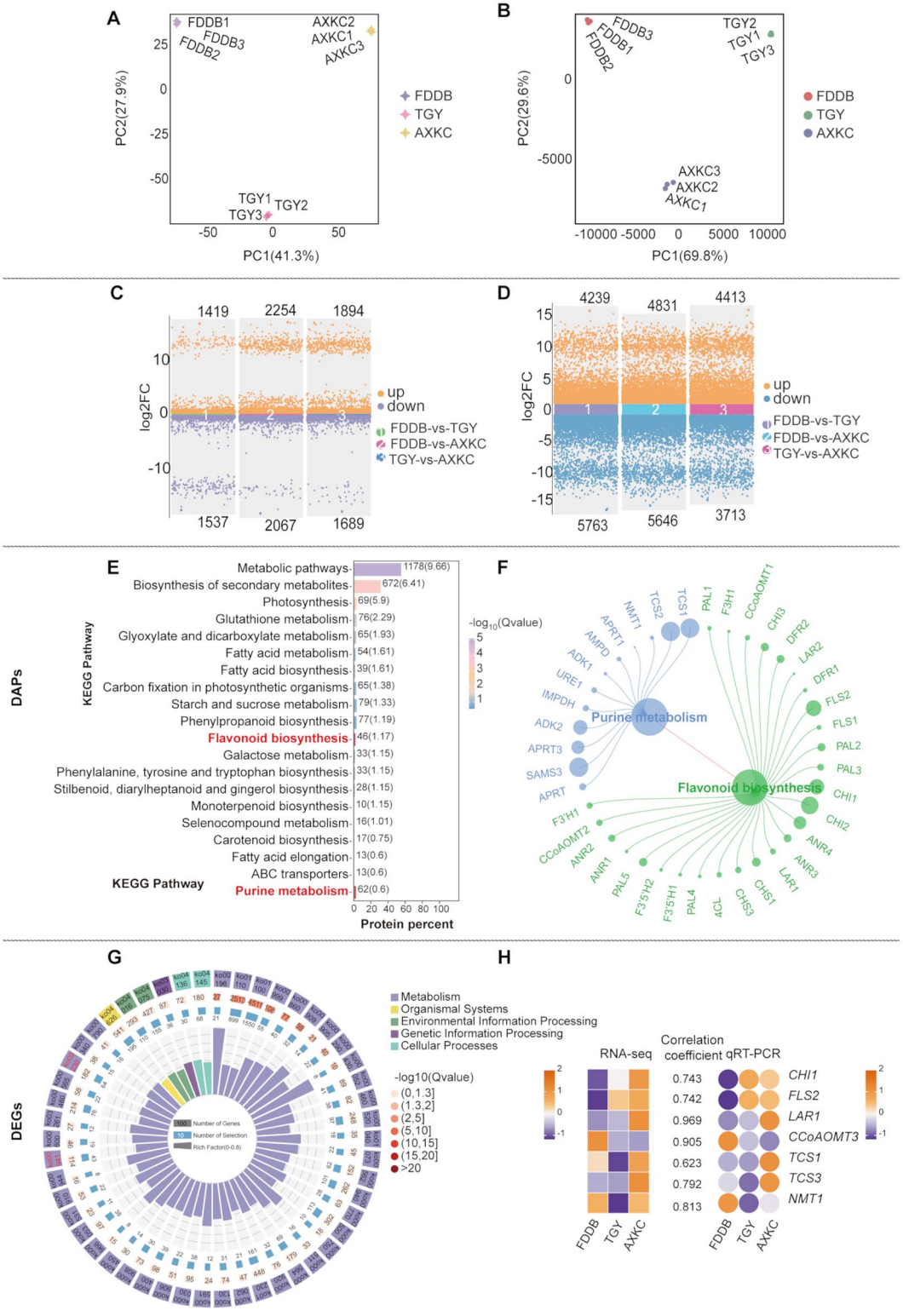

Fig. 2 (See legend on next page.)

(See figure on previous page.)

Fig. 2 Overview of proteomic and transcriptomic analysis of tea varieties. Sample relationship between the tested samples. (**A**) Proteomics data set. (**B**) Transcriptome sequencing data. Multiple group difference scatter plots: These plots list the number of up-regulated and down-regulated DAPs and DEGs based on pairwise comparisons. (**C**) Log2-transformed fold changes in DAPs abundance among different germplasms. (**D**) Log2-transformed fold changes in DEGs expression among different germplasms. Enrichment Analysis: (**E**) Enrichment analysis of proteomic DAPs. The top 20 enriched pathways are listed. KEGG terms related to theacrine and EGCG3"Me metabolism are highlighted in bold and red. (**F**) Circular dendrogram showing the abundance levels of DAPs in the purine metabolism and flavonoid biosynthesis pathways. The size of the circle indicates the abundance level of the enzyme. (**G**) DEGs are listed in a circle diagram with the top 50 KEGG enriched pathways. The first circle represents the Pathway ID, with different colors distinguishing KEGG_A_class. The second circle indicates the number of genes enriched in the Pathway ID in the background gene set. The third circle shows the number of genes in the target gene set enriched in the Pathway, and different colors distinguish up- and down-regulation. The fourth circle represents Gene Ratio, calculated as the number of genes in the target gene set enriched in the pathway divided by the number of genes in the background gene set enriched in the pathway. The Pathway ID of purine metabolism and flavonoid biosynthesis pathways were marked in red and bold. (**H**) Correlation analysis of 7 selected DEGs. The correlation coefficients of the RNA-seq data and qRT-PCR analyses for each gene are represented by the values between two heatmaps. FDDB: 'Fudingdabaicha', TGY: 'Tieguanyin', AXKC: 'Anxi kucha'

than mRNA. Enzymes directly catalyze chemical reactions in organisms and promote metabolites synthesis. Their abundance levels directly affect the accumulation of metabolites in the leaves of the three varieties.

At the protein and transcription levels, the changing trends of SAMS3, ADK1, APRT1, IMPDH and TCS1 in FDDB, TGY and AXKC leaves were consistent with theacrine content, with relatively high expression levels were relatively high in AXKC leaves. In the association analysis of targeted metabolites with proteome and transcriptome (Fig. 3G), the abundance levels of SAMS3, APRT1, IMPDH and TCS1 were significantly correlated with theacrine synthesis. This further illustrates the positive effects of genes and corresponding enzymes on theacrine synthesis in AXKC leaves.

Flavonoid biosynthesis pathway

Based on the enrichment of multiple analysis results in flavonoid biosynthesis pathways, we examined the DAPs and DEGs in these biosynthesis pathways in detail (Fig. 5). In the protein group, we found five PALs enzymes, with higher enzyme expression levels in FDDB leaves and the lowest enzyme expression levels in TGY leaves. A 4CL enzyme, the enzyme expression level was higher in FDDB and AXKC leaves. CHS1 was expressed at a higher level in AXKC leaves, while the enzymes CHI1, CHI2, CHI3, F3H1, FLS1 and FLS2 were expressed at high levels in both AXKC and TGY leaves. The CHI enzymes were responsible for synthesizing corresponding flavanols, which were then converted into flavonoids like epigallocatechin-3-glucose (EGCG precursor) by the FLS enzymes. The high expression of these enzymes facilitates the formation of more EGCG, providing the basic molecules needed for the accumulation of EGCG3"Me. In the results of target metabolites, EGCG content in AXKC and TGY leaves was higher, indicating that these enzymes positively regulate the synthesis of EGCG3"Me precursor, EGCG. The transcriptome data showed that the expression trends of the identified *CHIs* genes were consistent with those of the corresponding enzymes, demonstrating a positive regulatory effect. The expression level of F3'H1 enzyme was higher in FDDB and TGY leaves, while the F3'5'H2 enzyme expression level was higher in TGY and AXKC leaves. DFR1 was expressed at a higher level in FDDB leaves, and DFR2 showed higher abundance in TGY and AXKC leaves. ANR1 and ANR2 expression abundance was higher in FDDB and TGY leaves, whereas ANR3 and ANR4 enzymes had higher expression levels in AXKC leaves. The abundance levels of LAR1, LAR2, CCoAOMT1 and CCoAOMT2 were relatively high in AXKC leaves. These two enzymes have both indirect and direct effects on EGCG3"Me synthesis, and their high-level expression promotes the production of higher content of EGCG and EGCG3"Me, consistent with the results of targeted metabolites.

DEGs involved in the flavonoid biosynthesis pathway in the transcriptome data were identified, and the following genes were included. The *PALs* and *4CL2* genes were expressed at higher levels in FDDB leaves. The *C4H*, *CHS2*, *CHS3*, *CHI1*, *CHI2*, and *CHI4* were expressed at higher levels in TGY and AXKC leaves, especially in AXKC leaves. Most of the *F3'H* and *F3'5'H* genes were highly expressed in TGY and AXKC. *FLS1*, *FLS2*, and *FLS3* were up-regulated in TGY and AXKC leaves, and *FLS4* was up-regulated in FDDB. *DFR1*, *DFR3*, and *DFR4* were highly expressed in FDDB leaves, with only *DFR3* was up-regulated in AXKC leaves. *ANS* was up-regulated in FDDB leaves. *ANR3* was up-regulated in FDDB and AXKC leaves. *ANR1* and *ANR2* were up-regulated in TGY leaves. *LAR1* was expressed at a higher level in AXKC leaves, consistent with the enzyme expression abundance trend. Previous studies have found that CCoAOMT enzymes are involved in many plant secondary metabolic processes and affect the biosynthesis of lignin [34, 35]. In this study, *CCoAOMT3* was highly expressed in FDDB leaves.

CHI1, CHI2, FLS1, FLS2 and LAR1, genes and their corresponding enzymes were consistent with the changing trend of EGCG3"Me content in FDDB, TGY and AXKC leaves. In the association analysis of targeted metabolites with proteome and transcriptome (Fig. 4H), the abundance levels of CHI1, CHI2, FLS2 and LAR1 enzymes were significantly correlated with EGCG3"Me synthesis. This further illustrates the positive effects of

these genes and corresponding enzymes on EGCG3"Me synthesis in AXKC leaves.

Transcription factor regulations in theacrine and EGCG3"Me metabolism

Transcription factors (TFs) can specifically bind to the cis-acting elements in the promoter region of structural genes, regulating their transcriptional expression by either activating or inhibiting the promoter activity of target genes. It has been found that related enzymes/genes have a regulatory effect on the synthesis of theacrine and EGCG3"Me. To explore the upstream changes in the expression of these enzymes and genes, TFs were analyzed. TF annotation was performed using the PlantTFDB website https://planttfdb.gao-lab.org/. The proteomic data from this experiment were statistically analyzed, resulting in the annotation of a total of 46 TFs families. To further clarify the regulatory effects of TFs on the purine metabolic and the flavonoid metabolic pathways, the following analysis was performed. Firstly, previous studies have shown that bHLH, MYB, WRKY, and ERF can regulate these two pathways [18, 36]. The motif information of these TF bindings was obtained from the JASPAR database, and the transcriptional target genes were predicted using the MEME FIMO software. TFs and target genes with correlation (pearson > 0.8) were selected, and the TFs that played an up-regulated role were used to draw the network diagram.

In the purine metabolism pathway (Fig. 6B), one bHLH, two MYB, and two ERF transcription factor family members were identified to play an up-regulatory role. BHLH74 and MYB4 targeted and regulated *TCS2*, while CSTF50 (ERF) targeted and regulated *ADK3* and *IMPDH*, MYB4 and ERF4 also targeted and regulated *IMPDH*, and SRM1 (MYB) targeted and regulated *SAMS1*, *SAMS2*, *SAMS4*, and *SAMS5*. The expression levels of *IMPDH* and *TCS2* were consistent with the expression trends of CSTF50(ERF), ERF4, MYB4 and BHLH74 (Fig. 6A and C), which further demonstrating, the up-regulation of *TCS2* and *IMPDH* by these four TFs.

In the flavonoid biosynthesis pathway (Fig. 6E), two bHLH, two MYB, and one WRKY transcription factor family members were identified. BHLH74 and MYB4 targeted and regulated *FLS2*, MYC2 (bHLH) targeted and regulated *CHS2*, WRKY46 targeted and regulated *CHI1*, and SRM1 (MYB) targeted and regulated *FLS1*, *CHI1*, *CHI4*, *F3'5'H5* and *FLS2*. The expression levels of *FLS2*, *CHI1*, *CHI4*, and *F3'5'H5* were consistent with the expression trend of SRM1(MYB) (Fig. 6D and F), indicating the up-regulation of *FLS2*, *CHI1*, *CHI4*, and *F3'5'H5* by SRM1(MYB).

Discussion

Most alkaloids are known for their bitterness. Studies had shown that the bitterness of theacrine surpasses that of caffeine, suggesting that theacrine was a key compound responsible for the bitterness of Yunnan kucha and crude Pu-erh tea (Pt) [37]. In this study, AXKC tea leaves exhibited higher theacrine content, while TGY and FDDB showed barely detectable levels, consistent with previous findings [21]. Additionally, AXKC leaves had elevated levels of EGCG and EGCG3"Me, particularly EGCG, which was significantly higher than in the other two cultivars. This indicates a higher bitter compound accumulated in AXKC leaves. These bitter tastes can stimulate the body's oral secretions, promote digestion, and increase appetite [5]. AXKC is rich in theacrine and EGCG3"Me, suggesting it may have potential superior health benefits compared to other tea varieties. These compounds are also key contributors to the flavor of tea, warranting further study.

Relationship between theacrine content and gene/enzyme expression

SAMS is one of the key enzymes in the purine alkaloid metabolic pathway and acts as a methyl- donor in the synthesis of theacrine and other purine alkaloids biosynthesis [17]. The expression levels of *SAMS3* and the corresponding enzymes were upregulated in 'Anxi kucha' leaves, and the expression trend was consistent with the theacrine content. This was consistent with the result that the theacrine content was significantly positively correlated, further indicating that it had an important contribution to the synthesis of theacrine. Related studies have shown that blocking of IMPDH activity with ribavirin inhibited caffeine biosynthesis [38]. The expression abundance level of IMPDH enzyme was consistent with the purine alkaloid content of the three tea varieties, and the IMPDH enzyme had a very significant correlation with the theacrine content. TCS is a class of *N*-methyltransferase that catalyzes the methylation of *N*-1 and *N*-3 in tea plant. Previous studies have pointed out that *TCS1* plays a crucial role in the methylation of xanthosine at the 1-*N* position in caffeine biosynthesis [39]. At both the protein and transcriptional levels, TCS1 was upregulated in AXKC leaves, which may explain its moderate caffeine content. Recently, researchers identified three *N*-methyltransferases (NMTs), including a *N*-methyltransferase, *CkTcS*, as a key *N9*-methyltransferase that acted only on 1,3,7-trimethyluric acid but not on caffeine, and was involved in the biosynthesis of theacrine [6]. In 'Anxi kucha' leaves, the expression level of NMT2 enzyme was higher and it played a role in up-regulating expression.

At the protein and mRNA levels, the expression trends of SAMS3, APRT1, IMPDH, and TCS1 were consistent with the theacrine content in AXKC, TGY, and FDDB

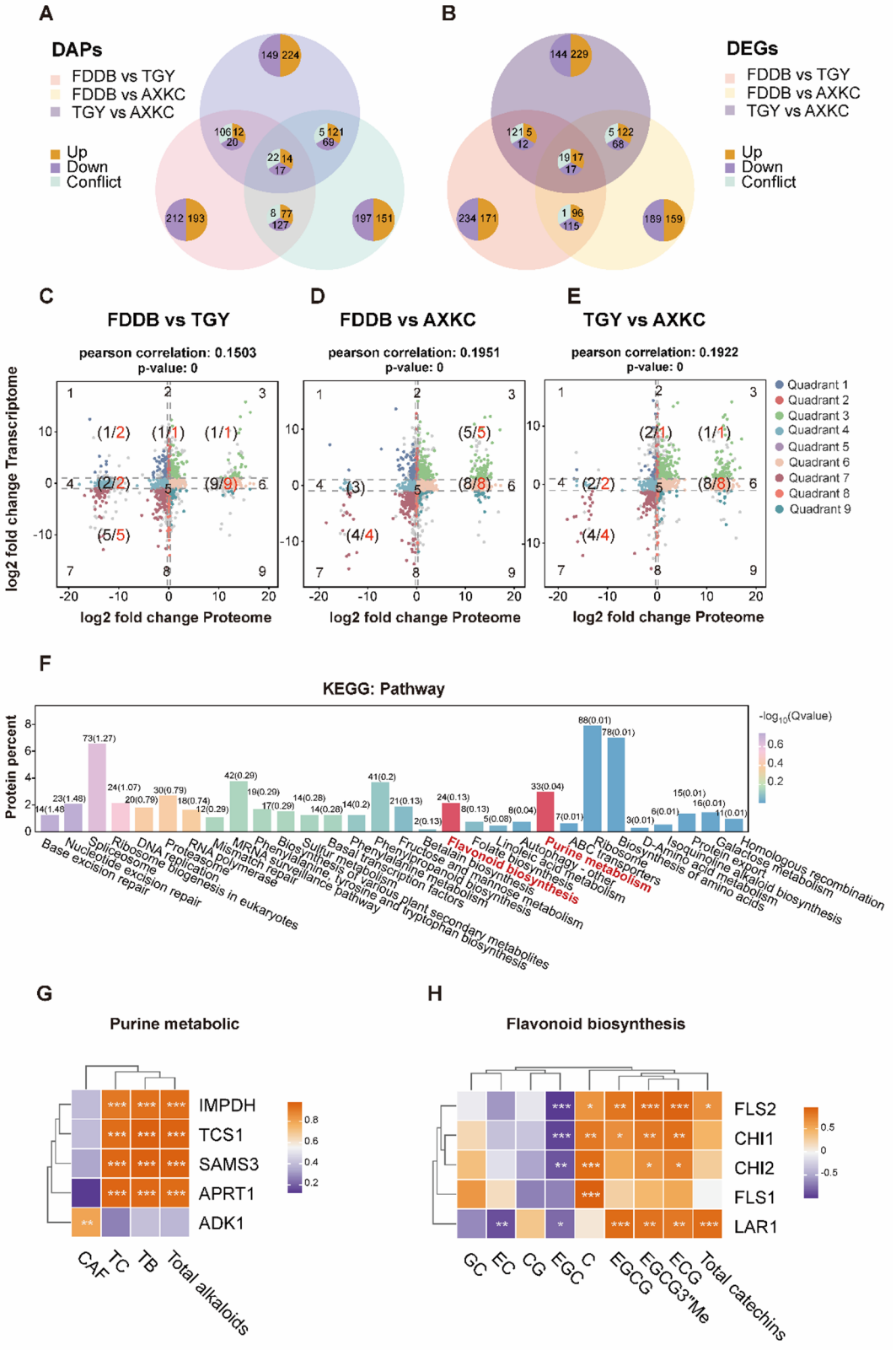

Fig. 3 (See legend on next page.)

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Fig. 3 Joint analysis of proteome, transcriptome, and metabolome. (**A**) DAPs shared by the three varieties in the Venn diagram analysis. (**B**) DEGs shared by the three germplasms in the Venn diagram analysis. Transcriptome-Proteome nine-quadrant diagram. (**C**) FDDB vs. TGY, (**D**) FDDB vs. AXKC, (**E**) TGY vs. AXKC. The numbers in the nine-quadrant diagram are common mRNA and protein numbers, where red represents protein. (**F**) Enrichment analysis of the top 30 KEGG in the 6th, 8th, and 9th quadrants of the transcriptome-protein nine-quadrant diagram, the purine metabolism and flavonoid biosynthesis pathways are highlighted in bold and red. Heat map of the correlation between enzymes upregulated in both the transcriptional and protein levels and targeted metabolites. (**G**) In the purine metabolism pathways. (**H**) In the flavonoid biosynthesis pathways. Statistical significance: $P >= 0.05$ no mark, "*" indicates the significant difference between the data ($0.01 < P < 0.05$), "**" indicates the significant difference between the data ($0.001 < P < 0.01$), "***" indicates the significant difference between the data Significance ($P <= 0.001$). FDDB: 'Fudingdabaicha', TGY: 'Tieguanyin', AXKC: 'Anxi kucha'

leaves, and their expression levels were higher in AXKC leaves. The results of the transcriptome-proteome-metabolome joint analysis showed (Fig. 3G) that there was a significant correlation between these enzymes and theacrine content. This may be the reason why more theacrine accumulates in AXKC leaves.

Relationship between EGCG3"Me content and gene/enzyme expression

The accumulation and composition of catechins were highly correlated with the expression levels of biosynthetic genes [40]. Research has shown that *CsCHI*, *CsF3H*, *CsDFR*, *CsANS*, and *CsANR* are key regulatory genes for catechin content [41]. F3'H and F3'5'H are key enzymes involved in forming dihydroxylated and trihydroxylated catechins [40]. The protein abundance of FLS is positively correlated with the total concentration of flavonol derivatives [42]. Catechin epimerization is mainly catalyzed by the enzymes LAR, ANS, and ANR [43]. The accumulation of EGCG3"Me is positively correlated with the expression levels of *CsDFR*, *CsLAR* and *CsCCoAOMT* [18].

At the protein and mRNA levels, CHI1 and CHI2 were highly expressed in AXKC and TGY leaves. Meanwhile, there was a significant positive correlation between the abundance levels of CHI1 and CHI2 enzymes and EGCG and EGCG3"Me contents. This indicates that the active regulation of CHI1 and CHI2 enzymes promotes the synthesis of EGCG and EGCG3"Me in targeted metabolites. The regulation of CHI enzyme provides intermediates for the subsequent synthesis of various catechins [41]. From these intermediates, several side branches are formed, each producing different categories of flavonoids. F3'5'H catalyzes dihydrokaempferol. There are two other branches: one is the F3'H catalysis-mediated branch for biosynthesis of C (catechin), EC (epicatechin), quercetin and its glycosyl derivatives. The other is the FLS-mediated branch for the biosynthesis of kaempferol and its glycosyl derivatives. In Fig. 5, the protein and transcriptional expression levels of F3'H were much lower than those of F3'5'H and FLS, suggesting that the F3'H-mediated branch is not the dominating branched pathway for flavonoid biosynthesis in tea leaves [44]. Among the targeted metabolites, the contents of C and EC were relatively small compared with other catechins (such as EGCG and ECG). FLS, with high levels of mRNA and protein, boosted the biosynthesis of flavonol glycosides. There is a significant positive correlation between FLS2, which has a higher enzyme abundance level, and EGCG and EGCG3"Me content.

Parallel to the biosynthesis of catechin compounds in tea leaves, at the protein and mRNA levels, LAR and CCoAOMT were highly expressed in AXKC leaves. In the correlation analysis with targeted metabolites (Fig. 4H), there was a highly significant positive correlation between LAR1 and the contents of EGCG and EGCG3"Me. In the real-time fluorescence quantitative PCR results, the correlation coefficients of *LAR1* and *CCoAOMT3* genes were both above 0.9, indicating high reliability of the results.

In general, CHI1, CHI2, FLS2, and LAR1, which were highly expressed at both protein and mRNA levels, play crucial roles in the flavonoid biosynthesis pathway. These genes and corresponding enzymes are the primary contributors to catechin biosynthesis.

Theacrine and EGCG3"Me transcription factor regulation in 'Anxi Kucha'

TFs regulate gene expression by specifically binding to promoters in downstream genes, modulating the biosynthesis of metabolites [18]. Many TFs are known to be involved in purine metabolism and flavonoid biosynthesis across different plants [18]. This study focused on TFs related to purine metabolism and flavonoid biosynthesis pathways. bHLH, MYB and ERF are related to the biosynthesis of purine alkaloids [36]. The expression trends of *TCS2* targeted by MYB4 and bHLH74 in the protein group were consistent, with both upregulated. MYB4, CSTF50 (ERF), and ERF4 upregulate the expression of IMPDH enzymes. In the correlation analysis of targeted metabolites, IMPDH and TCS2 enzymes were extremely significantly related to theacrine content.

Three R2R3-MYB proteins, including MYB11, MYB12, and MYB111, regulate flavonol biosynthesis by activating upstream genes such as *CHI* and *FLS*, while the MYB-bHLH-WD40 (MBW) complex activates the downstream genes in flavonoid pathway, regulating the biosynthesis of procyanidin and anthocyanins [45]. SRM1 (MYB) positively regulated *CHI1* and *FLS2*, and MYB4 and BHLH74 jointly positively regulated *FLS2*. Their expression levels at the protein and transcriptional were consistent with the trend of EGCG3"Me in the three tea varieties,

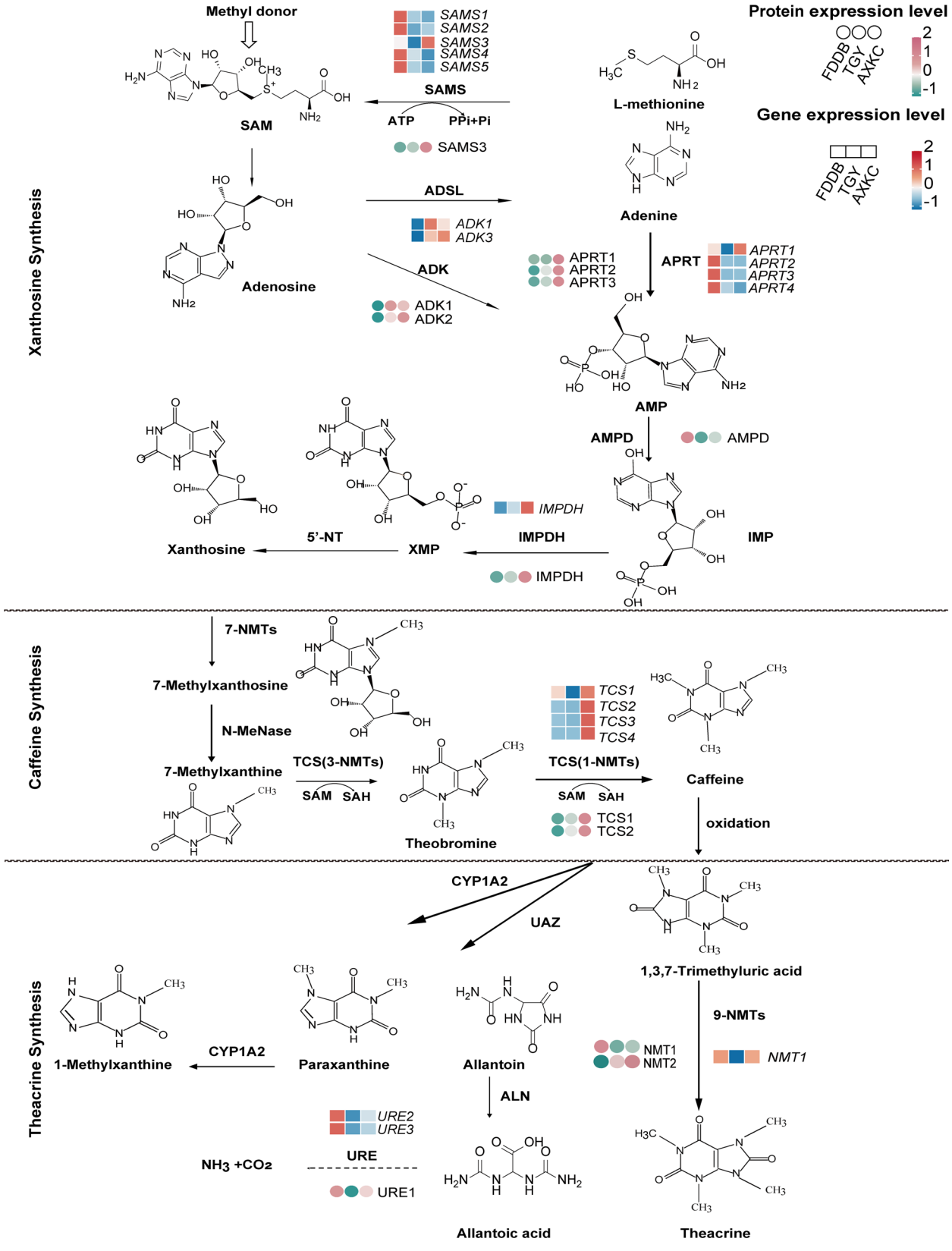

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Fig. 4 Purine metabolic pathway. The pathway consists of three main parts: the xanthosine synthesis pathway, the caffeine synthesis pathway and the theacrine synthesis pathway. Enzyme abbreviations are as follows: SAMS (S-adenosylmethionine synthetase); ADK (adenosine Kinase); APRT (adenine phosphoribosyltransferase); AMPD (adenosine monophosphate deaminase); IMPDH (inosine monophosphate dehydrogenase); 5′-NT (5′-nucleotidase); TCS (3-NMT and 1-NMT, tea caffeine synthase); CYP1A2 (cytochrome P450 family 1 subfamily apolypeptide 2); 9-NMT (9-methyltransferase); ALN (allantoinase); URE (urease). FDDB: 'Fudingdabaicha', TGY: 'Tieguanyin', AXKC: 'Anxi kucha'

and their expression levels were relatively high. In the correlation analysis of targeted metabolites, CHI1 and FLS2 enzymes were highly significantly correlated with EGCG3"Me content. They significantly contributed to the accumulation of EGCG3"Me in AXKC.

Compared with the same types of TFs transcripts reported in tea plants, we identified fewer bHLH, MYB, WRKY, and ERF isoforms, which may be due to the limitations of current protein identification technology [45]. MYB4, bHLH74, ERF4 and CSTF50 (ERF) positively regulated *IMPDH* and *TCS2*, which positively affected theacrine synthesis in AXKC SRM1 (MYB) positively regulated *CHI1* and *FLS2*, and MYB4 and BHLH74 jointly positively regulated *FLS2* to promote the accumulation of EGCG3"Me in AXKC. TFs played a key role in tea plant metabolism, affecting multiple biosynthetic pathways by regulating gene expression.

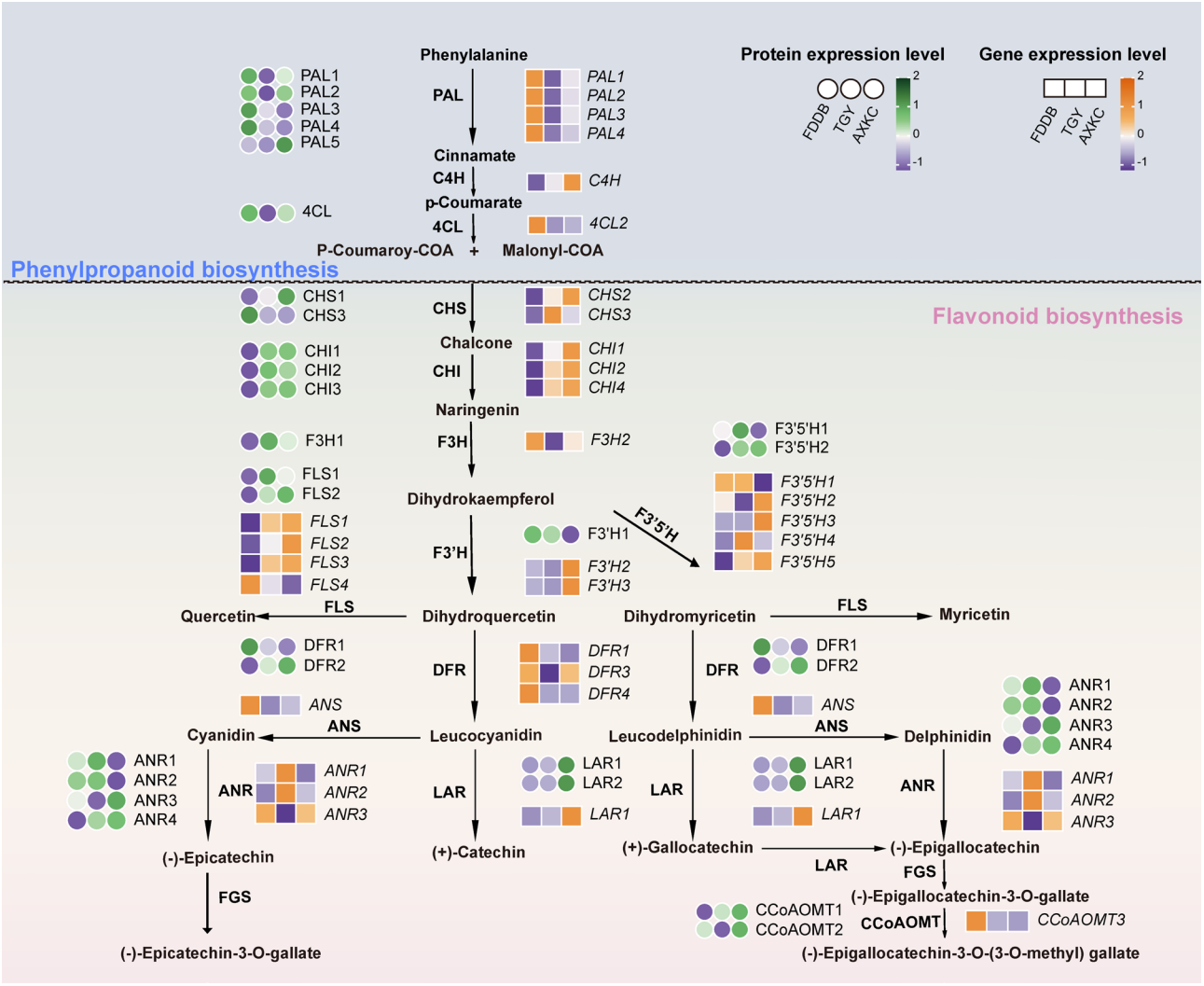

Fig. 5 Flavonoid biosynthesis pathway. Abbreviations of enzymes are as follows: PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; F3′H, flavonoid 3′-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavanol 4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; LAR, leucocyanidin reductase; FGS, flavan-3-ol gallate synthase; CCoAOMT, caffeoyl-CoA 3-*O*-methyltransferase. FDDB: 'Fudingdabaicha', TGY: 'Tieguanyin', AXKC: 'Anxi kucha'

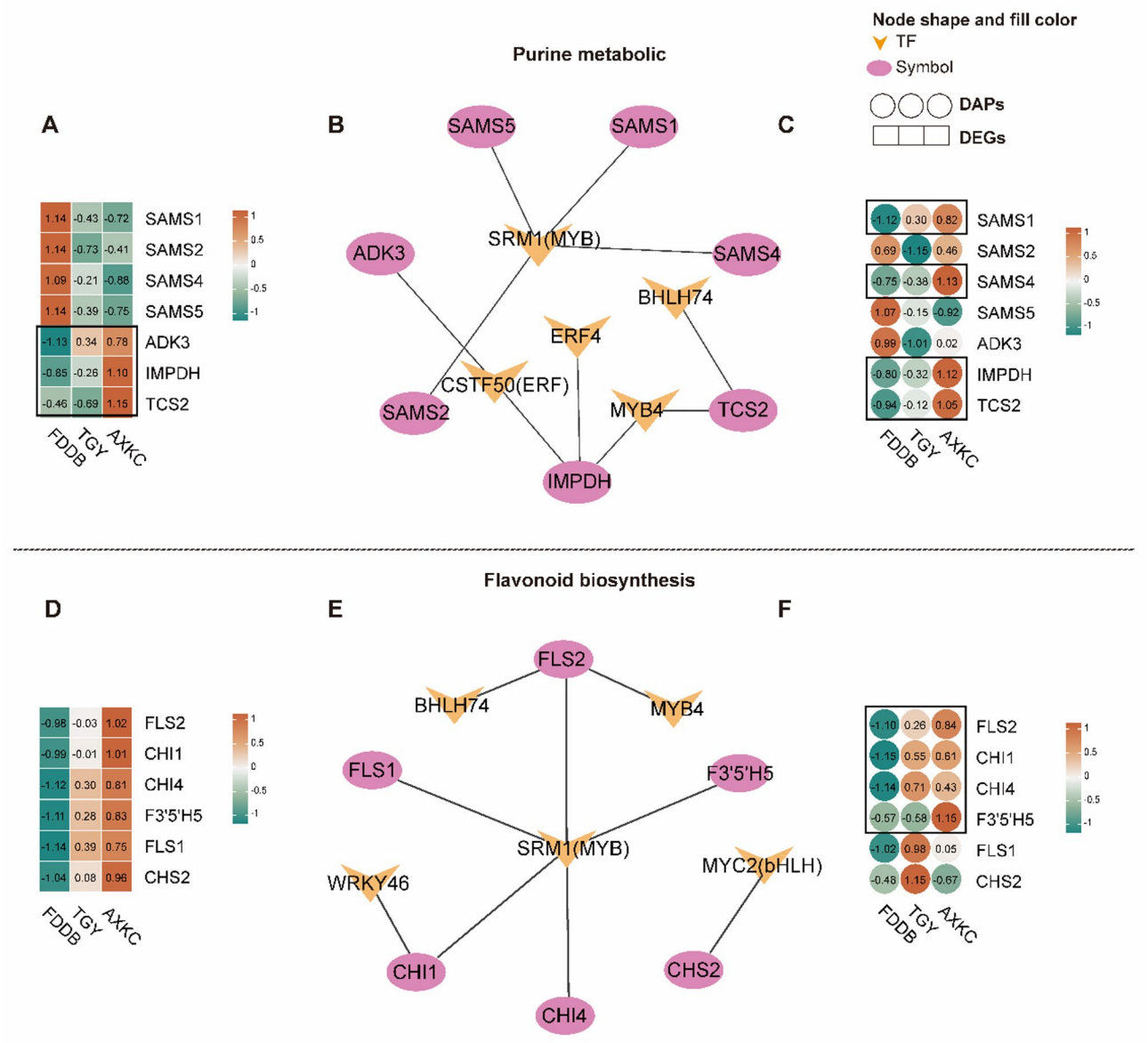

Fig. 6 Transcription factor analysis. Expression levels of targeted genes in the purine metabolism pathway in the proteome and transcriptome. (**A**) Gene expression heatmap. (**C**) Protein heat map expression. Expression levels of targeted genes in the flavonoid biosynthesis pathway in the proteome and transcriptome. (**D**) Transcriptomic expression. (**F**) Protein expression. (**B**) Transcription factors that upregulate the purine metabolism pathway. (**E**) Transcription factors that upregulate the flavonoid biosynthesis pathway. Orange icons represent transcription factors, and purple icons represent target genes. In the expression heat map, rectangles represent transcript levels (DEGs), and the circles represented protein levels (DAPs). FDDB: 'Fudingdabaicha', TGY: 'Tieguanyin', AXKC: 'Anxi kucha'

In conclusion, the integrated transcriptome-proteome-metabolome analysis showed that SAMS3, APRT1, IMPDH and TCS1 were the key rate-limiting enzymes promoting theacrine synthesis. Meanwhile, CHI1, CHI2, FLS2 and LAR1 were the key rate-limiting enzymes promoting EGCG3"Me synthesis. The analysis of transcription factors revealed that MYB4 and bHLH74 positively regulated the synthesis of theacrine and EGCG3"Me. AXKC is a brand-new and unique tea variety, providing valuable experimental materials for the subsequent cultivation of varieties rich in functional ingredients and the improvement of tea quality. Multi-omics analysis methods help us combine information from multiple species to explore plant metabolic pathways and their dynamic development. We found that the mutual regulation between transcription factors and enzymes plays an important role in the synthesis of theacrine and EGCG3"Me, which provides a comprehensive explanation for the complex network of metabolite biosynthesis and provides a rich data basis for molecular breeding,

which is of great significance for the cultivation of high-quality tea germplasm. At the same time, the exploration of this tea varieties also provides us with more excellent genetic resources for synthesizing theacrine and EGCG3"Me, which will lay a research foundation for the subsequent enzyme engineering synthesis of theacrine and EGCG3"Me.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06691-8.

Supplementary Material 1

Author contributions

Y.Z., M.G., W.Y., and N.Y. designed the research. L.L., S.G., Y.Z., Z.C., and J.Z. collected experimental samples. Y.Z. performed experiments and wrote the manuscript. S.W., W.G. analyzed the data. M.G., W.Y., S.W., and N.Y. provided valuable suggestions. W.Y., H.L., and N.Y. provided project funding. W.Y., N.Y. supervised and revised the manuscript. All authors reviewed and approved the manuscript.

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Data availability

Transcriptome data have been uploaded to the National Center for Biotechnology Information https://submit.ncbi.nlm.nih.gov/subs/sra/ via the SRA partner repository under the data set identifier PRJNA1179900.The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD062468 (https://www.ebi.ac.uk/pride/archive/projects/PXD062468/private).

Declarations

Ethics approval and consent to participate

All authors confirm that all the methods and experiments in this study, including the collection of plant material, complied with the institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Clinical trial number

Not applicable.

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