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Characterization of tea leaf metabolites dependent on tea (*Camellia sinensis*) plant age through ^1H NMR-based metabolomics

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Abstract

The chemical or metabolic compositions of tea (*Camellia sinensis*) varies according to numerous factors, such as geographical origin, cultivar, climate, plucking position, and horticultural practices. However, how the age of tea plants affects the metabolite compositions of tea leaves has not been reported yet. Therefore, we extended the metabolomic approach to the investigation of the age-related differences of tea leaf metabolites in the fresh leaves collected from tea plants aged 8 and 25 years. Multivariate statistical analysis with comprehensive metabolite profiles analyzed by ^1H NMR spectroscopy showed the clear metabolic differentiation between the fresh tea leaves from different ages of the tea plants. Of the various tea leaf metabolites varied according to the age of the tea plants, theanine, glutamine, catechin, and gallic catechin were uniquely dependent on the age of tea plants, demonstrating a difference of theanine metabolism between young and old tea plants. These results suggest that leaves from 25-year-old tea plants would still be worthy as a functional ingredient for the production in the food or cosmetic industry rather than quality-enhanced tea infusions for human consumption.

Keywords: *Camellia sinensis*, Tea, Age, Metabolomics, NMR

Introduction

Tea is one of the most widely consumed beverages in the world. The five types of teas, such as black, oolong, green, white, and pu-erh teas, are commonly available in the market, in which tea leaves are the typical ingredients [1, 2]. Tea has been known to have health benefits such as reduced cancer risk [3], neuroprotective effects [4], and anti-inflammatory activity with compounds rich in polyphenols, catechin and its derivatives, caffeine and theanine, and minor compounds, including amino acids, theaflavins, and thearubigins [5]. In addition to health benefits, modern cosmetic industries use tea as an

ingredient for improving the quality of cosmetics products, usually termed ‘cosmeceuticals’ [6].

The tea plant is a perennial and economic crop with a productive lifespan that can range up to 100 years [7]. Several studies stressed the importance of peak tea yields and their low productive-driver reasons. For example, the effects of a tea plant’s genotype and environment, management practices, and the interactions between the factors [8] on productivity have been reported, as well as the effect of tea plant age [9]. Notably, the age of tea plants has a substantial effect on tea yield and peak yields, which are optimum between the ages of 20 and 40 years and then decline afterward [10]. Therefore, the age of tea plants may be a topic of immense interest for research on the chemical composition of the tea leaf.

Metabolomic approaches have been reported as useful and promising tools to examine the changes in global metabolites of tea plants and potentially provide a better

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physiological understanding of the biochemical and cellular events [11, 12]. For instance, a recent proton nuclear magnetic resonance (^1H NMR) based global metabolic profiling study with green tea demonstrated that the tea metabolome was clearly dependent on growing area and hence was influenced by climatic conditions [13]. Moreover, a direct association between tea leaf metabolites and environmental factors, such as plucking seasons and climate variables, has been characterized by a ^1H NMR-based metabolomics approach [14]. Furthermore, a metabolomic approach revealed the metabolic dependence of tea leaves on plucking positions [15]. Although ^1H NMR-based metabolomic study with different types of teas provides much useful information for producing distinct tea products, the distinctions of tea leaf metabolites according to the age of tea plants remains unknown. Therefore, in the present study, we explored the age-related differences in tea metabolites in the fresh leaves collected from tea plants aged 8 and 25 years through ^1H NMR-based metabolomics approach.

Materials and methods

Origin of tea samples

Tea plants (*Camellia sinensis* var. Yabukita) of two different ages (8- and 25-year-old) analyzed in this study were grown at the same tea garden located in Seogwang (33° 18' 17.67" N, 126° 17' 42.97" E), Jeju-do, Republic of Korea. Fresh tea leaves were plucked at ten different parcels on May 14, 2015, and April 24, 2016, immediately kept in dry ice, sent to Chonnam National University (CNU, Republic of Korea), and stored at $-80\text{ }^\circ\text{C}$ until further analysis.

Extraction of tea samples

Tea leaves were extracted according to the protocol explained in the previously published article [16]. The frozen tea leaves without stems were ground with a mortar and pestle under liquid nitrogen. The ground tea was transferred into a plastic tube with a spatula and freeze-dried at $-80\text{ }^\circ\text{C}$ for 48 h. Then freeze-dried samples of 10 mg were dissolved in a mixture of methanol- d_4 (CD_3OD , 490 μL) and deuterium water (D_2O , 210 μL) in 1.5 mL Eppendorf tubes. After that, the mixture was sonicated at $25\text{ }^\circ\text{C}$ for 20 min followed by centrifugation at 13,000 rpm for 15 min at $10\text{ }^\circ\text{C}$. The resultant supernatants were transferred to 5-mm NMR tubes.

^1H NMR spectroscopic analysis of tea extracts

For ^1H NMR spectroscopic analysis, 550 μL of the supernatant was transferred into 5-mm NMR tubes, and a ^1H NMR spectrum was acquired on a Bruker Avance 700 spectrometer (Bruker Biospin, Rheinstetten, Germany)

operating at 700.40 MHz ^1H frequency and a temperature of 298 K, equipped with a cryogenic triple-resonance probe and a Bruker automatic injector. A one-dimensional (1D) NMR spectrum of tea leaf extract was acquired with the 1D nuclear Overhauser effect spectroscopy (NOESY) pulse sequence with water pre-saturation. The signal assignment of the tea leaf extract was facilitated by two-dimensional (2D) total correlation spectroscopy (TOCSY), heteronuclear single quantum correlation (HSQC), spiking experiments with reference compounds, and comparisons of chemical shifts reported in previous work or a database. Furthermore, one-dimensional statistical total correlation spectroscopy (STOCSY) [17] was also used for the signal assignment.

NMR data processing and multivariate statistical analysis

All ^1H NMR spectra derived from tea leaf extracts were corrected manually for the phase and baseline distortions using TOPSPIN software (Version 4.04, Bruker BioSpin, Rheinstetten, Germany), converted to ASCII format, and then imported into MATLAB (R2010b; The Mathworks, Inc., Natick, MA). The *icoshift* method [18] was applied to align the 1D NMR spectra in full resolution. After the alignment of the 1D NMR spectra, regions of the NMR spectra corresponding to the solvent methanol (3.36–3.40 ppm) and residual water (4.80–4.90 ppm) were removed. Probabilistic quotient normalization of the spectra using the median spectrum was carried out after total integral normalization to avoid dilution effects of samples and effects of metabolites [19]. The resultant datasets were then imported into SIMCA-P version 14.0 (Umetrics, Umeå, Sweden) and applied to a mean centering scaling method for multivariate statistical analysis. First, the principal component analysis (PCA), an unsupervised pattern recognition method, was performed to examine intrinsic variation in the dataset. After that, a supervised pattern recognition method, orthogonal projection on latent structure-discriminant analysis (OPLS-DA) [20] was used to extract maximum information on discriminant compounds from the spectra. An in-house MATLAB script developed by Imperial College London, UK, was used for pairwise comparison between two groups of classes in OPLS-DA loading plots, in which the loading plots corresponding to the correlation coefficients between variables and classes were combined by back-transformed loading together with variable weights. A color code visualized the concentration differences and discrimination weights between the classes in the OPLS-DA model that corresponds to the square correlation coefficient in the OPLS-DA loadings as described by [21]. A permutation test of 200 times repetitions along with seven-fold cross-validation was performed to validate the

OPLS-DA model. The Q^2 values generated from the permutation test were compared to the Q^2 values of the real model. If the maximum value of Q^2 from the permutation test was smaller than the Q^2 of the real model, the model was considered to be a predictable model. R^2X was used to evaluate possible overfitting of the model. Hence, the quality of the models is described by R^2X and Q^2 values. R^2X is defined as the proportion of variance in the data explained by the models and indicates goodness of fit, and Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability.

Statistical analysis

Statistical analyses of the data were conducted using the software package SPSS (IBM SPSS Statistics ver. 23; SPSS Corp., USA). Significance in the relative contents of tea leaf metabolites was evaluated by one-way analysis of variance (ANOVA) followed by Duncan’s multiple-range test. A probabilistic value of $P < 0.05$ was considered to be statistically significant. The integral area of the peaks identified in 1D 1H NMR that corresponded to non-overlapped individual metabolites was examined to quantify the relative comparisons of the levels of individual metabolites.

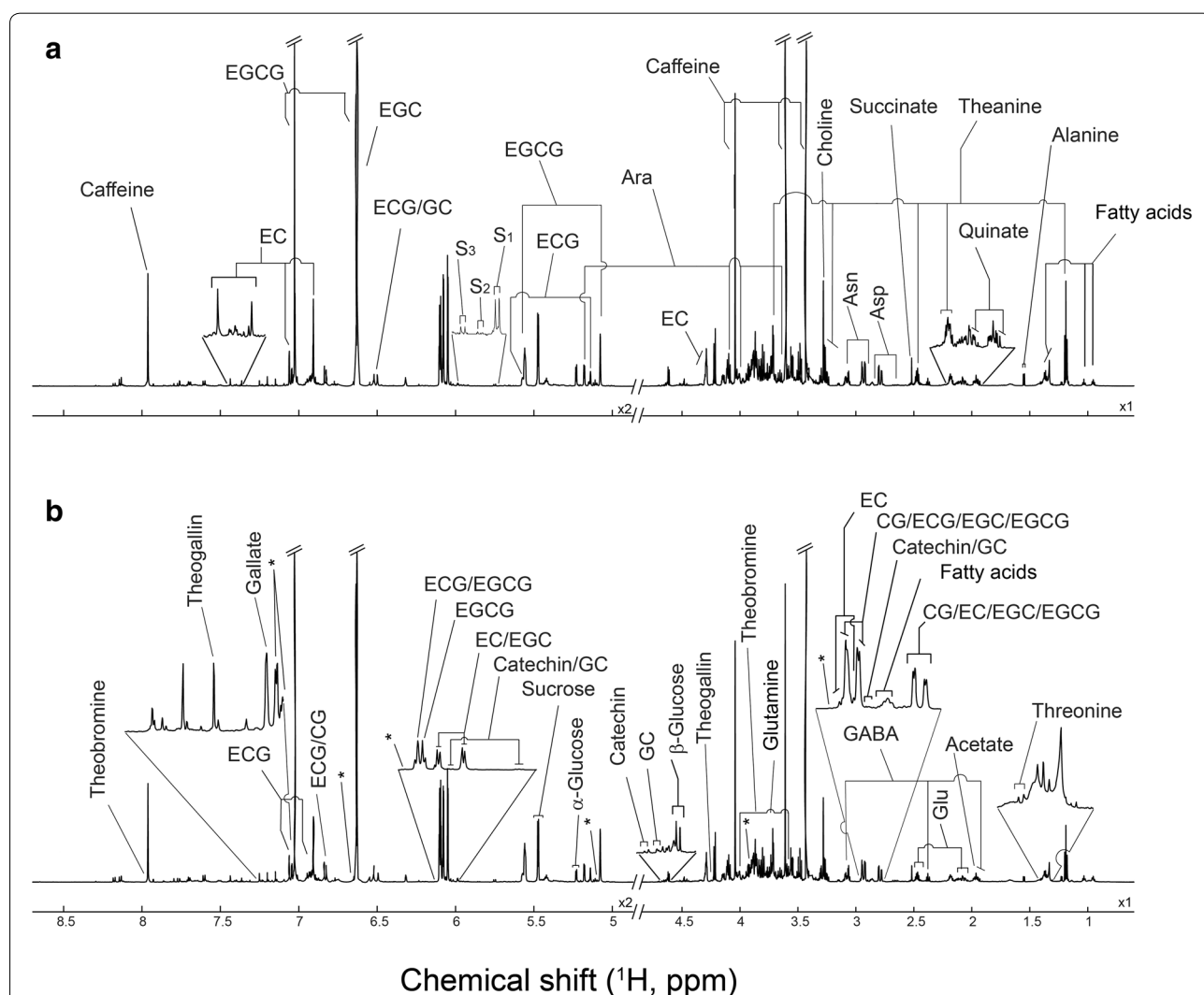


Fig. 1 Representative 1H 700 MHz NMR spectra of tea leaf extracts from 8-year-old (a) and 25-year-old (b) tea (*C. sinensis*) plants. The tea metabolites identified by 1H NMR spectroscopy include acetate, alanine, 2-O-(β -L-arabinopyranosyl)-myo-inositol (Ara), asparagine (Asn), aspartate (Asp), caffeine, catechin, choline, epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), fatty acids, γ -aminobutyric acid (GABA), gallate, galocatechin (GC), α -glucose, β -glucose, glutamate (Glu), glutamine, quinate, sucrose, succinate, theanine, theobromine, theogallin, and threonine. S1, S2, and S3 were tentatively assigned as sugar compounds. Asterisks represent epigallocatechin 3-O-(3''-O-methyl) gallate (EGCG3''Me)

Results

¹H NMR spectroscopic analysis of tea leaves

To understand the global metabolic dynamics in tea leaves as tea (*C. sinensis*) plants become aged, representative ¹H NMR spectra of tea leaves collected from 8-year-old (a) and 25-year-old (b) tea plants plucked in May 2015 and April 2016, respectively, are shown in Fig. 1. The 1D ¹H NMR spectra consisted of a diverse range of tea leaf metabolites, including acetate, alanine, 2-*O*-(β-L-arabinopyranosyl)-myo-inositol (Ara), asparagine, aspartate, caffeine, choline, fatty acids, gallate, γ-aminobutyric acid (GABA), α-glucose, β-glucose, glutamine, glutamate, quinate, sugars, sucrose, succinate, catechin, gallic acid (GC), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), epigallocatechin 3-*O*-(3''-*O*-methyl) gallate (EGCG3''Me), theanine, theobromine, theogallin, threonine, and valine. These tea leaf metabolites were assigned by spiking with the pure chemicals and also by comparing the data from the published literature [13–15, 22, 23]. The metabolites assignment was also validated by 2D TOCSY and HSQC NMR experiments, as described by previous studies [15, 22]. Additional file 1: Table S1 provides the chemical shifts of tea leaf metabolites and their corresponding multiplicity or coupling constant from tea leaves assigned by TOCSY and HSQC NMR experiments.

Metabolic differentiations of tea leaves according to plant age

A pattern recognition method by multivariate statistical analysis, such as principal component analysis (PCA) and orthogonal projection on latent structure-discriminant analysis (OPLS-DA), was employed for the entire ¹H NMR dataset for visualizing the global differences in tea leaf metabolites according to age of tea plant. An unsupervised PCA model was used to see the initial spectral features of the ¹H NMR dataset and the metabolic relationships between tea samples. The PCA model showed the metabolic dependence of tea leaves on growing vintage or year described by the first principle component with 67.3% variations and on the age of tea plants explained by the second principal component with 9.83% (Fig. 2a). These metabolic dependences were more clear in the OPLS-DA model, as shown in Fig. 2b. The tea leaves collected in 2015 and 2016 were further differentiated in the corresponding OPLS-DA models, as shown in Fig. 2c, d, respectively, which demonstrated strong dependences of tea leaf metabolites on the age of tea plants.

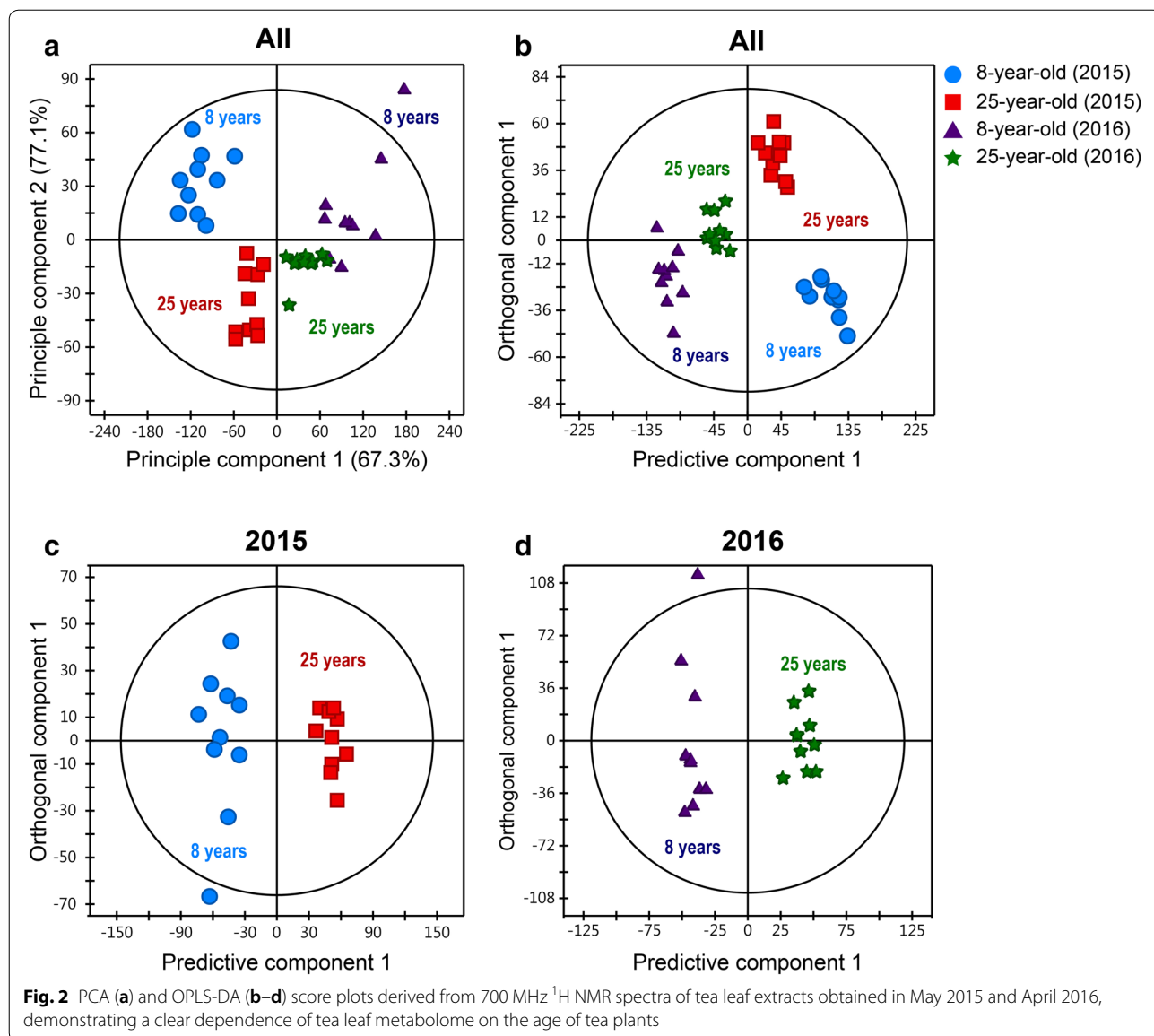
Identification of tea leaf metabolites associated with the age of tea plants

A pairwise OPLS-DA model was generated with one predictive and one orthogonal component to identify the

tea leaf metabolites responsible for metabolic differentiations according to the age of the tea plants (Fig. 3). Clear differentiation between the tea samples of different ages was observed in all OPLS-DA score plots with a high predictability (Q^2) and high goodness of fit (R^2X), which accounted for 0.60 and 0.85 between the tea leaves of the 8- and 25-year-old tea plants that were harvested in 2016 (Fig. 3a), and 0.66 and 0.93 between the tea leaves of the 8- and 25-year-old tea plants that were harvested in 2015 (Fig. 3c). All these OPLS-DA models were validated by permutation tests (Additional file 1: Fig. S2). The upper sections of the OPLS-DA loading plots represent the tea leaf metabolites that were higher in the old tea plants than in the young tea plants, whereas the lower sections were characterized by lower contents of tea leaf metabolites in the old tea plants (Fig. 3b, d). The different colors on the OPLS-DA loading plots explain the significant differences in metabolites responsible for differentiations between the young and old tea plants, and a correlation coefficient of the OPLS-DA plot greater than 0.45 was considered to be significant, as described in our previous studies [15]. The OPLS-DA loading plot with tea leaves between 8- and 25-year-old tea plants collected in 2016 showed lower levels of alanine (Ala), 2-*O*-(β-L-arabinopyranosyl)-myo-inositol (Ara), caffeine, epicatechin (EC), epigallocatechin (EGC), epigallocatechin 3-*O*-(3''-*O*-methyl) gallate (EGCG3''Me), leucine, α-glucose, β-glucose, glutamine (Gln), quinate, succinate, sucrose, theanine, theobromine in tea leaves from the 25-year-old tea plants (Fig. 3b). On the other hand, the tea leaf metabolites responsible for differentiations between 8- and 25-year-old tea plants plucked in 2015 were identified in the OPLS-DA loading plot (Fig. 3d). Therefore, tea leaves collected from 25-year-old tea plants in 2015 were characterized by lower levels of acetate, catechin, GABA, gallate, theanine, glutamine, and theogallin compared to 8-year-old tea plants (Fig. 3d). Quantitative differences of individual metabolites according to the age of tea plants were calculated from a total integral area of ¹H NMR spectra corresponding to each metabolite (Fig. 4).

Discussion

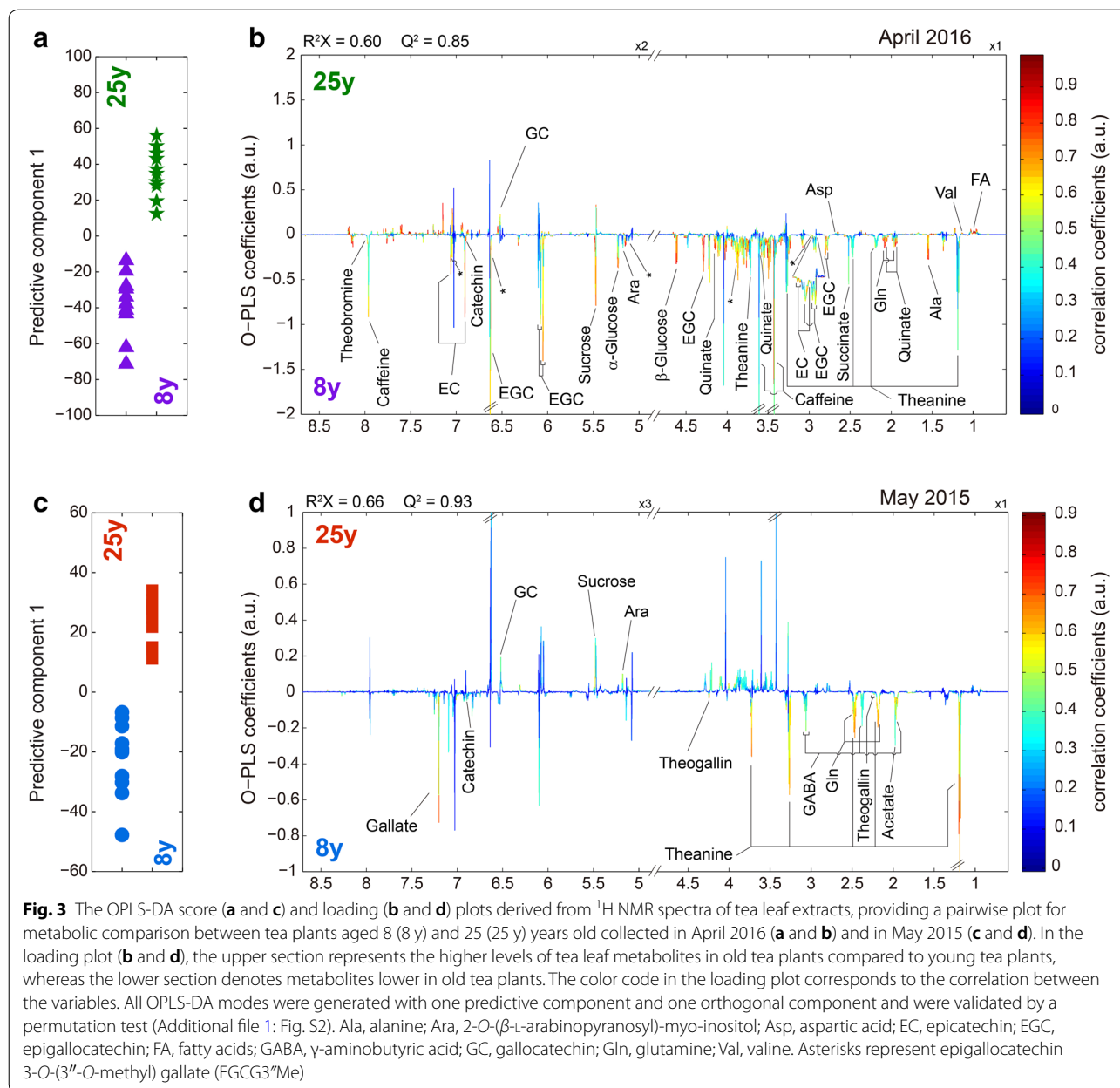
Metabolomics has already unveiled the metabolic profiles of tea leaves (*C. sinensis*), which were perturbed according to several factors, including plucking positions and seasons, climates, cultivation conditions, and cultivars [11, 13, 14, 22, 23]. However, information about the global metabolite differences between tea leaves according to the age of tea plants is very limited. Thus, in order to characterize the age-related metabolites in tea leaves, ¹H NMR-based metabolomics was employed with 8- and 25-year-old tea plants collected in 2015 and 2016.



Tea leaf metabolites associated with the age of tea plants were clearly different between 2015 and 2016, may be due to different plucking seasons of April in 2016 and May in 2015 (Fig. 3), even though the first harvest of the tea leaves is performed with unique criteria, such as amounts of total amino acids and the number of buds every year. Therefore, we speculate that the low rate of photosynthesis and then limited carbohydrate metabolism in old tea plants during April 2016, which were indicated by reduced glucose and sucrose, might reduce the synthesis of other tea metabolites, such as alanine, succinate, glutamine, theanine, EC, EGC, catechin, caffeine, and theobromine. However, many of the tea leaf metabolites not differ between 8- and 25-year-old tea plants during May 2015, perhaps because of different climate conditions between

April 2016 and May 2015. The associations between tea leaf metabolites and climate conditions in several years have been reported in our previous study [13].

Of the various tea leaf metabolites perturbed according to the age of the tea plants, theanine, glutamine, catechin, and gallic acid were uniquely dependent on the age of the tea plants (Fig. 4). Theanine in tea plants is produced from glutamic acid and ethylamine in shoots and converted into catechins in leaves [24, 25]. Therefore, high accumulations of theanine, glutamine, a precursor of glutamic acid, and catechin in leaves of young tea plants indicate reduced or lower metabolism of theanine in young tea plants than in old tea plants. On the other hand, these tea leaf metabolites would be highly metabolized in old tea plants, as evidenced by a high accumulation



of gallocatechin in leaves of old tea plants (Fig. 4P). The metabolite perturbations of tea leaves observed in different ages of tea plants in the current study should be distinct from those related to plucking positions because tea leaves collected according to plucking positions contain both young and old tea leaves [15]. For example, the first tea leaves of the five-tea leaf buds represent the youngest tea leaves, whereas the last tea leaves are the oldest tea leaves.

In conclusion, a unique metabolite marker in tea leaves to characterize old tea plants was found through a ¹H NMR-based metabolomics approach with 8- and 25-year-old tea plants. The tea leaf metabolites,

including theanine, glutamine, catechin and gallocatechin were strongly associated with the age of tea plants, demonstrating differences of theanine metabolism between young and old tea plants. The current study to characterize global metabolite differences of old tea plants and then assess their quality suggests that old tea plants would still be worthwhile for producing functional ingredients of various major catechin compounds, such as EC, EGCG, EGC, ECG, and EGCG3''Me, because their metabolisms were not dependent on the age of the tea plants, even though there are low amounts of theanine in their leaves.

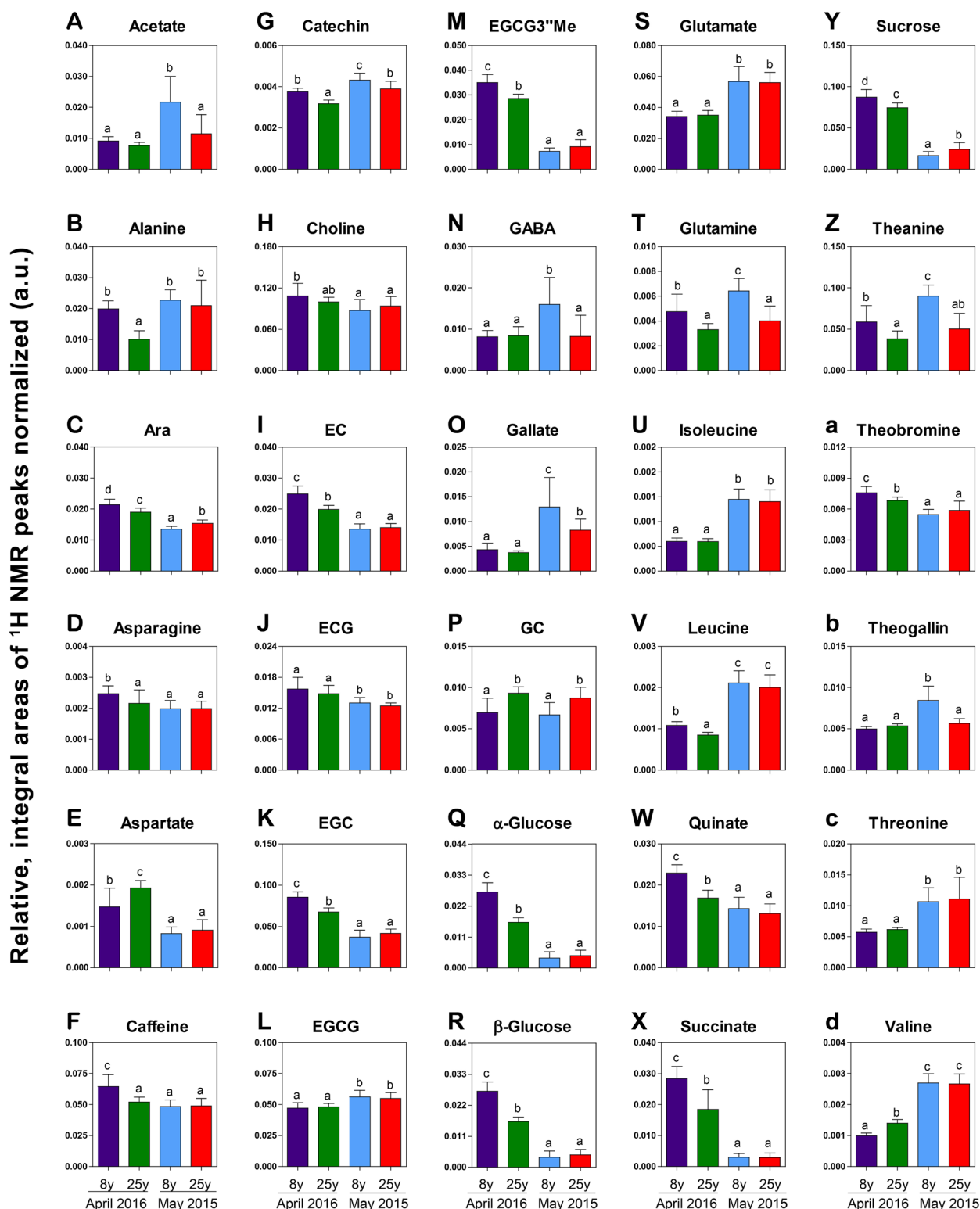


Fig. 4 Relative changes or variations of individual metabolites of fresh tea leaves from 8- and 25-year-old tea plants (April 2016) and 8- and 25-year-old tea plants (May 2015). Different small letters in the vertical graph bars of the leaves indicate significant differences between the samples, which were determined by Duncan's multiple range test at $P < 0.05$. The error bars were based on ten samples. Ara, 2-O-(β-L-arabinopyranosyl)-myo-inositol; GABA, γ-aminobutyrate; EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; EGCG3"Me, epigallocatechin 3-O-(3"-O-methyl) gallate

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s13765-020-0492-7>.

Additional file 1: Table S1. Assignments of Metabolites in the Representative ¹H NMR Spectra. **Fig. S1.** Representative ¹H 700 MHz NMR spectra of tea leaf extract from 8- and 25-year-old tea plants plucked on May 2015. **Fig. S2.** Permutation tests of PLS-DA models.

Abbreviations

CD₃OD: methanol-*d*₄; D₂O: deuterium water; Asn: asparagine; Asp: aspartate; ANOVA: one-way analysis of variance; Ara: 2-O-(β-L-arabinopyranosyl)-myo-inositol; EC: epicatechin; ECG: epicatechin gallate; EGC: epigallocatechin; EGCG: epigallocatechin gallate; EGCG3''Me: epigallocatechin 3-O-(3''-O-methyl) gallate; FA: fatty acids; GC: gallic acid; Glu: glutamine; ¹H NMR: proton nuclear magnetic resonance; HSQC: heteronuclear single quantum correlation; NOESY: nuclear Overhauser effect spectrometry; OPLS-DA: orthogonal projection on latent structure-discriminant analysis; PCA: principal component analysis; S1: sugar 1; S2: sugar 2; S3: sugar 3; STOCY: statistical total correlation spectroscopy; TOCSY: total correlation spectroscopy; 1D: one-dimensional; 2D: two-dimensional.

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Authors' contributions

MR, YSH and YRL analyzed the data and wrote the manuscript; KHH and MSL collected the tea materials; EHK performed the experiments; MR and YSH revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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