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Research Article

Metabolomic understanding of intrinsic physiology in *Panax ginseng* during whole growing seasonsHyo-Jung Lee^{1,☆}, Jaesik Jeong^{2,☆}, Alessander Couto Alves³, Sung-Tai Han⁴, Gyo In⁴, Eun-Hee Kim⁵, Woo-Sik Jeong^{6,**}, Young-Shick Hong^{1,*}¹ Division of Food and Nutrition, Chonnam National University, Gwangju, Republic of Korea² Department of Statistics, Chonnam National University, Gwangju, Republic of Korea³ Department of Epidemiology and Biostatistics, Imperial College London, London, UK⁴ R&D Headquarters, Korea Ginseng Corporation, Daejeon, Republic of Korea⁵ Protein Structure Group, Korea Basic Science Institute, Chungbuk, Republic of Korea⁶ Department of Food & Life Science, College of Biomedical Science & Engineering, Inje University, Gyeongsangnam, Republic of Korea

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ABSTRACT

Background: *Panax ginseng* Meyer has widely been used as a traditional herbal medicine because of its diverse health benefits. Amounts of ginseng compounds, mainly ginsenosides, vary according to seasons, varieties, geographical regions, and age of ginseng plants. However, no study has comprehensively determined perturbations of various metabolites in ginseng plants including roots and leaves as they grow.

Methods: Nuclear magnetic resonance (¹H NMR)–based metabolomics was applied to better understand the metabolic physiology of ginseng plants and their association with climate through global profiling of ginseng metabolites in roots and leaves during whole growing periods.

Results: The results revealed that all metabolites including carbohydrates, amino acids, organic acids, and ginsenosides in ginseng roots and leaves were clearly dependent on growing seasons from March to October. In particular, ginsenosides, arginine, sterols, fatty acids, and uracil diphosphate glucose–sugars were markedly synthesized from March until May, together with accelerated sucrose catabolism, possibly associated with climatic changes such as sun exposure time and rainfall.

Conclusion: This study highlights the intrinsic metabolic characteristics of ginseng plants and their associations with climate changes during their growth. It provides important information not only for better understanding of the metabolic phenotype of ginseng but also for quality improvement of ginseng through modification of cultivation.

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1. Introduction

Panax ginseng (*P. ginseng*), a member of the family Araliaceae, is one of the most important agricultural crops in the world. It is a slow-growing perennial herb plant [1]. Among 17 species of the *Panax* genus, *P. ginseng* (Korean ginseng), *Panax notoginseng* (Chinese ginseng, also called Sanchi ginseng), and *Panax quinquefolius* (American ginseng) are major commercial ginsengs [2]. Ginseng has been used as a traditional medicinal plant in many countries

including Korea and China for thousands of years [3]. The annual growth cycle of ginseng plants covers the budding stage and leaf expansion stage during spring, followed by the flowering stage, fruit stage, leaf loss stage, and root growing stage during autumn [4]. The aboveground part of ginseng plants shows clear morphological differences in different growth years during their growth periods. Among diverse parts of ginseng plants, roots are mainly used for medicinal purposes [5]. Many studies have reported diverse pharmacological effects of ginseng roots on human health,

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including immunostimulating, anticancer, antiaging, and antioxidative activities and cardiovascular control of blood pressure [6]. These pharmacological effects are explained by their multiple constituents, including ginsenosides, polysaccharides, polyacetylenic alcohols, and peptides [7].

The quality of ginseng plants is determined by amounts of triterpenoid saponins, also called ginseng saponins or ginsenosides, particularly in ginseng roots [8]. Structures of the ginsenoside vary according to the type of sugar moieties, site of sugar attachment, and number of sugars [9]. To date, more than 100 ginsenosides have been identified from *Panax* species [8]. Ginsenosides are divided into dammarane, oleanane, and ocotillol types depending on the skeleton of aglycone [8]. Dammarane-type ginsenosides have the structure of a trans-ring rigid tetracyclic skeleton. They are the main constituents in ginseng roots [8]. Dammarane-type ginsenosides are classified into two groups, protopanaxadiol and protopanaxatriol, according to positions the sugar moieties are attached to [9]. Sugar moieties in the protopanaxadiol group including Rb1, Rb2, Rb3, Rc, Rg3, Rh2, Rc, and Rd are attached to the C-3 and/or C-2 position. Protopanaxatriol groups feature sugar moieties attached to the C-6 and/or C-20 position. They are classified into Rf, Rg1, Rh1, and Rg2 [9,10]. Oleanane- and ocotillol-type ginsenosides are a minority. Contents of these ginsenosides depend on *Panax* species, their parts and age, and the extraction method [8]. Natural factors including soil types, climates, geographical location, and different production procedures also contribute to variations of ginsenoside contents [8,11]. These conditions are likely to affect the quality of ginseng during cultivation. However, comprehensive analysis of perturbations of ginseng metabolites during cultivation/growing or their associations with climatic conditions has not been reported yet.

Metabolomics seeks to identify and quantify metabolites of small molecules in the biological system by mass spectrometry coupled with a gas and liquid chromatography system and nuclear magnetic resonance (NMR) together with multivariate statistical analysis [12]. In particular, NMR is commonly used in metabolomics studies because it allows simultaneous identification and quantification of diverse metabolites with different chemical characteristics, including primary metabolites (sugars, organic acids, amino acids, and so on) and secondary metabolites (flavonoids, alkaloids, terpenoids, and so on) [13]. Thus, it has been widely used as an analytical approach in a variety of fields such as plant science, human health, and nutrition [14,15]. Recently, metabolomics studies have revealed that the ginseng metabolome varies

according to plant parts, geographical origins, and cultivation ages [16–19].

Because global metabolic consequences in ginseng plants during their growing seasons are poorly understood, the objective of this study was to explore metabolic variations of ginseng roots and leaves during their growing seasons from March to October using proton NMR (^1H NMR)–based metabolomics approach.

2. Materials and methods

2.1. Chemicals

Deuterium oxide (D_2O , 99.9% ^2H), 3-(trimethylsilyl) [2,2,3,3- $^2\text{H}_4$] propionate (TSP), and methanol- d_4 (CD_3OD , 99.8%) were purchased from Sigma (St. Louis, MO, USA). Pure compounds of ginsenosides, Rg1, Rg2s, Rg3s, Rb2, Rb1, Re, and Rf, used for spiking experiments were obtained from R&D Headquarters, Korea Ginseng Corporation (Daejeon, Republic of Korea).

2.2. Ginseng samples

All ginseng (*P. ginseng* C. A Meyer) samples used for the present study were grown in the same ginseng field and by the same farmer in Yeosu (37° 20' 51.1" N, 127° 30' 32.9" E), Gyeonggi-do, South Korea, under the same environmental conditions. They were harvested every morning in the last week of each month from March to September in 2017. The age of the ginseng plant was 6 years in 2017. To ensure biological replications, 10 different ginseng plants were obtained at different growing regions within the same ginseng field. After harvesting, all ginseng samples were immediately placed on dry ice and stored at -80°C until analysis.

2.3. Ginseng extraction and ^1H NMR spectroscopic analysis

Whole ginseng plants collected in the last week of every month were rinsed with cold water and divided into various parts, including main roots, rootlets, rootlet sides, stems, and leaves (Fig. 2). Each part of ginseng plants was then dried for 48 h using a freeze dryer. These freeze-dried ginseng samples were ground into powder using a pestle and mortar under liquid nitrogen. Then, 100 mg of freeze-dried ginseng powder sample was dissolved in a mixture of methanol- d_4 (CD_3OD , 490 μL) and deuterium water (D_2O , 210 μL) containing 0.05% (wt) TSP in a 1.5-mL Eppendorf tube. The mixture was sonicated at room temperature for 20 min and

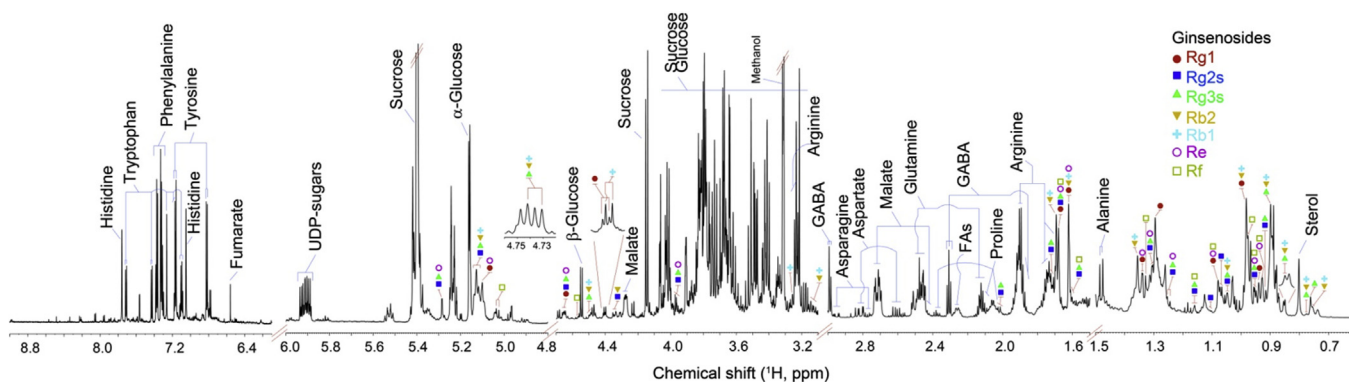


Fig. 1. Representative 700 MHz ^1H NMR spectrum of ginseng extracts and assignments of ginseng metabolites. These assignments of ginsenosides including Rg1, Rg2s, Rg3s, Rb2, Rb1, Re, and Rf were confirmed through spiking experiments with their pure compounds and 2D NMR experiments. FAS, fatty acids; GABA, γ -aminobutyrate; ^1H NMR, proton nuclear magnetic resonance; UDP-sugars, uracil diphosphate glucose-sugars.

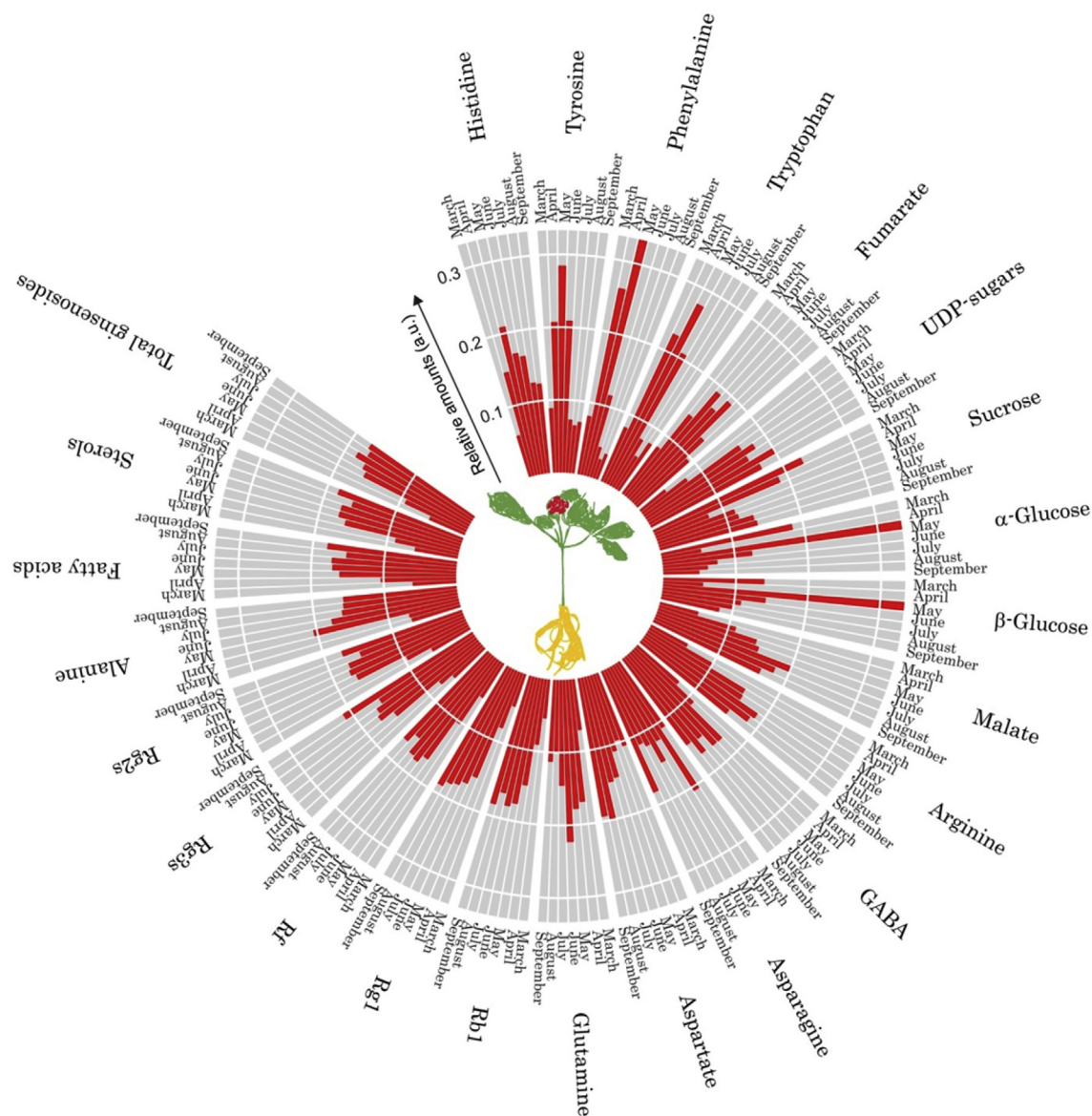


Fig. 2. Metabolome-wide pattern dependent on seasonal change in main roots of *Panax ginseng*. Amounts of each metabolite were determined by integration of ^1H NMR peaks. Because each metabolite in ginseng roots had totally different amounts, we considered data transformation to graphically show change patterns of metabolites all together in the current circus plot. Therefore, we took average values of 10 biological replicates for all ginseng plants and then calculated relative proportion of average values within each metabolite. Statistics for the difference of each metabolite are shown in Fig. 7. GABA, γ -aminobutyrate; UDP, uracil diphosphate.

then centrifuged at 13,000 rpm for 15 min at 10°C . The supernatant (600 μL) was then transferred into 5-mm NMR tubes. These extracts were analyzed using a Bruker Avance 700 spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 700.40 MHz ^1H frequency and a temperature of 298 K coupled with a cryogenic triple-resonance probe and a Bruker automatic injector. Methanol- d_4 in the aliquots provided a field-frequency lock. Signal assignment for representative samples was facilitated by two-dimensional total correlation spectroscopy and heteronuclear single-quantum correlation. In particular, ginsenoside compounds were identified through spiking experiments with their pure chemicals.

2.4. NMR data processing and multivariate statistical analysis

All one-dimensional (1D) NMR spectra of ginseng samples were phased. Their baselines were corrected using TOPSPIN software (version 3.2; Bruker Biospin, Rheinstetten, Germany), and then

converted to the ASCII format. ASCII format files were imported into MATLAB (R2010b; Mathworks, Inc., Natick, MA, USA). All spectra were aligned using the interval correlation shifting method [20]. Four regions of ^1H NMR spectra corresponding to TSP (-1.0 – 0.5 ppm), residual water (4.70 – 4.80 ppm), methanol (3.36 – 3.40 ppm), and others (8.6 – 10 ppm) were removed. The entire spectra were normalized by the probabilistic quotient normalization method to avoid dilution effects of extracts [21]. The resulting data sets were imported into SIMCA-P version 14.0 (Umetrics, Umea, Sweden) for multivariate statistical analysis. Two different analyses of a mean centering scaling method for pattern recognition were used. First, principal component analysis, an unsupervised pattern recognition method, was performed to visualize variance of global metabolites in data sets and detect outliers. Next, orthogonal partial least squares discriminant analysis (OPLS-DA), a supervised pattern recognition method, was used to maximize covariance between measured data (X variable) of peak intensities in NMR spectra and

response variable (Y variable) [22,23]. OPLS-DA loadings or coefficient plots were generated using MATLAB (Mathworks, Inc.) with scripts developed in-house at Imperial College London, UK. They were then used to detect distinctive metabolites between the two groups in the model with a color-coded correlation coefficient of each data point reported by Cloarec et al [24,25]. Qualities of these models were evaluated by R^2X and Q^2 values. R^2X was defined as the proportion of variance in the data described by these models to reveal goodness of fit. Q^2 was defined as the proportion of variance by the model to indicate predictability.

2.5. Statistical analysis

All statistical analyses of this study were performed using SPSS statistical software version 21 (SPSS Corp., Chicago, IL, USA). Duncan's multiple range tests of analysis of variance were applied to identify significant difference of the integral area of each metabolite in ginseng. Statistically significant difference was regarded at $P < 0.05$. The integral area of metabolite in the 1D 1H NMR spectrum was used to determine the amount of individual metabolite in ginseng roots and leaves.

3. Results

3.1. Metabolites of ginseng roots identified by 1H NMR spectroscopy

Representative 1D 1H NMR spectra obtained from extracts of ginseng main roots are shown in Fig. 1. Based on analysis of two-dimensional NMR experiments, 26 metabolites including alanine; arginine; aspartate; asparagine; gamma-aminobutyrate (GABA); fatty acids (FAs); fumarate; glutamine; α -glucose; β -glucose; ginsenosides, Radix g1, g2s, g3s, b2, b1, e, and f (referred as Rg1, Rg2s, Rg3s, Rb2, Rb1, Re, and Rf, respectively); histidine; malate; sterols; sucrose; phenylalanine; proline; tryptophan; tyrosine; and uracil diphosphate (UDP)-sugars were assigned. Assignments of all ginsenoside compounds were further validated through spiking experiments with their pure compounds. Fig. 2 shows metabolome-wide pattern in main roots of ginseng plants as they grew. Amounts of ginseng main root metabolites were markedly changed during whole growth of the ginseng plant. For example, the contents of all ginsenosides increased until May. Statistical dependences of metabolites of ginseng main roots are shown in Fig. 7.

3.2. Metabolic changes of ginseng roots depending on growing periods

Multivariate statistical analyses were applied to the entire data set of whole 1H NMR spectra of ginseng roots including main roots and lateral roots to conduct comparative interpretation and visualize metabolic discrimination among groups of ginseng roots collected at different growth stages from March to September (Figs. 3A–3C). These OPLS-DA models were validated by the permutation test (Fig. S1). At first, clear differentiations between main and lateral roots were observed (Fig. 3A), demonstrating distinct metabolic differences according to root parts. Although close classifications among ginseng roots harvested from June to September were observed both in main and lateral roots, ginseng roots harvested from March to June were markedly differentiated as shown in the OPLS-DA score plots (Figs. 3B and 3C). Climate parameters such as temperature, total sun exposure time, humidity, and total rainfall during cultivation of ginseng plants used for the present study are given in Figs. 3D and 3E. These results indicate that there are large changes of metabolite levels in ginseng roots from March to June during their growth and relationships between climate and ginseng metabolites. Clear metabolic differentiations in main roots

of ginseng between March and April (Fig. 4A), between April and May (Fig. 4C), between May and June (Fig. 4E), and between June and September (Fig. 4G) were observed in OPLS-DA score plots. These models had high fitness and predictabilities as indicated by their R^2X and Q^2 values, respectively. However, significant metabolic differentiations in main roots of ginseng between June and July, between July and August, or between August and September were not observed (data not shown). Metabolites in ginseng main roots responsible for differentiations between two growth stages (Figs. 4A, 4C, 4E and 4G) were identified from pairwise OPLS-DA loading plots (Figs. 4B, 4D, 4F and 4H). In the OPLS-DA loading plot (Fig. 4B), the upper section denotes higher levels of metabolites in main roots of ginseng harvested in April than those harvested in March, whereas the lower section indicates lower levels of metabolites in main roots of ginseng harvested in April. Main roots of ginseng harvested in April were characterized by higher levels of arginine, GABA, ginsenosides, glutamine, malate, histidine, phenylalanine, tryptophan, tyrosine, and UDP-sugars but lower levels of glucose and sucrose than those harvested in March (Fig. 4B). These changed levels of metabolites in main roots of ginseng between April and May were found to be similar to those between March and April. However, glucose levels were reversed (Fig. 4D). Indeed, levels of metabolites such as glucose and phenylalanine in ginseng main roots harvested in June were decreased compared with those harvested in May (Fig. 4F). Furthermore, levels of sucrose were significantly increased in ginseng main roots harvested in June (Fig. 4F). Interestingly, there were no changes in levels of ginsenoside compounds or amino acid levels, which continuously increased until May. For main roots of ginseng harvested in September, significant increases in sucrose level were found. However, levels of several amino acids such as arginine, glutamine, phenylalanine, tryptophan, and tyrosine were significantly lower than those harvested in June (Fig. 4H). These results of lateral roots of ginseng were similar to those of main roots of ginseng as shown in the OPLS-DA score and loading plots with high goodness of fit (R^2X) and strong predictability (Q^2) (Fig. S2). In the OPLS-DA score plot derived from 1H NMR spectra of ginseng lateral roots, clear differentiations between March and April (Fig. S2A), between April and May (Fig. S2C), between May and June (Fig. S2E), and between June and September (Fig. S2G) were observed. As observed in main roots, there was no significant difference in lateral roots between June and July, between July and August, and between August and September (data not shown). In OPLS-DA loading plots to find out growth stage-dependent metabolites in lateral roots of ginseng, lateral roots of ginseng harvested in April showed higher levels of arginine, ginsenosides, glutamine, malate, histidine, phenylalanine, tryptophan, and tyrosine than those harvested in March (Fig. S2B). Lower levels of glucose and sucrose were observed in lateral roots of ginseng harvested in April. However, there were no changes in levels of histidine, phenylalanine, and GABA (Fig. S2D). Lateral roots of ginseng harvested in June were characterized by higher levels of sucrose but lower levels of glucose and phenylalanine than those harvested in May (Fig. S2F). However, levels of ginsenosides or amino acids were not changed. Quantities of glutamine, phenylalanine, tryptophan, and tyrosine were markedly lower in lateral roots of ginseng harvested in September than in those harvested in June (Fig. S2H).

3.3. Metabolic differences between main and lateral roots of ginseng

It was interesting to observe metabolic differentiations between main and lateral roots of ginseng (Figs. 5A, 5C, and 5E). Diverse metabolites different between main and lateral roots of ginseng

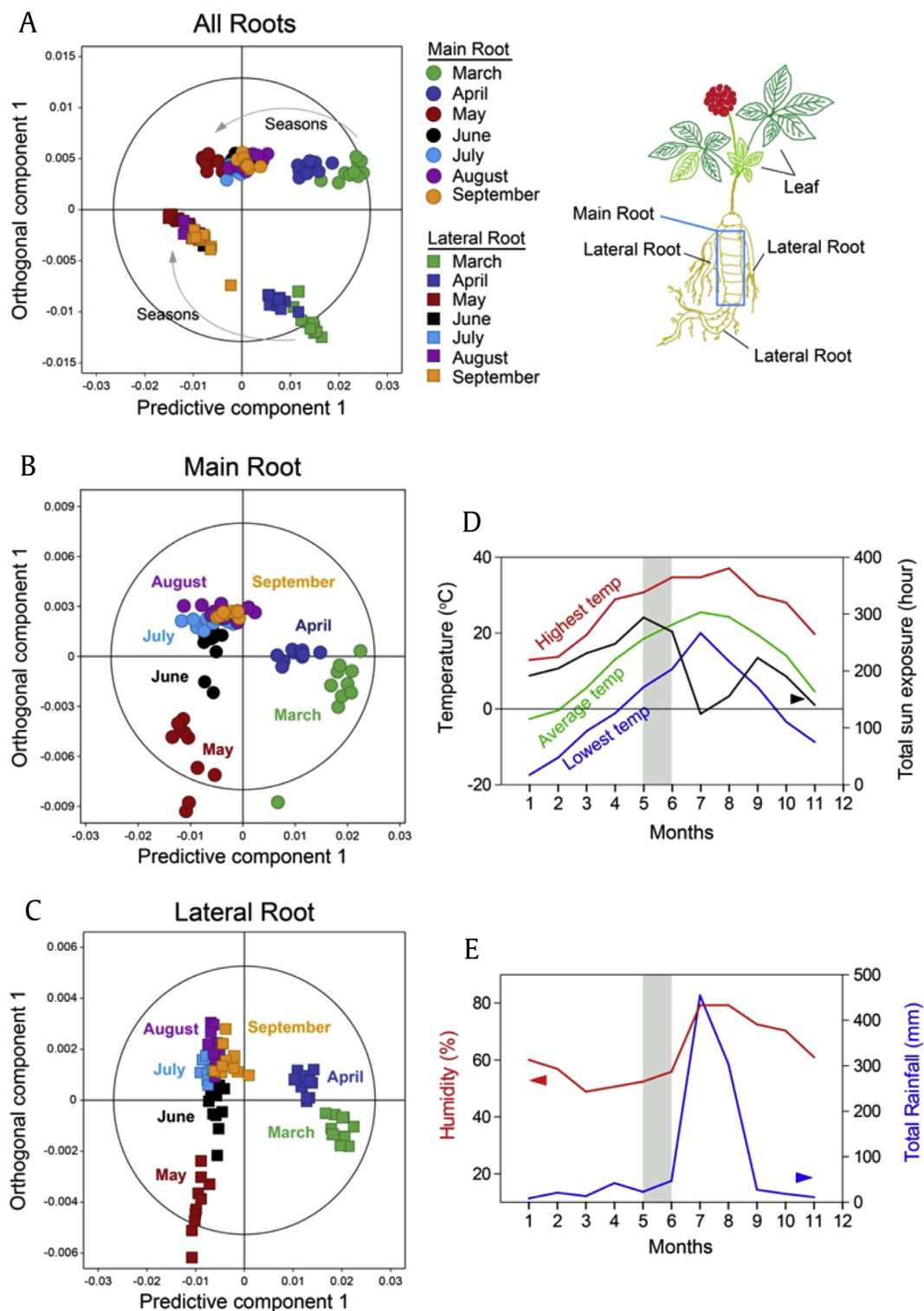


Fig. 3. Metabolic differentiations of ginseng roots and climatic conditions where ginseng plants are cultivated. OPLS-DA score plot of (A) all ginseng roots, (B) ginseng main roots and (C) lateral roots derived from ^1H NMR spectra of each ginseng root extract. These OPLS-DA models were validated by the permutation test repeated 200 times in the corresponding PLS-DA models (Fig. S1). Panels D and E show climate data at the region for cultivation of ginseng plants in 2017. In general, rainy season or monsoon start at the end of June in South Korea as shown by a drop of total sun exposure time and a large accumulation of total rainfall between June and July.

were identified at different growth stages, typically in March, June, and September, as shown in Figs. 5B, 5D, and 5F, respectively. Ginseng lateral roots harvested in all growth periods, including March, June, and September, were characterized by higher levels of

ginsenoside compounds, FAs, sterol, and arginine than ginseng main roots. However, levels of sucrose were significantly higher in ginseng main roots harvested in March, June, and September than in lateral roots (Figs. 5B, 5D, and 5F). Many studies have reported

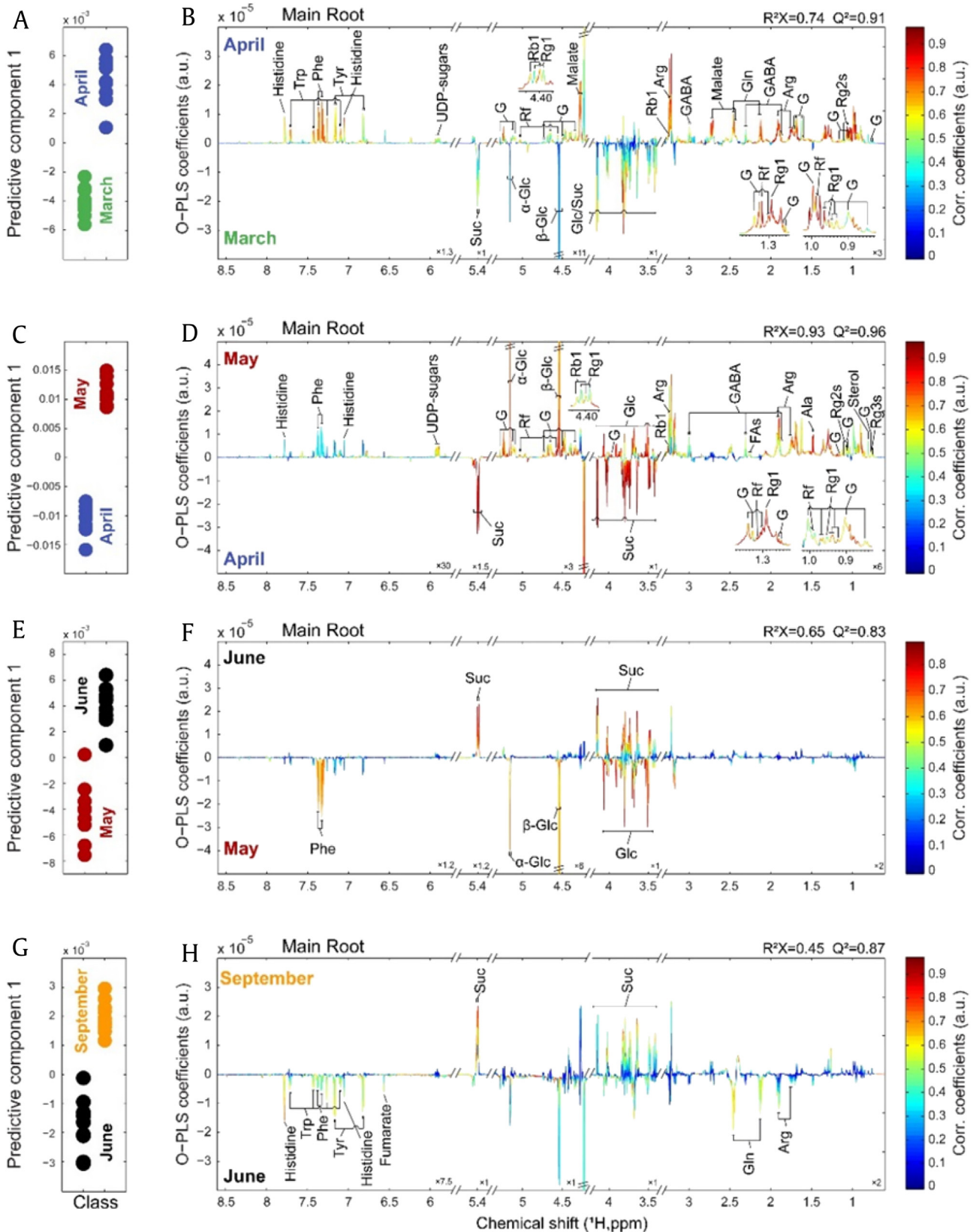


Fig. 4. Identifications of ginseng main root metabolites changed monthly. (A, C, E, and G) OPLS-DA scores and (B, D, F, and H) loadings plots derived from ¹H NMR spectra of ginseng main root extracts. (A and B) These OPLS-DA models were generated for pairwise comparisons between two harvesting seasons to identify metabolites differing between March and April. All OPLS-DA models were generated with one, for example, predictive and orthogonal component and validated by the permutation test repeated 200 times in corresponding PLS-DA models (data not shown). Ala, alanine; Arg, arginine; FAs, fatty acids; GABA, g-aminobutyrate; Glc, glucose; Gln, glutamine; G, ginsenoside; Phe, phenylalanine; Rg1, Rg2s, Rg3s, Rb1, and Rf, ginsenosides Radix g1, g2s, g3s, b1, and f; Suc, sucrose; Trp, tryptophan; Tyr, tyrosine; UDP-sugars, uracil diphosphate-sugars.

that levels of ginsenoside compounds in lateral roots are higher than those in main roots [26], consistent with the results of the present study.

3.4. Ginseng leaf metabolites and their variations according to growth

Representative ^1H NMR spectra of ginseng leaf extracts are shown in Fig. S3. Eleven metabolites were identified by ^1H NMR spectroscopy, including GABA; sucrose; α -glucose; β -glucose;

ginsenosides, Radix g1, e, and F2 (referred as Rg1, Re, and F2, respectively); succinate; kaempferol; and alanine. OPLS-DA score plots were applied to evaluate metabolic differences among groups of ginseng leaves harvested from May to September, showing clear metabolic differentiations (Fig. S4). These OPLS-DA models were validated by the permutation test repeated 200 times (Fig. S5). The results demonstrated significant variations of metabolites in ginseng leaves according to growth of ginseng plants during a single year. OPLS-DA score and loading plots for pairwise comparison between ginseng leaves harvested in May and June,

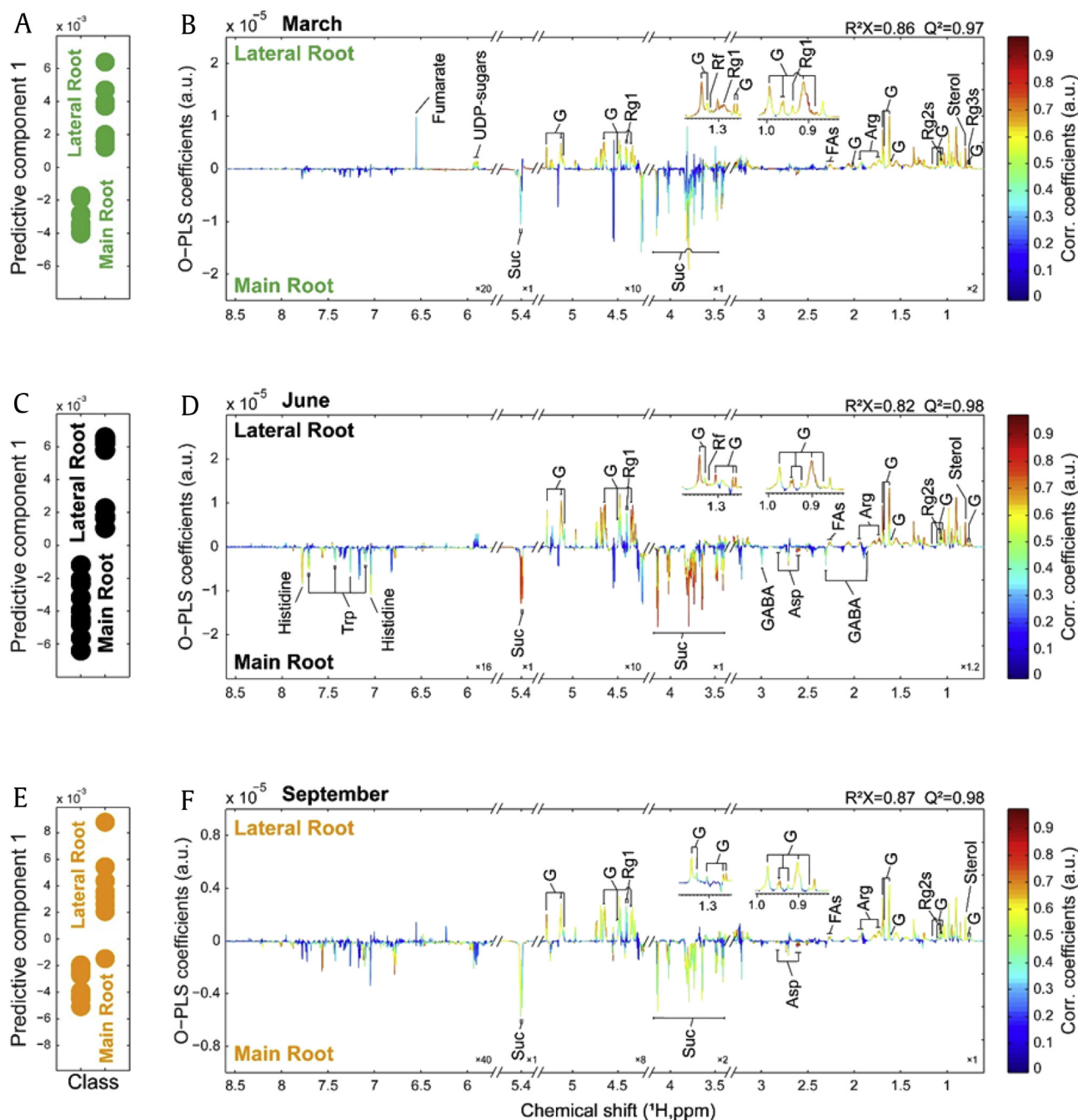


Fig. 5. Identifications of ginseng root metabolites different between main and lateral roots. (A, C, and E) OPLS-DA scores and (B, D, and F) loadings plots derived from ^1H NMR spectra of ginseng main and lateral root extracts for comparing metabolic differences between main root and rootlet at given harvesting seasons. All OPLS-DA models were generated with one, for example, predictive and orthogonal components and validated by the permutation test repeated 200 times in corresponding PLS-DA models (data not shown). Arg, arginine; Asp, aspartate; FAS, fatty acids; GABA, g-aminobutyrate; Glc; glucose, Gln, glutamine; G, ginsenoside; Rg1, Rg2s, Rg3s, and Rf, ginsenosides Radix g1, g2s, g3s, and f; Suc, sucrose; Trp, tryptophan; UDP-sugars, uracil diphosphate-sugars.

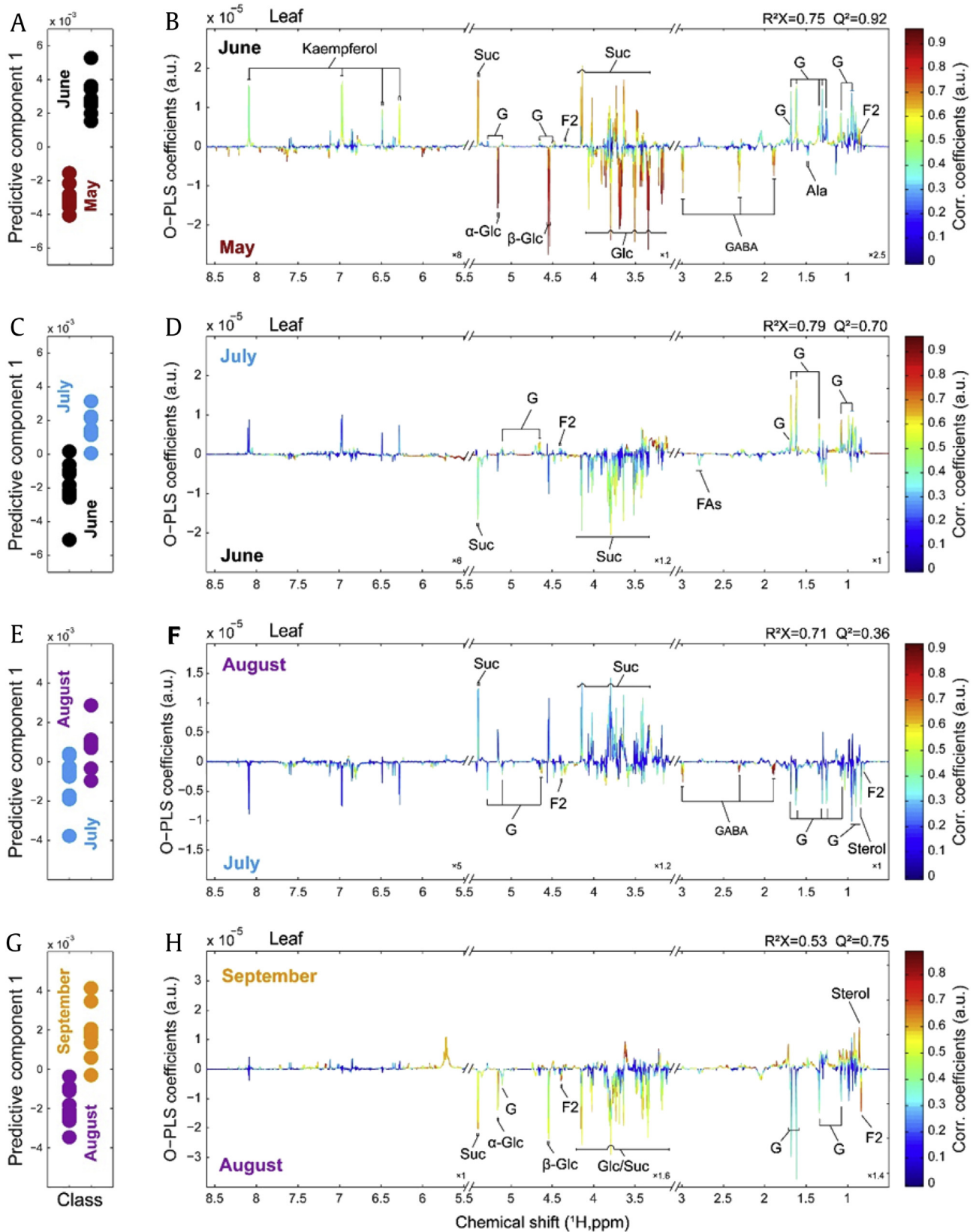


Fig. 6. Identifications of ginseng leaf metabolites changed according to cultivation time. (A, C, E, and G) OPLS-DA scores and (B, D, F, and H) loadings plots derived from ^1H NMR spectra of ginseng leaf extracts to compare metabolic differences between given harvesting seasons. All OPLS-DA models were generated with one, for example, predictive and orthogonal component and validated by the permutation test repeated 200 times (data not shown). Ala, alanine; FAs, fatty acids; F2, ginsenoside F2; GABA, g-aminobutyrate; Glc, glucose; G, ginsenoside; Suc, sucrose.

June and July, July and August, and August and September were generated (Fig. 6). Metabolic differentiations of ginseng leaves harvested in May and June were caused by lower levels of glucose, GABA, and alanine but higher levels of kaempferol, sucrose, and ginsenoside compounds in samples harvested in June than those harvested in May (Fig. 6B). In comparison with ginseng leaves harvested in June and July, levels of ginsenoside compounds and FAs were found to be higher in leaves collected in July than those collected in June, whereas levels of sucrose were lower in those collected in July (Fig. 6D). Higher levels of sucrose were observed in ginseng leaves harvested in August than those harvested in September, while lower levels of ginsenoside compounds, GABA, FAs, and sterol were found in samples harvested in July (Fig. 6F). In ginseng leaves harvested in September, levels of sucrose, glucose, and ginsenoside compounds were decreased except for sterol compared with samples harvested in August (Fig. 6H). Fig. 7 shows quantitative amounts of individual metabolites in ginseng main roots, calculated with the integral area of ^1H NMR peaks corresponding to individual metabolite with statistical significance. Quantitation results of individual metabolites in lateral roots and leaves of ginseng are shown in Figs. S6 and S7, respectively.

3.5. Metabolite variations of ginseng roots and leaves in October

Because all ginseng plants cultivated by the farmer who provided ginseng plants until September were completely harvested in September, ginseng plants grown until September and October were collected at another growing place to investigate metabolic perturbations in ginseng roots and leaves in October. As shown in the principal component analysis model generated with ^1H NMR spectra of ginseng main and lateral roots, no significant metabolic differentiation in main or lateral roots between September and October was observed (Fig. S8). However, ginseng leaves between September and October showed significant differentiation (Fig. S9), in which the OPLA-DA model was validated by the permutation test (Fig. S10). Interestingly, differences in glucose and sucrose levels in ginseng leaves were only observed between September and October, that is, levels of glucose and sucrose in ginseng leaves were decreased in October compared with those in September (Fig. S11). Fig. S12 shows relative amounts of glucose and sucrose in ginseng leaves collected in September and October with statistics.

4. Discussion

4.1. Sugars and their derivatives

In most plant leaves, glucose synthesized through photosynthesis is mainly converted to a form of sucrose or transported via phloem to developing organs such as growing young leaves, roots, and flowers [27,28]. These sugars play important roles in plant metabolism, growth, and development. Ginseng is well known as a perennial plant. Its leaves grow during spring and fall by October to store nutrients in roots during the winter season [4]. Previous studies have demonstrated that the energy required for maturation of ginseng roots could be obtained from decomposition of sucrose, leading to surface expansion of leaves because of active growth from decomposition of sucrose during spring [29]. It has been reported that the development of leaves in plants depends on photosynthetic capacity [30]. Therefore, significant reductions of sucrose levels in main and lateral roots of ginseng until May might reflect the decomposition of sucrose for growth or development of ginseng plants (Figs. 7T and S5T). The decomposition of sucrose was also evident by accumulations of UDP-sugars and FAs because of conversion of sucrose into UDP-glucose and fructose by sucrose synthase (Figs. 7E and 7X) [31–33]. As a result of plant metabolism,

UDP-glucose is related to the biosynthetic pathway of cellulose. It provides carbon source to diverse tissues of plants [33]. Fructose is associated with the synthesis of pyruvate and acetyl-CoA, the latter of which is a precursor of FAs (Fig. 7E) [34]. However, after May, sucrose levels in main and lateral roots were accumulated until September, although such accumulation was not marked. In our previous study, marked accumulation of sucrose has been observed in tea (*Camellia sinensis*) leaves grown under shade or dark conditions because of lack of photosynthesis [35]. In the present study, total sun exposure time started to decrease, whereas total rainfall increased in May (Figs. 3D and 3E). Considering that all ginseng samples used for the present study were collected at the end of each month, their variations in ginseng plants reflected metabolic consequence of growth for each month. Therefore, accumulation of sucrose in roots of ginseng after May likely demonstrates reduction of photosynthesis because of decreased sun exposure time and increased rainfall. Similar results were also observed in leaves of ginseng plants (Fig. S7). Reduced glucose in main roots, lateral roots, and leaves of ginseng plants might be due to limited photosynthesis and simultaneously poor conversion into sucrose (Figs. 7I and 7J, S6I, S6J, S7D, and S7E). Average temperature was not correlated with these changes in ginseng plants. As a result, amounts of carbohydrates, typically sucrose, accumulated during growth might affect the overall metabolism of ginseng plants in the next year, consequently affecting the quality of ginseng roots. These phenomena likely depend on climatic conditions in a given year.

4.2. Ginsenosides

There were dynamic seasonal variations for amounts of ginsenoside compounds in ginseng roots. Until May, ginsenoside compounds in ginseng roots were continuously synthesized (Figs. 7 and S6). However, there were no significant changes in levels of ginsenoside compounds after May. Ginseng plant is known to be a self-pollination plant. It begins to bloom in mid-May in Korea [36]. Many environmental factors including temperature, soil, and water can influence compositions and contents of ginsenoside compounds [11]. Ginsenoside biosynthesis is known to involve formation of squalene cyclization, oxidation, and glycosylation via mevalonic acid (MVA) and methylerythritol phosphate pathways [37]. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) is an enzyme that catalyzes the transition of HMG-CoA to MVA. Reaction of HMGR is the first rate-limiting step of the MVA pathway in plants [38]. Transcription and activity of HMGR in plants are influenced by diverse factors such as development, HMGR inhibitors, plant growth regulators, and light [39]. Previous studies have reported that expression of the HMGR gene in *Picrorhiza kurrooa* is inhibited by dark condition, whereas HMGR is accumulated at a high level under light condition [40]. Indeed, continuous exposure to darkness has resulted in a gradual decrease of expression of HMGR in 3-year-old ginseng leaves [41]. Therefore, decrease in HMGR expression can inhibit the growth of main root lengths and hamper the growth of lateral root [42]. Biosynthesis and accumulation of ginsenosides in ginseng plants are vitally affected by levels of various gene expressions at different developmental stages [4]. These results demonstrate that ginsenoside biosynthesis could be highly related to HMGR expression at the developmental stage and influenced by climatic conditions. Therefore, decreased sun exposure time and increased rainfall after May found in the present study might have contributed to inhibited synthesis of ginsenoside compounds in ginseng roots because of decreased photosynthesis (Figs. 3D and 3E), concurrently with accumulation of sucrose in roots and leaves (Figs. 7 and S7), as also described in fresh tea (*C. sinensis* L.) leaves grown under various shade conditions [35]. These results strongly suggest that levels of

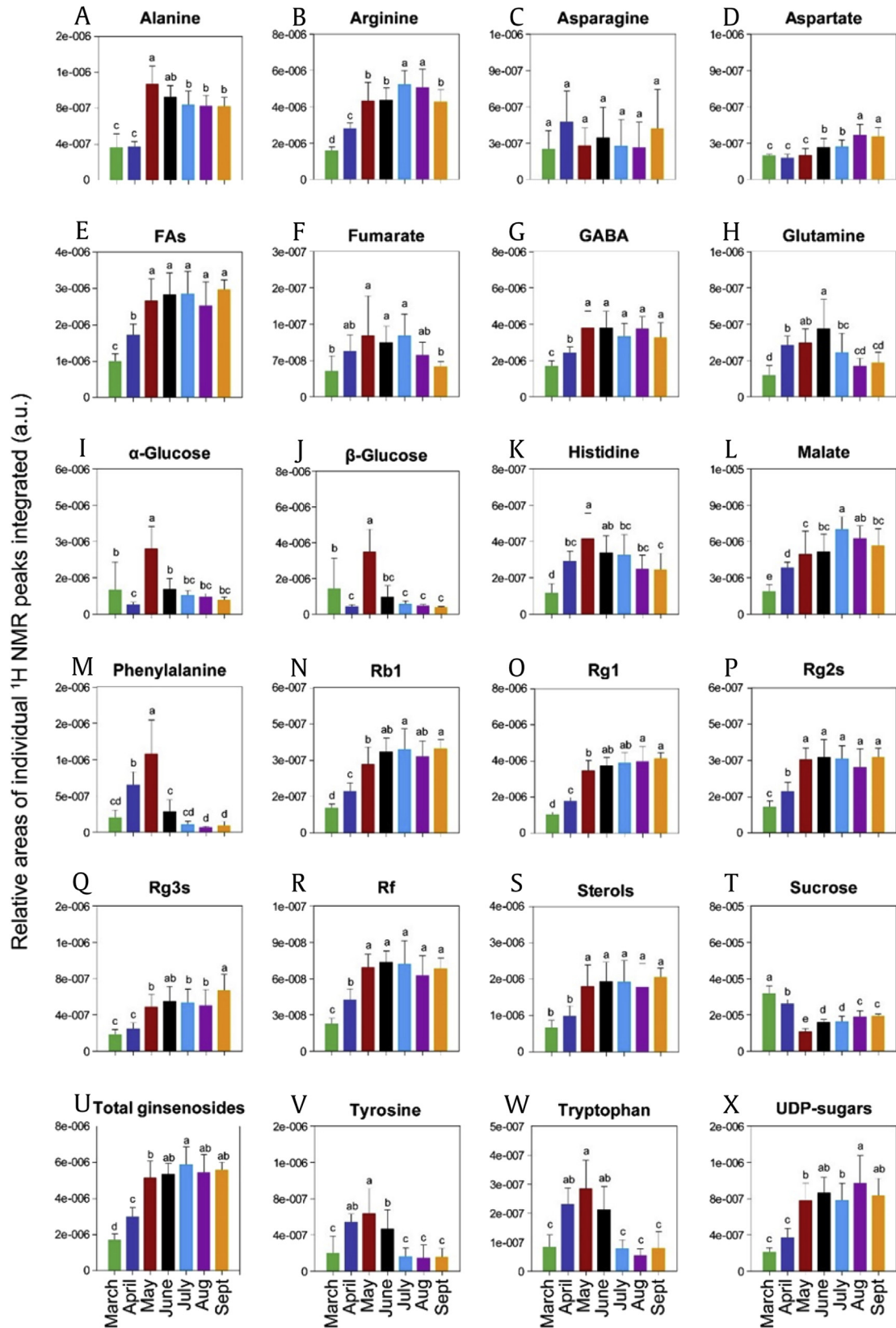


Fig. 7. Relative concentrations of ¹H NMR peaks corresponding to individual ginseng main root metabolites from March to September which were calculated following spectra normalization. The panels A–X represents alanine, Arginine, Asparagine, Aspartate, fatty acids (FAs), Fumarate, g-aminobutyrate (GABA), Glutamine, α-Glucose, β-Glucose, Histidine, Malate, Phenylalanine, Rb1, Rg1, Rg2s, Rg3s, Rf, Sterols, Sucrose, Total ginsenosides, Tyrosine, Tryptophan, and uracil diphosphate (UDP)-Sugars, respectively, Rg1, Rg2s, Rg3s, Rb1, and Rf, ginsenosides Radix g1, g2s, g3s, b1, and f.

ginsenoside compounds in ginseng roots depend on their developments closely linked to seasonal climatic conditions such as sun exposure time and rainfall. The contents of total ginsenosides including F2 and Re in ginseng leaves were not dependent on climates and seasons, possibly due to collection of leaves from May, for which further studies are needed.

4.3. Sterols

Plant sterols, also called phytosterols, belong to the family of isoprenoids. They play a central role in adapting the membrane of cells to temperature by controlling the flexibility of the membrane [43]. They also function as substrates of various secondary metabolites such as glycoalkaloids and saponins. They are associated with differentiation and proliferation of cells [44]. Metabolisms of sterols in ginseng main and lateral roots were similar to those of ginsenoside compounds as ginseng plants grew (Figs. 7S and 56S). Previous studies have reported that plant sterols and triterpenes share the same precursor, 2,3-oxidosqualene, that is produced through the MVA pathway [45]. Notable increases of ginsenoside compounds and sterols can be due to enhanced activity of squalene synthase which synthesizes squalene, a precursor of saponin, in ginseng roots [46,47]. It has been reported that mevalonate-5-pyrophosphate decarboxylase and farnesyl diphosphate synthase genes expressed in ginseng hairy roots contribute to accumulation of ginsenosides and sterols [48]. Although expression levels of mevalonate-5-pyrophosphate decarboxylase and farnesyl diphosphate synthase genes during seasonal cultivation of ginseng plants have not been reported, accumulations of sterols together with ginsenoside compounds in ginseng roots between March and May in the present study likely reflect increased expression levels of these genes that might also depend on seasons and climatic conditions.

4.4. Arginine

Arginine is a precursor of nitric oxide (NO) that is associated with many physiological processes of plants such as growth, development, and defense [49]. Cytokinin, a plant hormone, can lead to the synthesis of NO in tobacco, parsley, and *Arabidopsis* [50]. Moreover, arginine is involved in the synthesis of polyamine that is related to diverse physical responses such as development, aging, and stress in plants [51]. Owing to diverse roles of polyamines in cell metabolism and development, they are needed in large quantities for rapidly growing tissues. The biosynthesis of polyamine begins from arginine [51]. Therefore, increased levels of arginine in ginseng roots could be due to various biological responses or needs of growing ginseng plants. This might consequently contribute to active development of ginseng roots (Figs. 7B and 56B).

4.5. Aromatic amino acids

In plants, the shikimate pathway is known to play an important role in the biosynthesis of aromatic amino acids such as phenylalanine, tyrosine, and tryptophan as well as secondary metabolites such as flavonoids [52]. It is known that increased carbon flow through the shikimate pathway in the development process reflects the increasing demand for common protein biosynthesis [53]. Phenylpropanoid precursors phenylalanine and tyrosine for lignin biosynthesis are needed for seedling growth and shoot formation of the callus [54]. Auxin is one of the plant growth hormones, and indole-3-acetic acid is the major naturally occurring auxin in plants [55]. There are two major pathways for indole-3-acetic acid biosynthesis in plants: tryptophan (Trp)-independent and Trp-dependent pathways [56]. Trp-dependent auxin biosynthesis has

been shown to be essential for leaf formation, flower development, and other developmental processes [57]. Flavonoids are synthesized from phenylalanine or tyrosine sometimes [58]. Thus, synthesis and degradation pathways of aromatic amino acids are important for the production of flavonoids such as kaempferol [59]. Synthesis of flavonoids is strongly associated with environmental conditions. It is increased under various abiotic stresses [60]. According to climate data, decreased sun exposure time and increased rainfall after May might lead to several abiotic stresses in ginseng plants (Figs. 3D and 3E). The present study showed that levels of kaempferol in ginseng leaves were increased after May (Figs. 57F). However, kaempferol was not found in any ginseng roots throughout all growing seasons. This might be due to its too low amounts to be detected by NMR analysis. Therefore, levels of aromatic amino acids might be increased during ginseng plant growth until May but decreased after May to produce kaempferol because of increased abiotic stress caused by climate changes.

5. Conclusion

In conclusion, to date, metabolic characterization of ginseng plants during whole growing seasons has not been reported yet. The present study demonstrates that the metabolome of ginseng plants is clearly dependent on their growth and climatic conditions such as sun exposure time and rainfall. Comprehensive analysis of a wide range of ginseng plant metabolites and multivariate statistical analysis may provide important information about optimal harvesting time, highlighting that their intrinsic metabolism and metabolic traits might be potentially used for quality improvement of ginseng plants.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jgr.2019.04.004>.

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