Metabolomic Assessment Reveals an Elevated Level of Glucosinolate Content in CaCl₂ Treated Broccoli Microgreens

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Supporting Information

ABSTRACT: Preharvest calcium application has been shown to increase broccoli microgreen yield and extend shelf life. In this study, we investigated the effect of calcium application on its metabolome using ultra-high-performance liquid chromatography with mass spectrometry. The data collected were analyzed using principal component analysis and orthogonal projection to latent structural discriminate analysis. Chemical composition comparison shows that glucosinolates, a very important group of phytochemicals, are the major compounds enhanced by preharvest treatment with 10 mM calcium chloride (CaCl₂). Aliphatic glucosinolates (glucorucin, glucobrassicin, glucobrassicin, glucoraphanin, pentyl glucosinolate, and hexyl glucosinolate) and indolic glucosinolates (glucobrassicin, neoglucobrassicin, and 4-hydroxyglucobrassicin) were increased significantly in the CaCl₂ treated microgreens using metabolomic approaches. Targeted glucosinolate analysis using the ISO 9167-1 method was further employed to confirm the findings. Results indicate that glucosinolates can be considered as a class of compounds that are responsible for the difference between two groups and a higher glucosinolate level was found in CaCl₂ treated groups at each time point after harvest in comparison with the control group.

KEYWORDS: broccoli microgreens, metabolomics, glucosinolates, food quality, UHPLC-HRMS

INTRODUCTION

Glucosinolates (GLs) are an important group of phytochemicals present in Brassicaceae.¹ Broccoli (Brassica oleracea L. var. italica) is an economically important vegetable for consumption of both florets and young seedlings. It has been recognized that broccoli sprouts (3−5 day seedlings) are exceptionally rich in GLs.² Accumulating evidence indicates that GLs and their derivatives, such as isothiocyanate, contribute to plant stress tolerance and plant defense against pathogens.³,⁴ Furthermore, it is suggested that GLs are responsible for the protective effects against pancreas, lung, stomach, colon and rectal, and prostate cancer.⁵−⁷

Microgreens are 7−14 days old young edible greens produced from vegetables, herbs, or other plants, ranging in size from 5 to 10 cm long including stem and cotyledons (seed-leaves). In recent years, consumption of microgreens has increased along with consumer awareness and appreciation for their intense flavors, vivid colors, and phytonutrients.⁸−¹⁰ However, the low yield and the short shelf life of microgreens are limiting factors for the industry.¹¹,¹² Calcium chloride (CaCl₂) is proving to have a significant impact on the shelf life of various vegetables with the benefits of delaying aging or ripening, reducing postharvest decay, and improving the nutritional value. Calcium ions make cell walls less accessible to enzymes that cause softening or to cell wall degrading enzymes produced by fungal pathogens.¹³,¹⁴ Previously, we found that preharvest samples sprayed with 10 mM CaCl₂ increased the broccoli microgreen biomass by more than 50% and extended the shelf life significantly.¹⁵ However, the effect of calcium treatment on the inner chemical profile changes, especially GL contents, has never been studied.

Metabolomics is an emerging field of “omics” research that focuses on high-throughput characterization of small molecule metabolites in biological matrices.¹⁶,¹⁷ It has shown great usefulness in many areas of food science and nutrition research such as food component analysis,¹⁸−²⁰ food quality/authenticity assessment,²¹,²² food consumption monitoring, and physiological monitoring in food intervention or diet challenge studies.²³,²⁴ It is an important comparative tool for studying global metabolite levels of plant materials grown under different conditions. Untargeted metabolomic approaches are intended to measure as many metabolites as possible to obtain patterns or fingerprints of food samples.

Here, we report the analysis of the inner chemical changes of broccoli microgreens using a nontargeted metabolomic approach and the impact of calcium treatment on the GLs in microgreens on different days from preharvest growing to

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postharvest storage. The chemical profiles of broccoli microgreens were monitored for different growth periods and different shelf time after harvest. The goal of this study was to identify the differences in the chemical profiles for each experimental factor different group of samples and to further validate the findings quantitatively.

## MATERIALS AND METHODS

### Chemicals.
Formic acid and high-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were purchased from VWR International, Inc. (Clarksburg, MD). HPLC-grade water was prepared from distilled water using a Milli-Q system (Millipore Lab., Bedford, MA, USA). (−) Sinigrin hydrate (≥99.9%), sulfatase (from Helix pomatia), imidazole, and Sephadex A-25 chloride form were purchased from Sigma–Aldrich (St. Louis, MO, USA). Glucoerucin potassium salt, glucoerucin potassium salt, and glucoraphanin potassium salt were purchased from Chromadex (Irvine, CA, USA).

### Plant Materials and Sample Preparation for Metabolomic Study.
Broccoli microgreens were grown in a growth chamber (25 °C) as previously described. 15 Hydroponic “Sure seeds” were then spread evenly over the damp pad. The trays were kept in a growth chamber at 25 °C. During the first four days, the trays were covered, and the seeds were germinated in the dark. For the next six days, the seedlings were exposed to light with a light intensity of 42 μmol m−2 s−1 for 12 h/12 h (light/dark). The seedlings were sprayed once a day with H2O (pH 5.6) only or 10 mM CaCl2. Each treatment of microgreens has three trays as triplicate. Three trays of the microgreens were collected at the fourth day after sowing from each treatment group and labeled as “Preharvest Day 4.” All of the rest of the microgreens, including hypocotyl and cotyledons, were harvested on 10 days after sowing (labeled as “Harvest Day”) by cutting near the bottom of each hypocotyl with a pair of sterilized scissors. Harvested broccoli microgreens (0.01 kg each) were packaged in sealed bags (0.1 m × 0.1 m) prepared with polyethylene films of 16.6 μm, and 10 mL of the extract was used for each HPLC injection.

### Extration and Desulfation of GLs.
The extraction of GLs was performed following the ISO9167-1 method with slight modification. A 200 mg amount of broccoli microgreen powder was weighed into sintered glass tubes and extracted with 5 mL of methanol, for 15 min (IEC Clinical Centrifuge, Damon/IEC Division, Needham, MA, USA). The supernatant was filtered through 16.6 pmol s−1 m−2 for 12 h/12 h (light/dark). The seedlings were sprayed once a day with H2O (pH 5.6) only or 10 mM CaCl2. Each treatment of microgreens has three trays as triplicate. Three trays of the microgreens were collected at the fourth day after sowing from each treatment group and labeled as “Preharvest Day 4.” All of the rest of the microgreens, including hypocotyl and cotyledons, were harvested on 10 days after sowing (labeled as “Harvest Day”) by cutting near the bottom of each hypocotyl with a pair of sterilized scissors. Harvested broccoli microgreens (0.01 kg each) were packaged in sealed bags (0.1 m × 0.1 m) prepared with polyethylene films of 16.6 μm, and 10 mL of the extract was used for each HPLC injection.

### Qualitative and Quantitative Analysis of GLs.
GLs were determined using a UHPLC-HRMS method that simultaneously separates intact GLs and other phenolics. The separated intact GLs, hydroxycinnamic acids (chalconic acid derivatives and sinapic acid derivatives), and flavonols were identified following their MS2 ~ MS5 fragmentations, UV-visible spectra, and the order of elution previously described for similar acquisition conditions. The PDA signal at 229 nm was used to quantify the desulfo-GLs. The type and amount of desulfo-GLs in microgreen broccoli were calculated using known concentrations of standard sinigrin and recommended relative response factors. 25 The major GL compounds were quantified by integration of the peak areas using Xcalibur 2.1.

### LC-MS Data Pretreatment and Handling.
Acquired UHPLC-HRMS raw files were converted into mZXML format using Proteowizard 3.0. 3569 (http://proteowizard.sourceforge.net/), and then XCMS was employed for peak detection, noise filtering, and peak alignment. A two-dimensional data matrix was generated from XCMS including variable index (paired m/z~retention time), sample names (observations), and peak intensities. Multivariate analysis was performed on the output table from XCMS using Sigma 13 (Umetrics, Umeå, Sweden), where principal component analysis (PCA) was performed to visualize group clustering, trends, or outliers among the observations, and then, supervised orthogonal projection to latent structural discriminate analysis (OPLS-DA) was performed. Pareto scaling was used for data normalization before multivariate data analysis (MVDA). PC1 and PC2 scores were used for the PCA models as these principal components seemed to reflect the main variation and separation in data. The score plot of PCA was used to observe clustering and trends among all samples. The loadings plot of the first two principle components was applied to evaluate the variables causing the separation between the groups. The structures of the marker compounds were annotated by searching free databases of Scripps Metlin (http://metlin.scripps.edu/) using exact mass and MS/MS spectra and structures. Available standards were used for the confirmation of the identity of the compounds.

## RESULTS AND DISCUSSION

### Profiling of the Major Compounds in Broccoli Microgreens.
GLs and hydroxycinnamic acid derivatives are two major groups of compounds found in microgreen broccoli, which were reported in the matured broccoli. 27,28 In our previous study of other brassica microgreens, 3 it was found that hydroxycinnamic acid derivatives have good response under negative ionization mode. GLs are hydrophilic compounds with a sulfonate moiety in structure, which occur in nature as the anionic form. The most diagnostic ions were observed at m/z 259 and 275 in MS2 or MS3 spectra, produced by intraring rearrangement. This typical fragmentation can be used for fast characterization of GLs. 29~31 An UHPLC-MS profiling method

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under negative ionization mode with a data-dependent scan was developed for a comprehensive study of the GLs and other phenolic components, and it leads to the identification of 15 GLs, 3 acylated flavonolglucosides, 1 flavan 3-ol, 6 hydroxycinnamic acid derivatives, and 2 sugars in broccoli microgreens. A total of 15 GLs including 11 aliphatic GLs and 4 indolic GLs were identified (Figure S1, Supporting Information). The major ones were glucoerucin (ERN), glucoraphanin (RPN), glucobrassicin (GBRN), glucobrassicin (GBRN), and 4-methoxyglucobrassicin (MGBRN). Six hydroxyl cinnamoyl derivatives, including 1,2-disinapoylgentiobiose, 1,2-bis-O-sinapoyl-β-D-glucoside, 1,2,2′-trisinapoylgentiobiose, 2-feruloyl-1,2′-disinapoylgentiobiose 1-O-sinapoylglucose, and their isomers were identified by comparing the HRMS data with the previous reports for broccoli or other brassica vegetables.32−34 Other compounds such as catechin, acylated-kaemperol glycosides,35 and sugars were also found. Detailed accurate mass and mass fragmentation information can be found in Table S1 (Supporting Information).

Metabolomic Analysis of Different Stages of Microgreen Samples between CaCl2 Treated and Control Groups. As shown in Figure S1 (Supporting Information), it would be very difficult to find the differences by visual examination of the chromatograms between the CaCl2 treated microgreens and the control (H2O) group. A metabolomic approach was employed for characterization of the chemical difference between the groups. The original UHPLC-HRMS raw data led to 5107 ion features from 30 samples. The PCA based on the relative intensities of these ion features revealed the effects of CaCl2 treatment after pareto scaling (Figure 1A). The first two components accounted for 79.4% of the total variance. It showed no significant difference between the CaCl2 group and the control group (H2O) on the fourth day after sowing. They are grouped closely on the right of the PCA scoreplot. Another PCA was performed with postharvest samples only, as shown in Figure 1B. They are separated into two groups according to the postharvest times. The samples from harvest day (Day 0) and postharvest day 4 are clustered closely against the postharvest day 7 and day 14 groups on PC1, and the CaCl2 treated samples are separated with H2O treated samples on PC2. It suggested that the sampling time is another important factor for chemical composition of the samples. In order to define the effects on the CaCl2 treatment and to minimize other factors, a supervised OPLS-DA statistical model was utilized to focus on the discrimination of the CaCl2 treatment and H2O spray only. The OPLS-DA approach has been widely used in metabolomic studies on botanical materials with different growth conditions, species, locations, harvest times, and so forth.36−41 The OPLS-DA model resulted in one predictive and one orthogonal (1 + 1) component with the cross-validated predictive ability $Q^2(Y)$ 98.2% and the total explained variance $R^2(X)$ 58.0% (Figure 1C). In addition, a permutation test ($n = 100$) was carried out to evaluate the overfitting of the model. The discriminatory ion features were picked out by a variable importance projection (VIP) plot. The ion features with larger VIP values (more than 4) were...
considered more relevant for sample classification in this study, as shown in Figure 1D. After filtering out the ion features for adduct ions, isotope ions, and fragment ions, 12 constituents including 9 GLs, 2 sinapinic acid derivatives, and a hexose were considered as major marker compounds responsible for the group classification, as shown in Table 1. For example, ion feature (m/z 420.0448, t_R = 5.74 min) has a calculated elemental composition of C_{11}H_{20}O_{10}NS_{3} (3.4 ppm). The diagnostic ions at m/z 259 and 275 were observed in the MS^2 spectrum. It was identified as glucoraphanin, a compound previously reported in broccoli.42−44 Using the same approach, we conclude that the marker GL compounds are ERN, RPN, IBN, IVN, GBRN, neoglucobrassicin (NGBRN), OHGBRN, HXG, and PGN.

Table 1. Discriminatory Metabolites Responsible for the Group Classification between the CaCl_2 and H_2O Treated Groups

<table>
<thead>
<tr>
<th>ion feature (m/z, t_R)</th>
<th>VIP value</th>
<th>formula</th>
<th>possible identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>420.0448, 5.74</td>
<td>20.63</td>
<td>C_{11}H_{20}O_{10}NS_{3}</td>
<td>glucoraphanin</td>
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<tr>
<td>436.0398, 1.93</td>
<td>9.26</td>
<td>C_{12}H_{22}O_{10}NS_{3}</td>
<td>glucobrassicin</td>
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<tr>
<td>215.0317, 13.84</td>
<td>11.49</td>
<td>C_{6}H_{12}O_{6}Cl</td>
<td>hexose</td>
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<tr>
<td>959.2774, 46.75</td>
<td></td>
<td>C_{12}H_{23}NO_{9}S_{3}</td>
<td>trisnapoylgentiobioside</td>
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<tr>
<td>422.0244, 1.83</td>
<td>9.75</td>
<td>C_{11}H_{20}O_{10}NS_{3}</td>
<td>glucobrassin</td>
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<tr>
<td>406.0295, 3.56</td>
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<td>C_{13}H_{24}NO_{9}S_{2}</td>
<td>hexyl glucosinolate</td>
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<td>477.0632, 17.17</td>
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<td>463.0473, 3.49</td>
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<td>402.0883, 14.24</td>
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<td>C_{12}H_{23}NO_{9}S_{2}</td>
<td>pentyl glucosinolate</td>
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Verification of the Marker GLs by Targeted GL Analysis. The metabolomic approach suggested that the major marker compounds, which changed significantly by CaCl_2 treatment, were GLs. However, the comparison of marker compounds is based on peak area of the marker compounds. In order to confirm the findings from the nontargeted metabolomic approach, the targeted analysis of GLs in microgreen broccoli was carried out according to the guidelines established in ISO 9167-1.45 ISO 9167-1 specifies the method for the determination of GL content in rapeseed by quantifying the desulfo derivatives, using HPLC after sulfatase hydrolysis and anion ion-exchange concentration.

Before the lack of reference standards, the desulfo-GLs are confirmed using HRMS. The product ion at m/z 195.03 (C_{6}H_{11}O_{5}S^-) that represents the thioglucose group was used for fast identification of desulfo-GLs. Figure S2 (Supporting Information) shows the typical chromatogram for GL determination using the ISO method, and a total of 10 different GLs was identified together with the internal standard.

Figure 2. GL content in CaCl_2 (Ca) treated and control (H_2O) groups on different days.

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sinigrin. They were desulfo-IBN, desulfo-IVN, desulfo-RPN, desulfo-ERN, desulfo-PGN, desulfo-HXG, desulfo-OHBRN, and desulfo-NGBRN. The desulfo forms of two indolic marker GLs, GBRN and MGBRN, detected in the HPLC-HRMS profiling were not found using the ISO method. It may be due to that UV detection is much less sensitive compared to MS detection. The most abundant GLs are ERN followed by NGBRN and IVN. The GL profile in microgreen broccoli is different from that of broccoli sprouts but shows comparable total GL content.46–48

In comparison with the metabolomic approaches, the results show that aliphatic and indole GLs increase significantly after harvest but not for the preharvest samples in CaCl2 treated samples. As shown in Figure 2, the highest GL concentration was observed at preharvest samples for both CaCl2 and H2O treated samples. The total GLs are 56.99 μmol/g dry weight (dw) and 51.62 μmol/g dw detected in the CaCl2 treated and the control sample in the preharvest samples, respectively. After harvest, both the CaCl2 treated and the control group showed decreasing of total GLs. For the CaCl2 treated samples, the total GLs decreased to 39.24 μmol/g (by 33.0%) at day 14, while the GL samples in the control samples dropped to 23.84 μmol/g (by 50.5%). At each time point after harvest, the CaCl2 treated samples showed an elevated level of total GLs together with the higher individual GLs such as ERN, IBN, RPN, IVN, OHBRN, HXG, NGBRN, and PGN.

Accumulating evidence indicates that GL biosynthesis in plants can be upregulated by environmental stress signals and pathogen attacks.54 Calcium has been shown to be an important secondary messenger in the relay of developmental, environmental, and hormonal signals that regulate plant growth and development as well as responses to biotic and abiotic stresses.49–51 It has been reported the overexpression of a calcium/calmodulin-binding IQD1 leads to the expression of AtSR1/CaMTA3 resulted in the decreased GL levels and another calcium/calmodulin regulated transcription factor

**ASSOCIATED CONTENT**

**Supporting Information**

Typical HPLC chromatograms and the identification of the major compounds in broccoli microgreen (Figure S1 and Table S1); the extracted base peak (m/z 195) chromatogram of desulfo-GLs (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

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