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ARTICLE

Metabolic profiling of pale green and purple kohlrabi (*Brassica oleracea* var. *gongylodes*)

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Abstract Kohlrabi (*Brassica oleracea* var. *gongylodes*) is a dietary *Brassica* vegetable with noted health-beneficial properties associated with its numerous metabolites. The aim of this study was to elucidate phenotypic variation between the two cultivars through comprehensive analysis of the relationship of their primary and secondary metabolites. High-performance liquid chromatography (HPLC) and gas chromatography time-of-flight mass spectrometry (GC-TOFMS) are considered useful tools for profiling primary and secondary metabolites. A total of 45 metabolites, including organic acids, amino acids, sugars, and an amine, were identified in pale green and purple kohlrabies using GC-TOFMS-based metabolic profiling. The resulting data sets were analyzed by principal component analysis to determine the overall variation, and the purple and pale green vegetables were separated by the score plots generated. Additionally, HPLC analysis of anthocyanins in both cultivars revealed that green kohlrabies did not contain any anthocyanidins, while 11 anthocyanins were quantified in the purple ones. Cyanidin was the dominant anthocyanin found in the purple cultivar, with cyanidin-3-(feruloyl)-diglucoside-5-glucoside being the major one. This study suggests that GC-TOFMS and HPLC are suitable tools to determine metabolic connection among various metabolites and describe phenotypic variation between green and purple kohlrabies.

Keywords Anthocyanin · GC-TOFMS · HPLC · Kohlrabi · Metabolic profiling

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Introduction

Because of the continued interest in the promotion of human health, the consumption of *Brassica* vegetables, including cabbage (*Brassica oleracea* L. var. *captita*), brussels sprouts (*B. oleracea* var. *gemmifera*), broccoli (*B. oleracea* var. *italica*), kale (*B. oleracea* var. *acephala*), cauliflower (*B. oleracea* var. *botrytis*), kohlrabi (*B.oleracea* var. *gongylodes*), and mustard (*B. juncea* L.), which are associated with the prevention of numerous diseases including diabetes, cancer, and heart disease, has increased [1–6]. This beneficial effect is associated with anti-diabetic, anticancer, anti-inflammatory, and antioxidant

properties attributed to various metabolites including carbohydrates, amino acids, organic acids, vitamins, minerals, phenolic compounds, flavonoids, anthocyanins, carotenoids, and glucosinolates present in *Brassica* vegetables [7–16]. Metabolites are the end products and intermediates of complex metabolism. Primary metabolites are directly responsible for the physiological processes including normal growth, development, and reproduction in living organisms, and secondary metabolites are involved in plant defense and are used as medicines for human health [17–19].

Anthocyanins, a group of flavonoids, are regarded as important secondary metabolites because of their health-promoting activities including anti-hyperglycemic, antioxidant, anti-angiogenic, anti-inflammatory, anticancer, anti-influenza effects [20–23]. Of the approximately 17 aglycone types present in nature, peonidin, delphinidin, cyanidin, malvidin, pelargonidin, delphinidin, and petunidin are the most common in higher plants [24]. Individual anthocyanin has different properties according to the number of hydroxyl group; the number and nature of sugars added to the molecule; the location of sugar attachment; and the number and nature of aromatic or aliphatic acids added to these sugars within the molecule [25–27]. Cyanidin, a water-soluble pigment with the C6-C3-C6 structure, is the main aglycone in *Brassica* vegetables [12, 28, 29]. The nature and intensity of the color exhibited in plants are influenced by pH-dependent structural changes, which may result in red, violet or blue coloration [30–32].

Metabolic profiling is commonly defined as the measurement of metabolites of low molecular weight and intermediates produced through many biosynthetic and catabolic pathways in living organisms. Therefore, identification and quantification of numerous metabolites is necessary [33, 34]. GC-TOFMS is a reliable method for performing metabolic profiling since it has been successfully used to quantify and identify a broad range of metabolites such as sugars, sugar alcohols, organic acids, and amino acids in various research fields [35]. Primary metabolic profiling is closely correlated to plant phenotypes and involves important nutritional characteristics. Previously, we successfully performed GC-TOFMS-based metabolic profiling to classify samples of diverse biological quality, origin or status [17, 36–39]. Thus, GC-TOFMS could be considered to be a suitable platform for the identification and quantification of individual metabolites in pale green and purple colored kohlrabi, as it provides the high mass resolution and accuracy, high scan speed and good sensitivity [40, 41].

Kohlrabi is a biennial plant with an enlarged bulb-like, fleshy stem that is edible and occurs at its base [11, 42]. Several studies have described the health-promoting properties associated with the secondary metabolites of kohlrabi [11, 42–46]. In our previous study, a comparison of the secondary metabolites from the flesh and peel of the pale green and purple colored kohlrabi was made [14]. However, there are no reports on the phenotypic variation between pale green and purple kohlrabi in relation to their primary and secondary metabolites (Fig. 1), though vegetable classification is helpful for dietary guidance and assessment, based on color of vegetables. Thus, this current study aims to describe the relationship among the primary and secondary metabolites via HPLC analysis of their anthocyanins and GC-TOFMS-based analysis of amino acids, carbohydrates, sugar alcohols, organic acids, and an amine.

Materials and methods

Plant materials

The seeds of pale green and purple kohlrabies were purchased from Stokes Seeds Ltd. (St. Catharines, Canada) and then submerged in water for 1 day before germination in a greenhouse. The seedlings of the each cultivar were transferred and cultivated in the experimental field in Daejeon, Republic of Korea. The edible portion (enlarged bulb-like, fleshy stem) of the two cultivars was sampled after 10 weeks.

Anthocyanin extraction and analysis

The edible parts from the two kohlrabi cultivars were ground in the presence of liquid nitrogen and then freeze-dried in a lyophilizer. For each sample, 200 mg of the resultant powder was extracted with 2 mL of formic acid/water (5:95, *v/v*) and vortexed for 5 min. The sample was gently sonicated for 30 min and then centrifuged at

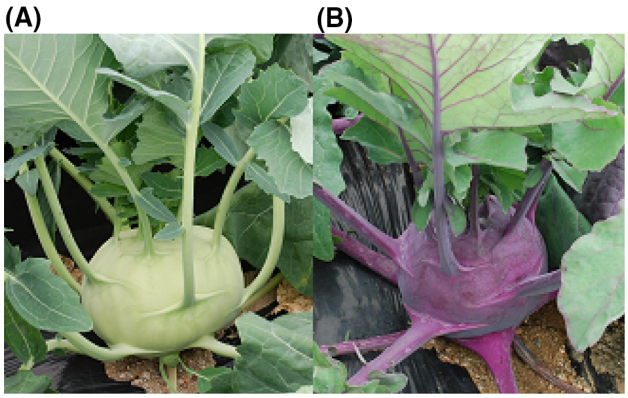


Fig. 1 Pale green (**A**) and purple (**B**) kohlrabi cultivars

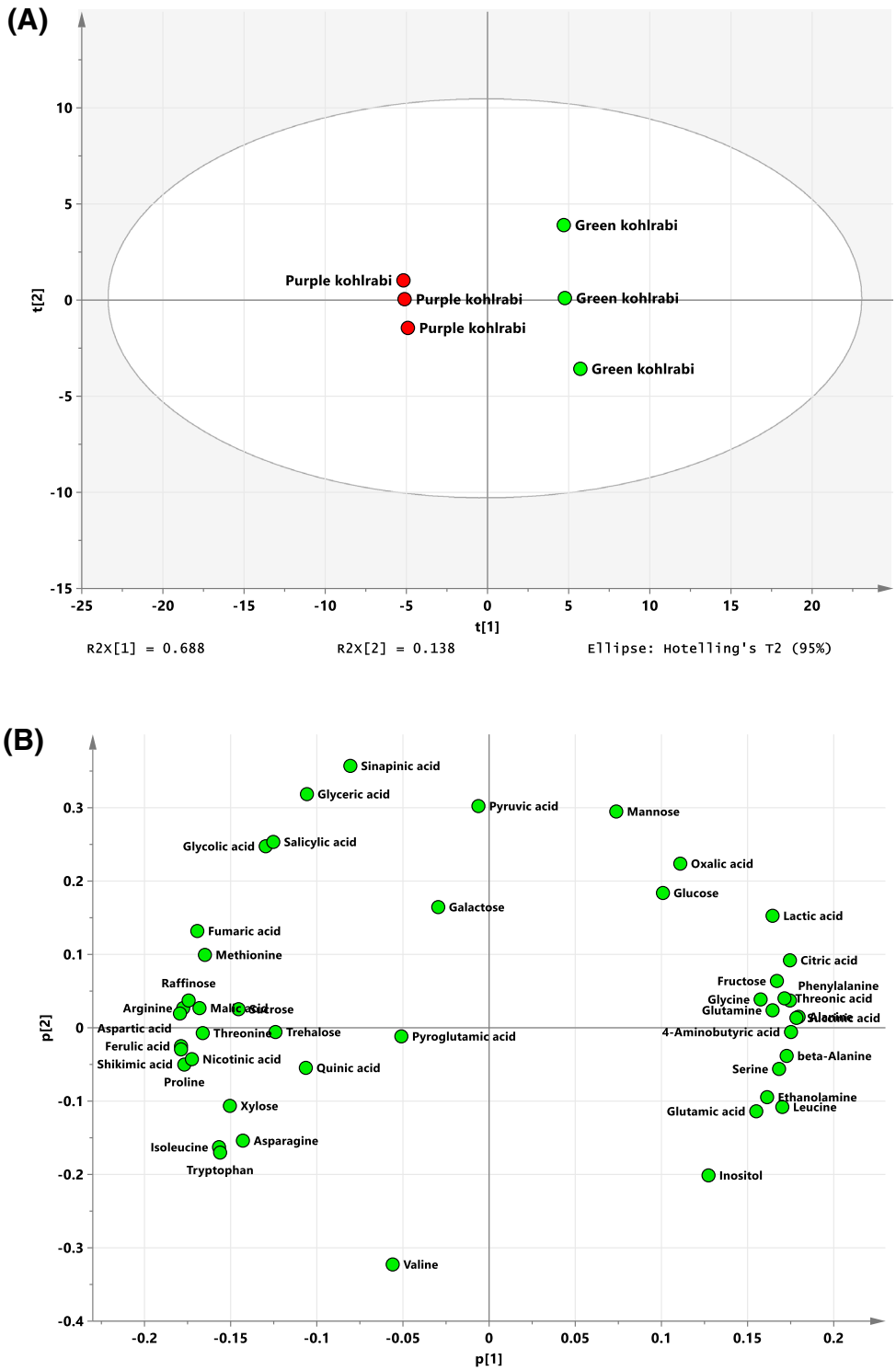
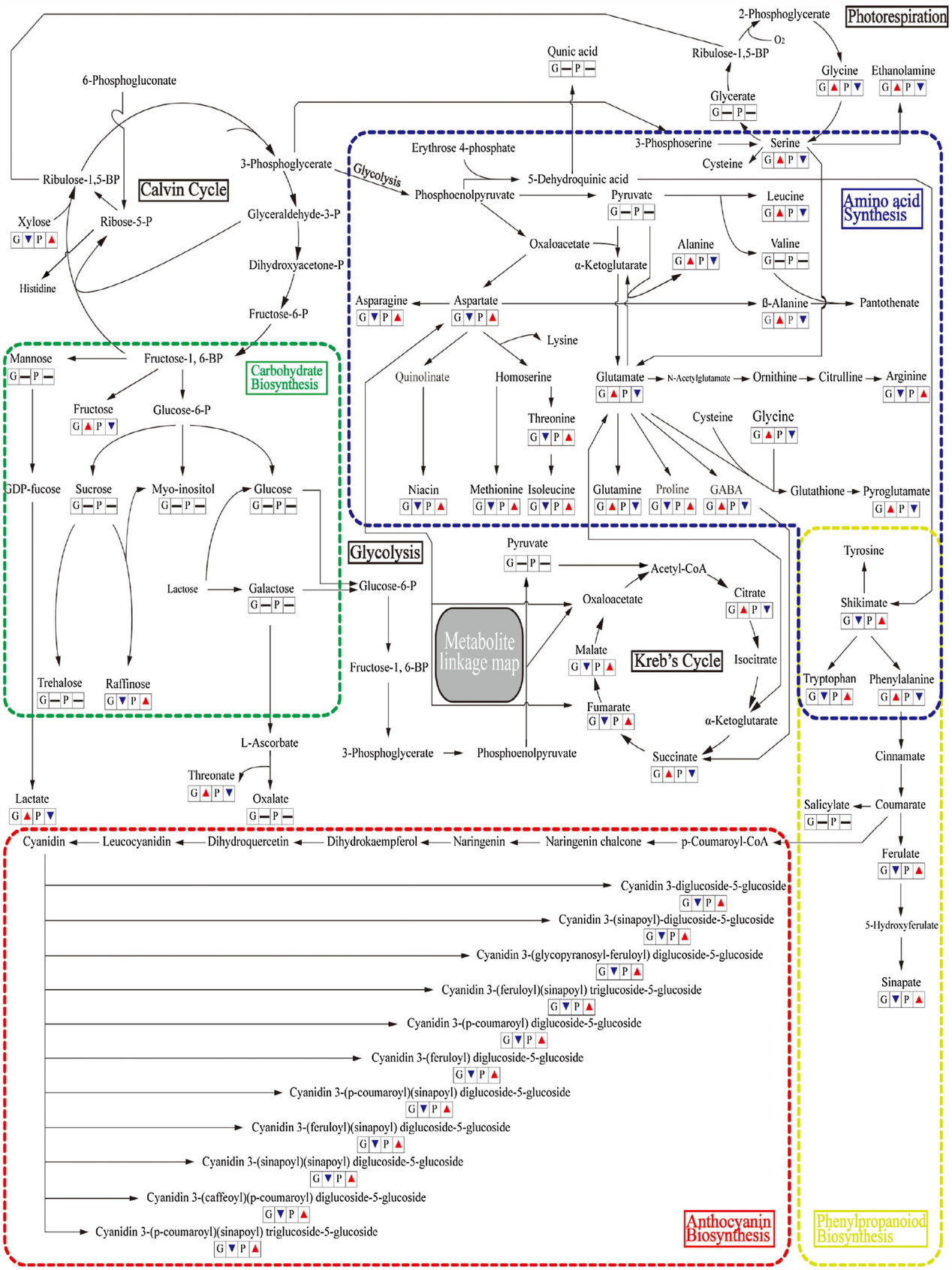


Fig. 2 Scores (**A**) and loading plots (**B**) of principal components 1 and 2 of the PCA results obtained from polar metabolite data of purple and green kohlrabi

8000 rpm and 4 °C for 15 min. Subsequently, the supernatant fluid was filtered via a 0.45 µm PTFE syringe filter (Merk Millpore, Daejeon, Korea) into a capped vial. The extractions were carried out in triplicates. First, 10 µL of the extracts were injected on a PerkinElmer Flexar HPLC (Shelton, CT, USA) equipped with a PDA LC detector for anthocyanin analysis. A Synergy 4 µ Polar-RP 80A (250 × 4.6 mm i.d., particle size 4 µm; Phenomenex, Torrance, CA, USA) column with a Security Guard Cartridge Kit (AQ C18, 4 × 3 mm i.d.; Phenomenex, Torrance, CA, USA) controlled at 40 °C and 520 nm was used to separate individual anthocyanins. The composition of


Photorespiration
2-Phosphoglycerate
O2
Ribulose-1,5-BP
Qunic acid
Glycine
Ethanolamine
Glycerate
6-Phosphogluconate
3-Phosphoserine
Serine
Cysteine
Erythrose 4-phosphate
3-Phosphoglycerate
Glycolysis
5-Dehydroquinic acid
Ribulose-1,5-BP
Calvin Cycle
Phosphoenolpyruvate
Pyruvate
Leucine
Amino acid Synthesis
Xylose
Ribose-5-P
Glyceraldehyde-3-P
Oxaloacetate
Alanine
Valine
α-Ketoglutarate
Dihydroxyacetone-P
Histidine
Asparagine
Aspartate
ß-Alanine
Pantothenate
Fructose-6-P
Lysine
Mannose
Fructose-1, 6-BP
Carbohydrate Biosynthesis
Quinolinate
Homoserine
Glutamate
N-Acetylglutamate
Ornithine
Citrulline
Arginine
Fructose
Glucose-6-P
Threonine
Cysteine
Glycine
GDP-fucose
Sucrose
Myo-inositol
Glucose
Niacin
Methionine
Isoleucine
Glutamine
Proline
GABA
Glutathione
Pyroglutamate
Glycolysis
Pyruvate
Tyrosine
Acetyl-CoA
Lactose
Galactose
Glucose-6-P
Oxaloacetate
Citrate
Shikimate
Metabolite linkage map
Malate
Kreb's Cycle
Isocitrate
Tryptophan
Phenylalanine
Trehalose
Raffinose
Fumarate
α-Ketoglutarate
Fructose-1, 6-BP
L-Ascorbate
Succinate
Cinnamate
3-Phosphoglycerate
Phosphoenolpyruvate
Threonate
Salicylate
Coumarate
Lactate
Oxalate
Cyanidin
Leucocyanidin
Dihydroquercetin
Dihydrokaempferol
Naringenin
Naringenin chalcone
p-Coumaroyl-CoA
Ferulate
Cyanidin 3-diglucoside-5-glucoside
5-Hydroxyferulate
Cyanidin 3-(sinapoyl)-diglucoside-5-glucoside
Cyanidin 3-(glycopyranosyl-feruloyl) diglucoside-5-glucoside
Sinapate
Cyanidin 3-(feruloyl)(sinapoyl) triglucoside-5-glucoside
Cyanidin 3-(p-coumaroyl) diglucoside-5-glucoside
Cyanidin 3-(feruloyl) diglucoside-5-glucoside
Cyanidin 3-(p-coumaroyl)(sinapoyl) diglucoside-5-glucoside
Cyanidin 3-(feruloyl)(sinapoyl) diglucoside-5-glucoside
Cyanidin 3-(sinapoyl)(sinapoyl) diglucoside-5-glucoside
Cyanidin 3-(caffeoyl)(p-coumaroyl) diglucoside-5-glucoside
Cyanidin 3-(p-coumaroyl)(sinapoyl) triglucoside-5-glucoside
Anthocyanin Biosynthesis
Phenylpropanoid Biosynthesis

◀**Fig. 3** A metabolic linkage map comparing primary and secondary metabolites of green and purple kohlrabi. The *up arrow* indicates that the mean value of the target metabolite is significantly higher (at $P < 0.05$). The *down arrow* indicates that the value of the metabolite is significantly lower (at $P < 0.05$). The horizontal bar means that mean values of the target compounds were not significantly different (at $P < 0.05$). A different letter (G and P) indicated green and purple kohlrabi, respectively

the mobile phase was as follows: eluent A: formic acid/water (5:95, *v/v*), and eluent B: formic acid/acetonitrile (5:95, *v/v*). The gradient program same as the previously published research [14] was employed: 0–8 min, 10% B; 8–13 min, 10–13% B; 13–15 min, 13% B; 15–18 min, 13–15% B; 18–25 min, 15% B; 25–30 min, 15–18% B; 30–35 min, 18% B; 35–40 min, 18–21% B; 40–45 min, 21% B; 45–45.1 min, 21–5% B; and 45.1–50 min, 5% B (total 50 min).

Polar metabolite extraction and analysis

GC-TOFMS was employed to identify and quantitate a variety of metabolites with low molecular weight in the kohlrabi cultivars. Polar metabolites were extracted using the procedure described by the previous study [36]. Chemicals and reagents, sample preparation procedures, methoxime-derivatization and trimethylsilyl etherification procedures, GC-TOFMS instrument operating conditions, analytical procedures for sample separation, and the identification and quantification of the molecules were carried out as previously described by the previous study [36]. A powdered sample was extracted with 1 mL of 2.5:1:1 (v/v/v) methanol/water/chloroform. Ribitol (60 µL, 0.2 mg/mL) was added an as internal standard (IS). Each derivatized sample (1 µL) was separated on a 30 m × 0.25-mm i.d. fused silica capillary column coated with 0.25-µm CP-SIL 8 CB low bleed (Varian Inc., Palo Alto, CA, USA). MS was performed using a Pegasus HT TOF mass spectrometer (LECO, St. Joseph, MI). ChromaTOF software (version 4.34; LECO, St. Joseph, MI, USA) was used for peak identification using an in-house library and reference compounds. Furthermore, several metabolites were identified by direct comparison with mass chromatogram of the sample with those of commercially available standard compounds acquired by a methoxime-derivatization and trimethylsilyl etherification procedures and GC-TOFMS analysis. All analytes were quantitatively calculated based on the peak area ratios relative to that of the I.S [17, 36, 43, 44].

Statistical analysis

HPLC data were statistically assessed using the Statistical Analysis System (SAS, system 9.4, 2013; SAS Institute, Inc., Cary, NC, USA). The significant difference among means was assessed by Duncan's multiple range test (DMRT). A probability value less than 0.05 ($P \leq 0.05$) was regarded as significant. All data were recorded as mean ± standard deviation of triplicate experiments. The relative quantitation data obtained by GC-TOFMS analysis were subjected to principal component analysis (PCA) accompanied by unit variance scaling (SIMCA-P version 13.0; Umetrics, Umeå, Sweden) to determine differences in the low molecular weight metabolite profiles in kohlrabi with respect to two colors. The PCA outputs were composed of score plots for indicating the sample distributions.

Results and discussion

Metabolite-specific profiling

Using this GC–MS approach, a total of 45 compounds, such as amino acids, organic acids, carbohydrates, sugar alcohols and an amine, were detected and normalized against the internal standard signal intensity. The resulting data were subjected to PCA of the two cultivars (Fig. 2). The first PCA component resolved the measured compound profiles of purple and green kohlrabi, describing 68.8% of the total variation. The 95% confidence interval of the modeled variation was defined by Hotelling's T2 region, representing an ellipse in the score plots [47]. To investigate the contributors to the principal components, the metabolic loadings in principal component 1 were compared. Shikimate and ferulate, involved in anthocyanin metabolism, were clustered on the left side of the loading plot, indicating that the values of those metabolites in purple kohlrabi were higher than those in green kohlrabi. In addition, alanine, β-alanine, leucine, GABA, serine, glutamine, glutamate, and glycine were clustered on the right side of the loading plot. The results suggest that the amino acids were higher in green kohlrabi than purple kohlrabi.

The most abundant metabolite in both cultivars was sugars. Sugars are critical primary nutrients, partially influencing the growth, development, and morphogenesis of plants. The comparison of carbohydrate levels between purple and green kohlrabi revealed that the level of total carbohydrates in green kohlrabi was 1.08 times higher than that in purple kohlrabi (Supplementary materials). In details, the fructose level in green kohlrabi was higher than that in purple cultivar while the levels of sucrose, raffinose and xylose were statistically higher than those of green cultivar. The quantities of glucose, galactose, myo-inositol, and trehalose were similar to the both cultivars.

A linkage map was constructed with 45 metabolites and 11 anthocyanins to understand metabolic connection between metabolites (Fig. 3). Sixteen proteinogenic and

three non- proteinogenic amino acids were detected in the two cultivars and the total amount of amino acids was higher in green kohlrabi. Specifically, the glutamate derivatives (glutamine, arginine, GABA, and pyroglutamate) and pyruvate-derived amino acids (alanine and leucine) were more abundant in green kohlrabi. Note that abundance of glutamate and glutamine (respectively, 1.20- and 1.54-fold higher in green kohlrabi), which are involved in nitrogen assimilation, [48] showed that the green kohlrabi had higher amount of N-compounds, including 19 amino acids and 1 amine, than the purple one. In addition, the larger pool of glutamate and alanine (19.38-fold), which act as important amino donors in photorespiration, [49] reflected that the levels of photorespiratory intermediates, the amino acids serine (1.19-fold) and glycine (1.15-fold), were also higher in green kohlrabi. However, purple kohlrabi contained more proline (1.76-fold) and arginine (2.72-fold) in comparison with green kohlrabi. Additionally, the oxaloacetate family of amino acids (aspartate, asparagine, threonine, methionine and isoleucine) was generally present in higher levels in purple kohlrabi.

Four Kreb's cycle intermediates were identified and quantified. Purple kohlrabi had the larger pools of malate (1.13-fold) and fumarate (1.26-fold), supported by the higher quantity of aspartate (3.00-fold). In contrast, citrate (1.95-fold) and succinate (2.00-fold) pools were higher in green kohlrabi, as explained by the higher level of glutamate (1.20-fold) and GABA (1.73-fold). The shikimate pathway is a main route for aromatic compounds, such as phenylalanine and tryptophan, in plants. These two primary metabolites serve as precursors for various secondary metabolites including flavonoids, glucosinolates, phenolic acids, and anthocyanins [50]. The levels of shikimate (2.07-fold) and tryptophan (1.51-fold) were higher in purple kohlrabi, reflecting that the relative downstream compounds, ferulate and sinapate (1.17-fold), were also more abundant in that cultivar. On the other hand, green kohlrabi contained the larger quantity of phenylalanine. In addition, ethanolamine, which is involved in phospholipid metabolism, [51] was also greater in green kohlrabi.

HPLC analysis of anthocyanin

The HPLC data revealed that the green kohlrabi did not contain any anthocyanins, while a total of 11 anthocyanins were detected in purple kohlrabi, which had a total anthocyanin level of 0.30 ± 0.01 mg/g dry weight (wt) (Table 1). Among the anthocyanin compounds detected, cyanidin-3-(feruloyl)-diglucoside-5-glucoside was the most dominant, and cyanidin-3-(*p*-coumaroyl)(sinapoyl)-triglucoside-5-glucoside and cyanidin-3-(caffeoyl)(*p*-coumaroyl)-diglucoside-5-glucoside were present in trace amounts. With the exception of cyanidin-3-diglucoside-5-glucoside, all of the anthocyanins detected were cyanidin glycosides to which phenolic acids including sinapic, caffeic, *p*-coumaric, and ferulic acids were attached. The identification of individual anthocyanins was confirmed by comparison with MS/MS data from our published research [14].

Anthocyanins, a class of phenylpropanoid-type pigments, are derived from carbohydrates generated during the process of photosynthesis through enzyme mediated reactions of shikimate and the phenylpropanoid biosynthetic pathway [52]. Most anthocyanins occurring in nature are present primarily in the form of anthocyanidins (aglycones) glycosylated with one or more sugar moieties [53]. In this

Table 1 Anthocyanin contents (mg/g dry wt.) in pale green and purple kohlrabis

No.[a]	RT[b] (min)	Trivial name	Pale green	Purple
1	11.15	Cyanidin 3-diglucoside-5-glucoside	0 b	0.03 ± 0.00 a
2	17.40	Cyanidin 3-(sinapoyl)-diglucoside-5-glucoside	0 b	0.01 ± 0.00 a
3	19.97	Cyanidin 3-(caffeoyl)(p-coumaroyl) diglucoside-5-glucoside	0	tr[c]
4	20.72	Cyanidin 3-(glucopyranosyl-feruloyl) diglucoside-5-glucoside	0 b	0.01 ± 0.00 a
5	26.26	Cyanidin 3-(p-coumaroyl)(sinapoyl) triglucoside-5-glucoside	0	tr
6	27.64	Cyanidin 3-(feruloyl)(sinapoyl) triglucoside-5-glucoside	0 b	0.01 ± 0.00 a
7	29.74	Cyanidin 3-(p-coumaroyl) diglucoside-5-glucoside	0 b	0.01 ± 0.00 a
8	31.73	Cyanidin 3-(feruloyl) diglucoside-5-glucoside	0 b	0.11 ± 0.00 a
9	38.43	Cyanidin 3-(p-coumaroyl)(sinapoyl) diglucoside-5-glucoside	0 b	0.01 ± 0.00 a
10	40.73	Cyanidin 3-(feruloyl)(sinapoyl) diglucoside-5-glucoside	0 b	0.07 ± 0.00 a
11	42.01	Cyanidin 3-(sinapoyl)(sinapoyl) diglucoside-5-glucoside	0 b	0.03 ± 0.00 a
Total			0 b	0.30 ± 0.01 a

[a] No, the elution order, [b] *RT* retention time, [c] *tr* trace

study, a phenotypic (color) difference between green and purple kohlrabi was determined to be due to the presence of anthocyanins. The purple cultivar contained a total of 11 anthocyanins, with cyanidin being the sole aglycone present. The anthocyanidin was glycosylated in various positions and acylated with aromatic acyl substituents including p-coumaryl, caffeoyl, feruryl, and sinapoyl groups. Previous studies on acylated anthocyanins noted that acylation improves the strength of their colors and confers increased stability to heat, light, pH, and SO_2. Acylated cyanindin glucosides are also more stable than their deacyl analogues [32, 54–57]. Higher levels of ferulate and sinapate in the purple cultivar would be necessary for acylation of the anthocyanins present in the purple cultivar.

Plant primary metabolism is defined as a collection of processes including the generation and utilization of a wide range of substances such as carbohydrates, fatty acids, nucleic acids, amino acids, and biopolymers for the survival of the plants. Secondary metabolism, however, accounts for biochemical reactions which are not essential to sustain plant life but play important roles in specific stages of growth and development, reproduction and defense [58–61]. In fact, the border between the primary and secondary metabolism is ambiguous, since intermediates synthesized by primary metabolism are also involved in pathways that generate secondary metabolites. Also, the overlapping interactions of the biochemicals ensure an interplay between the primary and secondary metabolism [61].

Carbon (C) and nitrogen (N) are the most important nutrients for conducting fundamental and normal cellular functions [62]. Carbohydrates, containing C, play a key role as an energy source and in plant metabolism. The total amount of carbons (represented mainly by sugars such as xylose, glucose, mannose, galactose, fructose, sucrose, trehalose, and raffinose) in the purple cultivar was lower than that in the green cultivar, which reflected on carbon and energy demand to support anthocyanin metabolism. Similarly, [63] reported that the enhanced alkaloid production in opium poppy cell cultures treated with a fungal elicitor showed more rapidly depleted pools of carbohydrates.

Nitrogen is the most important inorganic nutrient, being present in nucleic acids, amino acid, proteins, co-factors, and many secondary metabolites in plants [64, 65]. Amino acids are the first products originated from nitrogen assimilation and are synthesized by combination of ammonium and C-skeletons provided by photosynthetic products [62]. In plant species, the composition and concentration of free amino acids vary considerably with different nitrogen sources. In particular, the concentrations of individual free amino acids can be more easily modified than that of total free amino acids [66]. The current analysis of amino acids revealed that the total number (11) of amino acids found in the purple cultivar was lower than that in the green cultivar, reflecting intermediate or precursor supply to support anthocyanin acylation and metabolism. Lee and Finn [67] reported that the higher anthocyanin contents in Lingonberry (*Vaccinium vitis-idaea* L.) were associated with lower levels of total free amino acids. Additionally, higher levels of shikimate and tryptophan in the purple cultivar suggested greater accumulation of anthocyanins and phenolic acids. This is consistent with a prior study which revealed the high amounts of shikimate and anthocyanin in red- compared with white-colored buckwheat (*Fagopyrum esculentum*) flowers [68]. Also, the supply of exogenous shikimate enhanced anthocyanin production in maize roots [69].

The phenotypic variation between green and purple kohlrabies was determined by analyzing the correlation between the primary and secondary metabolites of the two cultivars through GC-TOFMS and HPLC analyses. The anthocyanins were considered the key factor in determining the color phenotype of the cultivars. The purple cultivar contained high levels of anthocyanins and low amounts of primary metabolites, reflecting the demand for carbon, energy and metabolic precursors to support its anthocyanin metabolism. Additionally, the high production of ferulate and sinapate might be involved in the acylation of anthocyanins. Lastly, the study confirmed that GC-TOFMS-based metabolite and HPLC-based anthocyanin profiling are useful approaches for determining metabolic interaction among a variety of metabolites and to describe phenotypic variation between green and purple kohlrabies.

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