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

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

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

16

17 INTRODUCTION

18 Tea is beneficial to humans for its numerous secondary metabolites.¹ It is normally made
19 from the young leaves of *Camellia sinensis* (L.) O. Kuntze, which belonging to the
20 section *Thea* (L.) Dyer, genus *Camellia* L. of the family *Theaceae*.² The characteristic
21 compounds in tea are theanine, purine alkaloids, polyphenols, and volatiles. Catechins
22 contribute 80–260 mg/g of dry weight in young tea shoots and are the principal
23 polyphenols.³ The main catechins in regular tea are (+)-catechin (C), (–)-epicatechin
24 (EC), (–)-epicatechin-3-gallate (ECG), (–)-epigallocatechin (EGC),
25 (–)-epigallocatechin-3-gallate (EGCG), and (+)-gallocatechin (GC).⁴ In regular tea,
26 EGCG is the most plentiful catechin, and caffeine is the main purine alkaloid. While the
27 chemical compounds of the young leaves of wild tea plants are diverse. For instance, in a
28 famous caffeine-free/theobromine accumulation tea plant in China, cocoa tea (*Camellia*
29 *ptilophylla* Chang, CCT) that originated from Guangdong Province, mainly contains
30 theobromine and (–)-gallocatechin-3-gallate (GCG) but low levels of caffeine and
31 EGCG.⁵ The feature of polyphenolic composition in *Camellia taliensis* (W. W. Smith)
32 Melchior is rich in 1,2-di-O-galloyl-4,6-O-(S)-hexahydroxydiphenoyl-β-D-glucose.⁶ Last
33 year (2017), procyanidin dimers and trimers were found in the tea plants of Puan tea⁷ and
34 *Camellia tachangensis* Chang,⁸ respectively.

35 Tea germplasm resources are the fundamental and useful materials for tea breeding
36 and potential strategic resources for the tea industry, indicating important significance for
37 scientific research and product innovation. An example is the albino tea cultivar ‘Baiye
38 1’, which contains high content of amino acids; it was discovered in Zhejiang Province,
39 China, which is the foundation of the local tea industry. This cultivar highlighted the

40 importance of discovering and developing novel tea germplasm resources. Cultivated tea
41 plants are usually found and utilized by humans. In marginal and remote mountainous
42 areas, some rare wild tea germplasms have not been discovered, which will provide
43 valuable genetic materials for tea breeding and special use. Hongyacha (HYC) is a wild
44 tea plant only distributed in the narrow mountain area at altitudes of 700–1000 m of
45 several neighboring villages in the southern region of Fujian Province, China (Figure
46 1A). The young leaves of most individuals are purple or light purple (Figure 1B). Local
47 people believe that drinking this tea can reduce internal heat, cure colds, and heal
48 stomach pains, etc. Thus, HYC is considered a local treasure. However, given its narrow
49 and special distribution, detailed information about HYC is lacking. The potential tea
50 germplasms should be comprehensively understood so that they can be utilized
51 effectively for breeding and production.

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

63 utilization of this rare wild tea germplasm.

64 **MATERIALS AND METHODS**

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

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

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

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

86 cloned using primer sets TCS1cDNA-F: 5'-CACTGCTGTGGCAGCTGGC-3' and
87 TCS1cDNA-R: 5'-CAACTTCTCATTTCTCCCAAC-3' as described previously.¹¹ Primer
88 sets TCS1P-F: 5'-TTGGGCAAGTTCGAGATTGT-3' and TCS1P-R:
89 5'-TACTTTCTCCTTCTCCTCTGT-3' were used for the amplification of the promoter
90 (from -757 bp to +67 bp). PCR were performed as follow conditions: 94 °C, 2 min; 35
91 cycles: 94 °C, 15 s; 53 °C, 25 s; 68 °C, 30 s; and final extension: 68 °C, 5 min. The target
92 band was separated in 1.2% agarose and extracted using a Gel Extraction Kit. The gene
93 was cloned into vector and sequenced.

94 **Activity of Recombinant Enzyme TCS1.** Vector construction, production of
95 recombinant enzymes and detection of enzymatic activities were conducted as previous
96 research.¹¹

97 RESULTS

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

109 Number of sepals was five, and pubescence on the outer side of sepal was absent (Figure
110 2D). Flower was small and diameter ranged from 1.5 cm to 2.9 cm (Figure 1C). Flowers
111 of HYC had five or six petals, and the inner petals were greenish (Figure 1C). Ovary
112 pubescence was present (Figure 2D). Length of the style varied from 0.5 cm to 1.0 cm.
113 Number of style splitting was three, and the position of style splitting was very high
114 (almost not splitting, Figure 2D). Fruits appeared globular, kidney-shaped or triangular.
115 The thickness of the pericarp was 6–9 mm (Figure 1D), and seeds appeared round.

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

121 **Chemical Compositions.** The purine alkaloids and catechins were determined using
122 an external reference method under a given HPLC condition (Figure 3). Table 2 shows
123 the spring (April) and fall (September) specific contents of nine compounds in three
124 different originating tea plants. For purine alkaloids, caffeine was plentiful in LJ43 at
125 32.67 (spring) and 27.03 (fall) mg/g dry weight, whereas only a small amount of
126 theobromine was found in LJ43. HYC and CCT contained high levels of theobromine
127 (more than 40 mg/g in two seasons), whereas caffeine was not detected. For tea
128 polyphenols, EGCG was the most abundant catechin, ECG, EGC, EC were next in
129 abundance, and little amounts of C, GC, and GCG were found in LJ43. By contrast, HYC
130 and CCT contained much more *trans*-catechins (C, GC, and GCG) and less *cis*-catechins
131 (EC, ECG, EGC, and EGCG) than LJ43. In HYC and CCT, GCG was the predominant

132 catechin in two seasons. Furthermore, three unique peaks (with the retention times of
133 18.24, 23.18, and 24.26 min) in HYC and CCT were found. These results showed that
134 HYC and CCT contained divergent chemical compositions compared with LJ43, whereas
135 the chemical profile of HYC was similar to CCT.

136 The high resolution mass spectrometry and tandem mass spectrometry were used to
137 tentatively characterize the compounds 1-3 (Figure 4). Compounds 1, 2 and 3 showed
138 $[M-H]^-$ parent ions at m/z at 761.1362, 787.1002 and 609.0892, respectively. The
139 molecular weights, retention times and MS/MS mass spectra of compounds 1, 2 and 3 in
140 HYC were same to the compounds of gallic acid-(4→8)-gallic acid gallate
141 (GC-(4→8)-GCG), 1,3,4,6-tetra-O-galloyl- β -D-glucopyranose (1,3,4,6-GA-glc) and
142 (-)-gallic acid-3,5-di-O-gallate (GC-3,5-diGA) in CCT reported previously.^{12,13} Their
143 fragmentation pathways were showed in Figure 5. In the fragmentation of compound 1,
144 m/z 609 was generated by neutral loss of dehydrogenated gallic aldehyde (152 Da) from
145 the precursor ion at m/z 761. Fragment ion at m/z 305 (deprotonated GC) was achieved
146 with the cleavage of the C-C (4→8) bond in m/z 609 or m/z 761. Fragment ion at m/z 465
147 was formed with the consecutive loss of 1,2,3-trihydroxybenzene (126 Da) and gallic
148 acid (170 Da) from the precursor ion at m/z 761. Other fragment ions are derived from the
149 further fragmentation of the fragment ions generated in MS/MS. By analysis of its
150 fragmentation patterns and with reference to the published mass spectra of procyanidin in
151 CCT,¹² compound 1 could be inferred to be GC-(4→8)-GCG. In the fragmentation of
152 compound 2, m/z 617 was generated by neutral loss of gallic acid (170 Da) from the
153 precursor ion at m/z 787. Further losses of dehydrogenated gallic aldehyde (152 Da) or
154 dehydrogenated gallic acid (168 Da) from m/z 617 produced fragment ions m/z 465 and

155 m/z 449, respectively. Neutral loss of gallic acid (170 Da) from the fragment ion m/z 465
156 produced the ion at m/z 295. The observation of consecutive gallic acid residue neutral
157 losses and deprotonated gallic acid (m/z 169) indicated that the compound 2 could be
158 inferred to 1,3,4,6-GA-glc. In the fragmentation of compound 3, neutral losses of
159 dehydrogenated gallic aldehyde (152 Da) and gallic acid (170 Da) from the precursor ion
160 m/z 617 produced fragment ions m/z 457 and m/z 439, respectively. The further
161 fragmentation of these two ions produced other fragment ions. Fragment ions at m/z 331
162 and m/z 305 (deprotonated GC) were generated by losses of 1,2,3-trihydroxybenzene(126
163 Da) and dehydrogenated gallic aldehyde (152 Da) from m/z 457, respectively. Fragment
164 ions at m/z 287 and m/z 269 were generated by losses of dehydrogenated gallic aldehyde
165 (152 Da) and gallic acid (170 Da) from m/z 439, respectively. By analysis of its
166 fragmentation patterns, compound 3 could be inferred to be GC-3,5-diGA.

167 **Molecular Characterization of Caffeine-free Accumulation.** To clarify the
168 molecular mechanism of caffeine-free accumulation in HYC, *TCS1* full-length cDNA
169 were cloned from HYC and CCT. The ORFs were 1,098 bp in length, and they encode
170 365 amino acids. Only two amino acids (Glu227Lys and Arg287His) were not the same
171 between HYC and CCT (Figure 6). For TCS1a cloned from *C. sinensis*, such as LJ43,
172 ORF was 1,110 bp in length, and it encoded 369 amino acids. All TCS1s contained the
173 conserved domains A, B', C, and YFFF.¹⁴ Comparing with TCS1a, the TCS1s of HYC
174 and CCT all had the Arg221His change; this acid residue had critical role for substrate
175 recognition in tea plant.^{14,15} To identify the allelic variations in the promoter region of
176 HYC and CCT, using the primer sets TCS1P-F and TCS1P-R, a set of 914 bp and 1032
177 and 734 bp fragments with 90–208 bp insertions/deletions (InDels) and initiation codon

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

183 **DISCUSSION**

184 In HYC growing areas, the natural teas from the young shoots of HYC are used to
185 boost the health of humans and heal or prevent illness. To date, information about the
186 chemical compositions of HYC is scant. In this study, we tentatively characterized the
187 chemical components of HYC by using HPLC and UPLC–MS. Interestingly, HYC has a
188 distinctly chemical profile compared with regular tea. In regular tea, the main purine
189 alkaloids and catechins are caffeine and *cis*-catechins; by contrast, HYC predominantly
190 contains *trans*-catechins, theobromine, and undetectable caffeine (Table 2). We also
191 found some rare compounds in HYC, such as GC-3,5-diGA, GC-(4→8)-GCG, and
192 1,3,4,6-GA-glc (Figure 3B). These three compounds are not discovered in young shoots
193 of *C. sinensis* but rich in HYC. GCG, the epimer of EGCG, plays a minor role in regular
194 tea for its low content. In HYC, GCG is the most abundant catechin. Previous studies
195 have found that GCG shows various biological activities including antibacterial¹⁶ and
196 cholesterol- and triglyceride-lowering activity.¹⁷ Moreover, GC-(4→8)-GCG is a
197 potential compound of antiangiogenic agent.¹² CCT extract demonstrates hypolipemic
198 activity,¹³ and inhibitions of hepatic steatosis and high fat diet-induced obesity.¹⁸
199 Moreover, CCT extract exhibits chemotherapeutic activities on human liver cancer and
200 prostate cancer.^{19,20} The chemical profile of HYC is similar to that of CCT (Figure 3).

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

202 Caffeine is a main purine alkaloid and central nervous system stimulant in regular
203 tea. However, high consumption of tea can cause harmful effects related to high caffeine
204 intake among sensitive people, such as insomnia, anxiety,²¹ reduction in bone mass,^{22,23}
205 and increased occurring rate of abortion during pregnancy.²⁴ A mean daily caffeine
206 consumption suggested for children younger than 18 years of age and adult consumers is
207 1 and 4 mg/kg body weight, respectively.²⁵ Supercritical carbon dioxide extraction and
208 hot water treatment have been utilized for decaffeination of tea.²⁶ However, industrial
209 decaffeination process can decrease bioactivities and affect the flavor of tea. High-quality
210 cultivars containing low caffeine content may supply a better alternative for tea lovers.
211 Thus, HYC is a naturally decaffeinated tea that may become a popular drink.

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

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

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

239 **ABBREVIATIONS USED:**

240 1,3,4,6-GA-glc, 1,3,4,6-tetra-O-galloyl- β -D-glucopyranose; C, (+)-catechins; CCT,
241 Cocoa tea; EC, (-)-epicatechin; ECG, (-)-epicatechin-3-gallate, EGC,
242 (-)-epigallocatechin; EGCG, (-)-epigallocatechin-3-gallate, GC, (+)-gallocatechin;
243 GC-3,5-diGA, (-)-gallocatechin-3,5-di-O-gallate; GC-(4 \rightarrow 8)-GCG,
244 gallocatechin-(4 \rightarrow 8)-gallocatechin gallat; GCG, (-)-gallocatechin-3-gallate; HYC,
245 Hongyacha; TCS, Tea caffeine synthase; TS, theobromine synthase.

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335 caffeine-containing tea species. *Food Funct.* **2014**, *5*, 1175-1185.
- 336

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 *TCSI* 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 *TCSI* allelic variation. The initiation codon (ATG) mutations
356 are shown by open boxes.

357

Tables

358 **Table 1.** Main specificities of the morphological characteristics between Hongyacha and
359 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

360

361 **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

362 ^a“one and a bud” young shoots were collected for making tea samples. ^bData are mean ±

363 SD (n = 3). ^cND, not detected; C, (+)-catechin; CAF, caffeine; EC, (-)-epicatechin;

364 ECG, (-)-epicatechin-3-gallate; EGC, (-)-epigallocatechin; EGCG,

365 (-)-epigallocatechin-3-gallate; GC, (+)-gallocatechin; GCG, (-)-gallocatechin-3-gallate;

366 TB, theobromine.

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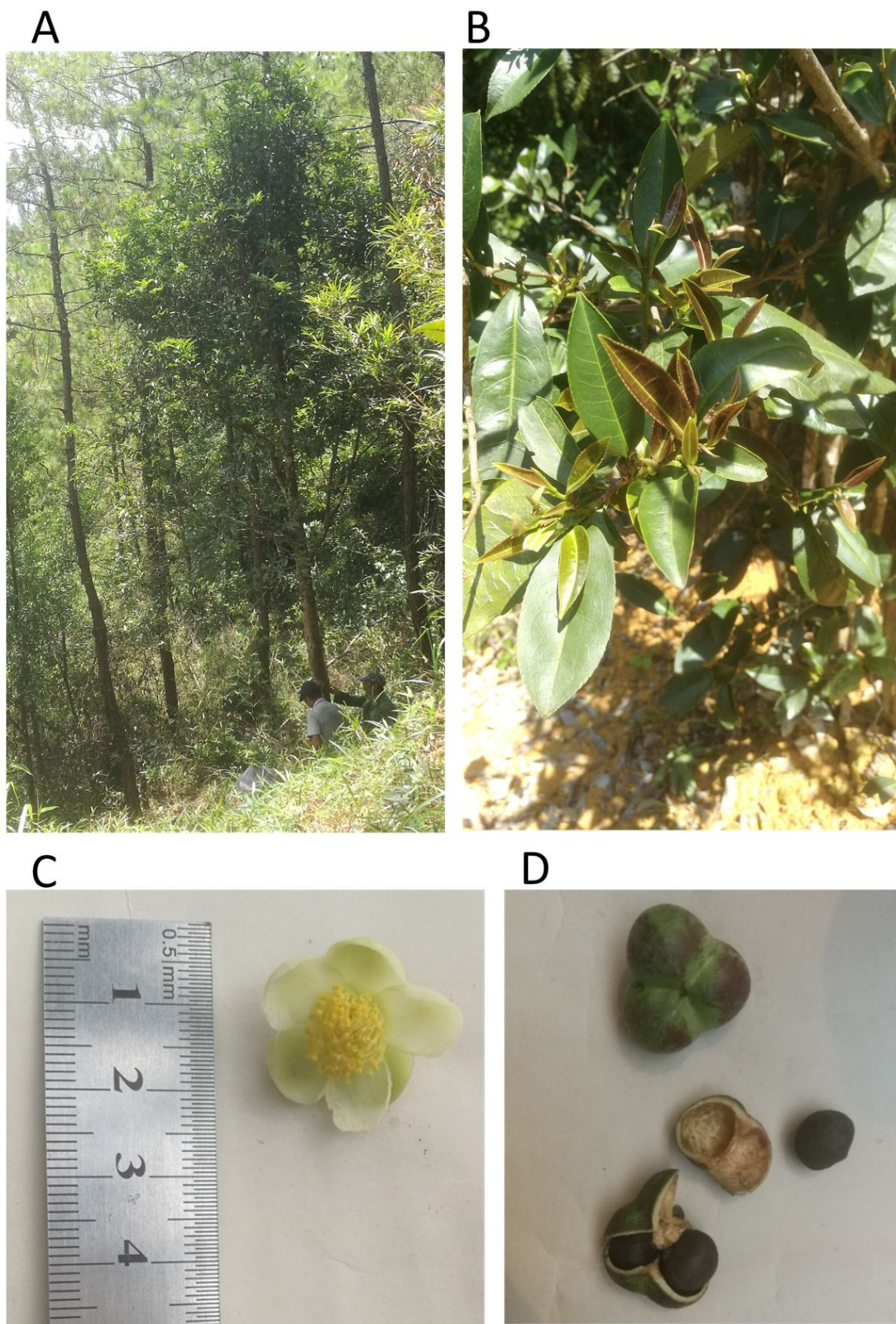
369 **Table 3.** Activity and substrate specificity of three different TCS1 recombinant
 370 enzymes^{a,b,c}

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 ^aData are mean ± SD (n = 3). ^bCCT, Cocoa tea; CS, caffeine synthase; HYC, Hongyacha;

372 ND, not detected; TS, theobromine synthase. ^cTCS1a was taken from reference.¹¹

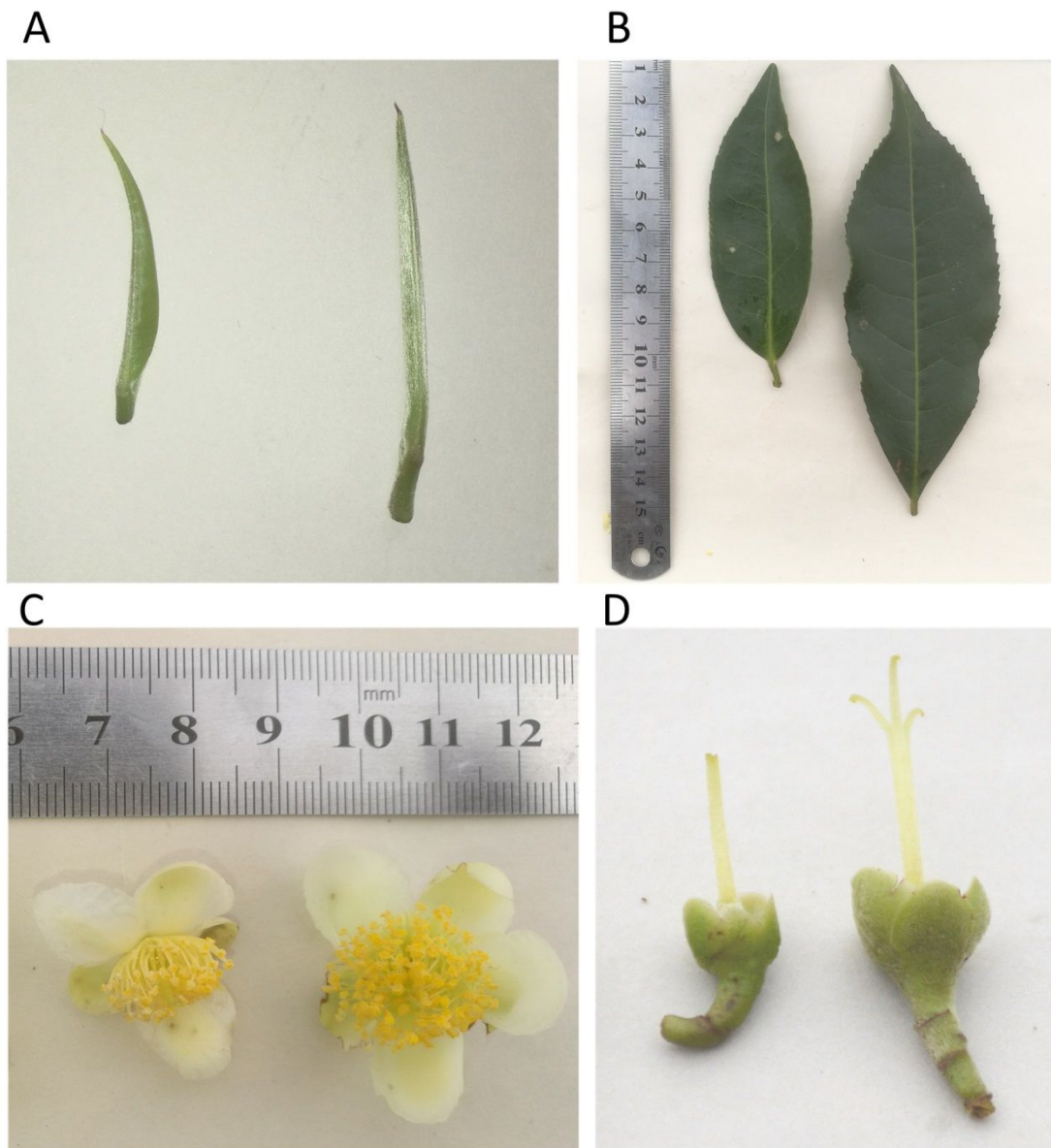
373 **Figure 1**



374

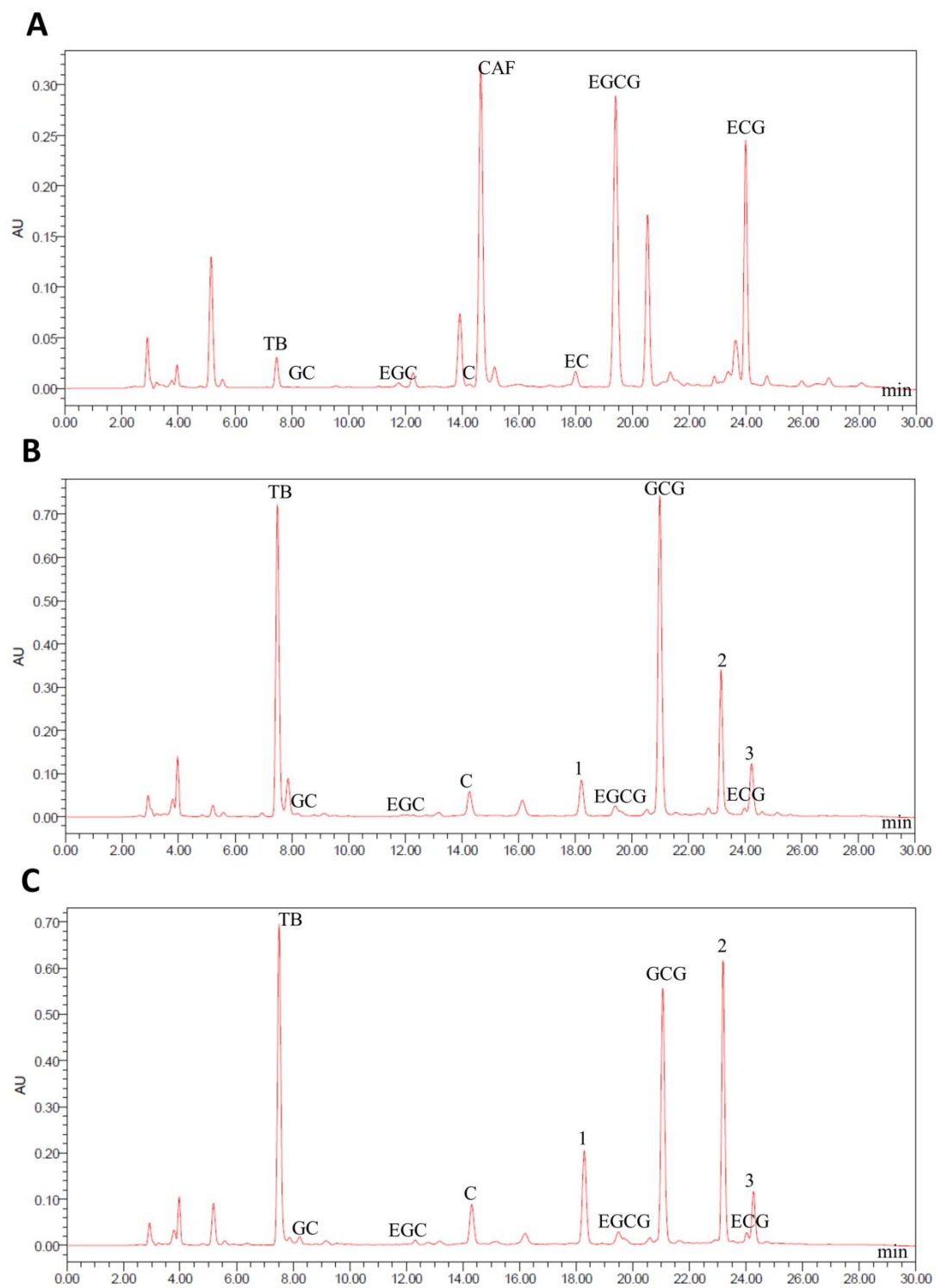
375

376 **Figure 2**



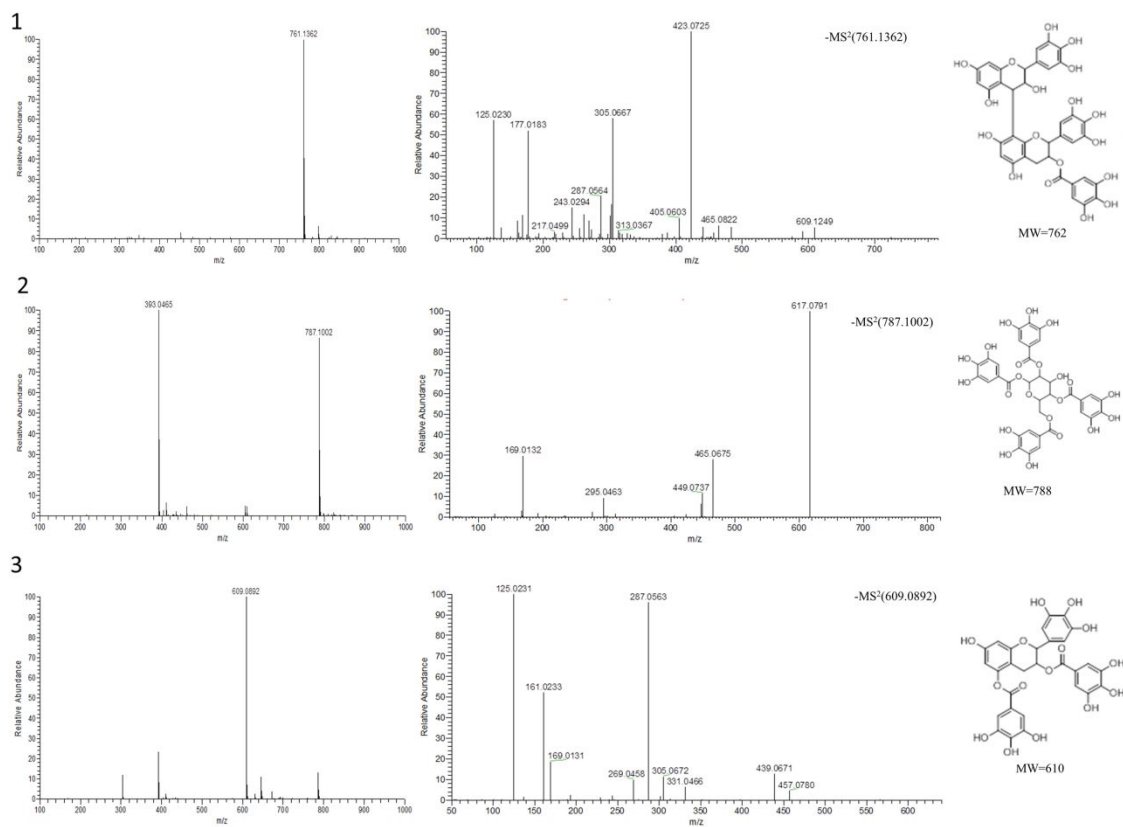
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379 **Figure 3**

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381

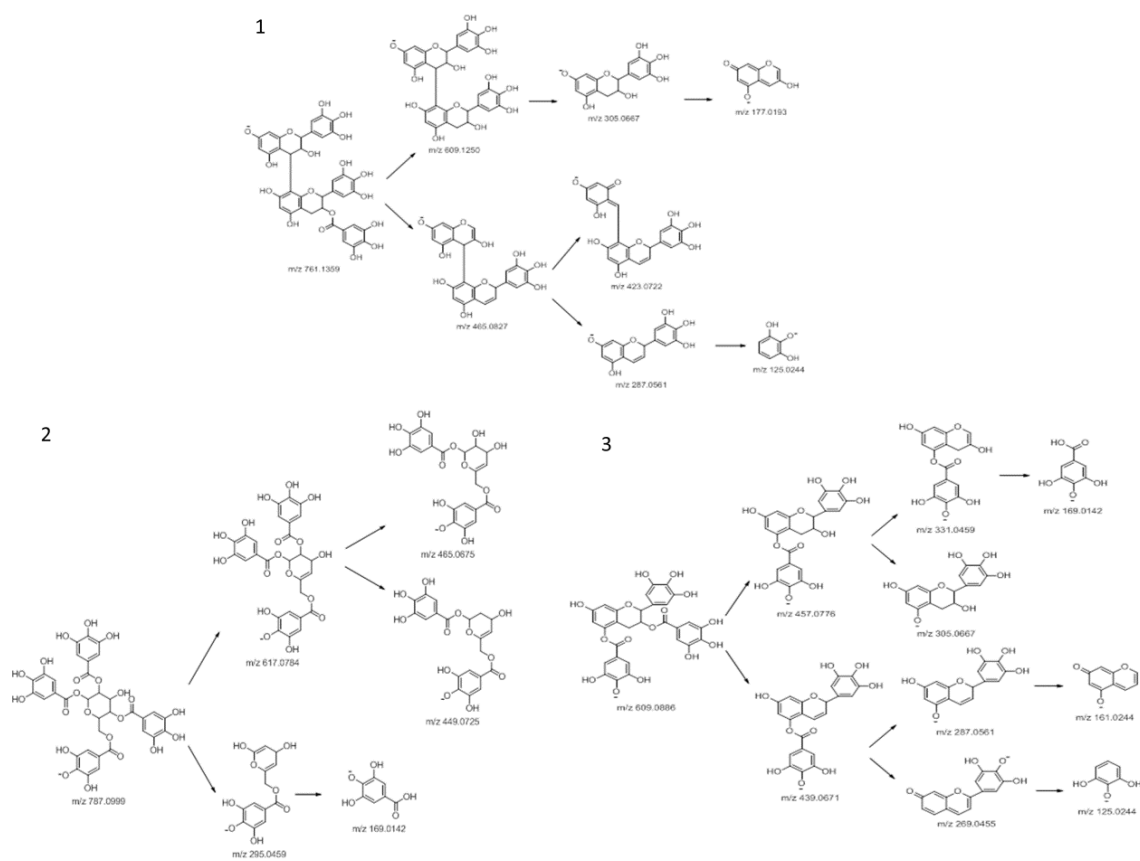
382 **Figure 4**

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384

385 **Figure 5**

386



387

388

389 **Figure 6**

TCS1a	MELATAGKVN EVLFMNRGEGESSYAQNS SFTQ QVASM AQ PALENA VETLFSRDFH.LQAL	59
HYCMGKVN EVLFMNRGEGEISYAQNS AFTQ KVASM AMPAL ENAVETLFSKDFHLL QAL	55
CCTMGKVN EVLFMNRGEGEISYAQNS AFTQ KVASM AMPAL ENAVETLFSKDFHLL QAL	55
Motif A		
TCS1a	NAADLGCAAGP NTFAVISTIKRM MEKKCRELNCQ TLELCVYLNDLFGNDFNTL FKGLSSE	119
HYC	TAADLGCAAGP NTFAVISTIKRM MEKKCRELYCQ TLELCVYLNDLFGNDFNTL FKGLSSQ	115
CCT	TAADLGCAAGP NTFAVISTIKRM MEKKCRELYCQ TLELCVYLNDLFGNDFNTL FKGLSSQ	115
Motif B'		
Motif C		
TCS1a	VIGNKCEEVPCYVMGV PGSFHGR LFF RNSLHLVHSSYSVHWLTQAPKGLTSREGLALNKG	179
HYC	VVGNKCEEVSCYVMGV PGSFHGR LFF RNSLHLVHSSYSVHWLTQAPKGLTSREGLALNKG	175
CCT	VVGNKCEEVSCYVMGV PGSFHGR LFF RNSLHLVHSSYSVHWLTQAPKGLTSREGLALNKG	175
YFF-region		
TCS1a	KIYISKTSPPVVR EAYLSQFHEDFTMFLNARSQEVVPNGCMVLIL GRQ CSDP SDMQSCF	239
HYC	KIYISKTSPPVVR EAYLSQFHEDFTMFLNARSQEVVPNGCMVLIL GRQ SSDP SEMESC	235
CCT	KIYISKTSPPVVR EAYLSQFHEDFTMFLNARSQEVVPNGCMVLIL GRQ SSDP SEMESC	235
★		
TCS1a	TWELLAMAI AELVSQGLIDEDKLD TFNIPSYFASIE EVK DIVERDGSFTIDHIEGF DLDS	299
HYC	TWELLAIAIA AELVSQGLIDEDKLD TFNVPSYWPSIE EVK DIVERDGSFTIDRLEGF FELDS	295
CCT	TWELLAIAIA AELVSQGLIDEDKLD TFNVPSYWPSI KEV KDIVERDGSFTIDHLEGF FELDS	295
★		
TCS1a	VEMQENDKWVRGEK FTKVVRAFTEPIISNQFGPEIMDKLYDKFTHIVVSDLEAKLPRTTS	359
HYC	LEMQENDKWVRGDK FAKMVRAFTEPIISNQFGHEIMDKLYDKFTHILVSDLEAELPRTTS	355
CCT	LEMQENDKWVRGDK FAKMVRAFTEPIISNQFGHEIMDKLYDKFTHILVSDLEAELPRTTS	355
TCS1a	IIIVLSKIDG	369
HYC	IIIVLSKIVG	365
CCT	IIIVLSKIVG	365

390

391

392 **Figure 7**

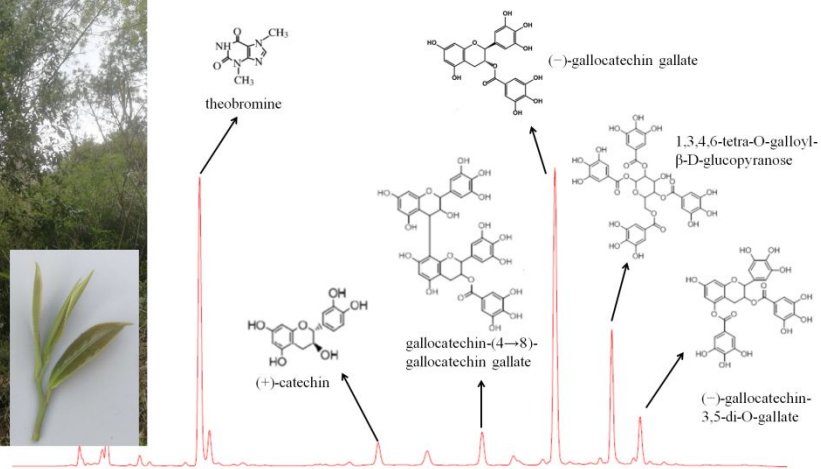
TCS1a	TTGGGCAAGTTCGAGATTGTACTAGCAAGATTTTAAACGCTAGCTGGGAGGGATTTTGGTTTGGTTGATTGGTATCTCATGATATAAATTTTAAATTTT	100
HYC	TTGGGCAAGTTCGAGATTGTACTAGCAAGATTTTAAAGGATAGCTGGGAGGGATTTTGGATTGGTTGATTGGTTCTCATGATATAAATTTTAAATTTT	98
CCT1	TTGGGCAAGTTCGAGATTGTACTAGCAAGATTTTAAAGGATAGCTGGGAGGGATTTTGGATTGGTTGATTGGTTCTCATGATATAAATTTTAAATTTT	98
CCT2	TTGGGCAAGTTCGAGATTGTACTAGCAAGATTTTAAAGGATAGCTGGGAGGGATTTTGGATTGGTTGATTGGTTCTCATGATATAAATTTTAAATTTT	98
TCS1a	ATTTTTTAAATTTTGGTTGGTTAAATATTTTGAATTTTTTTTTTCAAATCAGCTTTTTTTCGTATATATCAATCAGTCACCTTTTTCTTCTTATCT	200
HYC	.TTTTTTTTAAATTTTGGTTGGTTAAATTTTGAATTTTTTTTTTCAAATCAGCTTTTTTTCGTATATATCAATCAGTCACCTTTTTCTTCTTATCTCT	197
CCT1	.TTTTTTTTAAATTTTGGTTGGTTAAATTTTGAATTTTTTTTTTCAAATCAGCTTTTTTTCGTATATATCAATCAGTCACCTTTTTCTTCTTATCTCT	195
CCT2	.TTTTTTTTAAATTTTGGTTGGTTAAATTTTGAATTTTTTTTTTCAAATCAGCTTTTTTTCGTATATATCAATCAGTCACCTTTTTCTTCTTATCTCT	197
TCS1a	TTTCTCTCAATCATTTTTTTTCTCACACATCTACTCAAACTCAATAAAATATCAAATCATCCAAATCTCAAATTTTTTTTCAAAA	291
HYC	TTCTCTCAATCATTTTTTTTCTCACACATCTACTCAAACTCAATAAAATATCAAATCATCCAAATCTCAAATTTTTTTTCAAAA	297
CCT1	TTCTCTCAATCATTTTTTTTCTCTCCACATATCTACTCAAACTCAATAAAATATCAAATCATCCAAATCTCAAATTTTTTTTCAAAA	295
CCT2	TTCTCTCAATCATTTTTTTTCTCTCACACATATCTACTCAAACTCAATAAAATATCAAATCATCCAAATCTCAAATTTTTTTTCAAAA	297
TCS1a	TATACAACCAAACTAAAAAATTTCTAAACTCTCTCAAAAAAATATTTAAAAATTTATCTCAAAAACAAAACCAAAACAGCCCTTTT	387
HYC	TATACAACCAAACTAAAAAATTTCTAAACTCTCTCAAAAAAATATTTAAAAATTTATCTCAAAAACAAAACCAAAACAGCCCTTTT	395
CCT1	TATCCAACAAATTAATCAAAAAATTTCTAAACTCTCTCAAAAAAATATTTAAAAATTTATCTCAAAAACAAAACCAAAACAGCCCTTTT	395
CCT2	TATACAACCAAACTAAAAAATTTCTAAACTCTCTCAAAAAAATATTTAAAAATTTATCTCAAAAACAAAACCAAAACAGCCCTTTT	395
TCS1a	AGTTTCAAAAACGAAAAAAATTTTGGTTTAA.....TTGGACGTCA.....CGTGGCCTACTACTTACCAATAA..TAATAT..GTCATGT	465
HYC	AGTTTCAAAAACGAAAAAAATTTTGGTTTAA.....TTGGACGTCA.....CGTGGCCTACTACTTACCAATAA..TAATATCAACAATGA	476
CCT1	AGTTTCAAAAACGAAAAAAATTTTGGTTTAA.....TTGGACGTCA.....CGTGGCCTACTACTTACCAATAA..TAATATCAACAATGA	494
CCT2	AGTTTCAAAAACGAAAAAAATTTTGGTTTAA.....TTGGACGTCA.....CGTGGCCTACTACTTACCAATAA..TAATAT..ATCATGT	474
TCS1aTTT.....TATTTT.....TTTATCACTTAAATA.....AAATTTAATATCTCTTTTTT	514
HYCTTTCACTCCCAATAAATATCACTAATAGCACTAATACATTTAAAAAGTTAAAAA.....AAAAAATAACACACTCCA	555
CCT1	AACCAACAAATTTTCCCAAAAAATTTTGTCAAACCTGTAAACCAACCAAGTTTCCAAACAATCTCTCTCACACACATCAAATCAATCACTTTT	594
CCT2TTT.....TATTTT.....TTTATCACTTAAATA.....TTTATCACTG	495
TCS1a	TTAT.....TAA.....TTAAATACTTGTG.....TATCAC.....GTG.....CAA..	547
HYC	AGAC.....CAAACATCACATCAATTAATGACGGGACCCACTGCCACCTAATTAGTG.....GAAGC	612
CCT1	CCAAACTTTCTCTCAAAAATATTTTCAAACATCTTTTGAAAAAACAAAACCAACAATTTTTCATCTTTTATCTCAAACACATCTCTCGAAC	694
CCT2	T.....G.....	497
TCS1a	...AATCAA.....CCAATAATATCTCC.....AAAAAATACTTAACCTA.....GCGTA	592
HYC	ACTAATTAATGCTCCCAAGCATTTTTCTI.....AATAATATATCATGTTTCTAATTTTTTAAATCAGTGGCGTA	683
CCT1	ACAAACCAACATACCCCTTGATGTTTATGGAGCTCAGGTGGTACTACTTACCAATAAATAATATATCATGTTTCTAATTTTTTAAATCAGTGGCGTA	794
CCT2GCGTA	502
TCS1a	CCCGAGCCCCAGACTATAGAGGGCCCTCAGGCCATTATTCACATCACTGCTGTGGTAGCTGGCCCTTTGCTATAAAAAATTAGTGCTTTTCTGGTTAT	692
HYC	GCCTAACCTCCAGACTATAGATAGGCTTTTCAGGCATTATTCACATCACTACTGTGGTAGCTGGCCCTTTACTATAAAAAATTAGTGCTTTTCTGGTTAT	782
CCT1	GCCTAACCTCCAGACTATAGATAGGCTTTTCAGGCCATTATTCACATCACTACTGTGGTAGCTGGCCCTTTACTATAAAAAATTAGTGCTTTTCTGGTTAT	894
CCT2	GCCTAACCTCCAGACTATAGATAGGCTTTTCAGGCCATTATTCACATCACTACTGTGGTAGCTGGCCCTTTACTATAAAAAATTAGTGCTTTTCTGGTTAT	602
TCS1a	TCATATTCATAT.....CACTGCTGTGGCAGCTGGCCCTTTTGTCTATAAAAAATTACTTTTCCGACGAGGATGGAGCTAGCTACTGGGGAGGTGAA	786
HYC	TCATATTCATAT.....CACTGCTGTGGCAGCTGGCCCTTTTGTCTATAAAAAATTACTTTTCCGACGAGGATGGAGCTAGCTACTGGGGAGGTGAA	876
CCT1	TCATATTCATATTCATATCACTGCTGTGGCAGCTGGCCCTTTTGTCTATAAAAAATTACTTTTCCGACGAGGATGGAGCTAGCTACTGGGGAGGTGAA	994
CCT2	TCATATTCATAT.....CACTGCTGTGGCAGCTGGCCCTTTTGTCTATAAAAAATTACTTTTCCGACGAGGATGGAGCTAGCTACTGGGGAGGTGAA	696
TCS1a	CGAAGTGTGTTTCATGAACAGAGGAGAAGGAGAAAGTA	824
HYC	CGAAGTGTGTTTCATGAACAGAGGAGAAGGAGAAATTA	914
CCT1	CGAAGTGTGTTTCATGAACAGAGGAGAAGGAGAAATTA	1032
CCT2	CGAAGTGTGTTTCATGAACAGAGGAGAAGGAGAAATTA	734

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395 **TOC Graphic**

Hongyacha



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