JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Bioactive Constituents, Metabolites, and Functions

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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.8b03433 • Publication Date (Web): 10 Oct 2018

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Hongyacha, a Naturally Caffeine-free Tea Plant from Fujian, China

Ji-Qiang Jin[†], Yun-Feng Chai[†], Yu-Fei Liu[†], Jing Zhang[†],

Ming-Zhe Yao[†], and Liang Chen[†*]

[†]Tea Research Institute of the Chinese Academy of Agricultural Sciences, Key Laboratory of Tea Plant Biology and Resources Utilization; Ministry of Agriculture and Rural Affairs, 9 South Meiling Road, Hangzhou, Zhejiang 310008, China

*Corresponding Author

tel: +86 571 86652835, fax: +86 571 86650056.

E-mail: liangchen@tricaas.com

ABSTRACT: Hongyacha (HYC) is a type of new wild tea plant discovered in Fujian Province, China. This tea is helpful to the healing or prevention of disease in its original growing area. However, research on this tea is limited. Our results showed that HYC displayed obvious differences in its morphological characteristics compared with Cocoa tea (*Camellia ptilophylla* Chang), a famous caffeine-free tea plant in China. Theobromine and *trans*-catechins, but not caffeine and *cis*-catechins, were the dominant purine alkaloids and catechins detected in HYC. HYC might contain abundant gallocatechin-(4→8)-gallocatechin gallate, 1,3,4,6-tetra-O-galloyl-β-D-glucopyranose, and (−)-gallocatechin-3,5-di-O-gallate, which were not detected in regular tea. We also found that the *TCS1* of HYC was distinct, and the responding recombinant protein exhibited only theobromine synthase activity. The obtained results showed that HYC is a new kind of caffeine-free tea plant and may be used for scientific protection and efficient utilization in the future.

KEYWORDS: caffeine-free, chemical component, morphological characteristic, Hongyacha, tea caffeine synthase

INTRODUCTION

Tea is beneficial to humans for its numerous secondary metabolites.[1] It is normally made from the young leaves of *Camellia sinensis* (L.) O. Kuntze, which belonging to the section *Thea* (L.) Dyer, genus *Camellia* L. of the family *Theaceae*.[2] The characteristic compounds in tea are theanine, purine alkaloids, polyphenols, and volatiles. Catechins contribute 80–260 mg/g of dry weight in young tea shoots and are the principal polyphenols.[3] The main catechins in regular tea are (+)-catechin (C), (−)-epicatechin (EC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin-3-gallate (EGCG), and (+)-gallocatechin (GC).[4] In regular tea, EGCG is the most plentiful catechin, and caffeine is the main purine alkaloid. While the chemical compounds of the young leaves of wild tea plants are diverse. For instance, in a famous caffeine-free/theobromine accumulation tea plant in China, cocoa tea (*Camellia ptilophylla* Chang, CCT) that originated from Guangdong Province, mainly contains theobromine and (−)-gallocatechin-3-gallate (GCG) but low levels of caffeine and EGCG.[5] The feature of polyphenolic composition in *Camellia taliensis* (W. W. Smith) Melchior is rich in 1,2-di-O-galloyl-4,6-O-(S)-hexahydroxydiphenoyl-β-D-glucose.[6] Last year (2017), procyanidin dimers and trimers were found in the tea plants of Puan tea[7] and *Camellia tachangensis* Chang,[8] respectively.

Tea germplasm resources are the fundamental and useful materials for tea breeding and potential strategic resources for the tea industry, indicating important significance for scientific research and product innovation. An example is the albino tea cultivar ‘Baiye 1’, which contains high content of amino acids; it was discovered in Zhejiang Province, China, which is the foundation of the local tea industry. This cultivar highlighted the

importance of discovering and developing novel tea germplasm resources. Cultivated tea plants are usually found and utilized by humans. In marginal and remote mountainous areas, some rare wild tea germplasms have not been discovered, which will provide valuable genetic materials for tea breeding and special use. Hongyacha (HYC) is a wild tea plant only distributed in the narrow mountain area at altitudes of 700−1000 m of several neighboring villages in the southern region of Fujian Province, China (Figure 1A). The young leaves of most individuals are purple or light purple (Figure 1B). Local people believe that drinking this tea can reduce internal heat, cure colds, and heal stomach pains, etc. Thus, HYC is considered a local treasure. However, given its narrow and special distribution, detailed information about HYC is lacking. The potential tea germplasms should be comprehensively understood so that they can be utilized effectively for breeding and production.

In this paper, the morphological characteristics of HYC were analyzed to understand its botany features. High-performance liquid chromatography (HPLC) was carried out to determine the chemical compositions in HYC, which was compared with *C. ptilophylla* (CCT) and *C. sinensis* var. *sinensis*. Ultra-HPLC (UHPLC)–mass spectrometry (MS) was conducted to infer the unknown compounds. Interestingly, HYC was found to be a new kind of caffeine-free plant. Tea caffeine synthase (TCS) is a most important enzyme in caffeine biosynthetic pathway.[9] Thus, *TCS1* of HYC was cloned, and recombinant enzyme activity of TCS1 was analyzed to dissect the caffeine-free/theobromine accumulation mechanism. This study provides information about the morphological characteristics, chemical compositions, and molecular mechanism of caffeine-free accumulation of HYC, which is conducive to the scientific protection and efficient

utilization of this rare wild tea germplasm.

MATERIALS AND METHODS

Plant Materials. HYC and CCT were introduced from their original growing regions and currently preserved as tea germplasms in our institute at Hangzhou, Zhejiang Province. *C. sinensis* var. *sinensis* 'Longjing 43' (LJ43) was cultivated by our institution in 1987. To determine the chemical compositions, "one and a bud" young shoots in spring (April) and fall (September) were harvested from these tea plants. Samples were fixed with hot air at 120 °C for several minutes and then dried at 75 °C. The samples were kept frozen (−20 °C) until determination. Fresh tea samples were stored at −80 °C for RNA and DNA extractions.

Investigation of Morphological Characteristics. The young shoots, leaves, flowers and fruits of HYC and CCT were described and measured according to the International Union for the Protection of New Varieties of Plants (UPOV) Distinctness, Uniformity and Stability Test Guidelines for tea plant (TG/238/1) prepared by our research group.[10] In April, the characteristics of young shoots were investigated, and the leaves, flowers and fruits were investigated in November.

Sample Preparation and HPLC and UHPLC–MS Conditions. Sample preparation and HPLC conditions were similar to the description in our previous paper.[4] UHPLC–MS experiment was carried out on an UltiMate 3000 system (ThermoFisher Scientific, Bremen, Germany) coupled with Q-Exactive orbitrap mass spectrometer (ThermoFisher Scientific, Bremen, Germany). More UHPLC–MS conditions were listed in Support Information.

Molecular Cloning of *TCS1* cDNA and Promoter. Full-length cDNA of *TCS1* was

cloned using primer sets TCS1cDNA-F: 5'-CACTGCTGTGGCAGCTGGC-3' and TCS1cDNA-R: 5'-CAACTTCTCATTTCTCCCAAC-3' as described previously.[11] Primer sets TCS1P-F: 5'-TTGGGCAAGTTCGAGATTGT-3' and TCS1P-R: 5'-TACTTTCTCCTTCTCCTCTGT-3' were used for the amplification of the promoter (from −757 bp to +67 bp). PCR were performed as follow conditions: 94 °C, 2 min; 35 cycles: 94 °C, 15 s; 53 °C, 25 s; 68 °C, 30 s; and final extension: 68 °C, 5 min. The target band was separated in 1.2% agarose and extracted using a Gel Extraction Kit. The gene was cloned into vector and sequenced.

Activity of Recombinant Enzyme TCS1. Vector construction, production of recombinant enzymes and detection of enzymatic activities were conducted as previous research.[11]

RESULTS

Morphological Characteristics. The plant type of HYC was arbor, and its growth habit was semi-upright (Figure 1A). The date of "one and a bud" was in early April in its original growing area. Young leaf was purple or light purple (Figure 1B), and bud pubescence was sparse (Figure 2A). Leaf length ranged from 9.1 cm to 20.5 cm, and leaf width varied from 2.7 cm to 6.5 cm. Leaf shape was very narrow elliptic or narrow elliptic (Figure 1B). The number of vein pairs was 7–11. Leaves were green or dark green (Figure 1B). The leaf cross section was slightly folded upwards or flat (Figure 1B). The leaf upper surface was smooth or weakly rugose, and leaf texture was hard. Leaf base shape was acute or obtuse, and leaf apex shape was acute or acuminate. Depth of leaf serration was weak, and leaf margin undulation was absent or weak (Figure 1B). Time of full blooming was in early November. Length of pedicels varied from 0.3 cm to 0.9 cm.

Number of sepals was five, and pubescence on the outer side of sepal was absent (Figure 2D). Flower was small and diameter ranged from 1.5 cm to 2.9 cm (Figure 1C). Flowers of HYC had five or six petals, and the inner petals were greenish (Figure 1C). Ovary pubescence was present (Figure 2D). Length of the style varied from 0.5 cm to 1.0 cm. Number of style splitting was three, and the position of style splitting was very high (almost not splitting, Figure 2D). Fruits appeared globular, kidney-shaped or triangular. The thickness of the pericarp was 6−9 mm (Figure 1D), and seeds appeared round.

HYC displayed obvious differences in the morphological characteristics compared with CCT (Figure 2 and Table 1). For CCT, the bud was covered with dense pubescence, and pubescence on the outer side of the sepal was present and dense. Moreover, CCT presented medium leaf margin undulation and larger flower (diameter ranged from 2.8 cm to 3.7 cm). The position of style splitting in CCT was lower than that in HYC.

Chemical Compositions. The purine alkaloids and catechins were determined using an external reference method under a given HPLC condition (Figure 3). Table 2 shows the spring (April) and fall (September) specific contents of nine compounds in three different originating tea plants. For purine alkaloids, caffeine was plentiful in LJ43 at 32.67 (spring) and 27.03 (fall) mg/g dry weight, whereas only a small amount of theobromine was found in LJ43. HYC and CCT contained high levels of theobromine (more than 40 mg/g in two seasons), whereas caffeine was not detected. For tea polyphenols, EGCG was the most abundant catechin, ECG, EGC, EC were next in abundance, and little amounts of C, GC, and GCG were found in LJ43. By contrast, HYC and CCT contained much more *trans*-catechins (C, GC, and GCG) and less *cis*-catechins (EC, ECG, EGC, and EGCG) than LJ43. In HYC and CCT, GCG was the predominant

catechin in two seasons. Furthermore, three unique peaks (with the retention times of 18.24, 23.18, and 24.26 min) in HYC and CCT were found. These results showed that HYC and CCT contained divergent chemical compositions compared with LJ43, whereas the chemical profile of HYC was similar to CCT.

The high resolution mass spectrometry and tandem mass spectrometry were used to tentatively characterize the compounds 1-3 (Figure 4). Compounds 1, 2 and 3 showed $[M-H]^-$ parent ions at *m/z* at 761.1362, 787.1002 and 609.0892, respectively. The molecular weights, retention times and MS/MS mass spectra of compounds 1, 2 and 3 in HYC were same to the compounds of gallocatechin-(4→8)-gallocatechin gallate (GC-(4→8)-GCG), 1,3,4,6-tetra-O-galloyl-β-D-glucopyranose (1,3,4,6-GA-glc) and (−)-gallocatechin-3,5-di-O-gallate (GC-3,5-diGA) in CCT reported previously.[12,13] Their fragmentation pathways were showed in Figure 5. In the fragmentation of compound 1, *m/z* 609 was generated by neutral loss of dehydrogenated gallic aldehyde (152 Da) from the precursor ion at *m/z* 761. Fragment ion at *m/z* 305 (deprotonated GC) was achieved with the cleavage of the C-C (4→8) bond in *m/z* 609 or *m/z* 761. Fragment ion at *m/z* 465 was formed with the consecutive loss of 1,2,3-trihydroxybenzene (126 Da) and gallic acid (170 Da) from the precursor ion at *m/z* 761. Other fragment ions are derived from the further fragmentation of the fragment ions generated in MS/MS. By analysis of its fragmentation patterns and with reference to the published mass spectra of procyanidin in CCT,[12] compound 1 could be inferred to be GC- (4→8)-GCG. In the fragmentation of compound 2, *m/z* 617 was generated by neutral loss of gallic acid (170 Da) from the precursor ion at *m/z* 787. Further losses of dehydrogenated gallic aldehyde (152 Da) or dehydrogenated gallic acid (168 Da) from *m/z* 617 produced fragment ions *m/z* 465 and

m/z 449, respectively. Neutral loss of gallic acid (170 Da) from the fragment ion *m/z* 465 produced the ion at *m/z* 295. The observation of consecutive gallic acid residue neutral losses and deprotonated gallic acid (*m/z* 169) indicated that the compound 2 could be inferred to 1,3,4,6-GA-glc. In the fragmentation of compound 3, neutral losses of dehydrogenated gallic aldehyde (152 Da) and gallic acid (170 Da) from the precursor ion *m/z* 617 produced fragment ions *m/z* 457 and *m/z* 439, respectively. The further fragmentation of these two ions produced other fragment ions. Fragment ions at *m/z* 331 and *m/z* 305 (deprotonated GC) were generated by losses of 1,2,3-trihydroxybenzene(126 Da) and dehydrogenated gallic aldehyde (152 Da) from *m/z* 457, respectively. Fragment ions at *m/z* 287 and *m/z* 269 were generated by losses of dehydrogenated gallic aldehyde (152 Da) and gallic acid (170 Da) from *m/z* 439, respectively. By analysis of its fragmentation patterns, compound 3 could be inferred to be GC-3,5-diGA.

Molecular Characterization of Caffeine-free Accumulation. To clarify the molecular mechanism of caffeine-free accumulation in HYC, *TCS1* full-length cDNA were cloned from HYC and CCT. The ORFs were 1,098 bp in length, and they encode 365 amino acids. Only two amino acids (Glu227Lys and Arg287His) were not the same between HYC and CCT (Figure 6). For TCS1a cloned from *C. sinesis*, such as LJ43, ORF was 1,110 bp in length, and it encoded 369 amino acids. All TCS1s contained the conserved domains A, B', C, and YFFF.[14] Comparing with TCS1a, the TCS1s of HYC and CCT all had the Arg221His change; this acid residue had critical role for substrate recognition in tea plant.[14,15] To identify the allelic variations in the promoter region of HYC and CCT, using the primer sets TCS1P-F and TCS1P-R, a set of 914 bp and 1032 and 734 bp fragments with 90−208 bp insertions/deletions (InDels) and initiation codon

(ATG) mutations compared with *TCS1a* (824 bp) were amplified from HYC and CCT, respectively (Figure 7). Although high similarity in the *TCS1* cDNA sequence was observed between HYC and CCT, the promoter sequence significantly differed. For recombinant enzyme activity of TCS1, HYC and CCT showed only TS activities, and the TS activities were lower than TCS1a (Table 3).

DISCUSSION

In HYC growing areas, the natural teas from the young shoots of HYC are used to boost the health of humans and heal or prevent illness. To date, information about the chemical compositions of HYC is scant. In this study, we tentatively characterized the chemical components of HYC by using HPLC and UPLC–MS. Interestingly, HYC has a distinctly chemical profile compared with regular tea. In regular tea, the main purine alkaloids and catechins are caffeine and *cis*-catechins; by contrast, HYC predominantly contains *trans*-catechins, theobromine, and undetectable caffeine (Table 2). We also found some rare compounds in HYC, such as GC-3,5-diGA, GC-(4→8)-GCG, and 1,3,4,6-GA-glc (Figure 3B). These three compounds are not discovered in young shoots of *C. sinensis* but rich in HYC. GCG, the epimer of EGCG, plays a minor role in regular tea for its low content. In HYC, GCG is the most abundant catechin. Previous studies have found that GCG shows various biological activities including antibacterial[16] and cholesterol- and triglyceride-lowering activity.[17] Moreover, GC-(4→8)-GCG is a potential compound of antiangiogenic agent.[12] CCT extract demonstrates hypolipemic activity,[13] and inhibitions of hepatic steatosis and high fat diet-induced obesity.[18] Moreover, CCT extract exhibits chemotherapeutic activities on human liver cancer and prostate cancer.[19,20] The chemical profile of HYC is similar to that of CCT (Figure 3).

Thus, HYC tea is a potential beverage that is beneficial to one's health.

Caffeine is a main purine alkaloid and central nervous system stimulant in regular tea. However, high consumption of tea can cause harmful effects related to high caffeine intake among sensitive people, such as insomnia, anxiety,[21] reduction in bone mass,[22,23] and increased occurring rate of abortion during pregnancy.[24] A mean daily caffeine consumption suggested for children younger than 18 years of age and adult consumers is 1 and 4 mg/kg body weight, respectively.[25] Supercritical carbon dioxide extraction and hot water treatment have been utilized for decaffeination of tea.[26] However, industrial decaffeination process can decrease bioactivities and affect the flavor of tea. High-quality cultivars containing low caffeine content may supply a better alternative for tea lovers. Thus, HYC is a naturally decaffeinated tea that may become a popular drink.

We have found two low caffeine-accumulating molecular mechanisms in tea germplasms, i.e., *TCS1* encoded protein with only TS activity or *TCS1* with low expression level.[11] To survey the molecular characteristic underlying caffeine-free/theobromine accumulation in HYC, the *TCS1* in HYC was isolated, and the responding recombinant protein exhibited only TS activity, which was similar to the *TCS1* in CCT. In our previous study, diverse *TCS1* allelic variations have been detected among section *Thea* plants.[11] In the present study, the *TCS1* promoter sequence of HYC significantly differed compared with *TCS1a* and CCT (Figure 7). Our results showed that HYC exhibited a distinct *TCS1* allele with very low caffeine biosynthetic activity and pyramiding beneficial *TCS1* allele of HYC could improve the breeding of low caffeine cultivars.

HYC, a rare wild tea plant, is only distributed in the narrow mountain area of several

villages in Fujian Province. Although HYC has a similar chemical profile to CCT, the morphological characteristics (Figure 2 and Table 1) and sequence of *TCS1* promoter (Figure 7) of HYC and CCT clearly differed. The obtained results revealed that HYC was a new kind of caffeine-free tea germplasm with distinct constituents and special health properties. Given the increased interest in growing cultivated tea plant and lack of protection awareness among people in the growing areas of HYC, many wild tea plants are being eliminated and endangered. Nowadays, high trees, such as the tea plant in Figure 1A, are few. An effective protection and management plan is needed for understanding and utilizing these tea resources. Our results are useful for understanding of the morphological characteristics, chemical profile, and molecular mechanism of caffeine-free accumulation of HYC. As a newly and naturally decaffeinated tea plant found in China, HYC is gaining increasing attention and usage by the local government and businesses due to its distinct constituents and unique health benefits. Our work is helpful for the scientific protection and efficient utilization of this rare germplasm resource.

ABBREVIATIONS USED:

1,3,4,6-GA-glc, 1,3,4,6-tetra-O-galloyl-β-D-glucopyranose; C, (+)-catechins; CCT, Cocoa tea; EC, (−)-epicatechin; ECG, (−)-epicatechin-3-gallate, EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin-3-gallate, GC, (+)-gallocatechin; GC-3,5-diGA, (−)-gallocatechin-3,5-di-O-gallate; GC-(4→8)-GCG, gallocatechin-(4→8)-gallocatechin gallat; GCG, (–)-gallocatechin-3-gallate; HYC, Hongyacha; TCS, Tea caffeine synthase; TS, theobromine synthase.

Funding

247 This work was supported by the National Natural Science Foundation of China (No.
248 31670685), Earmarked Fund for China Agriculture Research System (CARS-19), the
249 Chinese Academy of Agricultural Sciences through the Agricultural Science and
250 Technology Innovation Program (CAASASTIP-2017-TRICAAS,
251 CAAS-XTCX2016016-5).

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337 **Figure captions**

338 Figure 1. Morphological characteristic of Hongyacha. A, plant type; B, leaves and young
339 shoots; C, flower; D, fruits and seeds.

340 Figure 2. Comparison of morphological characteristics between HYC (left) and CCT
341 (right). A, pubescence of bud; B, leaf margin undulation; C, flower diameter; D,
342 pubescence on outer side of sepal and position of style splitting.

343 Figure 3. HPLC chromatogram of catechins and purine alkaloids in three tea plants. A,
344 LJ43 (*C. sinensis* var. *sinensis)*; B, HYC; C, CCT (*C. ptilophylla*). Peak identification: C,
345 (+)-catechin; CAF, caffeine; EC, (–)-epicatechin; ECG, (–)-epicatechin-3-gallate; EGC,
346 (–)-epigallocatechin; EGCG, (–)-epigallocatechin-3-gallate; GC, (+)-gallocatechin; GCG,
347 (–)-gallocatechin-3-gallate; TB, theobromine. 1, 2, and 3 were three undetermined
348 compounds in HYC and CCT.

349 Figure 4. Parent ion and fragment ions of peaks 1–3 in negative ion mode.

350 Figure 5. Fragmentation pathways of compounds 1–3.

351 Figure 6. Comparison of *TCS1* amino acid sequences. The SAM-binding motifs (A, B',
352 and C) and "YFFF-region" conserved region are indicated by open boxes.[14] The amino
353 acid residue shown by a blue box has a critical role in substrate recognition.[14,15] The
354 different amino acids between HYC and CCT are indicated by a red asterisk.

355 Figure 7. Comparisons of *TCS1* allelic variation. The initiation codon (ATG) mutations
356 are shown by open boxes.

357

Tables

Table 1. Main specificities of the morphological characteristics between Hongyacha and Cocoa tea.

Morphological characteristics	Hongyacha (HYC)	Cocoa tea (CCT)
Young shoot: density pubescence of bud	sparse	dense
Leaf blade: undulation of margin	absent or weak	medium
Flower: diameter	small	medium
Flower: pubescence on outer side of sepal	absent	present
Flower: position of style splitting	high (almost not splitting)	high

Table 2. Contents of purine alkaloids and catechins in three kinds of tea (mg/g)[a,b,c]

Season	Compound	Longjing 43 (LJ43)	Hongyacha (HYC)	Cocoa tea (CCT)
Spring	TB	2.32 ± 0.01	55.16 ± 0.81	55.77 ± 1.46
	GC	1.22 ± 0.04	20.25 ± 4.65	48.06 ± 8.07
	EGC	9.11 ± 0.16	0.59 ± 0.03	1.52 ± 0.77
	C	1.32 ± 0.03	23.01 ± 1.03	36.55 ± 1.02
	CAF	32.67 ± 0.35	ND	ND
	EC	7.42 ± 0.11	ND	ND
	EGCG	62.79 ± 1.38	8.40 ± 0.85	11.59 ± 0.15
	GCG	ND	102.03 ± 2.91	80.72 ± 2.37
	ECG	29.10 ± 0.38	1.69 ± 0.03	2.81 ± 0.17
Fall	TB	0.75±0.00	44.23±0.04	40.12±0.13
	GC	3.85±0.09	28.14±0.87	49.66±0.05
	EGC	21.88±0.13	8.80±0.02	6.74±0.11
	C	1.48±0.05	25.79±0.14	43.62±0.21
	CAF	27.03±0.35	ND	ND
	EC	9.06±0.09	1.61±0.16	3.91±0.28
	EGCG	60.72±0.18	5.19±0.15	7.94±0.13
	GCG	ND	108.58±0.90	59.95±0.10
	ECG	15.61±0.05	1.86±0.05	3.83±0.45

[a]"one and a bud" young shoots were collected for making tea samples. [b]Data are mean ± SD (n = 3). [c]ND, not detected; C, (+)-catechin; CAF, caffeine; EC, (–)-epicatechin; ECG, (–)-epicatechin-3-gallate; EGC, (–)-epigallocatechin; EGCG, (–)-epigallocatechin-3-gallate; GC, (+)-gallocatechin; GCG, (–)-gallocatechin-3-gallate; TB, theobromine.

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369 **Table 3.** Activity and substrate specificity of three different TCS1 recombinant

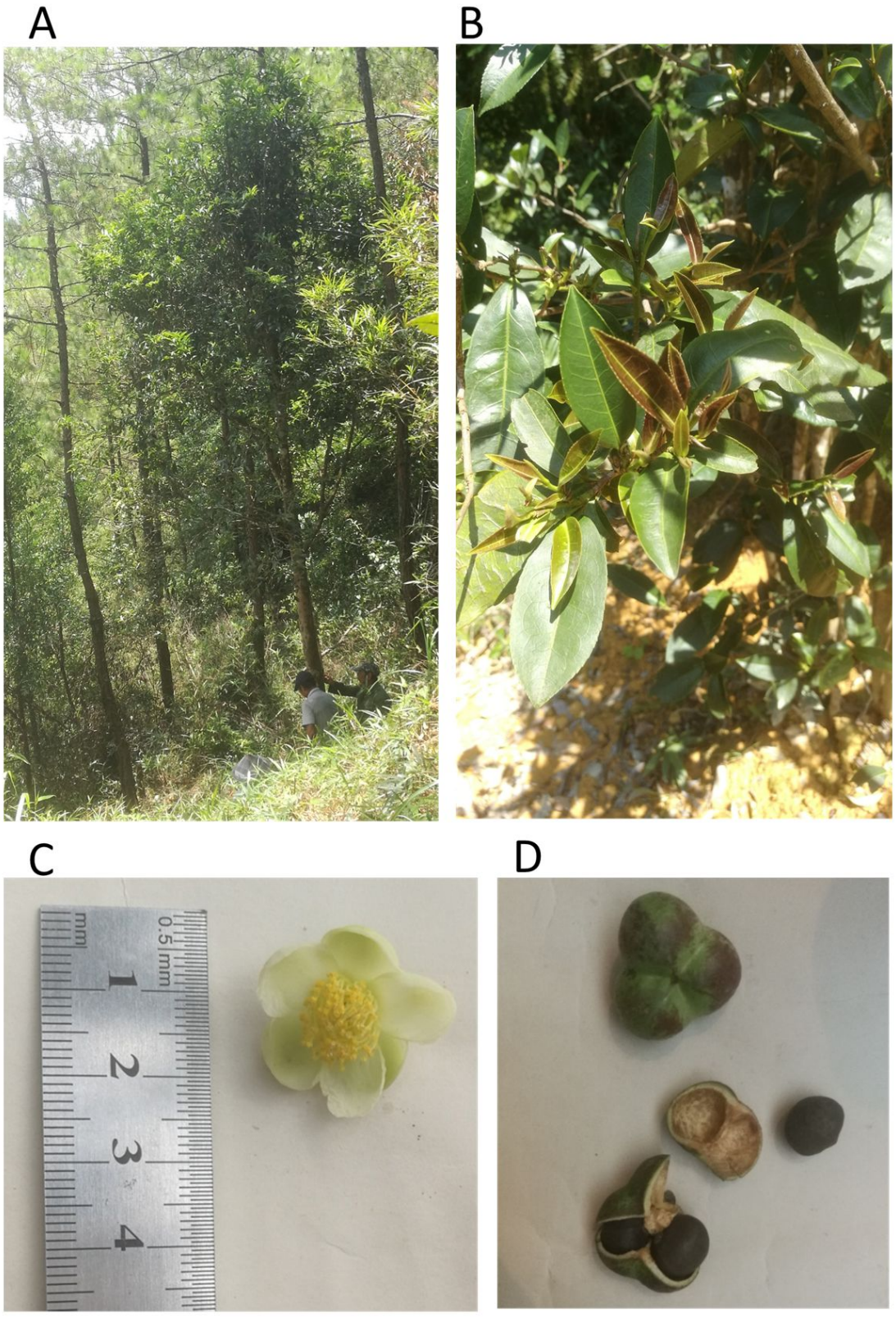
370 enzymes[a,b,c]

Recombinant enzyme	TS (pkat/mg)	CS (pkat/mg)	CS/TS（%）
TCS1a	111.9 ± 5.0	27.1 ± 0.1	24.2 ± 1.1
HYC	46.4 ± 3.8	ND	0
CCT	17.9 ± 0.9	ND	0
Methylated product	theobromine	caffeine	

371 [a]Data are mean ± SD (n = 3). [b]CCT, Cocoa tea; CS, caffeine synthase; HYC, Hongyacha;

372 ND, not detected; TS, theobromine synthase. [c]TCS1a was taken from reference.[11]

373 **Figure 1**



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376 **Figure 2**

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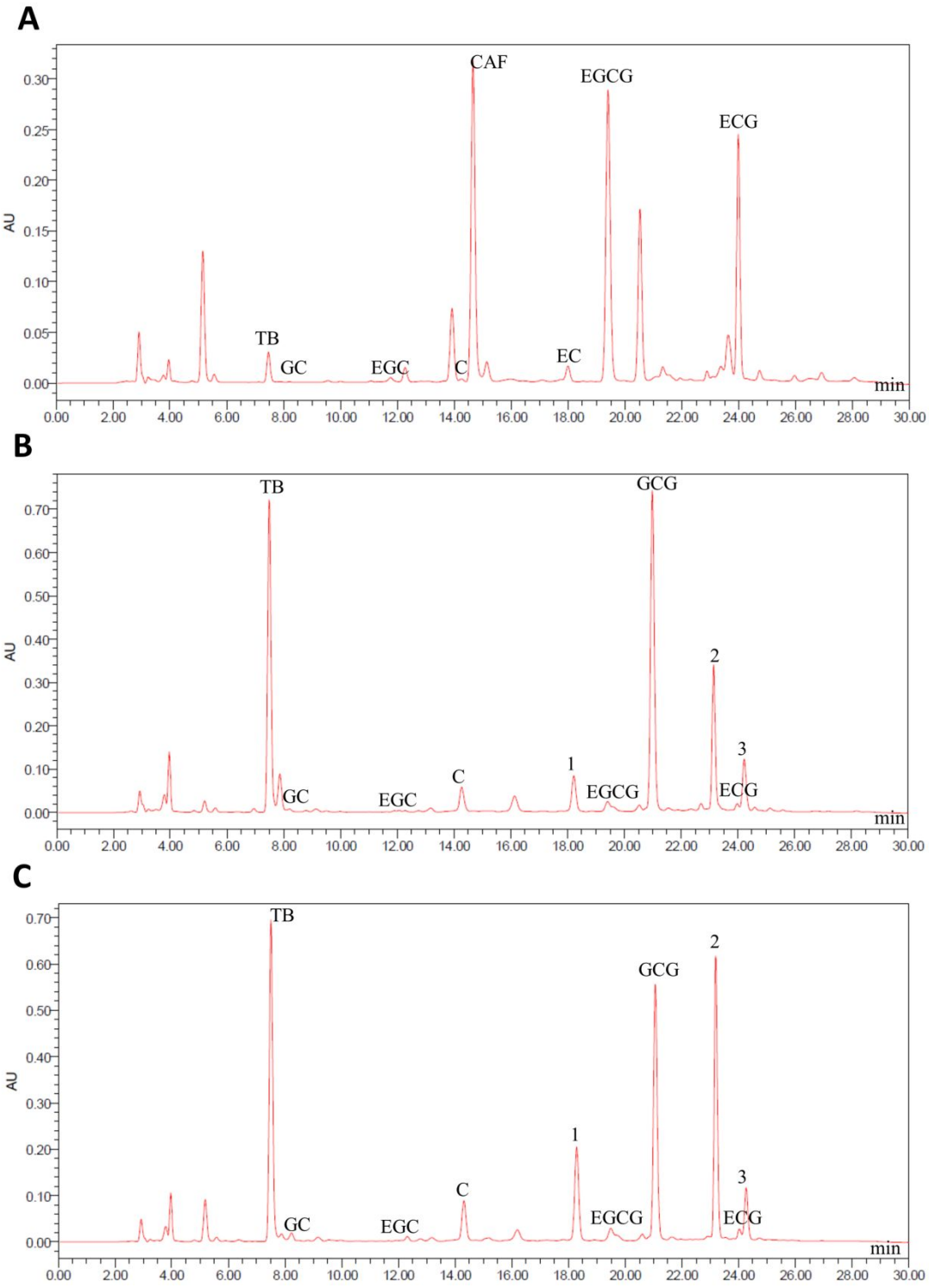
379 **Figure 3**



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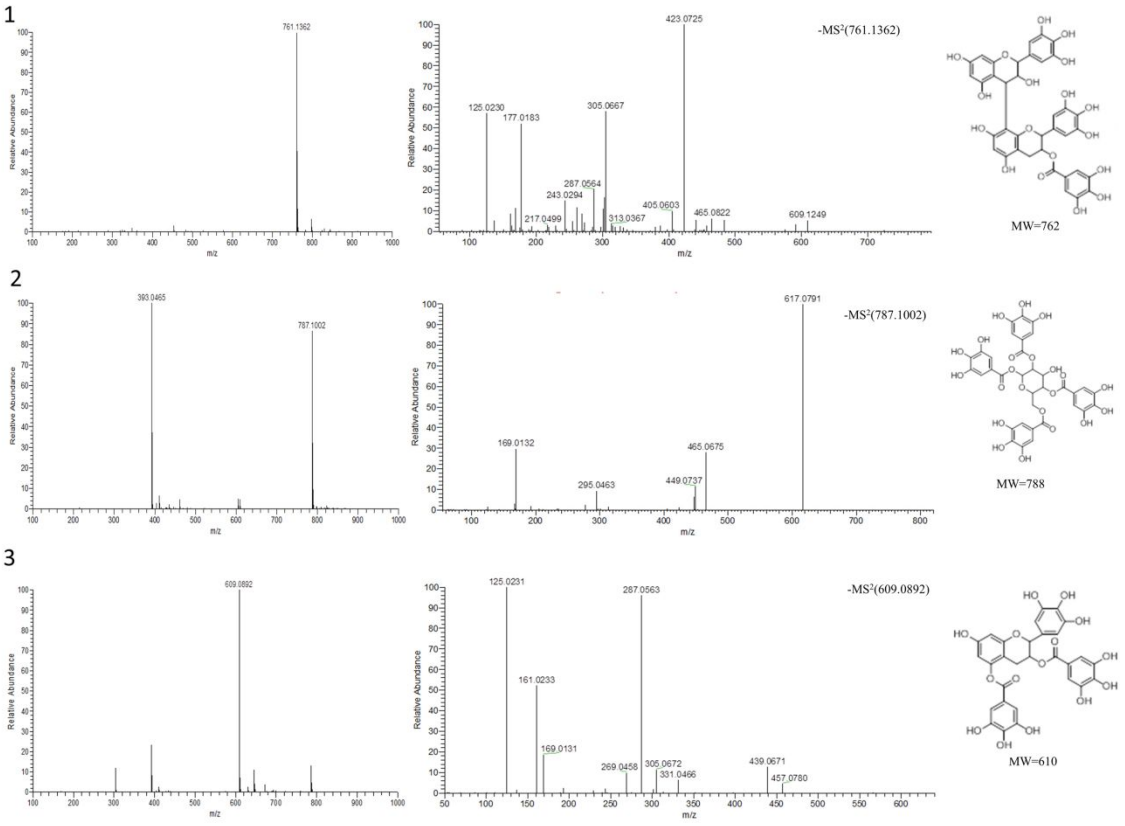
382 **Figure 4**



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385 **Figure 5**

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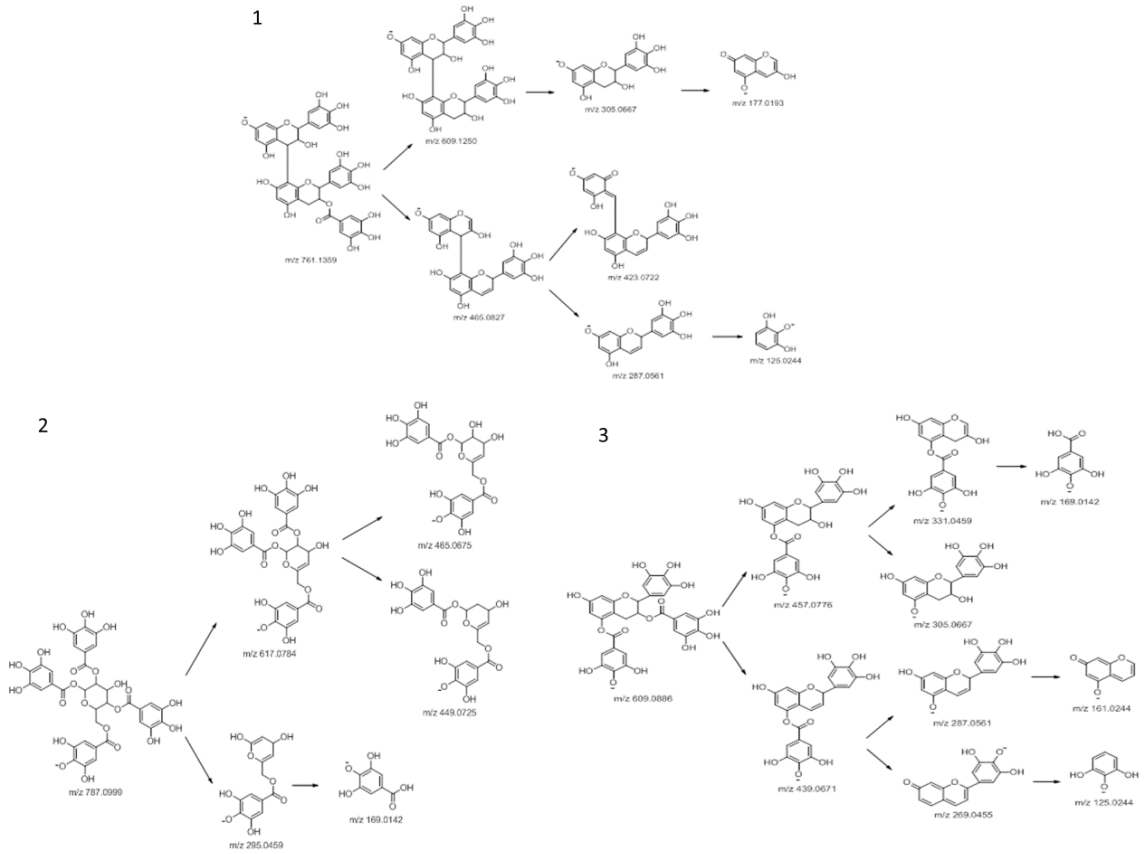

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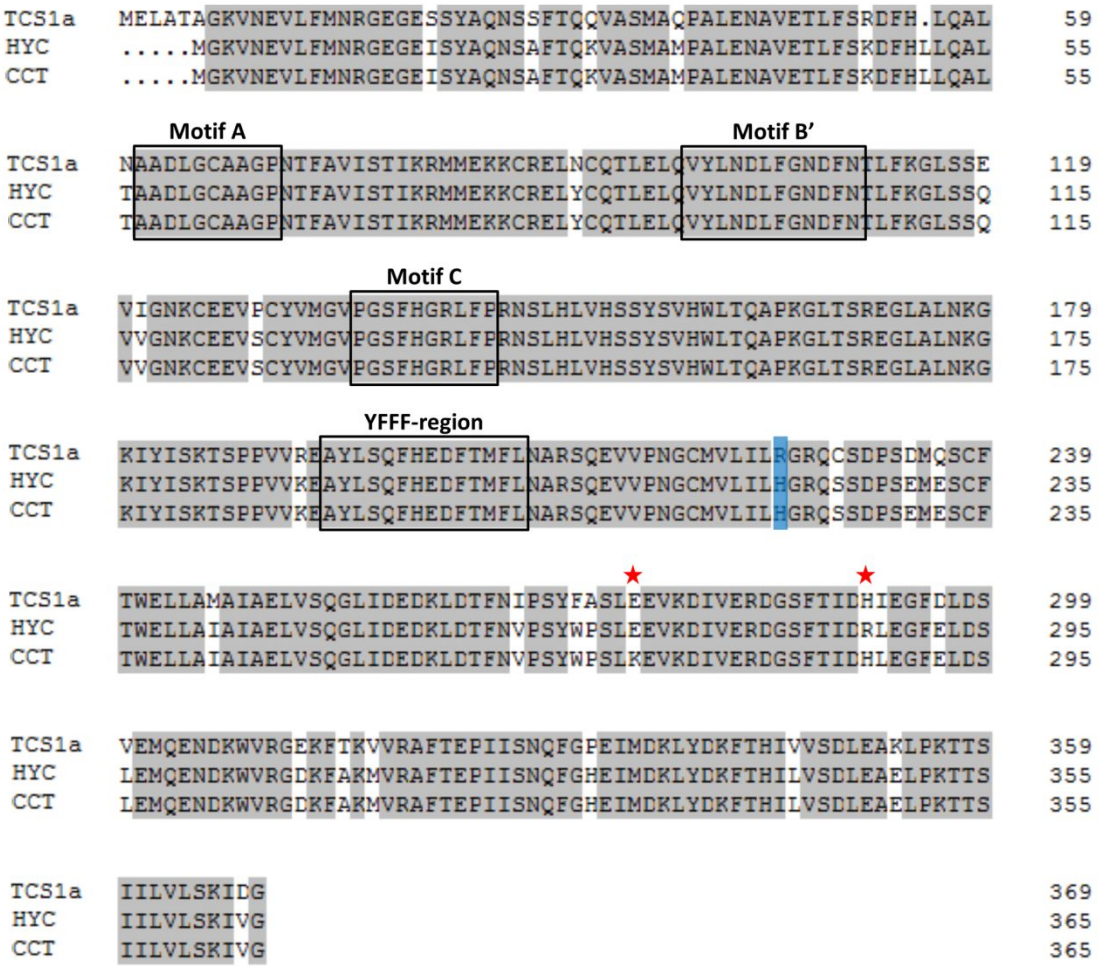
389 **Figure 6**



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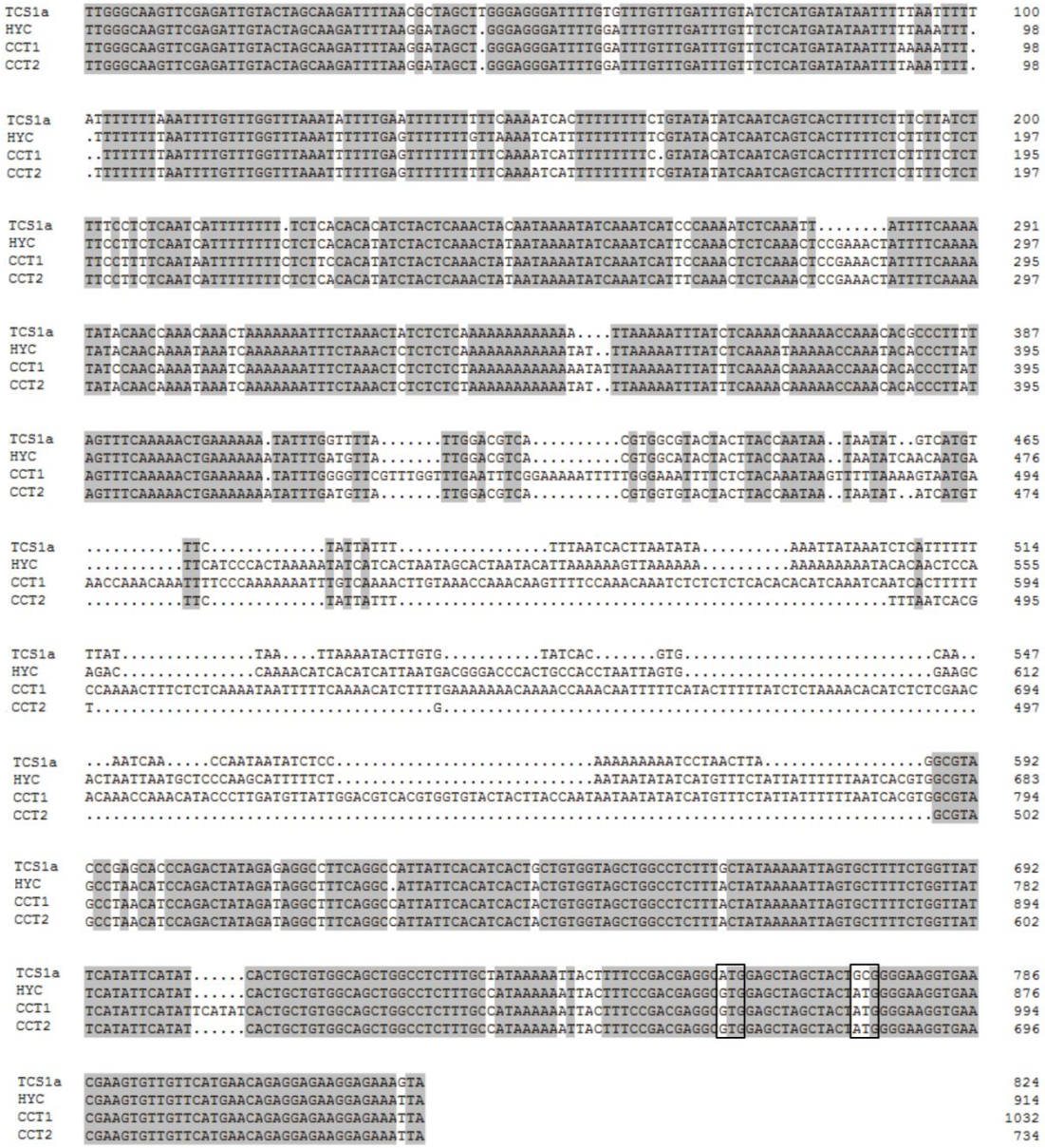
392 **Figure 7**



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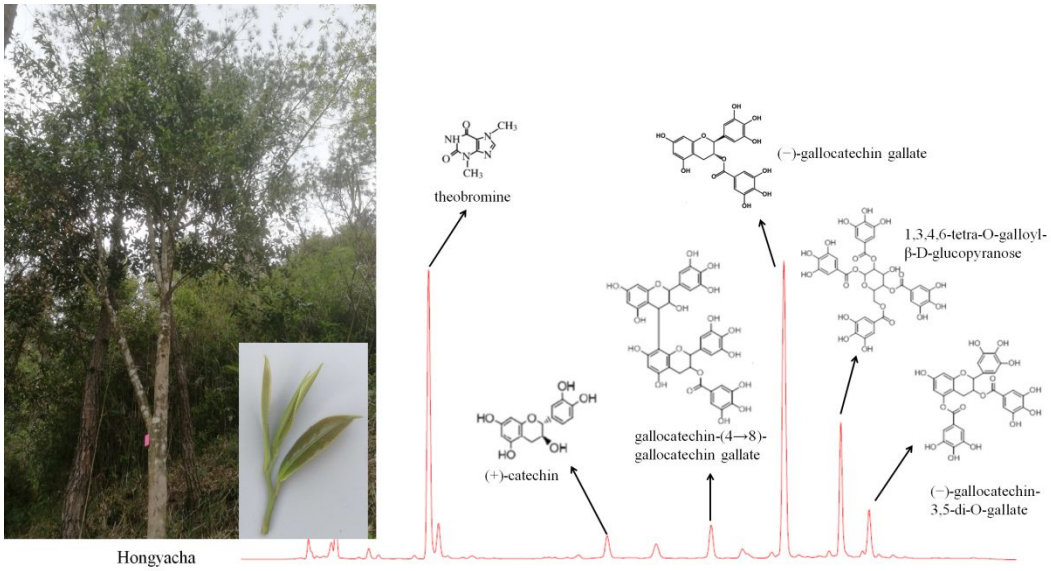
395 **TOC Graphic**



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