Determination of the chemical compounds of *Shuchazao* tea flowers at different developmental stages and in young shoots using $^1$H NMR-based metabolomics

Hong Ye $^1$ · Jingwei Hu $^{2,3}$ · Su Peng $^2$ · Wenming Zong $^4$ · Shuang Zhang $^1$ · Lin Tong $^1$ · Chen Cao $^1$ · Zenghui Liu $^1$ · Zhongwen Xie $^2$

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Abstract
The chemical compounds in tea leaves have been extensively explored in recent decades. However, the compounds in tea flowers have not been fully investigated. In present study, the main chemical compounds in tea flowers were identified at four developmental stages using non-targeted metabonomics based on proton nuclear magnetic resonance ($^1$H NMR) and an automatic amino acid analyzer, and compared with those in young tea shoots. The results showed significant differences in catechins, sugars, organic acids and amino acids between tea flowers and young shoots. The concentrations of epigallocatechin gallate, epigallocatechin, epicatechin, and caffeine were significantly lower ($p < 0.01$) and sugar content significantly higher ($p < 0.01$) in flowers than in young shoots. Caffeine and $\beta$-glucose gradually decreased and sucrose constantly increased during flower development; $\alpha$-glucose and fructose were most concentrated in the white bud and then decreased with flower development. Tea flowers contained more succinic acid, citric acid, and chlorogenic acid but less quinic acid and malic acid than young shoots. Both tea flowers and young tea shoots contained 20 common amino acids, including 7 essential ones. The concentration of amino acids was highest in the white bud (27.66 mg/g); young tea shoots contained significantly more L-theanine than tea flowers ($p < 0.01$). Our data indicate that the different stages of tea flowers have a set of characteristic chemical compounds and are potentially useful for functional foods.

Graphical abstract

Keywords Amino acids · Antitumor agents · Biosynthesis · Chemical composition · Tea flower · Young tea shoot

Hong Ye and Jingwei Hu equally contributed to this work.

Zhongwen Xie
zhongwenxie@ahau.edu.cn

|$^1$ Anhui Academy of Medical Sciences, 15 Yonghong Road, Hefei 230061, Anhui, People’s Republic of China

|$^2$ State Key Laboratory of Tea Plant Biology and Utilization, Anhui Agricultural University, 130 West Changjiang Road, Hefei 230036, Anhui, People’s Republic of China

|$^3$ Center for Biotechnology, Anhui Agricultural University, 130 West Changjiang Road, Hefei 230036, Anhui, People’s Republic of China

|$^4$ School of Engineering, Anhui Agricultural University, 130 West Changjiang Road, Hefei 230036, Anhui, People’s Republic of China
Introduction

The tea plant \textit{Camellia sinensis} (L.) O. Ktze. originated in the Yunnan-Guizhou plateau in China. Tea is an important crop in more than 30 countries and is one of the most consumed healthy beverages in the world. However, flowering of tea plants, a stage in their reproductive growth, consumes a large amount of carbohydrates and nutrients, negatively affecting tea shoot germination in the following year [1]. Therefore, tea flowers have been abandoned or suppressed as undesirable byproducts for a long time. In recent years, tea flowers mainly in a full bloom stage have received increasing attention. Previous researches have focused on isolation, identification, and analysis of certain families of nutrients and metabolites, and characterizations of biological activities in tea flowers [2]. Compared to tea leaves, tea flowers contain large amounts of protein, polysaccharide, saponin, and comparable amounts of total catechins, but are low in caffeine [2–4]. These compounds have been proven to play key roles in terms of antioxidation, anti-inflammation, antitumor, anti-obesity, anti-allergic activities, immunostimulation, and hypoglycemic effect [5–11]. Notably, the composition and content of the main chemical constituents of tea flower were closely related to flowering stages, species, place of origin, growth environment, growth cycles of the tea plant and so on [12–16]. \textit{Shuchazao} (\textit{Camellia sinensis} var. \textit{sinensis}) is a national premier tea cultivar and is widely cultivated in Eastern China. The genome of \textit{Shuchazao} has already been reported [17]. However, the differential metabolites between young shoots and flowers at the complete developmental stages are not reported yet. The chemical profiles are distinct between tea flowers and leaves, as well as different organs of the tea plant. It was reported that the metabolites combined with mineral distributions in flowers and leaves collected from a single tea plant were changed in autumn [4]. Traditionally, people prefer to pick young shoots from tea plants to manufacture tea. In Anhui Province of China, tea flowers bloom in autumn and young tea shoots are picked in spring. It is very curious to know the differences in chemical composition between flowers and young shoots in different seasons. We used proton nuclear magnetic resonance ($^1$H NMR), a widely used non-targeted metabolomics method, to analyze primary metabolites. $^1$H NMR-based tea metabolomics has been reported, but few reports focused on tea flowers [18, 19]. In this experiment, young tea shoots and tea flowers at four developmental stages were selected for $^1$H NMR metabolomics and multivariate statistical analysis. A set of differential metabolites between young shoots and flowers at each developmental stage was identified. In addition, amino acid composition between tea shoots and flowers was comparatively analyzed using an automatic amino acid analyzer. This work provides differentiated chemical compound information on tea flowers vs. young shoots. It also provides additional insight into the efficient use of tea flowers as potential functional foods.

Results and discussion

$^1$H NMR spectroscopic analysis

To illustrate the metabolic dynamics between young tea shoots and tea flowers, and between the four developmental stages of tea flowers, representative $^1$H NMR spectra of tea flowers and young tea shoots are shown in Fig. 1. Our 1D $^1$H NMR spectral analysis identified a diverse range of metabolites, including theanine, alanine (Ala), threonine (Thr), isoleucine (Ile), leucine (Leu), valine (Val), quinic acid, malic acid, sucinic acid, citric acid, arginine (Arg), chlorogenic acid, sucrose, $\alpha$-glucose, $\beta$-glucose, fructose, caffeine, epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), myo-inositol, 2-$\beta$-(1L-arabinopyranosyl)-myo-inositol (Ara), and $\beta$-coumaryl quinic acid. These metabolites were identified by adding pure chemicals and comparing the data with the published literature [20, 21]. The screened metabolites were subsequently validated through 2D COSY and HSQC NMR experiments. See Table 1 for the chemical shifts of metabolites and their corresponding multiplicity or coupling constants from tea shoots assigned by COSY and HSQC NMR experiments.

Comparative metabolomics of young tea shoots and tea flowers

For further elaboration on the differentiation of the chemical compounds among four developmental stage of tea flowers, principal component analysis (PCA) was conducted. The results showed that the first principal component accounted for 73.8%, while the second principal component accounted for 19.2%, and both accounted for a total of 93% (Fig. 2A), which included most of the chemical information. The results revealed that the samples of young tea shoots and green bud (stage I) clustered together on the negative axis. While white bud (stage II), full bloom (stage III), and decayed flower (stage IV) grouped together on the positive axis. The results indicated that the metabolites between stage I and late three stages were obviously deferent, and the metabolites between young tea shoots and late developmental stages of tea flowers were significantly differentiated.

To further illustrate the distinctive chemical components in tea flowers at the four developmental stages, orthogonal partial least squares discriminant analysis
Fig. 1  Representative $^1$H NMR spectra of extracts of young tea shoots (A) and tea flowers (B)

Table 1  Assignments of metabolites in the representative $^1$H NMR spectra

| Metabolites               | Chemical shift/ppm$^a$ and multiplicity                          |
|---------------------------|-----------------------------------------------------------------|
| 1  Val                     | 1.06–1.00 (m, CH$_3$)                                            |
| 2  Leu                     | 1.09 (t, CH$_3$, $J=7.3$ Hz)                                    |
| 3  Ile                     | 1.13 (dd, CH$_3$, $J=9.1, 7.3$ Hz)                              |
| 4  Theanine                | 1.12–1.07 (m, CH$_3$), 2.18–2.10 (m, CH$_2$), 2.38 (d, CH$_2$, $J=8.5$ Hz), 3.15 (m, CH$_2$) |
| 5  Ala                     | 1.47 (d, CH, $J=7.3$ Hz)                                         |
| 6  Thr                     | 1.31 (d, CH$_3$, $J=7.8$ Hz), 4.20 (s, CH)                      |
| 7  Quinic acid             | 1.90–1.78 (m, CH$_2$), 2.02 (dd, CH$_2$, $J=12.0$ Hz), 3.85 (s, CH), 4.07–3.98 (m, CH) |
| 8  Malic acid              | 2.38 (q, $\alpha$-CH$_2$, $J=7.0$ Hz)                           |
| 9  Succinic acid           | 2.44 (s, CH$_2$)                                                 |
| 10 Citric acid             | 2.57 (d, $\alpha$-CH$_2$), 2.69 (dd, $\alpha'$-CH$_2$, $J=12.3$ Hz) |
| 11 Caffeine                | 3.30 (s, CH$_3$), 3.49 (s, CH$_3$), 3.85 (s, CH$_3$), 7.82 (d, CH, $J=10.7$ Hz), |
| 12 Arg                     | 3.28–3.22 (m, CH$_2$), 3.49 (s, CH)                             |
| 13 myo-Inositol            | 3.30 (s, CH)                                                    |
| 14 Chlorogenic acid        | 3.85 (s, 6.97 (d, $J=5.9$ Hz)                                   |
| 15 Sucrose                 | 3.43 (q, CH, $J=9.5$ Hz), 3.72–3.64 (m, CH$_2$), 4.08 (s, CH), 5.39 (s, CH), |
| 16 Ara                     | 5.17 (s, 3.28–3.22 (m, CH$_3$), 3.72–3.64 (m, CH), 3.89 (s, 4.18–4.11 (m, OH)) |
| 17 EGC                     | 6.58 (s), 4.88 (d, $J=6.9$ Hz), 4.26 (d, $J=9.7$ Hz), 2.87 (d, $J=7.5$ Hz), 2.74–2.65 (m) |
| 18 EC                      | 2.87 (d, $J=7.5$ Hz), 2.74–2.65 (m), 4.26 (d, $J=9.7$ Hz), 4.81 (s) |
| 19 ECG                     | 2.87 (d, $J=7.5$ Hz), 3.01 (d, $J=7.2$ Hz), 5.08 (s), 5.47 (d, $J=2.4$ Hz), 6.05 (d, $J=9.6$ Hz), 6.97 (d, $J=5.9$ Hz), 6.87 (d, $J=8.2$ Hz), 6.78 (d, $J=7.9$ Hz) |
| 20 EGCG                    | 7.14 (s), 6.97 (d, $J=5.9$ Hz), 6.58 (s), 6.05 (d, $J=9.6$ Hz), 5.03 (d, $J=6.4$ Hz), 2.87 (d, $J=7.5$ Hz), 3.01 (d, $J=7.2$ Hz) |
| 21 $\beta$-Coumaryl quinic acid | 7.72 (s), 6.87 (d, $J=8.2$ Hz)                                  |
| 22 $\alpha$-Glucose       | 5.17 (s, CH), 3.53–3.50 (m, CH)                                 |
| 23 $\beta$-Glucose        | 4.57 (d, CH, $J=7.8$ Hz), 3.58 (q, CH, $J=8.3$ Hz)             |
| 24 Fructose                | 3.53–3.50 (m, CH$_2$)                                            |

$^a$Signals marked as bold were selected for relative integration
(OPLS-DA) was employed. The variable importance in the projection (VIP) value > 1.5 and \( p < 0.05 \) were selected for analysis. OPLS-DA results showed that samples of young tea shoots were clustered on the left-hand side, and samples from tea flowers of four developmental stages were clustered on the right-hand side of the coordinate axis (Fig. 2B). This indicated significant distinction of chemical profiles between young tea shoots and tea flowers at four stages. Chemicals such as sucrose, fructose, EGC, EGCG, EC, Ara, caffeine, theanine, \( \alpha \)-glucose, and \( \beta \)-glucose were identified as potential differential metabolites. Quantitative differences between individual metabolites in young tea shoots and tea flowers were calculated from the total integral \( ^{1}H \) NMR spectral area corresponding to each metabolite.

Compared with young tea shoots, tea flowers contained less catechin, caffeine, and Ara. As shown in Fig. 3, the monomer levels of EGCG, EGC, and EC were much higher in young tea shoots than in the four flowering stages. The ECG, Val, Leu, quinic acid, malic acid, myo-inositol, and \( p \)-coumaryl-quinic acid levels were also higher in young tea shoots than in tea flowers. However, the succinic acid, citric acid and chlorogenic acid levels in young tea shoots were slightly lower than those in tea flowers. The EGCG, EGC, ECG, EC, Val, Leu, quinic acid, malic acid, citric acid, and chlorogenic acid levels in the green bud were higher than those in the other three flowering stages. Compared with young tea shoots, tea flowers contained more sugar, including sucrose, glucose and fructose. The sucrose level gradually increased during the four developmental stages of tea flowers; the \( \alpha \)-glucose level was highest in the white bud stage, and then it gradually decreased; the \( \beta \)-glucose level was highest in the green bud stage, and then it decreased steadily; the fructose level was significantly lower in the green bud stage than in the other three stages.

Amino acid analysis of young tea shoots and tea flowers

A total of 20 free amino acids, including seven essential ones, were quantified. The results showed that theanine is a richest amino acid, which accounts for 80.41% of the total free amino acids detected. Young tea shoots contained significantly more theanine compared to tea flowers \( (p < 0.01) \). Young tea shoots also contained significantly more Gly, Ala, Tyr, and Leu than tea flowers \( (p < 0.05) \). However, tea flowers contained significantly more Asp, Arg, Pro, Glu, and His than young tea shoots \( (p < 0.05) \). During flower development, Asp, Lys, Arg, and Pro were highest in the green bud; Glu, Ile, Phe, and His were highest in the white bud; and Ser and Val were highest in the decayed flower (Fig. 3). The white bud contained the most essential amino acids, followed by young tea shoots, decayed flower, green bud, and full bloom. The different stages of tea flowers have a set of characteristic amino acids.

Catechins and caffeine

Our data showed that the metabolites were clearly different between Shuchazao tea flowers and young tea shoots. Catechins are the major components of polyphenolic compounds in tea leaves, which account for 12–24% of the dry weight of tea leaf. Catechin, caffeine, and some free amino acids are the main sources of the bitter taste, and are the decisive factors of tea quality [22]. It was reported that the contents of total catechins in tea flowers vary.
Determination of the chemical compounds of Shuchazao tea flowers at different developmental stages greatly from cultivars to cultivars [13]. According to Joshi, compared to tea leaves, tea flowers contain higher amounts of total catechins than tea leaves [9]. Jia et al. found that the contents of polyphenols such as catechin, EC, EGC, and procyanidine were considerably fewer in tea flowers than in leaves [4]. The variable concentration of total catechins in tea flowers can be explained by the variation in cultivar and season of collection.

This study found that young tea shoots contained more catechins, especially EGCG, EGC, EC, and caffeine, compared with tea flowers. This is consistent with the findings of Jia [4]. However, the ECG content gradually
Amino acids are substantial precursors of aromatic substances in tea leaves and have prominent functions in the aroma of tea. Green tea leaves harvested in spring contain more amino acid than those harvested in later seasons [23], indicating that young tea shoots have higher amino acid levels. Theanine is the main free amino acid in tea leaves. The new shoots of tea plants picked from March to May contain significantly more theanine than the shoots picked in September [24]. Wang et al. reported that the tea flower contained much higher content of free amino acids. Theanine was the most abundant amino acid in tea flower as tea, histidine became the second one [25]. However, our data showed that the concentration of amino acids was highest in the white bud, and young tea shoots contained significantly more L-theanine than tea flowers. Pro, Arg, and Phe exhibited higher concentration in the Stage I and II of tea flowers than that of young tea shoots. The difference between our results with Wang’s report may be due to the different samples used for measurement. Previous paper used the samples from processed teas and flowers, while ours samples are frozen fresh young tea shoots and tea flowers. The manufacture processes may change profile of amino acids.

Several recent papers have reported the roles of amino acids in plant flowering [25–27]. Ser, Pro, and Phe have been shown to be directly related to tea flowering [4]. Serine is an important amino acid that changes into ethanolicamine and choline, and phosphatidylethanolamine and phosphatidylcholine, in plants [28]. This study shows that during the course of tea flower development, tea flowers in the green bud stage contained the highest levels of Asp, Lys, Arg, and Pro, and the highest levels of Glu, Ile, Phe, and His in the white bud stage. Furthermore, the highest levels of Ser and Val were found in the decayed flower stage. From our data, serine continuously accumulated during tea flowering, which indicated that it may have a role in flower development [4]. Pro, which functions as a signal in flowering, can enhance flower development and induce changes in flowering time [29].

Organic acids and others

Tea contains nearly 30 organic acids, including mainly quinic acid, oxalic acid, malic acid, acetic acid, citric acid, tartaric acid, and ascorbic acid. These acids account for approximately 3% of the total dry weight [30]. Organic acids are an important determinant of tea quality. Studies examining the correlation between organic acid content and tea quality have reported that in the early stages of flowering, many compounds undergo oxidation in the tricarboxylic acid (TCA) cycle, to provide energy for flower development [31]. In our experiment, the succinic acid, citric acid, and chlorogenic acid levels were higher in tea flowers than in young shoots, possibly due to acceleration of the TCA cycle in the growth process of tea flowers. During flowering period, large amounts of organic acids are consumed. The chlorogenic acid concentration gradually increased from young tea shoots to the four flowering samples. Chlorogenic acid has a wide range of antibacterial effects. Therefore, this specific component of tea flowers has potential use in functional foods. Moreover, the quinic acid and malic acid levels were higher in young tea shoots than in tea flowers. Quinic acid could be one of the substrates of lignin polymerization; it accumulates at a faster rate in young tea leaves [32]. Yamamoto et al. proposed that the Ara level in young leaves decreased gradually during the growth process [33]. So it could be used as
a maker compound for developmental degree of tea leaves. Our data showed that young tea shoots contained much more Ara than tea flowers. This is consistent with the findings of Yamamoto.

Conclusion

Our data indicated significant differences in catechins, sugars, organic acids, and amino acids between Shuchazao tea flowers and young shoots. In addition, different stages of tea flowers have a set of characteristic chemical compounds. Chemicals such as sucrose, fructose, α-glucose, β-glucose, EGC, EGCG, EC, Ara, caffeine, quinic acid, malic acid, theanine, Gly, Ala, Tyr, Leu, Asp, Arg, Pro, Glu, and His were identified as potential differential metabolites between Shuchazao tea flowers and young shoots. The four developmental stages of tea flowers have potential use for functional food.

Experimental

Plant materials

Tea flowers and young tea shoots were collected from tea plant cultivar Shuchazao (Camellia sinensis var. sinensis) grown in the Wanzhong Comprehensive Experimental Station of Anhui Agricultural University, Lujiang County, Anhui Province, China. The sampling date for tea flower collection was November 11, 2019. Young tea shoots, comprising one bud and two leaves, were collected using the standard plucking method on April 24, 2020. Four developmental stage, stages, namely, green bud (stage I), white bud (stage II), full bloom (stage III), and decayed flower (stage IV) were harvested as tea flower samples (Fig. 4). The samples were stored with solid carbon dioxide during collection and then kept in a − 80 °C freezer after freeze-drying in the laboratory.

Chemicals

Deuterium water (D₂O, D 99.9%), methanol-d₄ (CD₃OD, D 99.8%), chloroform-d (CD₂Cl, D 99.8%), and NaOD (D 99.5%, 40% in D₂O) were obtained from McLean (Shanghai, China).

Sample extraction

¹H NMR: the freeze-dried samples were ground into powder using a mortar and pestle with liquid nitrogen. The powder sample was transferred into a plastic tube with a spatula. Then, 100 mg of the sample was dissolved in a mixture of methanol-d₄ (CD₃OD, 750 mm³), chloroform-D (D₂O, 1000 mm³), and buffer solution (KH₂PO₄ dissolved in D₂O, adjusted to pH = 6.0 with 1 M NaOD, containing 0.1% 2,2,3,3-3-(trimethylsilyl)propionic acid-d₄ sodium salt (TSP, 750 mm³) in 5 cm³ Eppendorf tubes. The mixture thus obtained was sonicated at 60 °C for 25 min and then centrifuged at 13,000 rpm for 15 min at 10 °C. The resultant supernatants were transferred into 5 mm NMR tubes.

Amino acids: the freeze-dried samples were ground into powder with liquid nitrogen, and 100 mg of the sample powder was transferred into 10-cm³ Eppendorf tubes. Then, 4 cm³ of 4% sulfosalicylic acid was added to the sample, and the mixture was sonicated for 30 min at 60 °C. The sample was set for 10 min, and then 1.5 cm³ of the supernatant was transferred into a 2 cm³ Eppendorf tube. Following centrifugation at 12,000 rpm for 40 min, the obtained supernatant was drained through a 0.22-µm filter membrane, and a volume of 20 mm³ was transferred into a brown sample bottle for further testing.

![Fig. 4 Young tea shoots and tea flowers in four developmental stages. Green bud (stage I), white bud (stage II), full bloom (stage III), and decayed flower (stage IV)](image-url)
**1H NMR spectroscopic analysis**

1H NMR analysis was performed using a 600 MHz NMR spectrometer (Agilent DD2 600 MHz NMR, Oxford, England) at room temperature with a 1H{13C/15N} 5 mm PFG automatable triple-resonance probe. The field-frequency lock depended on CD3OD. We used the WET1D pulse sequence of the Agilent library with shaped selective pulses to remove the residual water. Then, considering a spectral width of 9615.4 Hz with an acquisition time of 1.7 s and relaxation delay of 1.5 s, 256 transients were collected with 32,000 data points. 2D-NMR spectra usually adopted standard pulse sequences, including COSY (1H−1H correlation), HSQC (1H−13C direct correlation), and HMBC (1H−13C remote correlation).

**Amino acid data determination**

An automatic amino acid analyzer (Hitachi L-8900, Japan) was used for free amino acid determination. P/N 855-3507 chromatographic column specification: 4.6×60 mm, the packing in the separation column is 3 μm sulfonic acid cation exchange resin, reactor temperature 130 °C, column temperature 38 °C, sample injection volume 20 mm3. The flow rates were 0.35 cm3/min for the mobile phase and 0.3 cm3/min for the derivatization reagent. The detection wavelength of the first channel is 570 nm, the detection wavelength of the second channel is 440 nm, the sequential elution is 32 min, and the total analysis time of each sample is 53 min. The peak areas of compounds were compared with the amino acid standards. All measured compounds were used in three replicates and the results are presented as mean ± SD.

**NMR data processing and multivariate statistical analysis**

Mestrenova 14 software (Mestrelab Research, S.L., Spain) was used to analyze and process the data. The chemical shift range 0–9.0 ppm was integrated with an interval of 0.04 ppm. In the NMR spectrum, δ=4.70−4.90 ppm (residual water peak), 3.30−3.40 ppm (residual methanol peak), and 7.10−7.35 ppm (residual chloroform peak) were not integrated. The integrated data were then imported into SIMCA-P14.1 software (Umetrics, Umeå, Sweden) for PCA. PLS-DA and OPLS-DA were adopted to find the differential metabolites. SPSS 23 software (IBM SPSS Statistics ver. 23; SPSS Corp, USA) was used to analyze the differential metabolites through an independent samples t-test [34–36].

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00706-022-02928-6.

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