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Quality evaluation of green tea leaf cultured under artificial light condition using gas chromatography/mass spectrometry

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For an experimental model to elucidate the relationship between light quality during plant culture conditions and plant quality of crops or vegetables, we cultured tea plants (*Camellia sinensis*) and analyzed their leaves as tea material. First, metabolic profiling of teas from a tea contest in Japan was performed with gas chromatography/mass spectrometry (GC/MS), and then a ranking predictive model was made which predicted tea rankings from their metabolite profile. Additionally, the importance of some compounds (glutamine, glutamic acid, oxalic acid, epigallocatechin, phosphoric acid, and inositol) was elucidated for measurement of the quality of tea leaf. Subsequently, tea plants were cultured in artificial conditions to control these compounds. From the result of prediction by the ranking predictive model, the tea sample supplemented with ultraviolet-A (315–399 nm) showed the highest ranking. The improvement in quality was thought to come from the high amino-acid and decreased epigallocatechin content in tea leaves. The current study shows the use and value of metabolic profiling in the field of high-quality crops and vegetables production that has been conventionally evaluated by human sensory analysis. Metabolic profiling enables us to form hypothesis to understand and develop high quality plant cultured under artificial condition.

[**Key words:** *Camellia sinensis*; Plant factory; Metabolomics; Quality of green tea; Prediction model]

Technology for factories producing plant crops and vegetables in controlled environment (i.e., plant factories) has gathered much attention with the increasing concern about safety of food supply and some plant factories have already been operating commercially (1). However, crops from plant factory need a unique advantage over outdoor-grown crops to cover for their high production cost. Recently, leafy vegetables have been the main products of plant factory; utilizing the merits of year-round production and pesticide-free culture. In the future, through the development of plant growth control and/or useful component enrichment, more competitive crops are expected to appear. Many researches on plant culture using artificial light source have been reported that showed the possibility of producing highly competitive crops. These researches often focused on the relationship between light environment during culture and variation of growth or component of cultured plant (2,3). For instance, Yorio et al. showed that addition of blue light (400–500 nm) showed increase in dry weight of cultured spinach, radish, and lettuce compared with red light (660 nm) single irradiation condition (4).

Light quality was found to affect the increase in some medicinal compounds as well (5). Ohashi-Kaneko et al. (6) did research on the variation of chlorophyll, carotenoid, L-ascorbic acid, soluble sugar, and nitrate in plants of leaf lettuce, spinach, and komatsuna (Japanese mustard spinach) cultured under four types of light conditions using white, blue, and red fluorescent light. Hata et al. (7) cultured plants of sesame variety Gomazou under light conditions of three types with LED, and they reported that plants grown under blue light (470 nm) showed about 2-fold and 4-fold greater sesamin content than those grown under white light and red light (660 nm), respectively.

Although the effect of light environment during culture on change in biomass or a particular component has been reported by numerous researchers, there are limited results about the effect of light on change in sensory qualities of crops (e.g., taste, flavor, preference) to date (2,8). It could be assumed that the sensory qualities of crops are subjective and differ widely among individual crops in nature due to complex sensory attributes involving many components. In contrast to medicinal plants, it is difficult to determine the optimum culture conditions for maximizing the sensory qualities of crops because an increase in a particular component is not necessarily linked to the qualitative improvement of these crops. However, the improvement of the sensory qualities of crops is necessary to produce special high-quality crops in plant factories. Thus, in cultured crops, it is essential to develop a feedback system based on sensory analysis in order to determine the optimum light conditions.

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We have been mounting efforts to develop artificial culture techniques for high-quality crops with knowledge gained from plant metabolome analysis. Specifically, we have been performing multicomponent analysis and revealing characteristics of high quality crops in the market to find leads for constructing optimized artificial culture conditions. In addition, we have been measuring the effect of specialized artificial conditions on plant quality. Previously, we have reported our attempt to culture tea plant, *Camellia sinensis*, as an experimental model plant (9). In this approach, we focused on the amino-acid profiles of teas from a tea contest, and we performed metabolome profiling using high-performance liquid chromatography (HPLC) to construct a ranking predictive model to reveal the important amino acids in high-ranking teas. Based on this profiling, we designed an artificial culture condition to increase the important amino acids, and obtained artificial-cultured tea (AC tea) samples that were predicted to have very high quality. These results showed that a change in culture conditions (i.e., light quality during the late stage of the culture process) can significantly affect the amino-acid profile of tea leaf.

In the current study, we expanded the scope of analysis to various hydrophilic compounds including amino acids, and performed metabolome profiling of Japanese green tea using gas chromatography/mass spectrometry (GC/MS) multicomponent analysis reported previously (10). Subsequently, we measured the effect of light quality during the late stage of culture on plant metabolome and crop quality, and as a result, recognized the possibility of artificially culturing tea leaf for high-quality Japanese green tea.

MATERIALS AND METHODS

Tea samples Post-processed dry leaves of 36 ranked tea samples (spring-harvested first crop, called Ichi-ban-cha in Japanese) from the 2012 contest were analyzed. Japanese green teas are categorized by production process and in this study, teas in the category of Tencha (unground Matcha) were selected. Ranking of teas was determined by the total scores of the sensory tests, namely leaf appearance, leaf color in hot water, smell, color of the brew and its taste, as judged by professional tea tasters. Artificially cultured tea samples described below were prepared through the process of culture under artificial light. After harvest, raw tea leaves were immediately blanched and dried by using a microwave oven and stored at −80°C until analysis.

Reagents All chemicals used in this study were analytical grade. Methanol and chloroform used as extraction solvents were purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). Ribitol (diluted with deionized water to a concentration of 0.2 mg/mL) and pyridine used as a solvent were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methoxyamine hydrochloride was purchased from Sigma−Aldrich Co. (St. Louis, MO, USA). *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) was purchased from GL Science, Inc. (Tokyo, Japan).

Sample preparation and GC/MS analysis Dried tea leaves (15 mg) in 2-mL Eppendorf tubes were freeze-dried and ground with a Retsch ball mill (20 Hz, 1 min). Hydrophilic primary metabolites of green tea were extracted using a single-phase solvent mixture of MeOH, H_2O, and $CHCl_3$ in a ratio of 2.5/1/1 (v/v/v), respectively. The mixture was shaken for 5 min with 60 μL ribitol (as internal standard, added at the final concentration of 0.02 mg/mL) and centrifuged at 16,000 ×*g* for 3 min at 4°C. Subsequently, 900 μL of the supernatant was transferred to a 1.5-mL Eppendorf tube. After adding 400 μL of purified water (Wako Pure Chemical Industries, Ltd.), the mixture was vortexed and centrifuged. Four hundred microliter (400 μL) of the polar phase was then transferred to another 1.5-mL Eppendorf tube capped with pierced cap. The extract was dried in a vacuum centrifuge dryer until dry (overnight).

For derivatization, 100 μL of methoxyamine hydrochloride in pyridine (20 mg/mL-pyridine) was added as a first derivatizing agent. The mixture was incubated at 1200 rpm for 90 min at 30°C. Fifty microliter (50 μL) of the second derivatizing agent, MSTFA, was added and incubated at 1200 rpm for 30 min at 37°C. After derivatization, 200 μL of each sample was transferred to a crimp-top vial (Chromacol Ltd., London, UK) and analyzed by GC/MS.

For the annotation of metabolites and classification of tea grade, GC/MS analysis was performed using a GCMS-QP2010 ultra (Shimadzu Co., Kyoto, Japan) equipped with a 30 m × 0.25 mm i.d. fused silica capillary column coated with 0.25-μm CP-SIL 8 CB low bleed (Agilent Technologies, Inc., Palo Alto, CA, USA) and an AOC-20i autosampler (Shimadzu Co.). The injection temperature was 230°C. The helium gas flow rate through the column was 1.12 mL/min. The column temperature was held at 80°C for 2 min isothermally and then raised by 15°C/min to 330°C and held there for 6 min isothermally. The transfer line and the ion source temperatures were 250°C and 200°C, respectively. Ions were generated by a 70 kV electron impact (EI), and 20 scans per second were recorded over the mass range *m/z* 85−500.

Sample preparation and HPLC analysis The amino-acid content and composition of tea samples were measured by using HPLC method of Takayanagi et al. (11). All samples were ground into powder form; 0.1 g of each sample was extracted in 100 mL of purified water at 80°C for 30 min. After the addition of the internal standard (0.4 mg of norvaline), the extraction liquid was mixed with an *o*-phthalaldehyde (OPA) solution, which consisted of 0.143 g of OPA (Wako Pure Chemical Industries, Ltd.) dissolved in 10 mL of 0.1 M borate buffer at pH 10 and 0.25 mL of 2-mercaptoethanol (Kishida Chemical Co., Ltd.). The OPA solution was stored at 5°C and renewed weekly. Fifteen milliliter of the extraction mixture and the OPA solution was injected into Waters Alliance 2695 HPLC system (Waters Co., Milford, MA, USA) immediately after mixing, and amino acids derivatives were separated in an ODS column (4.6 × 150 mm, 5 μm particle size; GL Science Inc.) eluted with a gradient of solvent A (12% v/v ethanol, pH 6.0, adjusted with citric acid) and solvent B (50% v/v ethanol, pH 6.0, adjusted with citric acid). The gradient was linearly from 100% A to 100% B in 60 min, and the flow rate was 1 mL/min. The eluant was monitored in a Waters 474 fluorescence detector (Waters Co.; λex = 340 nm, λem = 455 nm), and the following free amino acids were detected: aspartic acid, glutamic acid, asparagine, serine, glutamine, arginine, theanine, and alanine. A standard solution for the preparation of the calibration curves, which contained a known concentration of each amino acid, was measured simultaneously, and the amino acids in tea samples were quantified by comparing peak areas with the standard.

The contents of catechins, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate were measured by the HPLC method based on Terada et al. (12), Suematsu et al. (13) and Horie et al. (14). Powdered tea samples were extracted with 50 mg/5 mL admixture of acetonitrile and purified water (50% v/v) at 170 spm shaking, room temperature, for 60 min. The extracts were then centrifuged at 3000 rpm for 10 min at 4°C. After the separation, 100 μL of the supernatant was filtered through a 0.45-μm filter (Minisart RC15; Sartorius Stedim Biotech GmbH, Göttingen, Germany) and analyzed by Prominence; CBM-20A (Shimadzu Co.). The separation column was TSKgel ODS-80TS (Tosoh Co., Tokyo, Japan), and elution solvents were solvent C (10 mM phosphate buffer; pH 2.1) and solvent D (100% acetonitrile) with a gradient. Initially, the gradient system was 99% solvent C and 1% D, followed by linearly changing to 15% D for 30 min, then kept in 10% C and 90% D for 10 min, and finally returned back to the initial state (99% C and 1% D) and kept for 25 min. Therefore, 1 cycle took 65 min in total. The flow rate was kept constant at 1 mL/min. Detection of analyte was accomplished with UV detection at 280 nm by using a SPD-20A UV/vis detector (Shimadzu Co.).

Multivariate analysis The raw chromatograms were converted to AIA (ANDI) file format by GCMS solution. Peak alignment, baseline correction, and normalization were performed using MetAlign (ver. 041011), and compound annotation was performed using the in-house software Aloutput2 (ver. 1.29). The result of auto-annotation by Aloutput2 was visually confirmed by comparison to the in-house mass chromatogram library. The artifact peaks from extraction solvents and antifoam reagents were also excluded from the data matrices. The obtained data matrices were subjected to a projection to latent structures (PLS) regression analysis by using Aloutput2 and SIMCA-P ver. 11.0 software (Umetrics AB, Umeå, Sweden). Moreover, peak deconvolution and compound identification were performed in the Automated Mass Spectral Deconvolution and Identification System (AMDIS) software. In addition, a series of linear alkanes ranging from C6 to C26 was analyzed under same GC conditions as references for retention indices (RI) and the RI values were compared with those from published literature.

Artificial culture conditions Tea seedlings (*C. sinensis*) used in all experiments were one-year-old rooted cuttings of the cultivar Yabukita. In each experiment, the same lot of plant was used, simultaneously acclimated and cultured in a hydroponic condition, to avoid confusion with variation across lots of plant or variation caused in preparation step.

First, tea seedlings were transplanted into hydroponic conditions in a glasshouse. The composition of the nutrient solution was determined according to the method described by Konishi et al. (15); it contained the macronutrients (mM) NH_4−N (2.1), NO_3−N (0.7), P (0.1), K (1.0), Ca (0.7), and Mg (1.0) and the micronutrients (μM) Fe (6.3; as EDTA salt), B (9.3), Mn (18.2), Zn (1.5), Cu (0.4), Mo (0.5), and Al (400). After a 3-week culture in 1/4 strength nutrient concentration, the seedlings were cultured in a full-strength solution. The culture solution was constantly aerated and fully renewed once every two weeks. Seedlings were acclimated to the hydroponic condition for at least seven weeks following transplantation and then used for the artificial culture experiment.

In the artificial culture experiment, seedlings were grown in growth chambers (LPH-220SPC, Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) with 15 plants per section. At the start of the cultivation process, the seedlings were in dormant condition. Culture conditions in each experiment are shown in Fig. 1. As for the basal condition, the plants were cultured through a 6-week dormancy period, the budbreak period (until new secondary leaf opening; normally 4−8 weeks), and a 4-week cover-culture period. The culture solution was constantly aerated and fully renewed once a week. The culture solution in the cultivation process under artificial light was nitrogen-rich, which contained the macronutrients (mM) NH_4−N (4.2),

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