Isolation and identification of spermidine derivatives in tea (Camellia sinensis) flowers and their distribution in floral organs

Title: Isolation and identification of spermidine derivatives in flowers of tea (*Camellia sinensis*) plants and their distributions in floral organs

Running title: Spermidine derivatives in tea flowers

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Abstract

BACKGROUND: Recently the flowers of tea (*Camellia sinensis*) have attracted more interest of researchers **because they have many bioactive compounds such as catechins etc.** The objective of this study was to investigate occurrences of some characteristic compounds in tea flowers.

RESULTS: A principal component analysis of metabolites using ultra performance liquid

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chromatography-time of flight mass spectrometry suggested a different metabolite profile between flowers and leaves of ***C. sinensis* var. Yabukita**. Four spermidine derivatives have been isolated from tea flowers. One of them was determined as N^1, N^5, N^{10}-tri-coumaroyl spermidine based on NMR, MS, and UV data. The other three spermidine derivatives were identified as feruoyl di-coumaroyl spermidine, coumaroyl di-feruoyl spermidine, and tri-feruoyl spermidine, respectively, based on MS^n evidences. The tri-coumaroyl spermidine as major spermidine conjugate was not detected in tea leaves. Furthermore, the spermidine conjugate decreases during floral development, and mainly occurs in anthers.

CONCLUSION: We provide the evidences that spermidine-phenolic acid conjugates occur in tea flowers with considerable amounts for the first time. Their occurrences in tea flowers will prompt us to reconsider the ecological roles of tea flowers. From an economic point of view, tea flowers might be suitable for raw materials in health-care food and pharmaceutical industries.

Keywords: anther; *Camellia sinensis*; conjugate; spermidine; tea flowers

INTRODUCTION

Tea (*Camellia sinensis*) is a plant commercially grown for beverage production. Beneficial health properties of **tea leaf extracts** and their phenolic compounds have been extensively studied.[1, 2] Recently, flowers of tea have attracted more interest of researchers because they have many bioactive compounds such as catechins with radical-scavenging effects,[3-5] nitric oxide suppressing effects in LPS-induced RAW 264.7 cells,[3] and

anti-proliferative and apoptotic effects in human breast cancer MCF-7 cells;[6] floratheasaponins with inhibitory activities on serum triglyceride elevation and the release of β-hexosaminidase from RBL-2H3 cells,[7,8] and polysaccharides with α-glucosidase inhibitory and amylase inhibitory activities.[9]

Up to date, relatively few studies have been performed on the differences in extensive metabolite profile between tea leaves and flowers. The previous reports were mostly focused on specific metabolite classes such as such as catechins, caffeine etc.[3] Recent advances in practical methodologies and affordable instrumentation to metabolite analysis allow us perform a rapid and systematical characterization of small molecule metabolites found in an organism, and it is possible to determine small differences in the metabolite composition between groups or treatments.[10] The objectives of this study are to compare the metabolite profile between tea flowers and leaves, and investigate occurrences of some characteristic compounds in tea flowers. In the present study we found a different metabolite profile between tea flowers and leaves by integrating the resolving power of ultra performance liquid chromatography-time of flight mass spectrometry (UPLC-TOFMS) and multivariate data analysis. **Moreover, several characteristic metabolites such as spermidine-phenolic acid conjugates present in tea flowers were identified, and their distributions in floral organs were investigated. Spermidine-phenolic acid conjugates are a widely distributed group of plant secondary metabolites that are concentrated in the floral parts of plants.[11-18] In the past few years, enormous strides have been made in understanding the diverse roles played by spermidine-phenolic acid conjugates in plants, including defense against wounding, pathogens, and insects, floral induction, flower formation, sexual**

differentiation, tuberization, cell division, and cytomorphogenesis (Reviewed by Facchini et al.[19]). In addition to these diverse functions in plants, the spermidine-phenolic acid conjugate, for example N^1, N^5, N^{10}-tri-coumaroyl spermidine, was found to appreciably inhibit HIV-1 protease.[20] In this study we firstly report the occurrence of this class of compounds in tea flowers. Since growth of tea flower was traditionally regarded as having the negative influence on the production of tea leaves, little attention has been paid so far to the ecological roles of flower in tea plants. The occurrences of the spermidine-phenolic acid conjugates in tea flowers will prompt us to reconsider the ecological roles of tea flowers.

MATERIALS AND METHODS

Sample preparation

The leaves (from 1st to 4th leaves) and flowers of *C. sinensis* var. Yabukita, which is the most popular variety of tea in Japan, were obtained from tea fields at the Center for Education and Research in Field Sciences, Shizuoka University (Fujieda, Shizuoka, Japan) in November from 2008 to 2010. The tea plants were fertilized four times per year, including N: P_2O_5: K_2O = 7.2: 3.6: 4.8 kg/1000 m^2 in February, 5.4: 1.8: 3.0 kg/1000 m^2 in April, 6.6: 1.2: 2.4 kg/1000 m^2 in June, and 7.2: 2.4: 6.0 kg/1000 m^2 in September. The samples were crushed to a fine powder using a Multi-Beads Shocker (2,000 rpm, 15 sec, Yasui Kikai Corporation, Japan) and stored at -80°C for analysis.

Analysis of endogenous metabolites by UPLC-TOFMS

To assess the differences in the composition of metabolites of tea flowers and leaves, 70% methanol-soluble constituents from the flowers and leaves were analyzed by

UPLC-TOFMS. Fifty mg (fresh weight) of finely powdered tissues were extracted with 1 mL of cold 70% methanol by vortexing for 1 min followed by an ultrasonic extraction on ice for 10 min, afterwards filtered through 0.45 μm membrane filter. Prior to Waters ACQUITY UPLC- LCT Premier XE^{TM} analysis, **the filtrate was diluted to 10 mL with 70% methanol. Two** μL of samples were injected onto an ACQUITY HSS T3 2.1 × 100 mm, 1.8 μm column. Sample and column temperatures were maintained at 4°C and 40°C, respectively. The samples were eluted using a flow rate of 0.3 mL/min applying a chromatographic gradient of two mobile phases (A: 0.1% aqueous formic acid; B: 0.1% formic acid in acetonitrile). The solvent B was maintained at 0% for 2 min, increased linearly from 0% to 25% at 10 min, and then to 98% at 13.5 min. Afterwards 98% of solvent B was maintained for 3.5 min and subsequently brought back within 0.1 min to 0% of the solvent and held for another 2.9 min to allow for column equilibration.

An electrospray source was used. Sample cone voltage was set at 30 V and capillary voltage was set at 3.0 kV. The source and desolvation temperatures were 120°C and 450°C, respectively. The desolvation and nebulizer gas flow rates were 900 L/h and 50 L/h, respectively. Spectra were collected in the negative ionization at a mass resolution of approximately 10,000 (full width half maximum). Data were acquired over the *m/z* range 70–1,000. The raw data from the UPLC-TOFMS analysis were transformed to peak tables using MarkerLynx, an application manager of MassLynx v. 4.1.

Isolation and purification of spermidine derivatives from tea flowers

The lyophilized and powdered tea flowers were extracted three times each with ethanol: water (70:30, v/v) under reflux for 120 min (solvent: tea flower powders = 10:1, v/w). The infusion was filtered with a 0.45 μm Millipore filter and concentrated under reduced

115 pressure to give the ethanolic extract of tea flowers, which was further separated by
116 liquid-liquid partitioning using chloroform, ethyl acetate and *n*-butanol successively, as
117 described previously.[5]
118 The ethyl acetate-soluble fraction was subjected to a Biotage Flash40 chromatography
119 system (ODS C-18 40 mm ID×15.0 cm, column volume= 188 mL), and the compounds
120 were eluted in order of decreasing polarity with a methanol gradient [MeOH-H_2O
121 (1:99→5:95→10:90→20:80→30:70→50:50→75:25, v/v) →MeOH] using the double
122 column volumes for every gradient step. From the 16 obtained fractions, fractions 13-16
123 were combined and further purified by preparative HPLC using a 10 mm× 250 mm, 5 μm
124 particle size, UG120Å C-18 reversed-phase column (Shiseido Co. Ltd., Japan) with the
125 solvent A (HCOOH-H_2O, 0.1:99.9, v/v) and the solvent B (MeCN), at 293 nm and a flow
126 rate of 4 mL/min. Elution was started under isocratic conditions of 12% of solvent B for 5
127 min, and followed by a linear increase of solvent B to 25% at 45 min, 60% at 65 min, 100%
128 at 75 min followed by an isocratic step of solvent B for 10 min. The fraction A (retention
129 time 55.8-57.2 min) was concentrated under vacuum and analyzed by an LCMS-2010 A
130 system (Shimadzu Cooperation, Tokyo, Japan) equipped with a 2.0 mm × 150 mm i.d., 5
131 μm particle size, UG120 C-18 reversed-phase column (Shiseido Co. Ltd., Japan). A total of
132 10 μL of the sample solutions was analyzed using gradient elution with the mobile phase of
133 MeCN: [HCOOH-H_2O, 0.1:99.9, v/v] (25:75, v/v) at a flow rate of 0.2 mL/min at 40°C for
134 50 min. UV-vis spectra were recorded between 200 and 600 nm for each chromatographic
135 peak. Optimized electrospray operating conditions were: dry gas 1.5 L/min, capillary
136 voltage 1.5 kV, dry temperature 250ºC. Four peaks were identified in the Fraction A (*See*
137 *results and discussion, Figure 2*). To further purify the four compounds, the Fraction A was

subjected to preparative HPLC using a 10 mm× 250 mm, 5 μm particle size, UG120Å C-18 reversed-phase column (Shiseido Co. Ltd., Japan) and the mobile phase of MeCN: [HCOOH-H_2O, 0.1:99.9, v/v] (25:75, v/v) at a flow rate of 4 mL/min, and 40°C. The purified compounds were identified by MS^n (Analyzer, ion trap; LTQ Orbitrap Discovery, Thermo Fisher Scientific) and NMR (JEOL JNM-LA 500 FT-NMR) using ^1H, ^{13}C, COSY, HSQC, and HMBC.

RESULTS AND DISCUSSION

Comparison of metabolite profiles of tea flowers and leaves

A principal component analysis of metabolites (*m/z* 70-1000) using UPLC-TOFMS demonstrated that significant differences exist between the metabolic profiles of leaves and flowers at different stages (Figure 1). In the past few years, enormous strides have been made in understanding the health properties of tea leaves and their bioactive compounds, especially catechins. In contrast to tea leaves, tea flowers contain comparable amounts of total catechins.[3] Moreover, other similar chemical compositions to tea leaves, for example, caffeine,[3] flavonol glycosides,[5]sugars,[9] and amino acids,[21] etc., were also identified in tea flowers. Different metabolic profiles between tea flowers and leaves in the present study suggest that some characteristic compounds may occur in tea flowers.

Identification and occurrences of spermidine derivatives in tea flowers

The four spermidine derivatives (compounds 1-4) were isolated and purified from tea flowers (Figure 2A). The compound 1 was determined as tri-coumaroyl spermidine based on MS^2 and MS^3 data (Figure 3) and NMR spectra as shown below. ^1H NMR (500 MHz, dissolved in CD_3OD): *p*-coumaroyl systems: δ 7.50-7.56 (m, H-7', 7'''), 7.35-7.48 (m, H-2',

2", 2"', 6', 6", 6"', 7"), 6.87 (m, H-8"), 6.72-6.83 (m, H-3', 3", 3"', 5', 5", 5"'), 6.36-6.44 (m, H-8', 8"'). Spermidine system: δ3.31-3.55 (m, H_2-2, 4, 6, 9), 1.60-1.93 (m, H_2-3, 7, 8). ^{13}C NMR (125 MHz, dissolved in CD_3OD): *p*-coumaroyl systems: δ169.2-169.4 (C-9', 9", 9"'), 160.5-160.7 (C-4', 4", 4"'), 144.4 (C-7"), 141.8-142.2 (C-7', 7"'), 130.9 (C-2", 6"), 130.6 (C-2', 6', 2"', 6"'), 127.9 (C-1"), 127.6-127.7 (C-1', 1"'), 118.5 (C-8"'), 118.2 / 118.4 (C-8'), 116.7 / 116.8 (C-3', 3", 3"', 5', 5", 5"'), 114.9 (C-8"). Spermidine system: δ47.6 / 49.0 (C-6), 45.7 /47.0 (C-4), 39.9 / 40.1 (C-9), 37.9 / 38.2 (C-2), 28.9 / 30.6 (C-3), 27.9 / 28.0 (C-8), 26.3 / 27.8 (C-7). The NMR data agree with those published in the literatures.[11, 20] The NMR spectra of the compound 2 were similar to those of the compound 1, except for the presence of methoxy group (δH 3.85-3.88 ,δC 56.4-56.6), suggest that the compound 2 contains feruoyl groups. Furthermore, based on MS^2 and MS^3 evidences (Figure 3), the compound 2 was determined as feruoyl di-coumaroyl spermidine. The NMR signals of the compound 2 were overlapped. Therefore, the positions of coumaroyl and feruoyl groups located in N^1, N^5, or N^{10} in the compound 2 are not completely assigned. The compound 3 and compound 4 were tentatively identified as coumaroyl di-feruoyl spermidine and tri-feruoyl spermidine, respectively, on the basis of MS^2 and MS^3 evidences (Figure 3). UV data of the compounds 1-4 (Figure 2B) agree with those published in the literature.[22]

Tri-coumaroyl spermidine (compound 1) as one major spermidine derivative in tea flowers decreased from 181.4 μg/g (fresh weight) at flower buds stage to 91.7 μg/g at full-open flowers stage during floral development (Figure 4A), and mostly occurred in anthers (271.6 μg/g, 82.5% contribution to 1 fully open flower), followed by filaments (20.6 μg/g, 9.2% contribution), petals (9.1 μg/g, 6.5% contribution), and other parts (8.2 μg/g, 1.9% contribution), successively (Figures 4B and C). Tri-coumaroyl

spermidine was not detected in tea leaves (Figure 4A). Several spermidine-phenolic acid conjugates have been isolated and identified in the floral parts of plants, such as *Corylus avellana*,[12] *Alnus glutinosa*, *Betula verrucosa*, *Pterocarya fraxinifolia*,[13] *Rosaceae*,[14] *Aphelandra*,[15] *Quercus dentate*,[16] *Pisum sativum*,[17] *Arachis hypogea*,[11] and *Fragaria* × *ananassa*.[18] Recently, spermidine hydroxycinnamoyl transferase, *O*-methyltransferases AtTSM1, and two cytochrome P450 enzymes CYP98A8 and CYP98A9 have been shown to be involved in the formation of the spermidine-phenolic acid conjugates.[22-24] Among these reports, the spermidine-phenolic acid conjugates are mainly found in pollen-accumulating anther part, which is consistent with the distribution in flowers of *C. sinensis* (Figures 4B and 4C). **Not all flower organs are equally employed in metabolite production, and spatial differences within a flower are quite common. Spatially-restricted production of secondary metabolite classes and specialized derivatives in flowers may take part in implementing the unique program of individual organs in the floral life cycle.[18] The accumulation of these compounds in anthers may be responsible for the pollen formation.[19]**

CONCLUSION

This study shows the different metabolite profiles between tea leaves and flowers. **Furthermore, four spermidine-phenolic acid conjugates were isolated and identified in tea flowers for the first time. The spermidine-phenolic acid conjugates occur with considerable amounts in tea flowers and highly accumulate in anthers. The information will help us to discover unknown ecological function of flowers in tea plants and will also contribute to the future application of tea flowers as raw materials**

in health-care food and pharmaceutical industries.

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Figure Legends

Figure 1 Principal component analysis of metabolites ($[M-H]^-$ *m/z* 70-1,000) of tea leaves and flowers at different stages.

Flower development was divided into 3 stages: at stage 1 the flower buds are closed, at stage 2 the flower is half open, and at stage 3 the flower is fully open. The principal component analysis was performed using combination of *m/z* and retention time in the spectra by MarkerLynx, an application manager of MassLynx v. 4.1.

Figure 2 Chromatogram (A), and UV spectrums (B) of the four compounds isolated from Fraction A (*See Materials and methods*).

Figure 3 Chemical structures of spermidine derivatives isolated from tea flowers and their identifications by MS^n.

The compounds 1-4 are shown in **Figure 2**. The positions of coumaroyl and feruoyl groups located in N^1, N^5, or N^{10} in the compounds 2-3 are not completely assigned.

Figure 4 Changes in tri-coumaroyl spermidine during floral developments and their distributions in floral organs of *C. sinensis* flowers.

The data are expressed as Mean ± S.D. (n=3). (A) N.D., not detected. (B) The "other part" is a mixture of carpels, receptacles, sepals, and pedicels. (C) The biomass contributions of

322 petals, filaments, anthers, and other parts to 1 flower are 42%, 26.4%, 18%, and 13.4%,
323 respectively. The relative ratio (%) was calculated based on the contribution of each floral
324 organ to 1 fully open flower.
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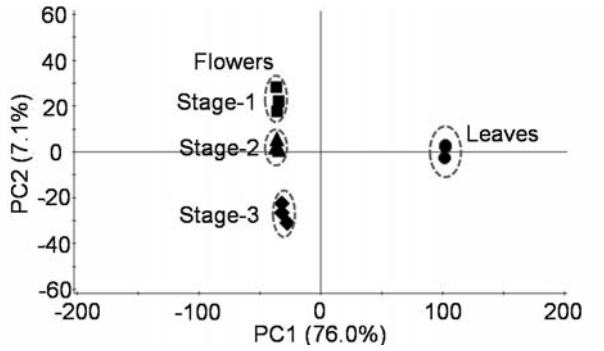


Figure 1 Ziyin YANG

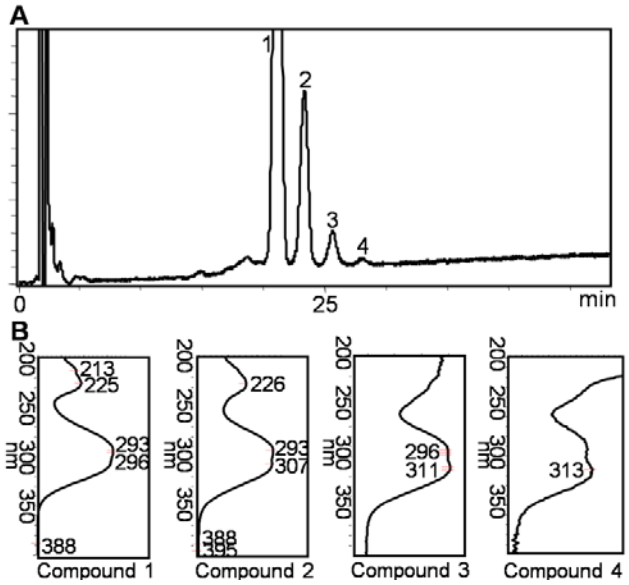
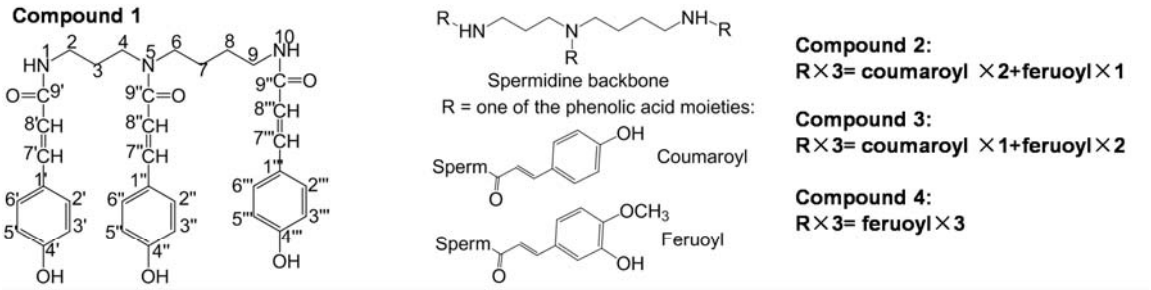
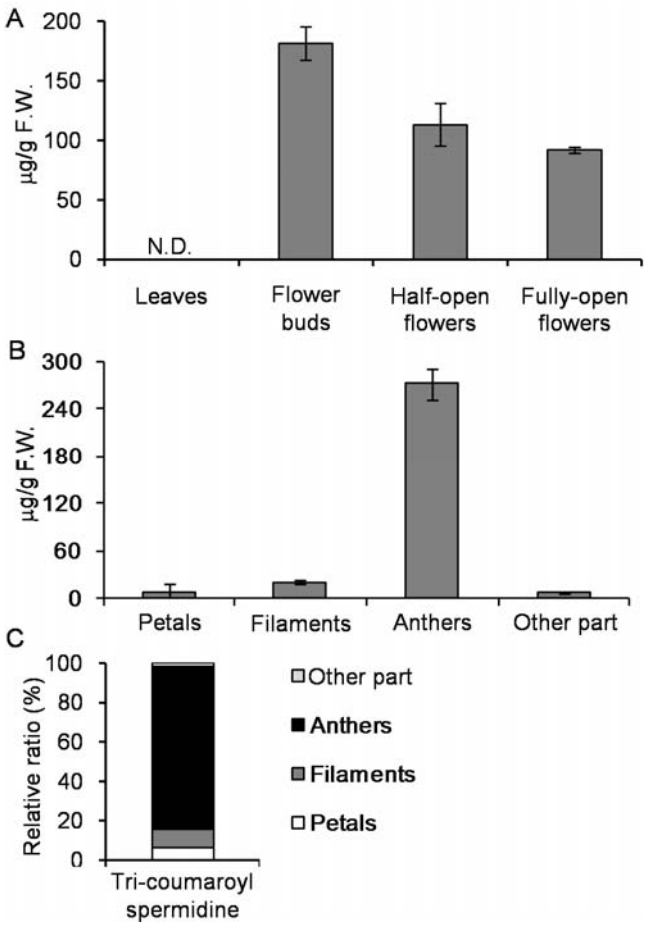


Figure 2 Ziyin YANG



Compound	*m/z* (-)	
	MS^2	MS^3
Compound 1 [M-H]⁻ 582	342, 436 [M-H-coum]⁻, 462	342/436/462→145 [M-H-coum-coum-sperm]⁻, 462→223, 342→256, 436/462→273, 342/462→299, 436/462→316, 436/462→342, 462→419
Compound 2 [M-H]⁻ 612	342, 372, 436 [M-H-Fer]⁻, 462, 466 [M-H-coum]⁻, 476, 492	466→135, 462/466/476→145 [M-H-coum-fer-sperm]⁻, 492→175 [M-H-coum-coum-sperm]⁻, 492→299, 462/466→316, 476→330, 462/492→342, 466→346, 492→372, 462→419, 476→433, 492→449
Compound 3 [M-H]⁻ 642	372, 466 [M-H-fer]⁻, 492, 496 [M-H-coum]⁻, 506, 522	466→135, 492→145 [M-H-fer-fer-sperm]⁻, 492/506/522→175 [M-H-coum-fer-sperm]⁻, 466/492→316, 492/522→329, 492→342, 466/522→346, 492/522→357, 492/522→372, 506→448, 492→449, 522→479, 506→463, 506→491
Compound 4 [M-H]⁻ 672	372, 496 [M-H-fer]⁻, 522, 536	372/496/522/536→175 [M-H-fer-fer-sperm]⁻, 372→314, 496→320, 372→329, 496→331, 372/496/522→346, 372/522→357, 522→372, 536→478, 522→479, 536→493, 522→507, 536→521

Figure 3 Ziyin YANG



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405 **Figure 4** Ziyin YANG