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Metabolic Evidence on Vintage Effect in Tea (*Camellia sinensis* L.) Plants

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Abstract

Recent metabolomics studies have reported diverse metabolites of tea depending on tea (*Camellia sinensis*) cultivars, cultivation conditions and geographical location. However, these studies were limited the effects of these conditions on metabolome of tea leaves in a single year. We explored the year-to-year variations in leaf metabolome of two tea (*C. sinensis*) cultivars over a period of five successive years from 2015 to 2019 to determine vintage tea products, such as in grapes or wines, and showed a clear metabolic differentiations of fresh tea leaves. Also, the best conditions of climate were suggested through an association of rainfall and sun-expose time with the metabolism of theanine in taste- or flavor-rich tea cultivar and of catechin compounds in EGCG3″Me-rich tea cultivar, thereby providing the potential vintage tea tailored to the cultivar. Since vintage wine is derived from grapes grown in a year under good climatic conditions, which provides high quality of wine in the best year, the current result highlights important information relevant to tea metabolome associated with climatic conditions in a specific year and the manufacture of vintage tea with unique quality.

Keywords Tea, *Camellia sinensis*, Vintage, Metabolomics, Climate, NMR

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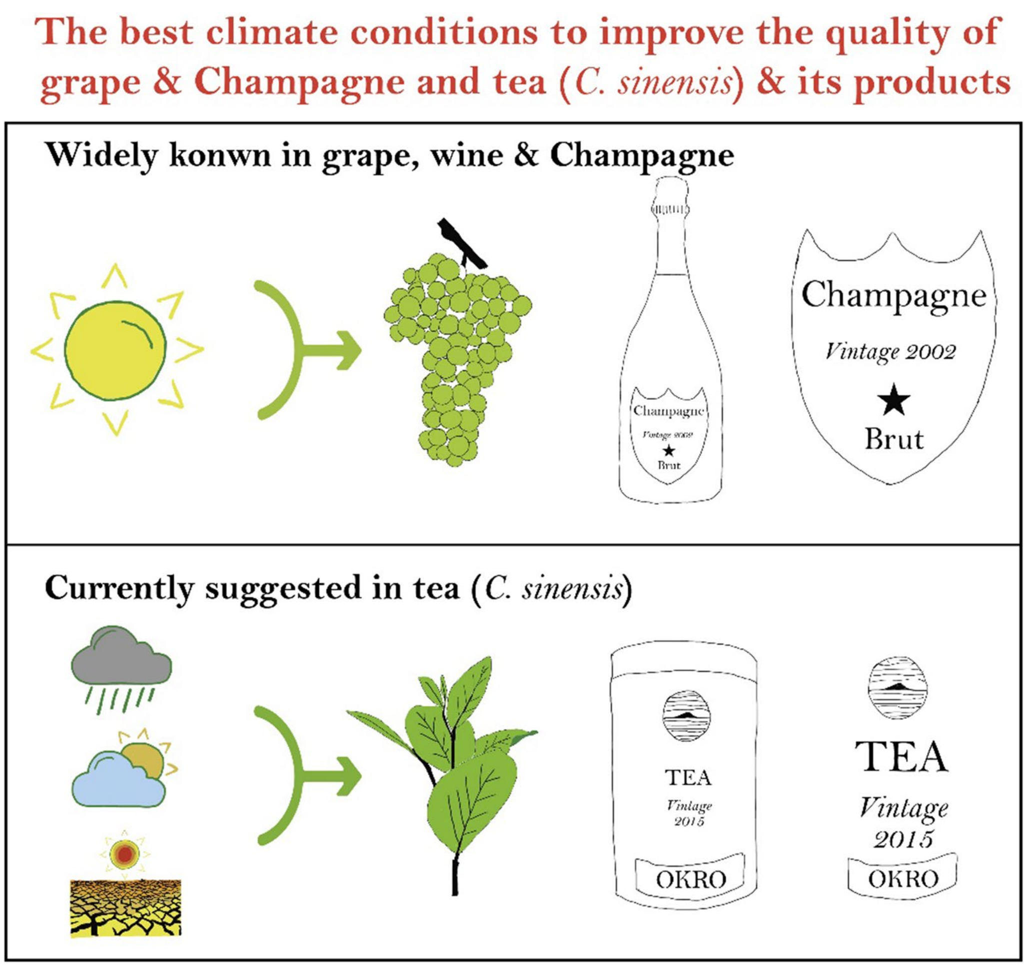
Graphical Abstract



Introduction

Tea (*Camellia sinensis*) is one of the most widely consumed beverages worldwide, due to its unique flavor, taste, and thirst-quenching properties [1]. Tea contains substantial amounts of antioxidative, anti-inflammatory, anticarcinogenic, and neuroprotective substances that contribute to putative health benefits and most likely ameliorate multiple health conditions such as obesity, diabetes, hypertension, heart disease, and cancer [2]. These putative health benefits are primarily attributed to the major bioactive components including catechin and its derivatives such as catechin gallate (CG), epicatechin (EC), epigallocatechin (EGC), epigcatechin-3-gallate (ECG), epigallocatechin-3- gallate (EGCG), and gallocatechin (GC) occurring in the tea. Besides, tea contains caffeine, theanine and other minor compounds including sugars, organic acids and amino acids [3]. These chemical compounds or metabolites collectively contribute to the taste of tea infusion for human consumption and biofunction-enhanced tea for tea-based cosmetics and food ingredients, based on genetic, environmental and cultivation factors [4, 5].

Metabolomics is a robust and unbiased analytical tool, which can be used to successfully unveils the comprehensive physiological and biochemical mechanisms in different food matrices [6, 7]. Several metabolomic studies have already reported the quantitative association between metabolite profiles and the quality of fresh tea leaves. The quality attributes of fresh tea leaves depend upon cultivars [8], shading [9] or light [10], pruning and unpruning [11], grafting [12], tea processing [13], plucking season [14, 15], plucking position [16], climate [17], and the country of origin [18]. In particular, using the ^{1}NMR-based metabolomics approach, the overall quality of tea leaves grown in three tea growing regions in South Korea was highlighted [19], in addition to that of tea grown in China, Japan, and South Korea [20] reflected by the metabolic response of tea leaves to climatic conditions. These studies suggest that the relationship between climatic factors and tea metabolites characterize the 'terroir' that has been globally utilized in viticulture [21] or wine science [22]. Moreover, the concept of 'terroir' refers to a complex interplay between environmental, genetic, and human factors in viticulture or wine science [23]. The winemakers or vintners have advanced climate metrics to produce 'vintage wine' that is manufactured from grapes grown and harvested in a single year of good climatic condition for grape ripening, which provides high-quality wine in a good year [24]. Studies suggest that a better evaluation of meteorological data in wine quality harvested in different vintages could lead to enhanced management of wine market, and the use of additional climate information meticulously provides reliable comprehensive wine metabolite data to determine the best wine and grape [25, 26]. To date, no study has reported the link between climatic factors and quality of tea (*C. sinensis*) grown in several years of vintage. Thus, an ^{1}H NMR-based metabolomic approach was used to explore the association of tea metabolome with climatic variables in tea plants grown in multiple years as well as in other natural products derived from plants.

In the present study, the metabolic variations of fresh tea leaves of two cultivars (*C. sinensis*) collected every year from 2015 to 2019, were investigated to identify 'vintage tea' that yields the best quality in a good year of vintage. Further, this study was designed to determine the key climatic factors associated with vintage tea.

Materials and methods

Chemicals

All chemical reagents used in the current study were "American Chemical Society (ACS) Reagent Grade". 3-(trimethylsilyl) [2,2,3,3-$^2H_4$] propionate (TSP, 97%) and solvent deuterium oxide (D_2O, 99.9% ^{2}H) were obtained from Sigma (St. Louis, MO, USA).

Tea samples and plucking time

Tea plants of two cultivars, *Camellia sinensis* var. sinensis cv. Jangwon 2 and Jangwon 3 were produced by asexual propagation from wild type tea cultivar, (*C. sinensis* var. Yabukita) at the same tea garden in the Seogwang area (33° 180′ 17.67″ N, 126° 17′ 42.97″ E), Jeju, South Korea. Metabolic phenotypes of these two cultivars of Jangwon 2 and 3 have been reported via comparison with wild type tea cultivar [8]. Fresh tea leaves of each cultivar were plucked randomly from 10 different locations or parcels throughout the tea farm between 2015 and 2019 to improve reliability of metabolic variations with biological replications. All the tea leaves were collected with dry ice immediately after plucking and stored at -80 °C until further analysis.

Information regarding plucking time, the first analysis and reanalysis of tea leaves by ^{1}H NMR spectroscopy are presented in Fig. 1. The initial plucking time of the tea leaves every year is determined by unique criteria, such as amounts of total amino acids and the number of buds [20]. Jangwon 2 and 3 tea cultivars was firstly plucked between 2015 and 2019 as follows: May 14, 2015; May 3, 2016; May 11, 2017; May 10, 2018, and May 10, 2019. However, in 2019, Jangwon 3 tea cultivar was firstly plucked in July, even though Jangwon 2 tea cultivar was plucked in May. All freeze-dried tea leaves of each cultivar were stored at − 80 °C after the first analysis by ^{1}H NMR spectroscopy in the plucked year and analyzed again in 2020.

Climatic parameters

Daily climatological parameters such as average temperature, total rainfall and total sun exposure time during the cultivation periods were considered as 'terroir' or important environmental factors for tea growing in the current study. Annual data on average temperature, total rainfall and total sun exposure time were also calculated from the daily climatic data to compare the usual climatic pattern in the tea-growing area, Jeju-do, South Korea between 2014 and 2019. All daily climatological data was obtained from the tea garden recorded by an automatic weather station.

Extraction of tea leaves

Tea leaves were extracted according to the protocol reported previously [27]. First, the frozen tea leaves were separated from their stems and ground with a pestle and mortar under liquid nitrogen. The ground tea leaves were transferred into a plastic tube, stored in a deep freezer for 24 h, and then freeze-dried for 48 h. Freeze-dried tea samples were stored at − 80 °C until analysis. For extraction,10 mg of freeze-dried samples were dissolved in a mixture of methanol-d_4 (CD_3OD, 490 μL) and deuterium water (D_2O, 210 μL) in a 1.5 mL Eppendorf tube. Th supernatants were extracted from the mixtures via sonication (25 °C for 20 min) followed by centrifugation (13000 rpm for 15 min at 10 °C). Finally, the resultant tea extracts or supernatants (550 μL) of the tea leaf extracts were transferred to 5 mm NMR tubes.

^{1}H NMR spectroscopic analysis of tea extracts

The ^{1}H NMR spectrum of tea leaf extract was acquired on a Bruker Avance 700 spectrometer (Bruker Biospin, Gmbh, Rheinstetten, Germany) operating at 700.40 MHz

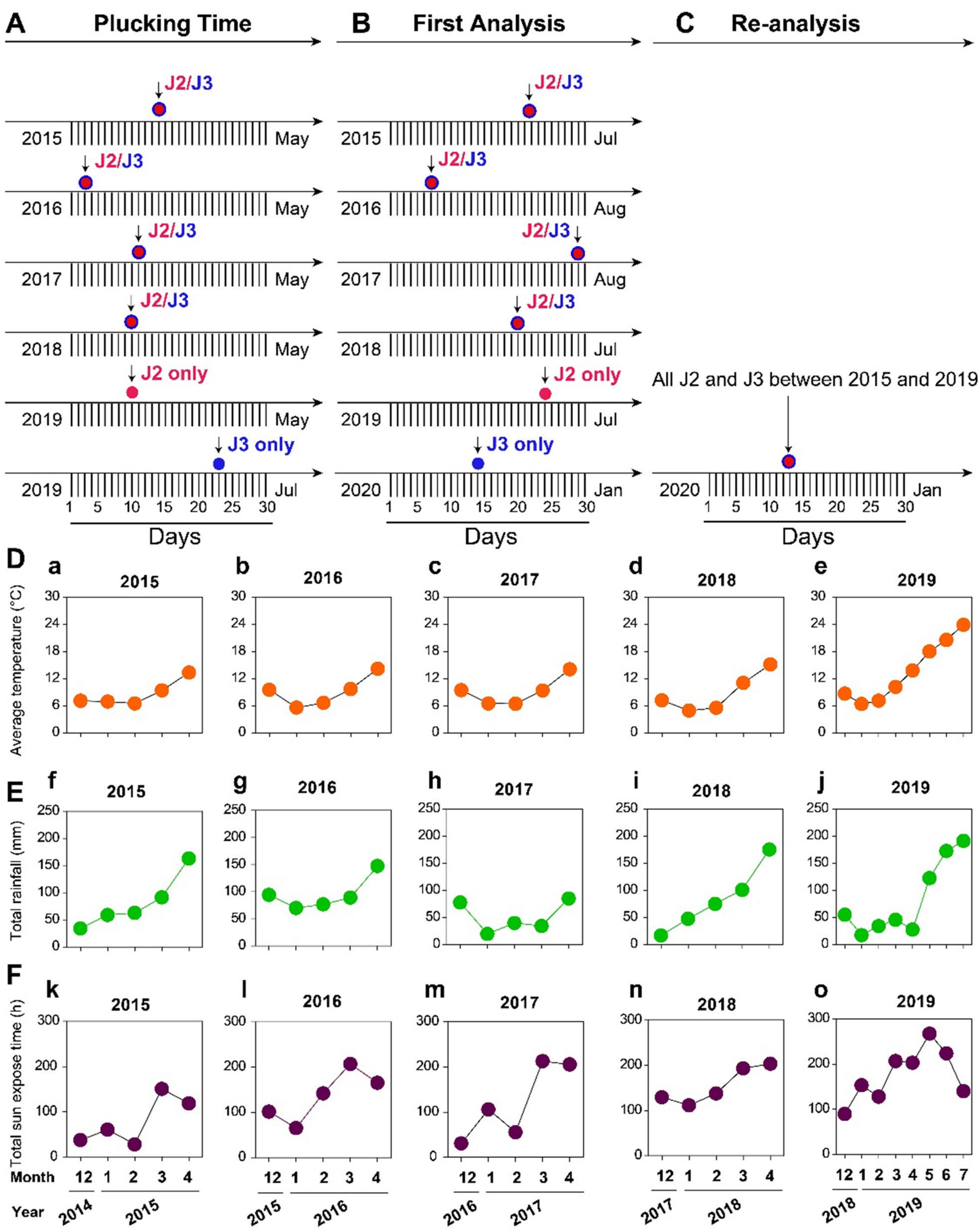


Fig. 1 Plucking time (**A**), the first analysis by ^{1}H NMR spectroscopy (**B**) of fresh tea leaves between 2015 and 2019, and the reanalysis of all tea leaves after storage at – 80 °C (**C**). Climate conditions of the tea growing areas in the Seogwang area of Jeju-do, South Korea (**D** – **F**). Monthly average temperature (℃, **D**), total rainfall (mm, **E**) and total sun exposure time (h, **F**) recorded from December to April in 2014–2019. All daily climatological data were recorded by an automatic weather station at the tea farm. J2, Jangwon 2 tea cultivar; J3, Jangwon 3 tea cultivar

^{1}H frequency and a temperature of 298 K, equipped with a cryogenic triple-resonance probe and a Bruker automatic injector. The one-dimensional (1D) NMR spectrum of tea leaf extract was acquired with 1D nuclear Overhauser effect spectrometry (NOESY) pulse sequence equipped with water presaturation. The signal assignment of 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 shift reported in previous works or database [8, 9, 16, 20]. Furthermore, one-dimensional statistical total correlation spectroscopy (STOCSY) was also used for the signal assignment [17].

NMR data processing and multivariate statistical analysis

All ^{1}H NMR spectra acquired from tea leaf extracts were adjusted manually for phase and baseline distortions utilizing TOPSPIN software (Version 4.0.7 Bruker Biospin, Rheinstetten, Germany) and then transformed into ASCII format followed by import into MATLAB (R2010b The Mathworks, Inc., Natick, MA). To align the 1D NMR spectra with full resolution, the interval Correlation Optimized shifting (*i*coshift) method [28] was applied without bucketing or binning. Following successful alignment, the spectral regions of 1D NMR corresponding to solvent methanol (3.36–3.40 ppm) and residual water (4.80–4.90 ppm) were removed. In order to avoid the dilution effects of tea extracts and the effects of metabolites, a total integral normalization of all NMR spectra was carried out followed by probabilistic quotient normalization of the NMR spectra using the median spectrum [29]. Subsequently, the resulting datasets, that consisted of the relative intensities of all ^{1}H NMR peaks, were imported into statistical software SIMCA-P version 15.02 (Umetrics, Umeå, Sweden) and subjected to a mean centering scaling method for multivariate statistical analysis. Initially, an unsupervised pattern recognition method known as the principal component analysis (PCA) was performed to analyze the intrinsic variation in the dataset. A supervised pattern recognition method, orthogonal projection on latent structure-discriminant analysis (OPLS-DA) was the performed to extract maximum information from the discriminant compounds based on the NMR spectra of tea extracts [30]. The pairwise comparisons between the two groups of classes visualized in OPLS-DA loading plots were created using MATLAB (The Mathworks, Inc.) with an in-house script developed by Imperial College London, UK. The OPLS-DA loading plots obtained corresponded to the correlation coefficients between variables and classes, and were combined via back-transformed loading together with variable weight. In the OPLS-DA model, a color code expressing the concentration variation and discrimination weights between the classes corresponds to the square correlation coefficient of the OPLS-DA loading as described by Cloarec et al., (2005) [31]. The OPLS-DA model was validated by a permutation test consisting of 200-fold repetitions along with a sevenfold cross-validation. The quality of the OPLS-DA model in the current study was assessed by the values of Q^2 and R^2X. The Q^2 values obtained 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 less than the Q^2 of the real model, the model was regarded as a predictable model. R^2X was used to evaluate the possible overfitting of the model. On the other hand, R^2X is defined as the proportion of variance in the data described by the models and indicates goodness of fit, and Q^2 denotes the proportion of variance in the data predicted by the model and indicates predictability.

Statistical analysis

Statistical analyses of the data were performed using the SPSS software package (IBM SPSS Statistics ver. 23; SPSS Corp., USA). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was conducted to ascertain the significance of the relative contents of tea metabolites. A probabilistic value of $P < 0.05$ was considered as statistically significant. The integral area of the peaks identified in 1D ^{1}H NMR, which corresponded to non-overlapped individual metabolites, was analyzed to quantify the relative levels of individual metabolites. Further, heat-map analysis with quantified levels of the individual lea metabolites was performed using MetaboAnalyst 4.0 [32], which is an open bioinformatics web site for metabolite data interpretation (https://metaboanalyst.ca/Metaboanalyst/home.xhtml).

Results and discussion

As metabolomics studies coupled with multivariate statistical analysis have reported the effects of vintage year on wine classification via a quantitative correlation between wine or grape metabolites and climatological data [25, 26, 33, 34], we identified comprehensive tea leaf metabolites by using structural data of 2D NMR experiments and the data from spiking experiments in our previous stuies [8, 9], and explored their associations with climatic conditions over five successive years.

Identification of global tea metabolites by ^{1}H NMR spectroscopy

In the current study, fresh leaves of two tea cultivars plucked in five successive vintages from 2015 to 2019 were analyzed by ^{1}H NMR spectroscopy to provide a multivariate dataset with global tea leaf metabolome.

The representative ^{1}H 700 MHz NMR spectra of tea leaf extracts from Jangwon 2 and 3 tea cultivars plucked in 2015 (**A**), 2016 (**B**), 2017 (**C**), 2018 (**D**), and 2019 (**E**) are presented in Additional file 1: Figs. S1 and S2 of the Supporting Information. A diverse range of tea metabolites including catechin and its derivatives, catechin gallate (CG), epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), epigallocatechin 3-gallate (EGCG), epigallocatechin-3-*O*-(3-*O*-methyl)-gallate (EGCG3"Me) and gallocatechin (GC), the most predominant theanine and caffeine, and other primary and secondary metabolites including acetate, alanine, 2-O-(*β*-L-Arabinopyranosyl)-myo-inositol (Ara), asparagine (Asn), aspartate (Asp), choline, unsaturated fatty acids (FAs), gallate, γ-aminobutyric acid (GABA), α-glucose, β-glucose, glutamate, isoleucine (Ile), leucine (Leu), quinate, sugars, sucrose, succinate, theobromine, theogallin, threonine, and valine (Val) were identified in 1D ^{1}H NMR spectrum. Tea leaf metabolites were assigned by spiking with the pure chemicals and also by comparing with published data. Moreover, the metabolite assignment was validated via 2D TOCSY and HSQC NMR as described in our previous studies [13, 16]. Furthermore, since NMR analysis of fresh tea leaves were carried out throughout 5 years, reproducibility of NMR analysis should be reliable for unbiased comparisons of the tea leaf metabolites analyzed every year. In the present study, decreased in the levels of fatty acids in the tea leaf stored for 5 years at − 80 °C were observed (Additional file 1: Fig. S5) possibly due to their auto-oxidation, but other tea leaf metabolites were not significantly changed. These results were discussed in the Supporting Information.

Metabolic differentiation of tea leaves according to tea cultivars and vintages

The pattern recognition methods based on multivariate statistical analysis such as PCA and OPLS-DA were applied to the entire ^{1}H NMR spectrum dataset to visualize the global metabolite variations of the two tea cultivars, Jangwon 2 (J2) and Jangwon 3 (J3), throughout the five successive vintage from 2015 to 2019 (Fig. 2). As shown in Fig. 2A, the PCA score plots with two principal components (PC1; 39.7% and PC2; 17.8%) reflected a discrete trend among tea samples, highlighting clear metabolic separation between Jangwon 2 and Jangwon 3 tea cultivars, and metabolic dependences of tea leaves on vintages between 2015 and 2019. Moreover, both in the two cultivars, the supervised OPLS-DA models revealed the greatest metabolic separation between the observation groups by eliminating the noncorrelated variation in *X* variables (metabolites) that is orthogonal to *Y* variables (year of vintage or cultivar) as shown in Fig. 2B. Distinct metabolites in tea leaves between the Jangwon 2 and 3 tea cultivars in the single year have been identified in our previous study [8]. The metabolic separations in OPLS-DA score plots within each tea cultivar might indicate significant effects of environmental conditions on metabolic variations for the five consecutive years (Fig. 2C and D).

Heatmaps were generated to determine the effects of different vintage years on metabolic changes in Jangwon 2 and Jagnwon 3 tea samples (Fig. 3). The colored box in the heatmap indicates significant differences among tea samples, in which red color indicates higher levels of metabolites and blue color refers lower amounts of metabolites, compared to mean value. The heatmap presented a clear intuitionistic visualization of various metabolites in Jangwon 2 tea cultivars harvested between 2015 and 2019 (Fig. 3A). For example, ECG, acetate, choline, amino acids such as alanine, GABA, glutamate, isoleucine, leucine, theanine, threonine and valine, and phenolic acids such as gallate, quinate, and theogallin were more abundant in 2015 vintage, whereas the amounts of catechin, GC, glutamine, caffeine, sugars (glucose and sucrose) and inositol glycoside (Ara) were the highest in 2019 vintage. An intuitionistic visualization of variation in tea leaf metabolites of Jangwon 3 cultivar plucked in 2015–2019 indicated that the chemical composition of tea leaves clearly depended on the year of vintage (Fig. 3B). Moreover, the heatmap showed that the diverse metabolites between the two tea cultivars was variably regulated. Thus, the heatmap results clearly indicates that the different climate conditions in multiple harvesting years affected tea metabolites differently.

To further identify the role of tea leaf metabolites in the metabolic differentiations of Jangwon 2 tea samples collected from 2015 to 2019, pairwise OPLS-DA models corresponding to ^{1}H NMR spectra of tea leaves were constructed (Fig. 4). The pairwise OPLS-DA models were effective in identifying the different metabolites between 2015 and 2016 vintage teas (Fig. 4A and B), 2016 and 2017 vintage teas (Fig. 4C and D), 2017 and 2018 vintage teas (Fig. 4E and F) and 2018 and 2019 vintage teas (Fig. 4G and H), which is explained by goodness of fit ($R^2X = 0.38$, 0.38, 0.45 and 0.71, respectively) and strong predictability ($Q^2 = 0.80$, 0.91, 0.93 and 0.95, respectively). OPLS-DA models were also generated to further identify diverse metabolites responsible for the differentiation of Jangwon 3 tea samples (Additional file 1: Fig. S6). All OPLS-DA models were validated using permutation tests reiterated 200-fold (Additional file 1: Figs. S8 and S9). Significant differences in tea leaf metabolites responsible for the differences between the tea samples were described by distinct colors in OPLS-DA loading plots, and a correlation coefficient higher than 0.45 was considered significant as explained in our previous studies [13, 16].

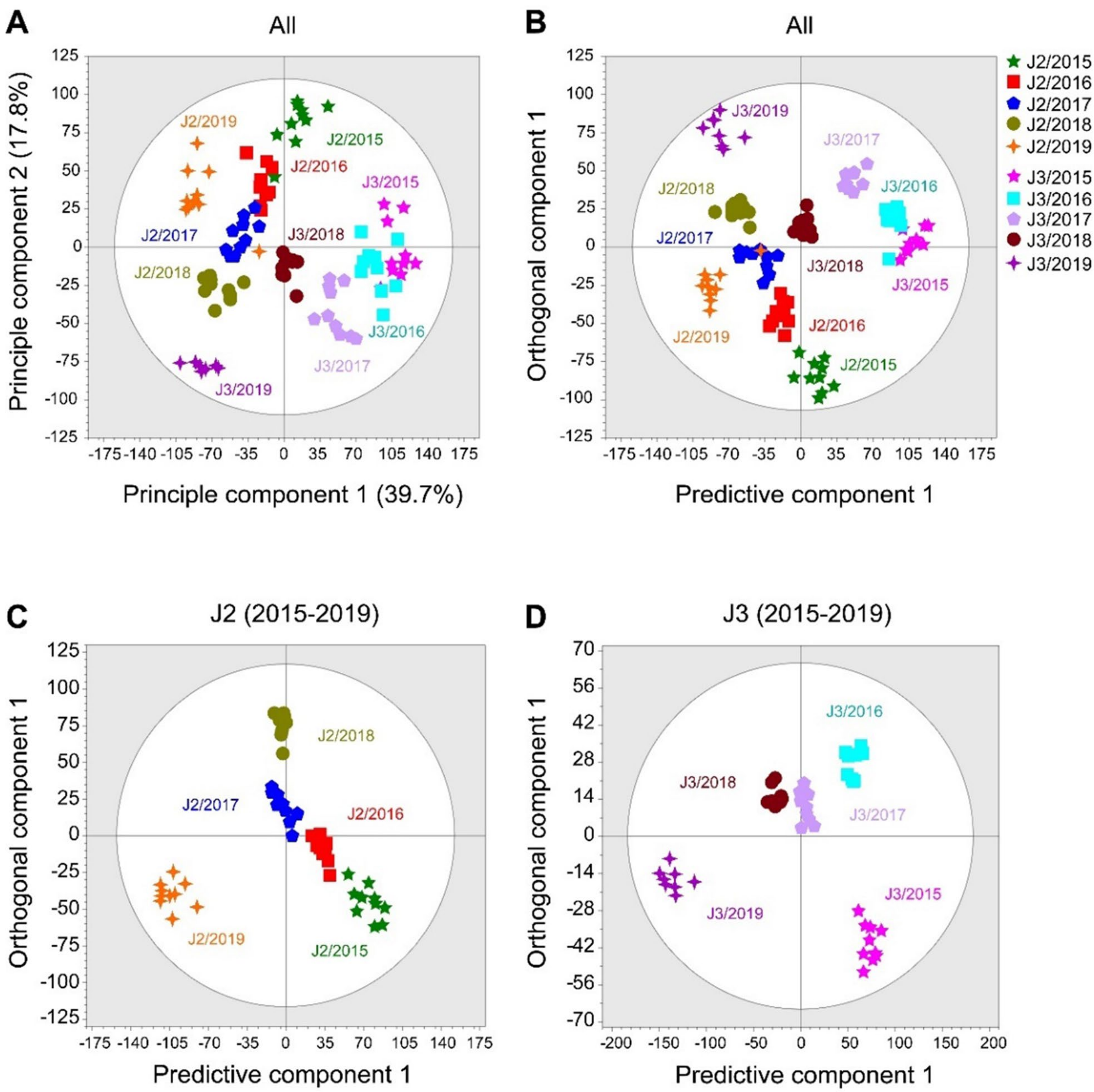


Fig. 2 Principal component analysis (PCA, **A**) and orthogonal partial least squares discriminant analysis (OPLS-DA, **B**, **C** and **D**) score plots derived from the first-analyzed ^{1}H NMR spectra of fresh tea leaf extracts obtained from Jangwon 2 (J2) and Jangwon 3 (J3) tea cultivars plucked in 2015 to 2019

In the case of Jangwon 2 tea cultivar, more levels of EC, EGC, catechin, sucrose, glucose, Ara, and asparagine, and lower levels of ECG, EGCG, gallate, theogallin, alanine, theobromine, isoleucine, leucine, valine, threonine, GABA, glutamine, choline and acetate were found in tea leaves plucked in 2016 vintage compared with 2015 vintage (Fig. 4B). Compared with tea leaves collected in 2017 vintage, the levels of EC, theobromine, and glucose were lower in tea leaves collected in 2016 vintage, whereas the levels of fatty acids, theanine, theogallin, quinate, acetate, glutamine, succinate, GABA, and gallate were higher (Fig. 4D). In the metabolic comparison between 2017 and 2018 vintages (Fig. 4F), the OPLS-DA loading plot showed lower levels of theanine, valine, alanine, quinate, acetate, theogallin, aspartate, glutamine, choline, caffeine, Ara, EGC, GC, EC,

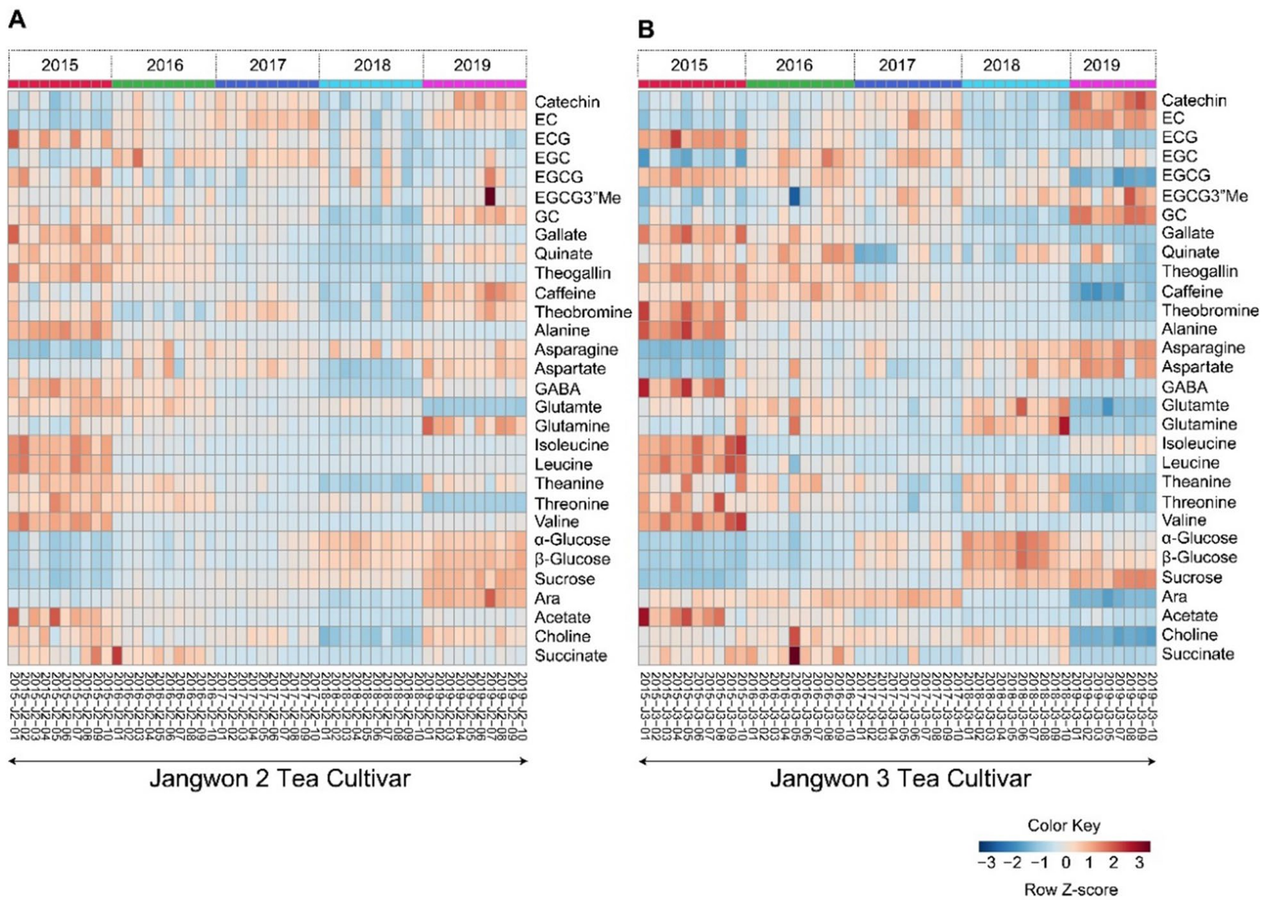


Fig. 3 Heatmap with quantified metabolites in Jangwon 2 (**A**) and Jangwon 3 tea (**B**) cultivars plucked between 2015 and 2019. Columns and rows represent individual metabolites and different tea samples, respectively. The relative abundance box (red indicates higher and blue denotes lower) is generated from integral areas of the peaks identified in 1D ^{1}H NMR corresponding to non-overlapped individual metabolites. Data scaling was based on mean-centered scale and similarity assessment for clustering was based on the Euclidean distance coefficient and the Ward linkage method. *Ara* 2-O-(*β*-L-Arabinopyranosyl)-myo-inositol, *EC* epicatechin, *ECG* epicatechin gallate, *EGC* epigallocatechin, *EGCG* epigallocatechin gallate, EGCG3″Me, epicatechin 3-*O*-(3-*O*-methyl)-gallate, *GABA* γ-aminobutyrate, *GC* gallocatechin

theogallin, and gallate in tea leaves collected in 2018 vintage compared with those in 2017 vintage. Further, the OPLS-DA loading plot revealed that tea leaves from 2018 vintage contained higher levels of ECG, fatty acids, glutamine, ad threonine, compared with 2019 vintage that was characterized by higher levels of theobrombine, caffeine, gallate, EC, GC, catechin, sucrose, glucose, Ara, choline, GABA, aspartate, succinate, theanine, acetate, quinate, alanine, and valine (Fig. 4H). Quantitative results of tea leaf metabolites of Jangwon 2 and Jangwon 3 tea cultivars during the five successive vintages from 2015 to 2019 are given in Fig. 5, together with their statistical analysis.

(See figure on next page.)

Fig. 4 OPLS-DA score (**A** – **G**) and loading (**B** – **H**) plots derived from ^{1}H NMR spectra of tea leaf extracts, providing a pairwise plot for metabolic comparison between leaves of Jangwon 2 tea cultivar harvested from vintage years 2015 and 2016 (**A** and **B**), 2016 and 2017 (**C** and **D**), 2017 and 2018 (**E** and **F**) and 2018 and 2019 (**G** and **H**). In the loading plot (**B**), the upper section represents metabolites higher in tea leaves from 2016 vintage compared to tea leaves in 2015 vintage, whereas the bottom section denotes metabolites lower in tea leaves in 2016 vintage tea plants. The color code in the loading plot corresponds to the correlation of the variables. All OPLS-DA modes were generated with one predictive component and one orthogonal component and validated by a permutation test (Additional file 1: Fig. S8). Ala, alanine; *Ara* 2-O-(*β*-L-Arabinopyranosyl)-myo-ino sitol, *EC* epicatechin, *ECG* epicatechin gallate, *EGC* epigallocatechin, *EGCG* epigallocatechin gallate, EGCG3″Me, epicatechin 3-*O*-(3-*O*-methyl)-gallate, *GABA* γ-aminobutyrate, *GC* gallocatechin, *Glu* glutamate, *Gln* glutamine, *Ile* isoleucine, *Leu* leucine, *ThG* theogallin, *Thr* threonine, *Val* valine

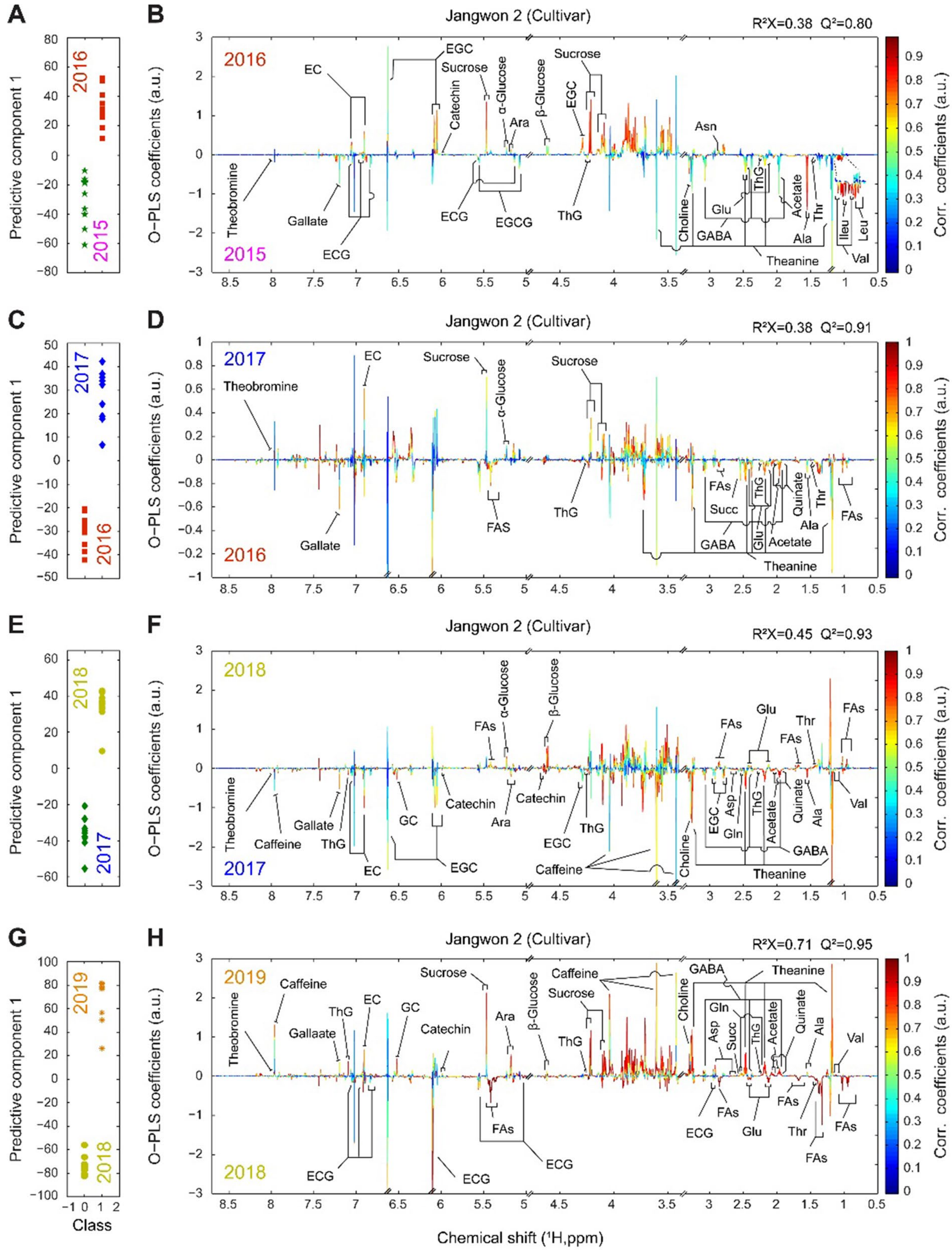


Fig. 4 (See legend on previous page.)

Differential metabolic response of two tea cultivars to climatic conditions in a given vintage

Identification and development of new tea cultivars with country-specific metabolic traits and taste properties are increasingly attracting the attention of researchers and the tea producers. In particular, the contents of catechins and theanine in various tea cultivars originating in different tea manufacturing countries widely varied and were mostly dependent on geographical origin, plucking position, climatic factors and tea cultivars [35, 36]. Metabotyping of three tea (*C. sinensis*) cultivars rich in taste (Jangwon 2), EGCG (Jangwon 1) and EGCG3"Me (Jangwon 3) has highlighted their intrinsic metabolic traits based on differences in the metabolisms of individual catechin and theanine compounds in each cultivar [8]. This study further reported distinct EGCG3"Me metabolism in Jangwon 3 tea cultivar compared with Chinese *C. sinensis* var. *sinensis* cv. Qingxin-wulong [36] and Japanese *C. sinensis* var. *assamica* cv. Benifuuki tea cultivar [37]. Hence, the current study further investigated the metabolic differences between Jangwon 2 and 3 tea cultivars grown in the each given year of the five successive years. Remarkably, a wide range of metabolites such as ECG, EGCG3"Me, caffeine, theobromine, theanine, glutamine, GABA, sucrose, Ara, acetic acid, and succinic acid were unique discriminators (ANOVA with post hoc, $P<0.05$) between Jangwon 3 and Jangwon 2 tea cultivars plucked in all given years from 2015 and 2019 (Fig. 5). In particular, the levels of ECG and EGCG3"Me were more abundant in Jangwon 3 tea cultivar during all the successive years, whereas the levels of sugars were higher in Jangwon 2 tea cultivar, indicating the distrinct intrinsic differences in metabolism between the two tea cultivars (Fig. 5C, F, X, Y, and Z).

Moreover, across the five successive years, the levels of theanine, glutamine, GABA, acetate, and succinate in Jangwon 2 tea cultivar were comparatively higher than in the Jangwon 3 tea cultivar except for 2018 (Fig. 5). These differences in the levels of catechin and its derivatives, theanine, and other metabolites between the two tea cultivars have been previously reported in a single year [8], which again demonstrated the intrinsic differences in metabolism of Jangwon 2 (the rich-taste) and 3 (rich in EGCG3"Me) tea cultivars and provided evidence supporting their individual metabolic characteristics. Furthermore, it was interesting to note that Jangwon 2 and 3 tea cultivars exhibited abnormal metabolic differences in 2018, compared with other years. For example, the theanine content of the leaves of Jangwon 3 tea cultivar was higher than that of Jangwon 2 tea cultivar, along with even higher amounts of catechin and its derivatives in Jangwon 3 tea cultivar (Additional file 1: Fig. S7). In general, catechins and theanine in the tea leaves have an inverse association as reported throughout plucking seasons [38] and between different tea cultivars in each vintage (Additional file 1: Fig. S7A–F), but does not in the tea leaves from 2018 (Additional file 1: Fig. S7H), which might be caused by abnormally and markedly high rainfall in 2018 (Fig. 1E). Indeed, the common reversal associations of catechin compounds with theanine between Jangwon 2 and 3 tea cultivars was also observed in 2019 (Additional file 1: Fig. S7J), even a very low rainfall in April of 2019. Therefore, it was concluded that Jangwon 2 and 3 tea cultivars might vary in their response to high rainfall, compared with drought conditions. This different metabolic responses between pruned and unpruned tea plants to abnormal climatic conditions of high rainfall was reported in our previous study [38].

Distinct metabolism in tea leaves according to climate in each vintage

We explored metabolic variations in two tea cultivars, Jangwon 2 and 3, over the five successive years, to determine the good vintage for tea leaves of high quality. Vintage is the year, especially in which wine of high quality, is produced and thus a wine of high quality or vintage wine is made from grapes harvested in a good year with good weather because sunny days give grapes the best chance of reaching full maturity and good ripeness. Year of production in wine, red and white wines, is commonly printed on the bottle when wine is produced from grapes picked in a single year. Therefore, year is always printed on the bottles of red and white wines, which not indicated 'vintage wine' produced in the good year. Indeed,

(See figure on next page.)

Fig. 5 Relative amounts of individual metabolites of fresh tea leaves from Jangwon 2 and Jangwon 3 tea cultivars collected in vintage years 2015, 2016, 2017, 2018 and 2019, respectively. The different small and capital letters in the vertical graph bars of each panel indicate significant differences in the level of each metabolite between the five vintage years of Jangwon 2 and Jangwon 3 tea cultivars, respectively. Asterisks represent significant differences in the levels of each tea metabolite between Jangwon 2 and Jangwon 3 tea cultivars in the same vintage year. Statistically significant differences in relative amounts of tea metabolites were determined by one-way ANOVA with Duncan's multiple range test at $P<0.05$ and paired *t*-test. Small and large letters denote statistical differences in Jangwon 2 and 3 tea cultivars, respectively, and asterisks indicate statistical differences between Jangwon 2 and 3 tea cultivars in the given vintage. Ala, alanine; *Ara* 2-O-(*β*-L-Arabinopyranosyl)-myo-inositol, *EC* epicatechin, *ECG* epicatechin gallate, *EGC* epigallocatechin, *EGCG* epigallocatechin gallate, EGCG3"Me, epicatechin 3-*O*-(3-*O*-methyl)-gallate, *GABA* γ-aminobutyrate, *GC* gallocatechin

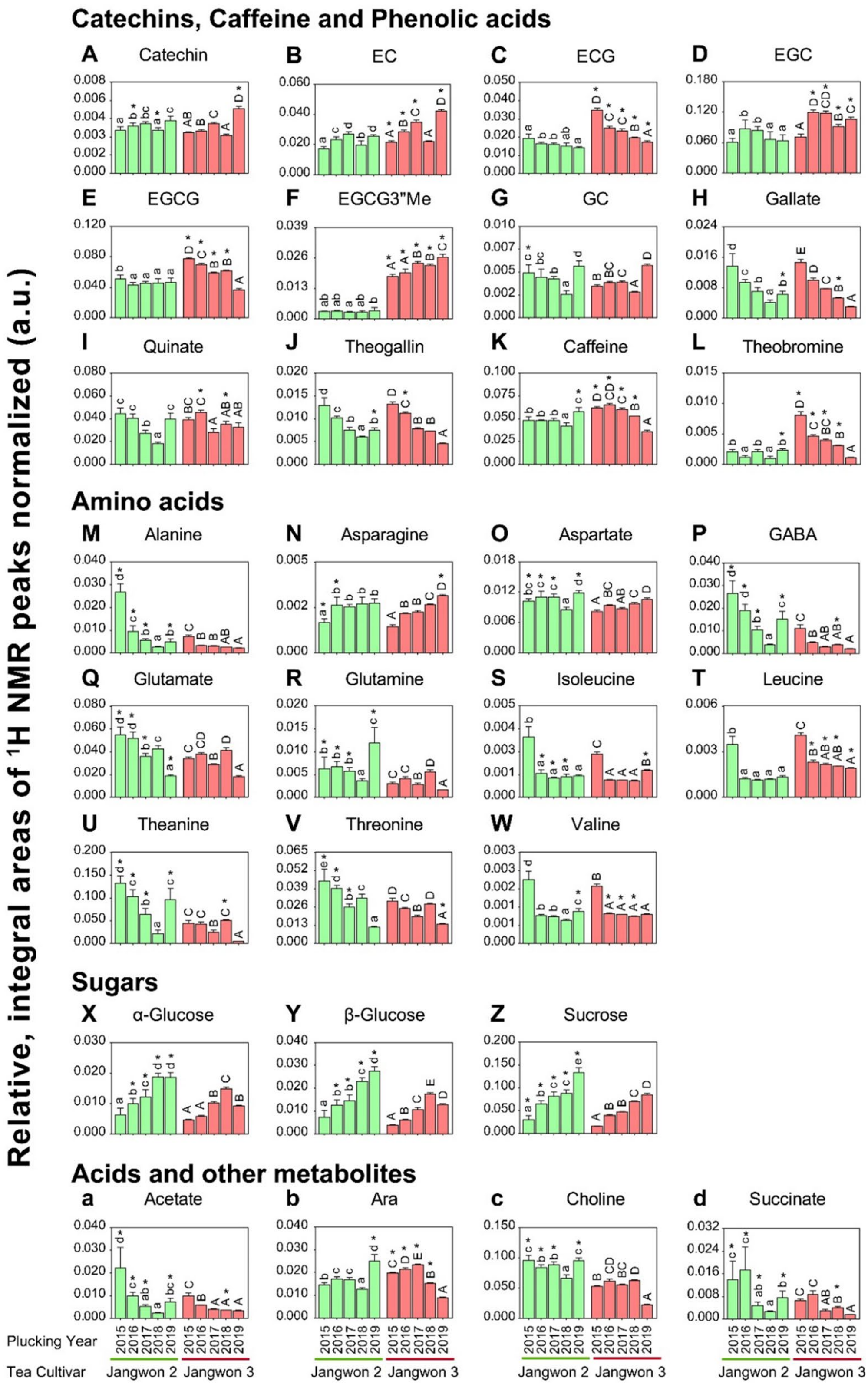


Fig. 5 (See legend on previous page.)

information on vintage of wines depends on description of wine producers. However, in the case of Champagne wine, production year of non-vintage Champagne is not printed on the bottles but displayed together with 'vintage' term like 'Vintage 2019' when Champagne is produced with grapes picked in the best year of climatic conditions. Baciocco et al. [24] have reported that, in the comparisons between consensus vintage rankings of red and white wines from 1961 to 2009 in Bordeaux, France, good vintages exhibited higher heat accumulation and lack of rainfall, and suggested that mean maximum temperature was an important discriminator between good and poor vintages.

In the current study, we dedicated the metabolism of theanine in Jangwon 2 tea cultivar across the five successive years because the tea cultivar is rich in theanine. As described in our previous study [39], the theanine content was low in 25-year-old than in 8-year-old tea (*C. sinensis*) plants. However, the theanine levels in the current study were not dependent on the age of the tea plants, which varied during the five successive years from 2015 to 2019, in both Jangwon 2 and 3 tea cultivars. These findings demonstrated that metabolic variations in tea leaves during the five successive years mainly affected by climatic conditions, rather than the age of tea plants. The lowest amounts of theanine were found in tea leaves of Jangwon 2 cultivar plucked in 2018 (Fig. 5U). At April in 2018, the highest rainfall and the longest sun exposure time were comparable with other vintages (Fig. 1E). These climatic conditions were negatively associated with the amounts of theanine and thus could be a bad weather for Jangwon 2 tea cultivar. In 2015 of the five successive years, the highest amounts of theanine were positively be associated with high rainfall under short sun exposure time. Moreover, positive correlations between theanine levels and extreme low rainfall, close to drought, during spring of plucking season in 2019 were also observed in Jangwon 2 tea cultivar. Theanine is an amino acid responsible for the full-bodied flavor, sweetness and exotic taste of tea. Theanine is known to be degraded by light and thus its levels are increased in shady growing conditions [9]. Furthermore, in summer season of July, large reductions of theanine and elevations of GC, EGCG, ECG, EGC, EC, and catechin in fresh tea leaves collected both from pruned and unpruned tea (*C. sinensis*) plants demonstrates active metabolism of theanine to catechin derivatives by light [38]. The relationship between theanine metabolism and climatic conditions have been previously reported in fresh tea (*C. sinensis*) leaves harvested in three different tea growing areas in a single year during spring season of April [19], which was partly consistent with the results conducted in the current study in a single tea growing region over five successive years. As a result, the climatic conditions of high rainfall under short sun exposure time, and of drought likely lead to inhibition of theanine metabolism into catechin derivatives and would thus be the best vintage for taste- or flavor-rich tea cultivar of Jangwon 2. Interestingly, these positive associations were further found in the metabolisms of alanine, GABA, succinate, acetate, quinate, theogallin and gallate in Jangwon 2 tea cultivar across the five successive vintages, as highlighted in Fig. 6.

In the case of Jangwon 3 tea cultivar that was developed by asexual propagation via cuttings from wild tea cultivar (*C. sinensis* var. Yabukita) [8] and thus is rich in EGCG3"Me, we focused on metabolism of EGCG3"Me in the tea cultivar. Notably, high amounts of EGCG3"Me in the leaves of Jangwon 3 tea cultivar were observed across five successive years, compared to those of Jangwon 2 cultivar (Fig. 5F), as previously reported in a single year [8]. In general, tea leaves are first plucked in May every year (Fig. 1A). However, the plucking time of Jangwon 3 tea cultivar in 2019 was largely delayed for 2 months because its growth retardation was caused by abnormal drought conditions as shown in Fig. 1Ej. This phenomenon could be due to scarcity of water in the soil that reduced photosynthesis, growth and survivability of tea plants grown under abnormal drought conditions [40]. Nevertheless, increased accumulation of EGCG3"Me, as well as catechins, EC and GC, were observed in the leaves of Jangwon 3 tea cultivar harvested in July, 2019 (Fig. 5), likely due to normal climatic conditions in the harvesting time even though drought condition occurred in spring season. Interestingly, long sun expose time during harvesting time was positively associated with accumulations of EGCG3″Me in Jangwon 3 tea cultivar between 2015 and 2019, which was independent on rainfall as indicated in climatic data and results on amounts of EGCG3″Me. Plant physiological responses, such as the control of closing and opening of stomata are adjusted to maintain water loss during drought at the expense of carbon starvation or the risk of hydraulic failure [41]. Previous studies have reported that drought conditions change the metabolism of tea leaves and thus affect the tea quality positively or negatively. For example, Wang et al. [42] have reported that drought stress resulted in the dehydration and wilting of tea (*C. sinensis*) leaves, which significantly reduced the quality of tea leaves as demonstrated by decreased amounts of total polyphenols, catechins, caffeine, theanine and some free amino acids along with increased amounts of total flavonoids. In contrast, Scott et al. [43] have reported that the effects of drought on the non-volatile compounds such as theanine, catechins and methylxanthines were insignificant. It was clear that drought conditions in 2019 did not affect growth of Jangwon 2 tea cultivar, but largely affected of growth Jangwon

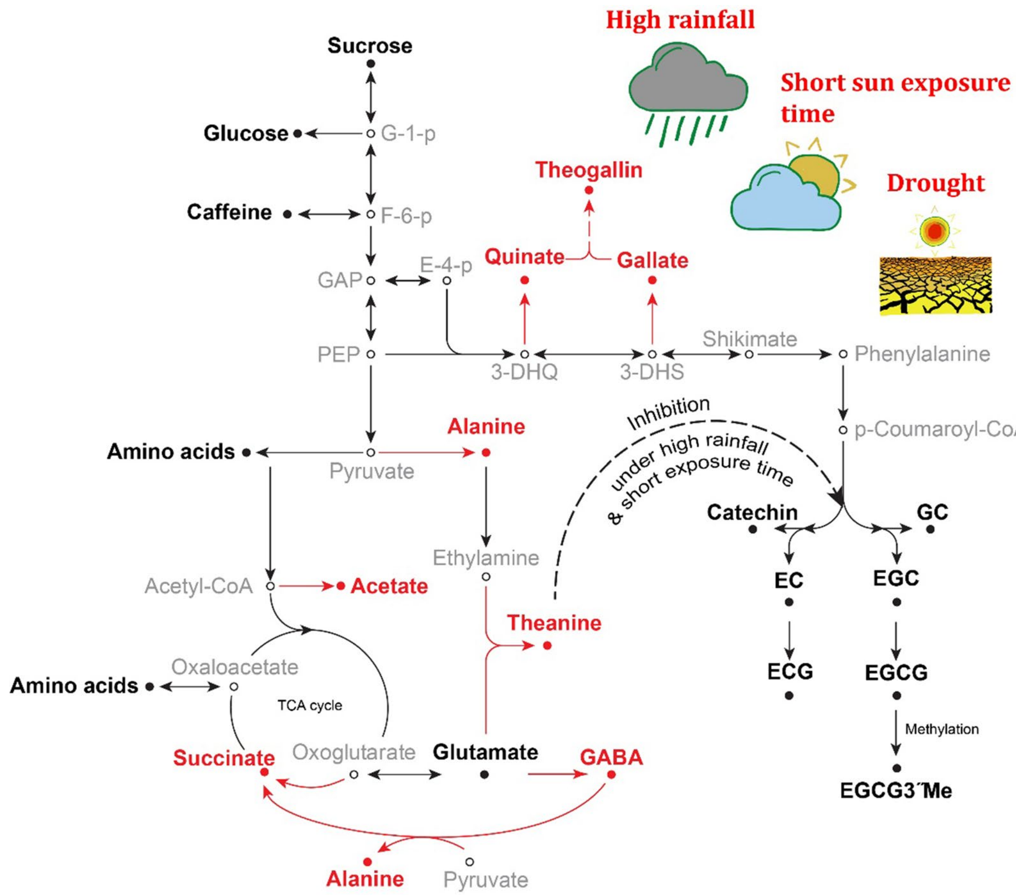


Fig. 6 Highlight of tea leaf metabolites accumulated under the best climate conditions of high rainfall under short sun exposure time or drought for taste- or flavor-rich tea cultivar (Jangwon 2 tea cultivar). Metabolites with bold letter indicate tea leaf metabolite observed in the current study and in red color were found to be dependent on the climate conditions during five successive years

3 tea cultivar, which may suggest the variability of two tea cultivars that are physiologically and metabolically dissimilar in response to drought conditions.

In conclusion, the present ^{1}H NMR-based metabolomics approach dedicated to investigate variations in tea leaf metabolome of the two tea (*C. sinensis*) cultivars over five successive years, and thus determine vintage tea products. The two tea cultivars Jangwon 2 and 3 had different metabolic responses toward climatic conditions in each year of the five successive years. For example, drought conditions seriously affect the growth of Jangwon 3 tea cultivar, a EGCG3"Me-rich cultivar, but not of Jangwon 2, a taste-rich cultivar. Indeed, high rainfall under short sun expose time may be the best conditions for Jangwon 2 cultivar with the highest theanine amounts, and long sun expose time for Janewon 3 cultivar with the highest EGCG3″Me amounts. Therefore, the interplay between climatic conditions and tea leaf metabolism over five successive year suggests a possible production of 'vintage tea' in individual tea cultivar.

Abbreviations

CD_3OD	Methanol-d_4
D_2O	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	Unsaturated fatty acids
GC	Gallocatechin
Glu	Glutamine
^{1}H NMR	Proton nuclear magnetic resonance
HSQC	Heteronuclear single quantum correlation
Ile	Isoleucine
Leu	Leucine
NOES	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
STOCSY	Statistical total correlation spectroscopy

TOCSY	Total correlation spectroscopy
Val	Valine
ID	One-dimensional
2D	Two-dimensional

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-023-00841-y.

Additional file 1: Fig. S1. Representative 1H 700 MHz NMR spectra of tea leaf extracts obtained from Jangwon 3 tea cultivar collected in May 2015, May 2016, May 2017, May 2018, and July 2019. **Fig. S2**. Representative 1H 700 MHz NMR spectra of tea leaf extracts obtained from Jangwon 2 tea cultivar collected in May 2015, May 2016, May 2017, May 2018, and July 2019. **Fig. S3**. PCA and OPLS-DA derived from 1H NMR spectra of leaf extracts obtained from Jangwon 2 (J2) and Jangwon 3 (J3) tea cultivars from 2015 to 2019. **Fig. S4**. OPLS-DA score and loading plots derived from 1H NMR spectra of leaf extracts from Jangwon 2 and Jangwon 3 tea cultivars. **Fig. S5**. 1H NMR spectra corresponded to unsaturated fatty acids first analyzed in 2015 and reanalysis after storage of dried tea leaves at -80 °C from Jangwon 2 and Jangwon 3 tea cultivars. **Fig. S6**. OPLS-DA score and loading plots derived from 1H NMR spectra of tea leaf extracts between leaves of Jangwon 3 tea cultivar harvested from vintage years 2015-2019. **Fig. S7**. OPLS-DA score and loading plots derived from 1H NMR spectra of tea leaf extracts between leaves of Jangwon 2 and 3 tea cultivar harvested from vintage years 2015-2019. **Fig. S8**. Permutation tests of PLS-DA models obtained from Jangwon 2 tea cultivar. **Fig. S9**. Permutation tests of PLS-DA models obtained from Jangwon 3 tea cultivar. **Fig. S10**. Permutation tests of PLS-DA models between Jangwon 2 and 3 tea cultivar in each year.

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Declarations

Competing interests
The authors declare that they have no competing interests.

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