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ARTICLE

Metabolomics of differently colored Gladiolus cultivars

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Abstract Gladiolus (*Gladiolus grandiflora* Hort.) plants are a perennial favorite, known for their beautiful flowers of many different colors. In this study, we determined metabolic differences between seven Gladiolus cultivars with differently colored flowers by monitoring anthocyanin, carotenoid, and hydrophilic metabolites. We detected nine anthocyanins in the seven cultivars. Only the 'Violetta' cultivar contained all nine anthocyanins and it exhibited the highest anthocyanin content, whereas no anthocyanins were detected in the 'New Wave' (white color) or 'Limoncello' (yellow color) cultivars. In addition, we detected seven carotenoids, the contents of which varied significantly among the cultivars depending on the flower color. 'Limoncello' exhibited the highest levels of carotenoids. Of the seven carotenoids, β-carotene and lutein accumulated in the most cultivars. In addition, we identified 43 metabolites using gas chromatography–time-of-flight mass spectrometry. The levels of organic acids and sugars in the 'New Wave' cultivar differed significantly from those in the 'Violetta' and 'Limoncello' cultivars with a P value < 0.01. Thus, our results may help in understanding the metabolic differences between differently colored Gladiolus cultivars and should be useful in future databases.

Keywords Anthocyanin · Carotenoid · Gladiolus cultivars · Hydrophilic metabolites · Metabolomics

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Introduction

Gladiolus (*Gladiolus grandiflora* Hort.) plants are among the world's most important horticultural plants and belong to a family of around 250–300 flowering plants in South Africa, with some cultivars being found in Central Asia, Western and Central Europe, and in Northwest and Eastern Africa (Goldblatt and Manning 1998). They belong to the Iridaceae family (Order Asparagales) which is named for Irises and, includes well known flowers such as Freesia, Gladiolus, and Crocus. This family is perennial and has a bulb, corm, or rhizome.

Gladiolus plants are grown commercially in the United States (Florida and California), Holland, Italy, France, Poland, Bulgaria, Brazil, India, Australia, and Israel (Narain 2004). Gladioli grow suitably in borders, flowerbeds, and containers, and they have great value as cut flowers. A number of cultivars have been derived from the common Gladiolus (*G. hortulanus*) and they produce flowers that are bi- or tri-colored (Narain 2004).

In general, varying flower colors are preferred and a very important component of the horticulture business. Flower color is determined mainly by anthocyanins. A few researchers have reported on flower anthocyanins of Gladiolus cultivars. Seven anthocyanins, including pelargonidin 3-*O*-rhamnosylglucoside, peonidin 3-*O*-rhamnosylglucoside-5-*O*-glucoside, pelargonidin 3-*O*-rhamnosylglucoside, peonidin 3-*O*-rhamnosylglucoside-5-*O*-glucoside, pelargonidin 3-*O*-diglucoside-5-*O*-glucoside, cyanidin 3,5-di-*O*-glucoside, delphinidin 3,5-di-*O*-glucoside, and delphinidin 3-triglucoside, were isolated from 10 Gladiolus cultivars by Shibata and Nozaka (1963). More than 10 anthocyanins from 10 to 12 cultivars were described by Yatomi and Arisumi (1968) and Arisumi and Kobayashi (1971), respectively. Akavia et al. (1981) analyzed 18 anthocyanins from nine Israeli Gladiolus cultivars, using high performance liquid chromatography (HPLC). Furthermore, Takemura et al. (2004) reported that purple flowers of the Japanese Gladiolus cultivar 'Ariake' have malvidin 3,5-di-*O*-glucoside as the major anthocyanin, two minor malvidin glycosides, and three major flavonols, kaempferol, 3-*O*-sophoroside, kaempferol 3-*O*-rutinoside, and quercetin 3-*O*-rutinoside. However, they mentioned that anthocyanins have rarely been detected in recent cultivars (e.g., 'Ariake', *G.* × *grandiflora*). Jones and McConchie (1995) demonstrated that wilting, pigment degradation, and petal collapse cause senescence of flower petals.

To investigate the metabolite differences of Gladiolus flowers from different species and to identify significant metabolites, the contents of low-molecular-weight metabolites, such as organic acids, sugars, and amino acids in the flowers, was determined using gas chromatography–time-of-flight mass spectrometry (GC-TOFMS). Anthocyanins and carotenoids play an important role as secondary metabolites and are involved in the nutritional characteristics of the plants (Hoekenga 2008; Aharoni and Galili 2011). The GC-TOFMS-based analytical methods are highly sensitive for the detection of metabolites derived from primary metabolism of plants (Aharoni and Galili 2011).

Metabolomics is a useful tool for determining phenotypic variation in biological samples (Steuer 2006). This study aimed to identify the primary metabolites and bioactive secondary metabolites from Gladiolus. Thus, we profiled abundant hydrophilic primary metabolites using GC-TOFMS, and quantified carotenoids and anthocyanins using HPLC, from seven differently colored Gladiolus cultivars. The data obtained were then subjected to Pearson's correlation analysis, principal component analysis (PCA), orthogonal partial least squares discriminant analysis (OPLS-DA), and hierarchical clustering analysis (HCA) to identify the relationships between metabolite contents. The analysis of Gladiolus anthocyanins has been described in only a few publications (Shibata and Nozaka 1963; Yatomi and Arisumi 1968; Arisumi and Kobayashi 1971; Akavia et al. 1981) and, to our knowledge, no previous reports have been published on carotenoid analysis or the metabolic profiling of Gladiolus. Therefore, this study focused on metabolic profiling and the quantification of carotenoids, as well as anthocyanins, from various modern Gladiolus cultivars.

Materials and methods

Sample preparation

Corms of Gladiolus cultivars were purchased from Woori Seed Co., Ltd (Guacheon, Korea) and plants were cultivated on the experimental farm of Chungnam National University (Daejeon, Korea). The plant materials were harvested in August 2013 and were pulverized in a mortar and pestle in liquid nitrogen (N) for the analysis of anthocyanins, carotenoids, and for metabolic profiling. Three flowers of each were used for metabolite, anthocyanin, and carotenoid analysis.

Anthocyanin extraction and analysis

Anthocyanin analysis was performed according to the protocol of Park et al. (2011), with slight modifications. Briefly, the filtrate was analyzed by high performance liquid chromatography (HPLC), using a refractive index (RI) and photodiode array (PDA) detectors (Agilent

Technologies, Palo Alto, CA, USA) equipped with a Synergi 4 μm POLAR-RP 80A column (250 × 4.6 mm, i.d., particle size 4 μm; Phenomenex, Torrance, CA, USA). Column temperature of 30 °C, flow rate of 1.0 mL/min, injection volume of 20 μL, and detector wave length of 520 nm were studied to optimize peak separation. The mobile phase consisted of water (A) and acetonitrile (B). Samples were eluted with the following gradient: 0 min, 5 % B; 10 min, 20 % B; 20 min, 20 % B; 20.1 min, 5 % B; 30 min, 5 % B. On the basis of the retention time and peak area, individual anthocyanins were quantified with respect to the standard compounds cyanidin 3-*O*-glucoside (Fujicco Co., Ltd, Kobe, Japan), malvidin 3-*O*-glucoside chloride (Chromadex, Kobe, Japan), pelargonidin 3-*O*-glucoside chloride (Extrasynthèse, Genay, France), and expressed in milligrams per gram dry weight. All HPLC analysis experiments were conducted in triplicate.

Carotenoid and polar metabolite analysis

Carotenoids were extracted and measured according to the procedure published by Park et al. (2014). GC-TOFMS was used to detect and measure the low-molecular-weight compounds in the Gladiolus samples. The extraction of polar metabolites was performed as described by Kim et al. (2007). The sample preparation methods, chemicals and reagents, methoxime (MO) derivatization and trimethylsilyl (TMS) etherification procedures, GC-TOFMS instrument operating conditions, analytical methods for the separation of the samples, and the scanning and detection of the compounds were implemented as described in previous publication (Kim et al. 2007). The mass data were analyzed using ChromaTOF software. Our previous mass data agreed with the present data (Kim et al. 2013; Park et al. 2013).

Statistical analysis

Selected ions were used for quantification (Table S1). The quantification data of 43 metabolites were subjected to principal component analysis (PCA; SIMCA-P version 13.0 software; Umetrics, Umeå, Sweden) to evaluate the differences between groups of multivariate data. OPLS-DA was subsequently used to improve the separation. In addition, we performed an external validation to test the validity the OPLS-DA models. For the predictions, two samples were removed randomly from each cultivar and the OPLS-DA prediction model was performed without them. The data file was scaled with unit variance scaling before all the variables were subjected to PCA and OPLS-DA. We performed the Student's *t*-test and Duncan Multiple Range Test using SAS 9.2 software (SAS Institute, Cary, NC, USA). MULTIEXPERIMENT VIEWER version 4.4.0 software (http://www.tm4.org/mev/) was used for the analysis of heat map and HCA visualization of the correlation coefficients.

Results and discussion

Anthocyanin content in differently colored Gladiolus flowers

In this study, analysis of the flowers of the Gladiolus cultivars (Fig. 1) identified nine anthocyanins: cyanidin 3-*O*-rutinoside-5-*O*-glucoside, petunidin 3-*O*-rutinoside-5-*O*-glucoside, petunidin 3-*O*-glucoside, pelargonidin 3-*O*-rutinoside-5-*O*-glucoside, pelargonidin 3,5-di-*O*-glucoside, malvidin 3,5-di-*O*-glucoside, malvidin 3-*O*-rutinoside, pelargonidin 3-*O*-glucoside, and malvidin 3-*O*-glucoside. Arisumi and Kobayashi (1971) isolated 13 anthocyanins, including pelagonidin 3,5-di-*O*-glucoside, pelargonidin 3-*O*-rhamnosylglucoside, peonidin 3-*O*-rhamnosylglucoside, peonidin 3,5-di-*O*-glucoside, malvidin 3-*O*-rhamnosyl-glucoside, malvidin 3,5-di-*O*-glucoside, malvidin 3-*O*-rhamnosylglucoside-5-*O*-glucoside, petunidin 3,5-di-*O*-glucoside, petunidin 3-*O*-rhamnosylglucoside-5-*O*-glucoside, pelargonidin 3-*O*-glucoside, malvidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, and petunidin 3-*O*-rhamnosylglucoside. In addition, Akavia et al. (1981) reported 18 anthocyanins from nine Israeli Gladiolus cultivars.

'Violetta' accumulated more anthocyanins than any of the other cultivars tested. It contained five anthocyanins (petunidin 3-*O*-rutinoside-5-*O*-glucoside, petunidin 3-*O*-glucoside, pelargonidin 3,5-di-*O*-glucoside, malvidin 3,5-di-*O*-glucoside, and malvidin 3-*O*-glucoside); however, the other four anthocyanins were not detected in this cultivar. 'Rose Supreme' accumulated three anthocyanins (pelargonidin 3-*O*-rutinoside-5-*O*-glucoside, pelargonidin 3,5-di-*O*-glucoside, and pelargonidin 3-*O*-glucoside); however, the other six anthocyanins were absent in this cultivar. 'Esta Bonita' contained two anthocyanins (petunidin 3-*O*-glucoside, pelargonidin 3-*O*-rutinoside-5-*O*-glucoside), whereas 'Mon Amour' only had the anthocyanin malvidin 3,5-di-*O*-glucoside. Notably, none of the nine anthocyanins were detected in either 'New Wave' or 'Limoncello'. 'Violetta' exhibited the highest accumulation of anthocyanins of all the cultivars tested. Among the anthocyanins detected in 'Violetta', pelargonidin 3,5-di-*O*-glucosides were present at the highest concentration of 8.04 mg/g dry weight (DW). The concentration of pelargonidin 3,5-di-*O*-glucoside in 'Violetta' was 67 and 3.54 times higher than in 'Rose Supreme' and 'Plumtart',

Fig. 1 Flowers of different Gladiolus cultivars. (**A**) New wave; (**B**) Mon amour; (**C**) Limoncello; (**D**) Rose supreme; (**E**) Esta Bonita; (**F**) Plumtart; (**G**) Violetta. *Scale bars* are 30 mm

respectively. The accumulation of malvidin 3,5-di-*O*-glucoside in 'Violetta' was 1.24 times higher than in 'Plumtart'. Pelargonidin 3-*O*-glucoside accumulated only in 'Violetta' and 'Rose Supreme', whereas petunidin 3-*O*-rutinoside-5-*O*-glucoside was found only in 'Violetta' and 'Plumtart'. Petunidin 3-*O*-glucoside was found in 'Violetta', 'Plumtart', and 'Esta Bonita'. 'Violetta' contained a petunidin 3-*O*-rutinoside-5-*O*-glucoside concentration that was three times higher than that in 'Plumtart'. In contrast, 'Plumtart' contained a slightly higher concentration of petunidin 3-*O*-glucoside than 'Violetta' (Table 1). Lastly, pelargonidin 3-*O*-rutinoside-5-*O*-glucoside accumulated only in 'Violetta' and 'Rose Supreme'. Malvidin 3,5-di-*O*-glucoside, as the major anthocyanin, along with two minor malvidin glycosides and three major flavonols from the purple flowers of the Japanese Gladiolus 'Ariake', were isolated by Takemura et al. (2008). Recently, Takemura et al. (2008) isolated and identified anthocyanins from Gladiolus cultivars such as 'Beijing', 'Juku-gaki', 'Fado', 'Ben Venuto Marches', and 'Viletta'. Our results are in accordance with those from previous studies.

Carotenoids in differently colored Gladiolus flowers

Analysis of the flowers of different Gladiolus cultivars identified seven types of carotenoids (Table 2, Fig S1). The levels of carotenoid content varied significantly, based on the flower color of the cultivars. From the analysis of flowers of different Gladiolus cultivars, all carotenoid types accumulated in large quantities in 'Limoncello' were compared to the other cultivars. Among the seven carotenoids, lutein and β-carotene accumulated in large amounts in all cultivars, especially in 'Limoncello' and 'Esta Bonita'. The lutein content in the Gladiolus cultivars ranged from 2.57 to 66.05 μg/g DW. In particular, the concentration of lutein in 'Limoncello' was 25.7 times

Table 1 Anthocyanin contents in different Gladiolus cultivars (mg/g dry weight)

Peak no.	RT (min)	Anthocyanin[a]	Rose Supreme	Plumtart	Violetta	New Wave	Limoncello	Mon Amour	Esta Bonita
1	8.73	Cyanidin 3-*O*-rutinoside-5-*O*-glucoside	ND[b]	ND	0.29 ± 0.01a	ND	ND	ND	ND
2	9.12	Petunidin 3-*O*-rutinoside-5-*O*-glucoside	ND	0.19 ± 0.01b	0.56 ± 0.01a	ND	ND	ND	ND
3	9.54	Petunidin 3-*O*-glucoside	ND	0.33 ± 0.02a	0.32 ± 0.00a	ND	ND	ND	0.03 ± 0.00b
4	9.88	Pelargonidin 3-*O*-rutinoside-5-*O*-glucoside	0.14 ± 0.00b	ND	0.52 ± 0.01a	ND	ND	ND	0.10 ± 0.00c
5	10.68	Pelargonidin 3,5-di-*O*-glucoside	0.12 ± 0.00c	2.27 ± 0.10b	8.04 ± 0.12a	ND	ND	ND	ND
6	11.17	Malvidin 3,5-di-*O*-glucoside	ND	1.91 ± 0.06b	2.36 ± 0.03a	ND	ND	0.05 ± 0.00c	ND
7	11.69	Malvidin 3-*O*-rutinoside	ND	ND	0.40 ± 0.01a	ND	ND	ND	ND
8	12.39	Pelargonidin 3-*O*-glucoside	0.02 ± 0.00b	ND	0.67 ± 0.02a	ND	ND	ND	ND
9	13.46	Malvidin 3-*O*-glucoside	ND	0.27 ± 0.08b	1.34 ± 0.04a	ND	ND	ND	ND
		Total	0.27	4.97	14.5	ND	ND	0.05	0.12

[a] Within each column, values followed by the same letters are not significantly different at $P \leq 0.05$, using duncan multiple range test (n = 3)

[b] ND, not detected

higher than in 'Plumtart'. The lutein content did not differ significantly between 'Limoncello' and 'Esta Bonita'.

The variation in lutein content was much higher between other cultivars. The lutein content of 'Limoncello' was 24.3, 18.4, and 16.0 times higher than in 'Violetta', 'New Wave', and 'Rose Supreme', respectively. The second highest carotenoid accumulation was that of β-carotene and its content also varied widely among the cultivars. The accumulation of β-carotene was significantly higher in 'Limoncello', 'Esta Bonita', and 'Mon Amour' than in the other cultivars. The β-carotene content ranged from 1.17 to 49.66 μg/g DW among the Gladiolus cultivars. 'Limoncello' contained the highest quantity of β-carotene, which was 42.4 times higher than the lowest β-carotene content in 'Violetta'. The β-carotene content in 'Violetta' was 31.4, 25.0, and 21.0 times higher than in 'Plumtart', 'New Wave', and 'Rose Supreme', respectively.

Next to lutein and β-carotene, the most highly accumulated carotenoid was 13-*cis*-β-carotene. The accumulation of this carotenoid followed a pattern similar to those of lutein and β-carotene. The content of 13-*cis*-β-carotene ranged from 0.38 to 6.53 μg/g DW among the Gladiolus cultivars. Similar to the contents of other carotenoids, 'Limoncello' contained the highest quantity of 13-*cis*-β-carotene, with a content 17.2 times higher than that of the cultivar with the lowest content, 'Violetta'. 'Limoncello' also had a similar amount of 13-*cis*-β-carotene compared with that of 'Plumtart', 'New Wave', and 'Rose Supreme'. The accumulation trend of carotenoid contents was similar between the different Gladiolus cultivars. Like lutein, 13-*cis*-β-carotene, and β-carotene, the content of 9-*cis*-β-carotene was significantly higher in the 'Limoncello', 'Esta Bonita', and 'Mon Amour' cultivars than in the other cultivars. The 9-*cis*-β-carotene content in the Gladiolus cultivars ranged from 0.08 to 4.72 μg/g DW. The concentration of 9-*cis*-β-carotene in 'Limoncello' was 59 times greater than that in 'Violetta'. The accumulations of β-cryptoxanthin, α-carotene, and zeaxanthin were lower compared with those of other carotenoids, irrespective of the type of cultivar tested.

β-cryptoxanthin was not detected in either 'Violetta' or 'Plumtart'. In contrast, 'Limoncello' accumulated the highest amount of β-cryptoxanthin, which was 38 times higher than the corresponding amounts in 'New Wave' and 'Rose Supreme', followed by 'Esta Bonita'. 'Limoncello' had an 8.1 times higher amount of β-cryptoxanthin compared with that in 'Mon Amour'. No α-carotene was detected in 'New Wave', 'Violetta', or 'Rose Supreme'. 'Limoncello' accumulated the highest α-carotene level, with a content 5.5 times higher than that of 'Plumtart'. 'Limoncello' had 3 and 1.6 times higher α-carotene content than 'Mon Amour' and 'Esta Bonita', respectively.

Among the carotenoids monitored, zeaxanthin accumulated to the lowest level. While no zeaxanthin was present

Table 2 Carotenoid contents in different Gladiolus cultivars (μg/g dry weight)

Color	Lutein[a]	Zeaxanthin	β-cryptoxanthin	α-carotene	13-*cis*-β-carotene	β-carotene	9-*cis*-β-carotene	Total
New Wave	3.58c	0.77c	0.07d	ND[b]	0.49d	1.99d	0.21d	7.11
Violetta	2.72c	ND	ND	ND	0.38d	1.17d	0.08d	4.35
Plumtart	2.57c	ND	ND	0.40d	0.42d	1.58d	0.13d	5.1
Rose Supreme	4.14c	0.31d	0.07d	ND	0.56d	2.36d	0.21d	7.65
Mon Amour	21.81b	0.69c	0.33c	0.73c	1.43c	9.95c	0.96c	35.9
Esta Bonita	62.11a	1.01b	1.82b	1.35b	2.61b	16.03b	1.86b	86.79
Limoncello	66.05a	1.21a	2.66a	2.20a	6.53a	49.66a	4.72a	133.03

[a] Within each column, values followed by the same letters are not significantly different at $P \leq 0.05$, using duncan multiple range test ($n = 3$)

[b] ND, not detected

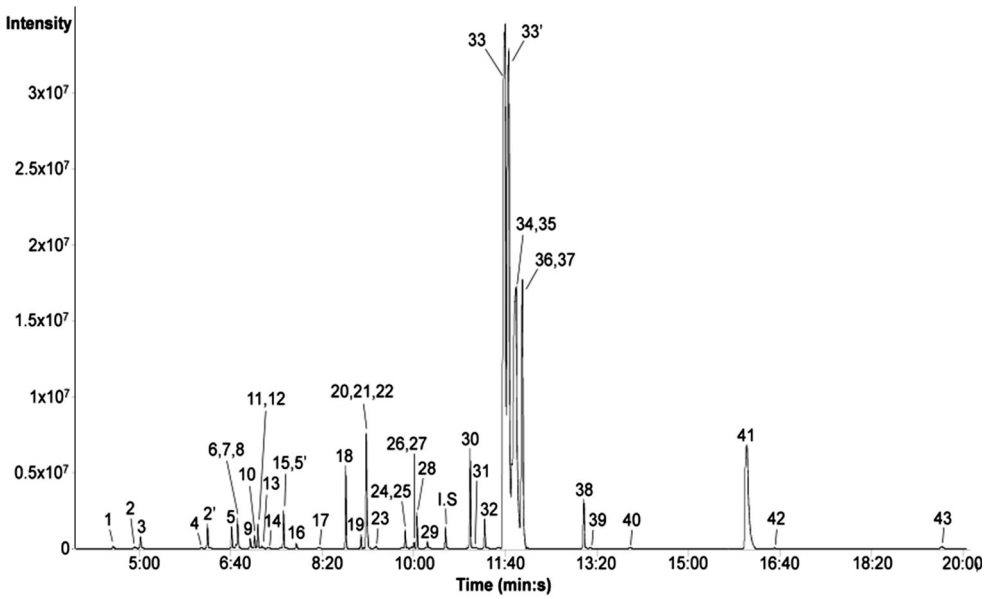

Fig. 2 Selected ion chromatogram of metabolites extracted from Gladiolus as MO/TMS derivatives separated on a 30 m × 0.25 mm I.D. fused-silica capillary column coated with 0.25-μm CP-SIL 8 CB low bleed. Peak identification: *1* lactic acid; *2* valine; *3* alanine; *4* glycolic acid; *2'* valine; *5* serine; *6* ethanolamine; *7* glycerol; *8* leucine; *9* isoleucine; *10* proline; *11* nicotinic acid; *12* glycine; *13* succinic acid; *14* glyceric acid; *15* fumaric acid; *5'* serine; *16* threonine; *17* β-alanine; *18* malic acid; *19* aspartic acid; *20* methionine; *21* pyroglutamic acid; *22* 4-aminobutyric acid; *23* threonic acid; *24* arginine; *25* glutamic acid; *26* phenylalanine; *27* *p*-hydroxybenzoic acid; *28* xylose; *29* asparagine; *30* glutamine; *31* shikimic acid; *32* citric acid; *33* fructose; *33'* fructose; *34* galactose; *35* glucose; *36* mannose; *37* mannitol; *38* inositol; *39* ferulic acid; *40* tryptophan; *41* sucrose; *42* trehalose; *43* raffinose; *IS* internal standard (ribitol)

in 'Violetta' or 'Plumtart', the amount of zeaxanthin in 'Limoncello' was four times greater than in 'Rose Supreme', and was also significantly higher than in 'Mon Amour', 'New Wave', or 'Esta Bonita'.

The total carotenoid contents of the differently colored Gladiolus flowers ranged from 4.35 to 133.03 μg/g DW. 'Limoncello' had 30.6 times more 9-*cis*-β-carotene than 'Violetta'. 'Limoncello' contained 26.1, 18.7, 17.4, 3.7, and 1.53 times more carotenoids than 'Plumtart', 'New Wave', 'Rose Supreme', 'Mon Amour', and 'Esta Bonita', respectively. Harborne (1988) reported that the orange color of the flowers of the 'San Marino' cultivar was due to pelargonidin glycosides and carotenoids. In addition, the 'Jester' and 'Princess' cultivars, which have yellow flowers, had very small amounts of pelargonidin glycosides. However, in this study, pelargonidin glycoside was not detected in 'Limoncello', which has a yellow-colored flower. Therefore, the yellow color is due to carotenoids and chlorophylls, or chlorophyll alone (Takemura et al. 2008).

Metabolic profiling of Gladiolus cultivars using GC-TOFMS analysis

The identification and profiling of primary metabolites using GC-TOFMS analysis allows clear discrimination between Gladiolus genotypes. We used GC-TOFMS to

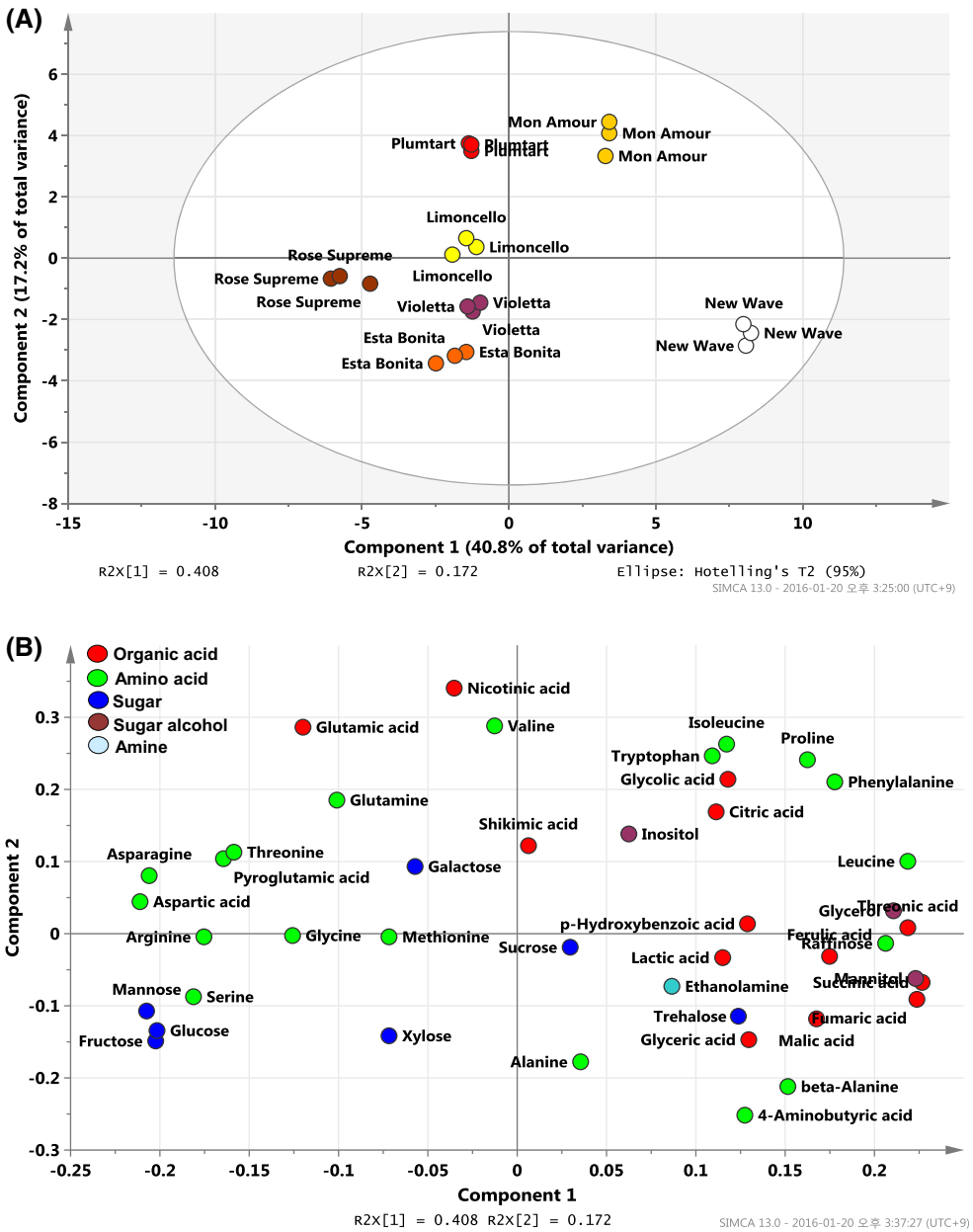

Fig. 3 Scores (**A**) and loading plots (**B**) of principal components 1 and 2 of the PCA results obtained from polar metabolite data for different Gladiolus cultivars. The *ellipse* represents the Hotelling's T2 with 95 % confidence in score plot

identify and measure the low-molecular-weight hydrophilic compounds in the differently colored samples. Using the GC–MS technique, we determined the contents of primary metabolites such as organic acids, amino acids, and sugars and identified a total of 43 metabolites among the seven Gladiolus cultivars (Fig. 2; Table S1). The retention times and the fragment patterns of the detected metabolites, as illustrated in Table S1, were consistent with data presented by Kim et al. (2013).

PCA analysis provides clear confirmation and evidence of differences in the identified compounds between the samples (Cinzia et al. 2006). Core primary metabolites could be used as a tool for metabolite discrimination between genotypes (Tarpley et al. 2005). The internal standard signal intensities obtained from the 43 metabolites were normalized based on PCA analysis to determine the variation in metabolite profiles between the cultivars (Fig. 3). The principal components (PC) can be presented graphically as a score plot (Kim et al. 2007). PCA accounted for 58.0 % of the total variation within the data set and indicated the absence of significant variation between the cultivars tested. The first PC accounted for 40.8 % of the total variation, resolving the metabolite profiles of 'New Wave' and other cultivars.

Hotelling's T2 region, shown as an ellipse in the score plots, defined the 95 % confidence interval of the modeled

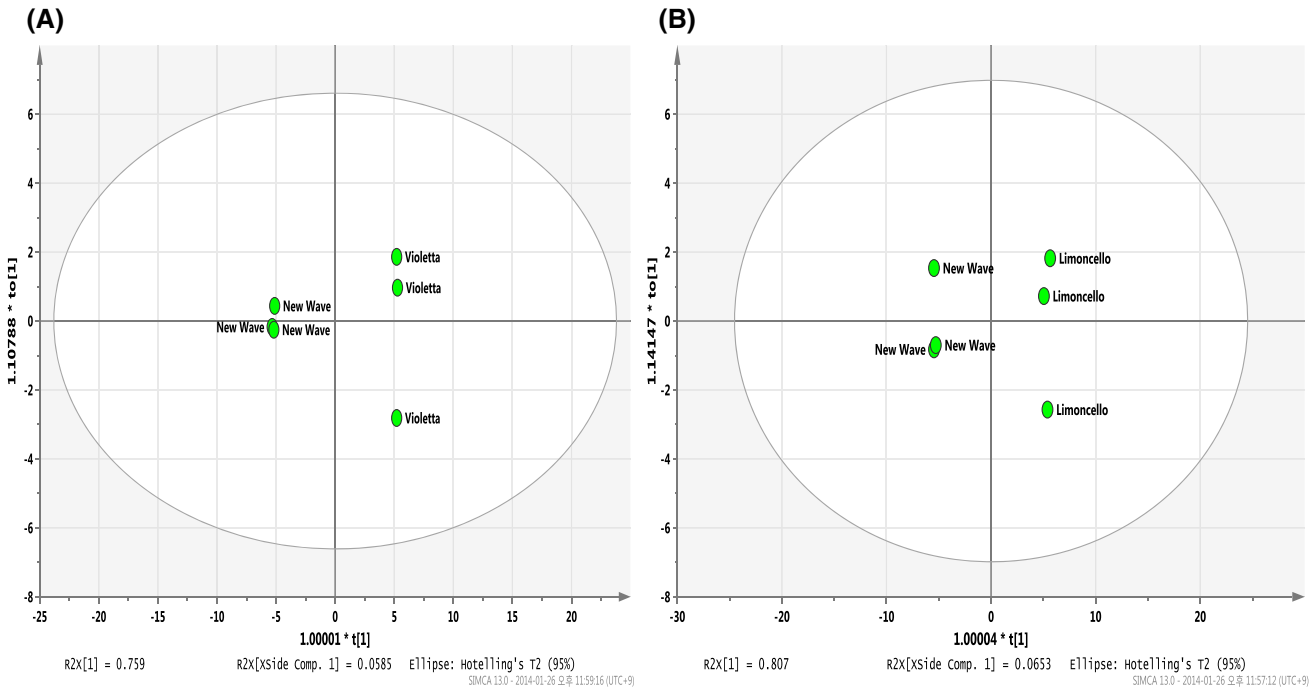

Fig. 4 OPLS-DA score plots derived from GC-TOFMS data sets for 'New Wave' and 'Violetta' (**A**) and 'New Wave' and 'Limoncello' (**B**). The *ellipse* represents the Hotelling's T2 with 95 % confidence in score plot

variation. Q^2 is defined as the proportion of variance in the data predicted by a model. A large Q^2 value ($Q^2 > 0.5$) indicates good predictability. In this study, the Q^2 value for the PCA model was 0.439. The corresponding loading plot shows the metabolites responsible for separation on the score plot. Sugars such as mannose, fructose, and glucose were clustered on the left side of the loading plot, indicating that the levels of the sugars in 'New Wave' were lower than those in the other cultivars. Anthocyanins were more concentrated in 'Violetta' than in the other cultivars, while carotenoids were concentrated mainly in 'Limoncello'. This variation was attributed primarily to sugars and organic acids, for which the corresponding loadings were negative for mannose, fructose, and glucose, but positive for threonic, succinic, fumaric, and glyceric acids. The loadings indicated that the sugars were more concentrated in 'Limoncello' and 'Violetta' than in 'New Wave', whereas most organic acids were more concentrated in the 'New Wave' cultivar. Other than cellular components, secondary metabolites are the main building blocks of the components derived from primary metabolism. Acetyl coenzyme A (acetyl-CoA), shikimic acid, mevalonic acid, and 1-deoxyxylulose 5-phosphate are the key factors for the synthesis of secondary metabolites. In contrast, we reported that green cauliflower contained high levels of carotenoids, but low levels of organic acids involved mainly in the reactions of the tricarboxylic acid (TCA) cycle (Park et al. 2013).

OPLS-DA can be used as an appropriate tool to identify markers for classification. We applied OPLS-DA methods to identify the main metabolites responsible for the separation between 'New Wave' and 'Limoncello or 'Violetta' (Fig. 4). Variable importance in the projection (VIP) is a weighted sum of squares of the OPLS weight, and a value >1 is generally used as a criterion to identify the variables important in the model. The significantly distinguishing metabolites are summarized according to VIP >1 and $P < 0.01$ from Student's *t*-test (Table 3). These findings suggest that the primary metabolism differed significantly between the differently colored Gladiolus flowers. The intermediates in sucrose metabolism, the TCA cycle (malic acid, citric acid, succinic acid, and fumaric acid), and photorespiration (glyceric acid) were found to be the significant components in creating the OPLS-DA mode Tables (3, 4). Bao and Cormier (1991) reported that high sucrose and low nitrate concentrations could affect control of anthocyanin production in pigmented cells of *Vitis vinifera* suspension cultures. Sugars might lead to the activation or inactivation of anthocyanin biosynthetic pathway gene expression. Sugar-dependent upregulation of the anthocyanin synthesis pathway is sucrose specific (Cinzia et al. 2006). Also, sucrose and mannitol treatments increase carotenoid content in citrus juice sacs in vitro (Zhang et al. 2012). The concentration of shikimate was higher in 'Limoncello' and 'Violetta' than in 'New Wave'. Shikimate links primary metabolism and phenylpropanoid

Table 3 Differential metabolites between 'Violetta' and 'New Wave' derived from the OPLS-DA model of GC-TOFMS analysis

Metabolite	VIP[a]	*P* value[b]	FC[c]
Glycerol	1.14622	<0.0001	0.39 ± 0.01
Proline	1.14604	<0.0001	0.35 ± 0.02
Mannitol	1.14551	<0.0001	0.11 ± 0.01
Fumaric acid	1.14440	<0.0001	0.40 ± 0.01
Succinic acid	1.14356	<0.0001	0.32 ± 0.01
Glyceric acid	1.14232	<0.0001	0.56 ± 0.02
Threonic acid	1.14120	<0.0001	0.51 ± 0.02
β-Alanine	1.14095	<0.0001	0.44 ± 0.02
Alanine	1.13665	0.0001	0.48 ± 0.03
Aspartic acid	1.13602	0.0002	1.76 ± 0.07
Leucine	1.13579	0.0002	0.59 ± 0.03
Galactose	1.12958	0.0004	1.73 ± 0.11
Xylose	1.12645	0.0005	1.28 ± 0.05
Ethanolamine	1.12403	0.0006	0.68 ± 0.02
Malic acid	1.12349	0.0007	0.71 ± 0.02
4-Aminobutyric acid	1.11955	0.0009	0.67 ± 0.03
Arginine	1.11789	0.0010	5.73 ± 0.09
Glycolic acid	1.11771	0.0010	0.52 ± 0.04
Raffinose	1.11744	0.0010	0.34 ± 0.06
Asparagine	1.11270	0.0014	1.55 ± 0.08
Methionine	1.11203	0.0014	1.46 ± 0.07
Isoleucine	1.10897	0.0017	0.81 ± 0.04
Shikimic acid	1.10456	0.0021	2.27 ± 0.29
Phenylalanine	1.09513	0.0031	0.62 ± 0.02
Fructose	1.09141	0.0035	1.27 ± 0.07
Glutamic acid	1.08607	0.0042	1.35 ± 0.06
Glucose	1.07551	0.0058	1.21 ± 0.07
Mannose	1.07509	0.0059	1.21 ± 0.07

[a] Variable importance in the projection (VIP) was obtained from the OPLS-DA model with a threshold of 1.0

[b] *P* value obtained from Student's *t*-test

[c] Fold change (FC) between 'Violetta' and 'New Wave'. The values are expressed relative to 'New Wave' and presented as the mean ± SD of determinations from three independent samples

Table 4 Differential metabolites between 'Limoncello' and 'New Wave' derived from OPLS-DA model of GC-TOFMS analysis

Metabolite	VIP[a]	*P* value[b]	FC[c]
Glycerol	1.11310	<0.0001	0.46 ± 0.01
Fumaric acid	1.11218	<0.0001	0.24 ± 0.02
Mannitol	1.11155	<0.0001	0.22 ± 0.01
Glyceric acid	1.11068	<0.0001	0.61 ± 0.01
Succinic acid	1.11031	<0.0001	0.32 ± 0.01
Inositol	1.10926	<0.0001	0.74 ± 0.01
Malic acid	1.10909	<0.0001	0.42 ± 0.01
Threonine	1.10819	<0.0001	1.37 ± 0.02
Threonic acid	1.10787	<0.0001	0.54 ± 0.02
Aspartic acid	1.10735	<0.0001	1.70 ± 0.04
Ethanolamine	1.10580	<0.0001	0.49 ± 0.01
4-Aminobutyric acid	1.10538	<0.0001	0.45 ± 0.01
Serine	1.10533	<0.0001	1.47 ± 0.03
β-Alanine	1.10512	0.0001	0.56 ± 0.01
Glutamic acid	1.10014	0.0002	1.78 ± 0.07
Citric acid	1.09942	0.0003	1.48 ± 0.02
Leucine	1.09314	0.0005	0.63 ± 0.05
Pyroglutamic acid	1.09160	0.0006	1.63 ± 0.09
Raffinose	1.09115	0.0006	0.33 ± 0.02
Proline	1.08205	0.0012	0.67 ± 0.07
Phenylalanine	1.08051	0.0014	0.52 ± 0.02
Fructose	1.07875	0.0015	1.21 ± 0.04
Asparagine	1.07792	0.0016	1.45 ± 0.05
Shikimic acid	1.06457	0.0029	1.77 ± 0.17
Nicotinic acid	1.05830	0.0037	1.27 ± 0.04
Isoleucine	1.05551	0.0041	0.82 ± 0.05
Glycolic acid	1.05234	0.0045	0.70 ± 0.02
Mannose	1.04909	0.005	1.19 ± 0.06
Glutamine	1.04432	0.0058	1.59 ± 0.18
Methionine	1.02875	0.0086	1.28 ± 0.07
Glucose	1.02274	0.0098	1.18 ± 0.08

[a] Variable importance in the projection (VIP) was obtained from the OPLS-DA model with a threshold of 1.0

[b] *P* value obtained from Student's *t*-test

[c] Fold change (FC) between 'Limoncello' and 'New Wave'. The values are expressed relative to 'New Wave' and presented as the mean ± SD of determinations from three independent samples

anthocyanin biosynthesis. Sucrose and shikimate concentrations affect flower color.

To further confirm the performance of these models, 67 % of the samples were selected randomly as training samples. Prediction parameters of the remaining 33 % of the samples using OPLS-DA models were established with the training samples: New Wave and Violetta: $R^{2}Y_{\mathrm{cum}} = 1.0$, $Q^{2}Y_{\mathrm{cum}} = 0.999$; New Wave and Limoncello: $R^{2}Y_{\mathrm{cum}} = 1.0$, $Q^{2}Y_{\mathrm{cum}} = 0.993$. A correlation coefficient ($R^{2}Y$) indicates the goodness of model fit. $Q^{2} > 0.9$ indicates an excellent predictive model.

The differences between various plant metabolites, especially primary and secondary metabolites, derived from biological samples were analyzed by correlation analysis. In this study, Pearson's correlation analyses and HCA identified the association between the 43 metabolites in Gladiolus and its contents (Fig. 5). Camacho et al. (2005) claimed that a positive correlation results from an equilibrium situation. Similarly, in this study, fructose levels were positively correlated with glucose ($r = 0.9723$, $P < 0.0001$) and mannose ($r = 0.9628$, $P < 0.0001$). Also, TCA cycle intermediates, such as malic acid, citric acid,

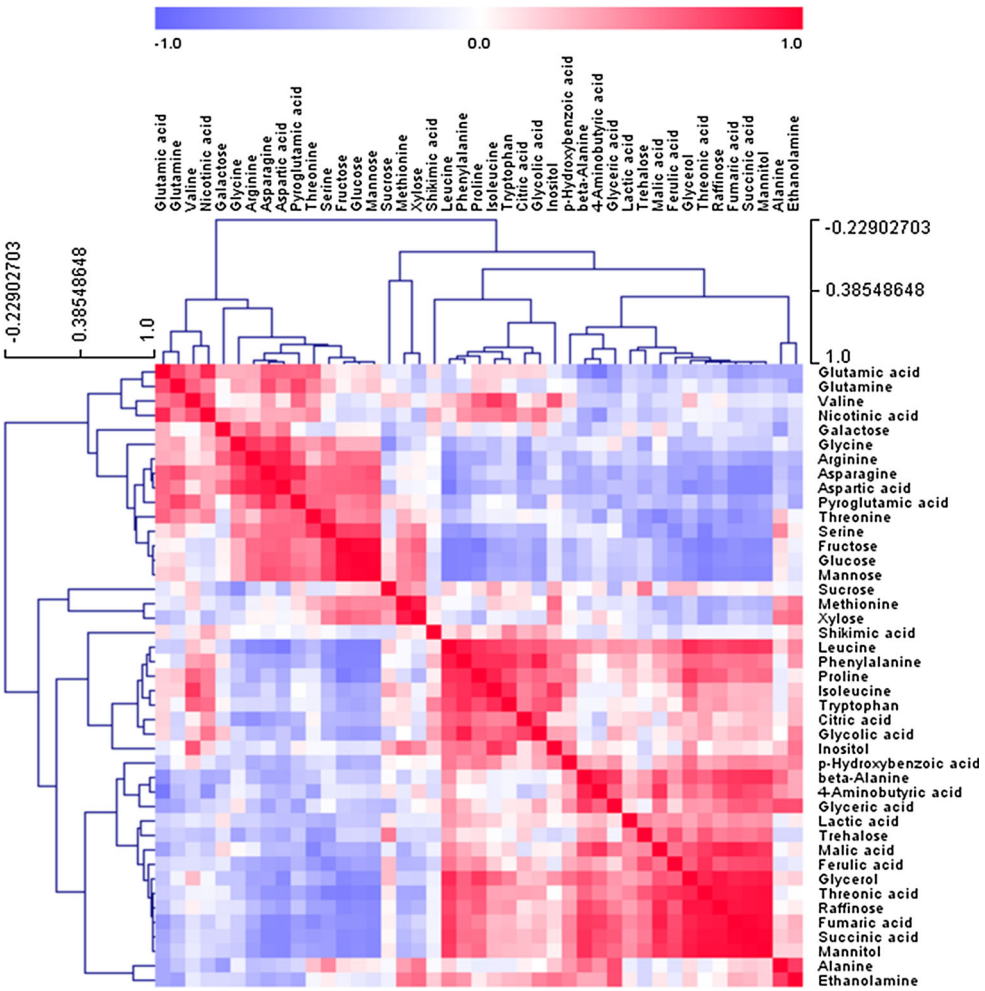

Fig. 5 Correlation matrix of metabolites from seven Gladiolus cultivars. *Each square* indicates the Pearson's correlation coefficient of a pair of compounds, and the values of the correlation coefficients are represented by the intensity of *blue* or *red colors*, as indicated on the *color* scale

succinic acid, and fumaric acid, exhibited positive relationships. The analytical results indicated that the fumaric acid content was significantly and positively correlated with levels of succinic acid ($r = 0.9846$, $P < 0.0001$). Thus, HCA provided that most of the compounds clustered on the basis of their biochemical nature (e.g., citric acid cycle).

Flowers rely predominantly on a supply of sucrose to produce energy in the form of ATP and for synthesis of macromolecules and organic compounds involved in primary and secondary plant metabolism. A previous study claimed that TCA cycle intermediates are connected to flavonol biosynthesis and cellular catabolism (Muhlemann et al. 2012). In this study, we performed anthocyanin, carotenoid, and metabolic profiling to determine the metabolic differences in seven Gladiolus cultivars with differently colored flowers. The amounts of anthocyanins and carotenoids, and the metabolic profiles varied widely among the seven differently colored cultivars. We performed PCA and OPLS-DA to identify differences between the Gladiolus cultivars. 'Violetta' Gladiolus had higher levels of both shikimic acid, a precursor for the phenylpropanoid biosynthetic pathway, and anthocyanin than the other cultivars. In addition, the significant contributors to a discrimination model for white ('New Wave'), yellow ('Limoncello'), white and purple ('Violetta') cultivars were similar Tables (3, 4). We previously reported that flavonoid content exhibits a positive correlation with carotenoid content in colored rice grains (Kim et al. 2010).

GC-TOFMS-based metabolic profiling is an important separation tool to detect plant metabolites of the central pathways of primary metabolism in a sensitive manner. In this study, we quantified hydrophilic primary metabolites, together with carotenoids and anthocyanins, from seven differently colored Gladiolus cultivars. This metabolite profiling would provide a powerful tool for identifying metabolic networks connecting primary and secondary metabolism in plants.

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