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Flavonol Glycosides and their Distribution in the Perianths of *Gladiolus × grandiflora* Cultivars

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Abstract Fifteen flavonol glycosides were isolated from the perianths of two *Gladiolus × grandiflora* cultivars "Ariake" and "Christmas" by various chromatography. Of their flavonols, eight glycosides were identified as kaempferol 3-*O*-sophoroside, 3-*O*-glucoside, 3-*O*-rutinoside and 3-*O*-glucosyl-(1→2)-rhamnoside, quercetin 3-*O*-rutinoside and myricetin 3-*O*-rutinoside, 3-*O*-glucoside and 3-*O*-rhamnoside by UV spectra, LC-MS, acid hydrolysis, ^{1}H and ^{13}C NMR, and TLC and HPLC comparisons with authentic samples. Other seven flavonols were characterized as kaempferol 3-*O*-pentosylglucoside, 3-*O*-rhamnosylrhamnosylglucoside and 3-*O*-glucosylrhamnoside, laricitrin 3-*O*-diglucoside, 3-*O*-rhamnosylglucoside and 3-*O*-rhamnosylhexoside, and syringetin 3-*O*-rhamnosylglucoside. Flavonol composition of the perianths of 89 *Gladiolus × grandiflora* cultivars including 12 purple, 13 reddish purple, 13 pink, 19 red, 2 orange, 16 white, 11 yellow and 3 green flower cultivars was surveyed by HPLC. Moreover, correlation between flower colors and flavonol glycosides was discussed.

Key words: flavonol glycosides, flower colors, *Gladiolus* cultivars, kaempferol glycosides.

Introduction

Gladiolus is the largest genus in the family Iridaceae, mainly distributed in Africa, especially southern Africa, the coast of the Mediterranean, central Europe, the Arabian Peninsula and western Asia, and consists of ca. 250 species (Goldblatt, 1996; Goldblatt and Manning, 1998). It is one of the most important plants in horticulture today, both as an ornamental garden subject and as a cut-flower horticultural crop plants, and many cultivars are bred.

Flower anthocyanins of *Gladiolus × grandiflora* L. cultivars has been reported by some authors. Twenty-five anthocyanins were isolated and characterized as pelargonidin 3-*O*-glucosude, 3-*O*-rhamnosylglucoside, 3,5-di-*O*-glucoside, 3-*O*-diglucoside-5-*O*-glucoside and 3-*O*-rhamnosylglucoside-5-*O*-glucoside, and cyanidin, peonidin, delphinidin, petunidin and malvidin 3-*O*-glucosides, 3-*O*-rhamnosylglucosides, 3,5-di-*O*-glucosides and 3-*O*-rhamnosylglucoside-5-*O*-glucosides (Shibata and Nozaka, 1963; Yatomi and Arisumi, 1968; Arisumi and Kobayashi, 1971; Akavia *et al.*, 1981; Takemura *et al.*, 2005). Takemura *et al.* (2008) isolated eleven anthocyanins from the flowers of six *Gladiolus × grandiflora* cultivars and identified as pelargonidin 3-*O*-rutinoside, 3,5-di-*O*-glucoside and 3-*O*-rutinoside-5-*O*-glucoside, cyanidin and peonidin 3,5-di-*O*-glucosides and 3-*O*-rutinoside-5-*O*-glucosides, petunidin 3,5-di-*O*-glucoside, and malvidin 3-*O*-glucoside, 3,5-di-*O*-glucoside and 3-*O*-rutinoside-5-*O*-glucoside. Moreover, antho-

cyanin composition in the perianths of 84 *Gladiolus* × *grandiflora* cultivars was surveyed by high-performance liquid chromatography (HPLC) and divided into 18 groups according to the anthocyanin pattern.

Other flavonoids in *Gladiolus* species and *G.* × *grandiflora* cultivar were reported from the flowers and leaves of *G. tristis* L. and flowers of *G.* × *grandiflora* cultivar, i.e., kaempferol, quercetin, myricetin, laricitrin and syringetin 3-*O*-galactosides from *G. tristis* (Williams *et al.*, 1986), and kaempferol 3-*O*-sophoroside and 3-*O*-rutinoside, and quercetin 3-*O*-rutinoside from *G.* × *grandiflora* (Takemura *et al.*, 2005). On the other hand, two *C*-glycosylflavones, isoorientin 7-*O*-rhamnosylglucoside and 7-*O*-rhamnosylarabinosylglucoside, two flavone *O*-glycosides, luteolin and tricin 5-*O*-glucosides, a flavonol *O*-glycoside, quercetin 3-*O*-galactoside, were isolated from the leaves of *G. tristis* (Williams *et al.*, 1986). Foliar flavonoids were also found in 10 *Gladiolus* species, i.e., *G. byzantinus* Mill., *G. carmineus* C.H.Wright, *G. delenii* Van-Geel, *G. floribundus* Hort., *G. illyricus* Koch, *G. italicus* Mill., *G. papilio* Hook.f., *G. scallyi* Baker, *G. segetum* Ker-Gawl. and *G. triphyllus* Bertol., and characterized as kaempferol, quercetin and/or isorhamnetin glycosides, together with apigenin, luteolin and tricin glycosides (Williams *et al.*, 1986). In this paper, we describe the isolation and identification of the perianths of two *Gladiolus* × *grandiflora* cultivars "Ariake" and "Christmas". Moreover, flavonoid composition of 89 *Gladiolus* cultivar flowers was surveyed by HPLC.

Materials and Methods

Plant materials

Eighty-nine *Gladiolus* × *grandiflora* cultivars (see Table 1) were cultivated in the nursery of the Tsukuba Botanical Garden, National Museum of Nature and Science, Tsukuba, Ibaraki Pref., Japan for plant materials. One cultivar "Ariake" was cultivated in Ogawa Town, Ibaraki Pref., Japan.

General

Analytical HPLC was performed with Shimadzu HPLC systems using Pegasil ODS (I.D. 6.0 × 150 mm, Senshu Scientific Co., Ltd., Tokyo) at a flow-rate of 1.0 ml min^{-1}. Detection wavelength was 350 nm. Eluents were MeCN/H_2O/H_3PO_4 (22 : 78 : 0.2) for glycosides and MeCN/H_2O/H_3PO_4 (35 : 65 : 0.2) for aglycones. Liquid chromatograph-mass spectra (LC-MS) was performed with Shimadzu LC-MS systems using Shim-pack VR ODS (I.D. 2.0 × 150 mm, Shimadzu) at a flow-rate of 0.2 ml min^{-1}, ESI^+ 4.5 kV and ESI^- 3.5 kV, 250°C. Eluent was MeCN/H_2O/HCOOH (18 : 82 : 1). Acid hydrolysis was performed in 12% HCl, 100°C, 30 min. After shaking with diethyl ether, aglycones migrated to organic layer, and sugars were left in aqueous mother liquor. Sugar identification was performed by paper chromatography using solvent systems, BBPW (*n*-BuOH/benzene/pyridine/H_2O = 5 : 1 : 3 : 3) and BTPW (*n*-BuOH/toluene/pyridine/H_2O = 5 : 1 : 3 : 3).

Thin layer chromatography (TLC) was performed with Cellulose F plastic plate (Merck, Germany) using solvent systems, BAW (*n*-BuOH/HOAc/H_2O = 4 : 1 : 5, upper phase), BEW (*n*-BuOH/EtOH/H_2O = 4 : 1 : 2.2), 15% HOAc and Forestal (HOAc/HCl/H_2O = 30 : 3 : 10). NMR spectra (^1H and ^{13}C NMR, ^1H-^1H COSY, ^1H-^1H NOESY, HMBC and HSQC) were measured in DMSO-d_6 or pyridine-d_5 at 500 MHz (^1H NMR) and 125 MHz (^{13}C NMR).

Extraction and isolation

Fresh perianths of *Gladiolus* × *grandiflora* cultivars were extracted with MeOH. After concentration, the extracts were applied to preparative paper chromatography (PC) using solvent systems, BAW, 15%HOAc and then BEW. Roughly isolated flavonoids were further purified by preparative HPLC and then applied to Sephadex LH-20 column chromatography using solvent system, 70% MeOH. The flavonoids were obtained as pale yellow powders or MeOH solution.

Table 1. Flavonol composition in the perianth of *Gladiolus* × *grandiflora* cultivars

Cultivars & Strains	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Purple cultivars															
Ariake	+	+	t	+ +	+	+	t	t	t	t	+ +	+	+	+	+
Blue Frost	+	+	t	+ +		+	t	t	t		t		t	+	
Blue Lagoon	+	+		+	t	+ +					+ +		+	t	
Brisbane	t	t		+	t	+					+ +		t	t	t
Ten-shu	+	+	t	+ +	t	+ +					+ +		+ +	+	
Blue Isle	+	+	t	+	+	+					+ +		+	+	
Blue Nile	t	t		+	t	t					+ +		t	+	
Sei-un	+	+		t	t	+ +					+ +	+			
Violetta	+ +	+ +	t	t	t	+ +					+ +		+	+	
PBI No.60	+	+	t	+ +	t	+ +	t				+ +		+	+	
PBI No.304	t	t		+	+	+					+ +		+	+	
PBI No.332	+	+		+	t	+					+ +		+	+	
Reddish purple cultivars															
Ama	t	t	t	+	t	+		t	t		+	t	+	+	
Blue Sky	+	+	t	+	t	+		t			t	t	+	t	t
Fusou	t	t	+ +	t	t					t	+ +		+	+	
Mai-hime				+	t	t		t			+ +	t	t	t	
Blue Mountain	t	t	t	+	t	+	t	t	t		t		t	t	
Feather Hill	t	t	+	t	t	t					+ +	t	t	t	
Madrid	t	t		t		+					+ +		+	+	
Marches	t	t	t	+ +	t	t	t				+ +		+	+	+
New Orleans	t	t	t	+	t	+					+ +		+	+	t
Purple Star	t	t		t	t	+					+ +	t	+		
Obelisk				t	t	t	t				+	t	t		
Wind Song				+	t	t					+ +	+			
PBI No.510	t	t		+ +	t	+	t				+ +		+	+	
Pink cultivars															
Ben Venuto				+ +						t	+		+	+ +	
Fado	t	t		t	+ +						+ +	+			
Hana-kasumi	t	t		+	t						+ +	t	+	+ +	
High Style				+	+						+ +	t	t	+	
Naples				t	+						+ +	t		t	
Normandy				t	+ +						+ +	+			
Ou-ka				+	+				t		+ +		+	+ +	+
Rose Maiden	+	+		+	+				+		+	+		+	
Shiho-no-asa				+	t	t	t				+ +		+	+	
Silver Shadow				+							+ +		+	+	
Vadi Napori				t	+						+ +	t	t	t	
Wine and Roses				+		t	t				+ +		t	+	t
PBI No.38	t	t	t	+					+		t		t	+ +	t
Red cultivars															
Ali Baba											+				
Beijing				t							+ +		+	t	t
Drummer				+		t	t	t			+ +		+		
Eternity				+							+ +		t	+	
Jessica				+				t			+ +		+	t	
Jigoku-mon				+ +							+ +		+	+	
Juku-gaki											+ +			t	
Marilyn Monroe				+ +		+					+ +		+		
Mascagni				+							+ +		t	t	
Match Point				t	+						+ +	+			
Shin-ku											+ +	t			
Shuku-ho				+	+						+ +		t	t	
Spich-and-Span				t	t						+ +	t	t	+	t
Tahiti				+							+ +			t	
Tellesia				+							+ +		t	+	
Traderhorn				t							+			t	

Table 1. Continued.

Cultivars & Strains	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
True Love				++							t		+	t	
Whistler											++		t	+	t
Zorro					+						+	+			
Orange cultivars															
San Marino				t							++		t	++	t
Orange Emperor				+							++		+		
White cultivars															
Colorado	+	+		++	t		+		+		+	t	+	++	
Flevo Eyes	t	t	+	+						+		t	+	++	
Fuji-no-yuki											++				
Popcorne				+	+			+						++	t
Prospector				+							+		+	+	
Princess Summer				t							++				
Richmond				+										++	
Sai-un				+							++		+	+	
Snow Mass				+				t			++		t	+	
Sophie				+										+	
Tan-cho				+							++		+	t	
Vega				+											
White Friendship				+							+		+	+	
White Giant				++							++		t	+	
White Surf				+							t		t	++	
Wine and Pearl				+				+			+	t	t	++	
Yellow cultivars															
Bostoque				+				+			++		t	++	+
Christmas				+							t			++	
Falster														++	
Jacksonville Gold				+							++		t	+	
Jester				t							++		t	++	
Kin-sei				+							++			+	t
Morning Gold				+							++				
Princess Margaret Rose				+				+			++		t	++	+
San-rei	+	+		++				+			+			++	
Topaz				+							++			+	
PBI No.120				++	t	t				t	+		t	++	
Green cultivars															
Green Isle				+			t	t	+		+		++	++	+
Green Star				t				t			++		+	+	t
Ryoku-fu				+							+		+	++	++

1 = laricitrin 3-*O*-diglucoside, **2** = myricetin 3-*O*-rutinoside, **3** = myricetin 3-*O*-glucoside, **4** = kaempferol 3-*O*-sophoroside, **5** = quercetin 3-*O*-rutinoside, **6** = laricitrin 3-*O*-rhamnosylglucoside, **7** = kaempferol 3-*O*-pentosylglucoside, **8** = myricetin 3-*O*-rhamnoside, **9** = laricitrin 3-*O*-rhamnosylglucoside, **10** = kaempferol 3-*O*-rhamnosylrhamnosylglucoside, **11** = kaempferol 3-*O*-rutinoside, **12** = syringetin 3-*O*-rhamnosylglucoside, **13** = kaempferol 3-*O*-glucoside, **14** = kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside, and **15** = kaempferol 3-*O*-glucosylrhamnoside.

++ = major, + = presence, and t = trace.

Identification

Flavonoids were characterized by UV spectral survey according to Mabry *et al.* (1970) and Markham (1982), LC-MS, acid hydrolysis, ^{1}H and ^{13}C NMR, and/or TLC and HPLC comparisons with authentic samples. TLC, HPLC, UV, LC-MS and NMR data of the isolated flavonoids were as follows.

Laricitrin 3-*O*-diglucoside (**1**). TLC (Rf): 0.46 (BAW), 0.52 (BEW), 0.51 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 light yellow. HPLC (retention time, Rt) (min.): 4.21. UV: λmax (nm) MeOH 259, 365; +NaOMe 272, 330sh, 405 (inc.); +$AlCl_3$ 267, 316, 362, 426; +$AlCl_3$/HCl 268, 315, 361, 415sh; +NaOAc 271, 334sh, 403; +NaOAc/H_3BO_3 263, 305sh, 390. LC-MS: *m/z* 657 $[M+H]^+$ (molecular ion peak, laricitrin + 2 mol glucose).

Laricitrin (aglycone from **1**, **6** and **9**). TLC (Rf): 0.45 (BAW), 0.45 (BEW), 0.27 (Forestal); color UV (365 nm) bright yellow, UV/NH_3 yellow. UV: λmax (nm) MeOH 253, 374; + NaOMe decomposition; + $AlCl_3$ 266, 311, 367, 454; + $AlCl_3$/HCl 260, 310, 362, 428; + NaOAc 269, 328, 407; + NaOAc/H_3BO_3 259, 390. LC-MS: *m/z* 333 $[M+H]^+$ (molecular ion peak).

Myricetin 3-*O*-rutinoside (**2**). TLC (Rf): 0.43 (BAW), 0.48 (BEW), 0.33 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 orange yellow. HPLC (Rt) (min.): 4.27. UV: λmax (nm) MeOH 260, 360; + NaOMe decomposition; + $AlCl_3$ 269, 316, 362, 427; + $AlCl_3$/HCl 269, 314, 361; + NaOAc 268, 301sh, 404; + NaOAc/H_3BO_3 261, 380. LC-MS: *m/z* 627 $[M+H]^+$ (molecular ion peak, myricetin + each 1 mol of glucose and rhamnose), *m/z* 481 $[M-146+H]^+$ (fragment ion peak, myricetin + 1 mol glucose), and *m/z* 319 $[M-308+H]^+$ (fragment ion peak, myricetin).

Myricetin 3-*O*-glucoside (**3**). TLC (Rf): 0.44 (BAW), 0.52 (BEW), 0.16 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 orange yellow. HPLC (Rt) (min.): 4.84. UV: λmax (nm) MeOH 254, 360; + NaOMe decomposition; + $AlCl_3$ 269, 316, 362, 427; + $AlCl_3$/HCl 264, 312, 362; + NaOAc 269, 323, 395; + NaOAc/H_3BO_3 260, 300sh, 380. LC-MS: *m/z* 481 $[M+H]^+$ (molecular ion peak, myricetin + 1 mol glucose), *m/z* 319 $[M-162+H]^+$ (fragment ion peak, myricetin).

Kaempferol 3-*O*-sophoroside (sophoraflavonoloside, **4**). TLC (Rf): 0.60 (BAW), 0.63 (BEW), 0.57 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 dark greenish yellow. HPLC (Rt) (min.): 4.92. UV: λmax (nm) MeOH 266, 296sh, 350; + NaOMe 275, 325, 397 (inc.); + $AlCl_3$ 274, 304, 352, 397; + $AlCl_3$/HCl 275, 302, 346, 393; + NaOAc 274, 308, 386; + NaOAc/H_3BO_3 266, 296sh, 353. LC-MS: *m/z* 633 $[M+H+Na]^+$, 609 $[M-H]^-$ (molecular ion peaks, kaempferol + 2 mol glucose), *m/z* 449 $[M-162+H]^+$ (fragment ion peak, kaempferol + 1 mol glucose), *m/z* 287 $[M-324+H]^+$ (fragment ion peak, kaempferol). ^1H NMR (500 MHz, pyridine-d_5) δ_H 13.40 (1H, *s*, 5-OH), 8.46 (2H, *d*, $J = 8.9$ Hz, H-2′,6′), 7.40 (2H, *d*, $J = 8.9$ Hz, H-3′,5′), 6.74 (1H, *d*, $J = 1.8$ Hz, H-8), 6.68 (1H, *d*, $J = 1.8$ Hz, H-6), 6.62 (1H, *d*, $J = 7.6$ Hz, 3-glucosyl H-1), 5.60 (1H, *d*, $J = 7.6$ Hz, 2″-glucosyl H-1), 5.29 (1H, *d*, $J = 19.8$ Hz, 3-glucosyl H-2), 4.50 (1H, *m*, 3-glucosyl H-6a), 4.48 (1H, *m*, 2″-glucosyl H-6a), 4.43 (1H, *m*, 3-glucosyl H-6b), 4.41 (1H, *m*, 2″-glucosyl H-6b), 4.31 (1H, *m*, 3-glucosyl H-3), 4.30 (1H, *m*, 2″-glucosyl H-3), 4.29 (1H, *m*, 3-glucosyl H-4), 4.28 (1H, *m*, 2″-glucosyl H-4), 4.17 (1H, *m*, 2″-glucosyl H-2), 3.97 (1H, *m*, 3-glucosyl H-5), 3.97 (1H, *m*, 2″-glucosyl H-5). ^{13}C NMR (125 MHz, pyridine-d_5) (kaempferol) δ_C 157.1 (C-2), 134.2 (C-3), 178.7 (C-4), 162.6 (C-5), 99.8 (C-6), 165.5 (C-7), 94.3 (C-8), 156.4 (C-9), 105.0 (C-10), 121.8 (C-1′), 131.8 (C-2′,6′), 116.1 (C-3′,5′), 161.5 (C-4′); (3-*O*-glucose): δ_C 105.7 (C-1), 83.6 (C-2), 78.2 (C-3), 71.1 (C-4), 78.7 (C-5), 62.3 (C-6); (2″-*O*-glucose): δ_C 99.5 (C-1), 75.9 (C-2), 78.1 (C-3), 70.8 (C-4), 78.4 (C-5), 62.0 (C-6).

Quercetin 3-*O*-rutinoside (rutin, **4**). TLC (Rf): 0.53 (BAW), 0.57 (BEW), 0.42 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 yellow. HPLC (Rt) (min.): 5.45. UV: λmax (nm) MeOH 258, 264sh, 360; + NaOMe 273, 326, 410 (inc.); + $AlCl_3$ 274, 431; + $AlCl_3$/HCl 268, 301, 362, 394sh; + NaOAc 273, 326, 402; + NaOAc/H_3BO_3 262, 294sh, 379.

Laricitrin 3-*O*-rhamnosylglucoside (**6**). TLC (Rf): 0.46 (BAW), 0.53 (BEW), 0.37 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 light yellow. HPLC (Rt) (min.): 5.68. UV: λmax (nm) MeOH 255, 264sh, 360; + NaOMe 271, 324, 416 (inc.); + $AlCl_3$ 270, 313, 368, 433; + $AlCl_3$/HCl 270, 311, 363, 400sh; + NaOAc 268, 325, 416; + NaOAc/H_3BO_3 261, 304, 383. LC-MS: *m/z* 641 $[M+H]^+$, 639 $[M-H]^-$(molecular ion peaks, laricitrin + each 1 mol of glucose and rhamnose), *m/z* 495 $[M-146+H]^+$ (fragment ion peak, laricitrin + 1 mol glucose), *m/z* 333 $[M-308+H]^+$ (fragment ion peak, laricitrin).

Kaempferol 3-*O*-pentosylglucoside (**7**). TLC (Rf): 0.67 (BAW), 0.68 (BEW), 0.56

(15%HOAc); color UV (365 nm) dark purple, UV/NH_3 dark greenish yellow. HPLC (Rt) (min.): 6.11. UV: λmax (nm) MeOH 266, 296sh, 348; +NaOMe 275, 324, 397 (inc.); +$AlCl_3$ 274, 305, 357, 394sh; +$AlCl_3$/HCl 273, 304, 351, 394sh; +NaOAc 274, 311, 390; +NaOAc/H_3BO_3 267, 299sh, 352. LC-MS: *m/z* 581 $[M+H]^+$ (molecular ion peak, kaempferol+each 1 mol of glucose and pentose), *m/z* 449 $[M-132+H]^+$ (fragment ion peak, kaempferol+1 mol glucose), *m/z* 287 $[M-294+H]^+$ (fragment ion peak, kaempferol).

Myricetin 3-*O*-rhamnoside (myricitrin, **8**). TLC (Rf): 0.53 (BAW), 0.72 (BEW), 0.33 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 orange yellow. HPLC (Rt) (min.): 6.23. UV: λmax (nm) MeOH 254, 264sh, 356; +NaOMe decomposition; +$AlCl_3$ 315, 363, 420; +$AlCl_3$/HCl 313, 360; +NaOAc 269, 320sh, 383; +NaOAc/H_3BO_3 255, 300sh, 374. LC-MS: *m/z* 465 $[M+H]^+$ (molecular ion peak, myricetin+1 mol rhamnose), *m/z* 319 $[M-146+H]^+$ (fragment ion peak, myricetin).

Laricitrin 3-*O*-rhamnosylhexoside (**9**). TLC (Rf): 0.59 (BAW), 0.65 (BEW), 0.33 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 light yellow. HPLC (Rt) (min.): 7.00. UV: λmax (nm) MeOH 260, 310sh, 363; +NaOMe 273, 320, 406 (inc.); +$AlCl_3$ 315, 362, 430; +$AlCl_3$/HCl 314, 361; +NaOAc 270, 322, 404; +NaOAc/H_3BO_3 262, 300sh, 392. LC-MS: *m/z* 641 $[M+H]^+$ (molecular ion peak, laricitrin+each 1 mol of hexose and rhamnose), *m/z* 495 $[M-146+H]^+$ (fragment ion peak, laricitrin+1 mol hexose), *m/z* 333 $[M-308+H]^+$ (fragment ion peak, laricitrin).

Kaempferol 3-*O*-rhamnosylrhamnosylglucoside (**10**). TLC (Rf): 0.70 (BAW), 0.83 (BEW), 0.69 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 dark greenish yellow. HPLC (Rt) (min.): 7.16. UV: λmax (nm) MeOH 266, 296sh, 344; +NaOMe 274, 326, 391 (inc.); +$AlCl_3$ 274, 305, 354, 390sh; +$AlCl_3$/HCl 275, 303, 347, 390sh; +NaOAc 274, 306, 375; +NaOAc/H_3BO_3 266, 300sh, 351. LC-MS: *m/z* 741 $[M+H]^+$ (molecular ion peak, kaempferol+1 mol glucose+2 mol rhamnose), *m/z* 595 $[M-146+H]^+$ (fragment ion peak, kaempferol+each 1 mol of glucose and rhamnose), *m/z* 287 $[M-454+H]^+$ (fragment ion peak, kaempferol).

Kaempferol 3-*O*-rutinoside (nicotiflorin, **11**). TLC (Rf): 0.62 (BAW), 0.68 (BEW), 0.43 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 dark greenish yellow. HPLC (Rt) (min.): 7.45. UV: λmax (nm) MeOH 266, 296sh, 350; +NaOMe 275, 324, 400 (inc.); +$AlCl_3$ 274, 305, 356, 394; +$AlCl_3$/HCl 274, 302, 349, 390; +NaOAc 274, 317, 394; +NaOAc/H_3BO_3 266, 296sh, 354. LC-MS: *m/z* 595 $[M+H]^+$, 593 $[M-H]^-$ (molecular ion peaks, kaempferol+each 1 mol of glucose and rhamnose), *m/z* 449 $[M-146+H]^+$ (fragment ion peak, kaempferol+1 mol glucose), *m/z* 287 $[M-308+H]^+$ (fragment ion peak, kaempferol). ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 12.63 (1H, *s*, 5-OH), 8.04 (2H, *d*, J=8.9 Hz, H-2′,6′), 6.94 (2H, *d*, J=8.8 Hz, H-3′,5′), 6.47 (1H, *d*, J=2.0 Hz, H-8), 6.26 (1H, *d*, J=2.0 Hz, H-6), 5.37 (1H, *d*, J=7.6 Hz, glucosyl H-1), 4.43 (1H, *d*, J=1.1 Hz, rhamnosyl H-1), 1.04 (3H, *d*, J=6.2 Hz, rhamnosyl CH_3). ^{13}C NMR (150 MHz, DMSO-d_6): (kaempferol) δ_C 156.8 (C-2), 133.5 (C-3), 177.7 (C-4), 161.5 (C-5), 99.1 (C-6), 164.4 (C-7), 94.1 (C-8), 157.2 (C-9), 104.3 (C-10), 121.2 (C-1′), 131.2 (C-2′,6′), 115.4 (C-3′,5′), 160.2 (C-4′); (3-*O*-glucose) δ_C 101.7 (C-1), 74.5 (C-2), 76.3 (C-3), 70.3 (C-4), 76.1 (C-5), 67.2 (C-6); (6″-*O*-rhamnose) δ_C 101.1 (C-1), 70.7 (C-2), 70.9 (C-3), 72.1 (C-4), 68.6 (C-5), 18.1 (C-6).

Syringetin 3-*O*-rhamnosylglucoside (**12**). TLC (Rf): 0.53 (BAW), 0.57 (BEW), 0.43 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 light yellow. HPLC (Rt) (min.): 8.23. UV: λmax (nm) MeOH 252, 264sh, 307sh, 360; +NaOMe 266, 331, 422 (inc.); +$AlCl_3$ 315, 364, 410sh; +$AlCl_3$/HCl 313, 362, 410sh; +NaOAc 266, 274sh, 329, 429; +NaOAc/H_3BO_3 262, 305sh, 363. LC-MS: *m/z* 655 $[M+H]^+$ (molecular ion peak, syringetin+each 1 mol of glucose and rhamnose), *m/z* 509 $[M-146+H]^+$ (fragment ion peak, syringe-

tin + 1 mol glucose), *m/z* 347 [M − 308 + H]$^+$ (fragment ion peak, syringetin).

Syringetin (aglycone from **12**). TLC (Rf): 0.58 (BAW), 0.58 (BEW), 0.47 (Forestal); color UV (365 nm) bright yellow, UV/NH_3 yellow. UV: λmax (nm) MeOH 255, 371; + NaOMe decomposition; + $AlCl_3$ 266sh, 315, 360; + $AlCl_3$/HCl 266sh, 313, 360; + NaOAc 270, 326, 406; + NaOAc/H_3BO_3 260sh, 379. LC-MS: *m/z* 347 [M + H]$^+$ (molecular ion peak).

Kaempferol 3-*O*-glucoside (astragalin, **13**). TLC (Rf): 0.77 (BAW), 0.81 (BEW), 0.33 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 dark greenish yellow. HPLC (Rt) (min.): 9.20. UV: λmax (nm) MeOH 266, 350; + NaOMe 275, 324, 398 (inc.); + $AlCl_3$ 272, 305, 357, 390sh; + $AlCl_3$/HCl 271, 303, 351, 390sh; + NaOAc 274, 310, 388; + NaOAc/H_3BO_3 267, 353.

Kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside (**14**). TLC (Rf): 0.69 (BAW), 0.70 (BEW), 0.58 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 dark greenish yellow. HPLC (Rt) (min.): 10.35. UV: λmax (nm) MeOH 265, 345; + NaOMe 274, 323, 388 (inc.); + $AlCl_3$ 272, 304, 357, 390sh; + $AlCl_3$/HCl 271, 301, 355, 390sh; + NaOAc 274, 310, 381; + NaOAc/H_3BO_3 265, 345. LC-MS: *m/z* 595 [M + H]$^+$ (molecular ion peak, kaempferol + each 1 mol of glucose and rhamnose), *m/z* 287 [M − 308 + H]$^+$ (fragment ion peak, kaempferol). ^{1}H NMR (500 MHz, pyridine-d_5): δ_H 13.23 (1H, *s*, 5-OH), 8.00 (2H, *d*, *J* = 8.9 Hz, H-2′,6′), 7.29 (2H, *d*, *J* = 8.5 Hz, H-3′,5′), 6.75 (1H, *d*, *J* = 2.1 Hz, H-8), 6.74 (1H, *d*, *J* = 1.8 Hz, H-6), 6.34 (1H, *brs*, rhamnosyl H-1), 5.28 (1H, *d*, *J* = 7.6 Hz, glucosyl H-1), 5.07 (1H, *d*, *J* = 2.4 Hz, rhamnosyl H-2), 4.61 (1H, *dd*, *J* = 3.4 and 8.9 Hz, rhamnosyl H-3), 4.48 (1H, *dd*, *J* = 2.2 and 12.1 Hz, glucosyl H-6a), 4.42 (1H, *dd*, *J* = 4.6 and 11.9 Hz, glucosyl H-6b), 4.29 (1H, *m*, glucosyl H-3), 4.28 (1H, *m*, glucosyl H-4), 4.25 (1H, *m*, rhamnosyl H-5), 4.22 (1H, *m*, rhamnosyl H-4), 4.09 (1H, *t*, *J* = 8.2 Hz, glucosyl H-2), 3.95 (1H, *m*, glucosyl H-5), 1.42 (3H, *d*, *J* = 5.8 Hz, rhamnosyl CH_3). ^{13}C NMR (125 MHz, pyridine-d_5): (kaempferol) δ_C 157.9 (C-2), 135.5 (C-3), 178.8 (C-4), 162.9 (C-5), 99.9 (C-6), 165.9 (C-7), 94.6 (C-8), 157.7 (C-9), 105.4 (C-10), 121.6 (C-1′), 131.5 (C-2′,6′), 116.4 (C-3′,5′), 161.8 (C-4′); (3-*O*-rhamnose) δ_C 102.5 (C-1), 82.6 (C-2), 72.3 (C-3), 73.7 (C-4), 71.7 (C-5), 18.2 (C-6); (2″-*O*-glucose): δ_C 107.7 (C-1), 75.8 (C-2), 78.4 (C-3), 71.1 (C-4), 78.6 (C-5), 62.3 (C-6).

Kaempferol 3-*O*-glucosylrhamnoside (**15**). TLC (Rf): 0.84 (BAW), 0.85 (BEW), 0.43 (15%HOAc); color UV (365 nm) dark purple, UV/NH_3 dark greenish yellow. HPLC (Rt) (min.): 11.65. UV: λmax (nm) MeOH 266, 347; + NaOMe 275, 324, 400 (inc.); + $AlCl_3$ 274, 304, 355, 390sh; + $AlCl_3$/HCl 274, 303, 355, 390sh; + NaOAc 275, 308, 385; + NaOAc/H_3BO_3 267, 354. LC-MS: *m/z* 595 [M + H]$^+$ (molecular ion peak, kaempferol + each 1 mol of glucose and rhamnose), *m/z* 287 [M − 308 + H]$^+$ (fragment ion peak, kaempferol).

Results and Discussion

Identification of the flavonols from Gladiolus × grandiflora *flowers*

Flavonol **14** was obtained from *Gladiolus × grandiflora* cultivar “Christmas” flowers as pale yellow powder. UV spectral properties of **14** showed that this compound is a flavonol having free 5-, 7- and 4′-hydroxyl and a substituted 3-hydroxyl groups (Mabry *et al.*, 1970). Kaempferol, glucose and rhamnose were liberated by acid hydrolysis of **14**. The attachment of each 1 mol of glucose and rhamnose to 3-position of kaempferol was shown by the appearance of molecular ion peak, *m/z* 595 [M + H]$^+$ on LC-MS. However, since fragment ion peak, *m/z* 449 [M − 146 + H]$^+$, did not appear, it was presumed that **14** is kaempferol 3-*O*-glucosylrhamnoside but not rhamnosylglucoside. In the NMR spectra, the proton and carbon signals were assigned by ^{1}H-^{1}H COSY, NOESY, HMQC and HMBC. In the ^{1}H NMR spectrum, each one of glucosyl and rhamnosyl anomeric proton signal at δ_H 5.28 and 6.34 appeared, together with six aromatic proton signals (H-6, H-8, H-2′,6′ and

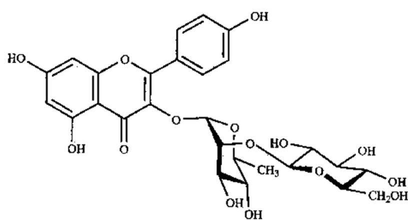

Fig. 1. Kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside.

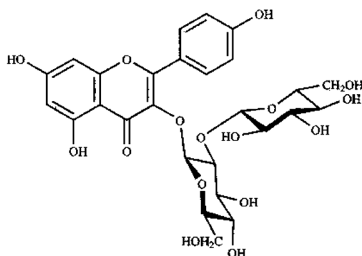

Fig. 2. Kaempferol 3-*O*-sophoroside.

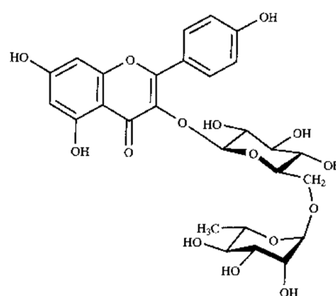

Fig. 3. Kaempferol 3-*O*-rutinoside.

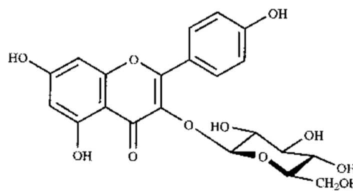

Fig. 4. Kaempferol 3-*O*-glucoside.

H-3′,5′) derived from kaempferol. Of their anomeric protons, rhamnosyl H-1 (δ_H 6.34) correlated with the C-3 of kaempferol (δ_C 135.5) by HMBC, showing that rhamnose was attached to 3-position of kaempferol. On the other hand, the interglycosidic linkage of **14** was shown as 3-*O*-glucosyl-(1→2)-rhamnose from HMBC correlation between glucosyl anomeric proton at δ_H 5.28 and the C-2 signal of 3-*O*-rhamnose at δ_C 82.6. It was shown by the coupling constant (J = 7.6 Hz) of glucosyl anomeric proton and appearance as *brs* of rhamnosyl anomeric proton that glucose and rhamnose are in the β-pyranose and α-pyranose, respectively. Thus, **14** was identified as kaempferol 3-*O*-β-glucopyranosyl-(1→2)-α-rhamnopyranoside (Fig. 1). Kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside has previously been reported from *Ginkgo biloba* L. leaves (Ginkgoaceae) (Hasler and Sticher, 1992).

Flavonoids **4**, **11** and **13** were characterized as kaempferol 3-*O*-glycosides by UV spectral survey according to Mabry *et al.* (1970). Glucose, and glucose and rhamnose were liberated by acid hydrolysis of **4** and **13**, and **11**, respectively, as glycosidic sugars. The attachment of 2 mol glucose, and each 1 mol of glucose and rhamnose to kaempferol was determined by LC-MS, i.e. appearance of molecular ion peaks, *m/z* 611 and *m/z* 595 from **4** and **11**. Finally, **4**, **11** and **13** were identified as kaempferol 3-*O*-sophoroside (Fig. 2), kaempferol 3-*O*-rutinoside (Fig. 3) and kaempferol 3-*O*-glucoside (Fig. 4) by TLC and HPLC comparisons with authentic samples from *Dianthus caryophyllus* L. flowers (Iwashina *et al.*, 2010), *Osyris alba* L. fruits (Iwashina *et al.*, 2008) and *Cyrtomium* spp. fronds (Iwashina *et al.*, 2006), respectively. The identification of **4** and **11** was followed by NMR spectral survey.

Acid hydrolysis of **2**, **3** and **8** liberated glucose and/or rhamnose as glycosidic sugars, together with myricetin as aglycone. Their UV spectral properties were those of myricetin 3-*O*-glycosides (Markham, 1982). In LC-MS survey of **2**, molecular ion peak, *m/z* 627 $[M+H]^+$ appeared, together with fragment ion peaks, *m/z* 481 $[M-146+H]^+$ and 319 $[M-308+H]^+$, showing the compound is myricetin 3-*O*-rhamnosylglucoside. On the other hand, since molecular ion peaks, *m/z* 481 and 465 $[M+H]^+$ appeared from **3** and **5**, respectively, the glycosides are myricetin 3-*O*-monoglucoside and 3-*O*-monorhamnoside. Finally, **2**, **3** and **8** were identified as

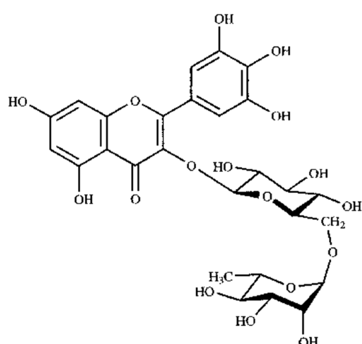

Fig. 5. Myricetin 3-*O*-rutinoside.

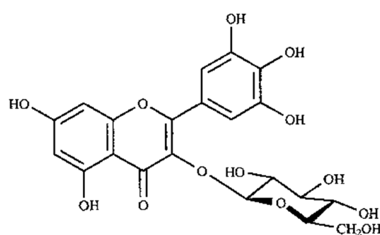

Fig. 6. Myricetin 3-*O*-glucoside.

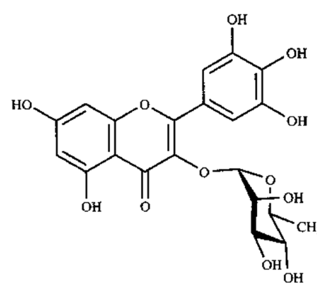

Fig. 7. Myricetin 3-*O*-rhamnoside.

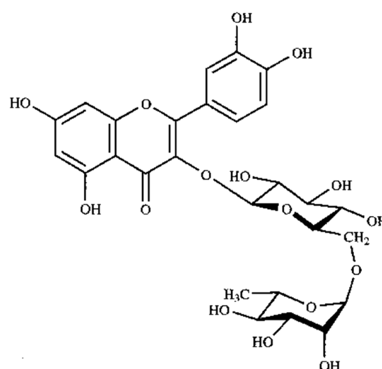

Fig. 8. Quercetin 3-*O*-rutinoside.

myricetin 3-*O*-rutinoside (Fig. 5), myricetin 3-*O*-glucoside (Fig. 6) and myricetin 3-*O*-rhamnoside (Fig. 7) by TLC and HPLC comparisons with authentic samples from *Cyrtomium microindusium* Sa.Kurata fronds (Iwashina *et al.*, 2006), *Corylopsis* spp. leaves (Iwashina *et al.*, 2012) and *Myrica rubra* Sieb. et Zucc. bark (Hattori and Hayashi, 1931), respectively.

Flavonoid **5** was shown to be flavonol having free 5-, 7-, 3′- and 4′-hydroxyl, and a substituted 3-hydroxyl groups by UV spectral survey according to Mabry *et al.* (1970). Practically, quercetin was produced by acid hydrolysis, together with the sugars, glucose and rhamnose. Thus, original glycoside was identified as quercetin 3-*O*-rutinoside (Fig. 8) by TLC and HPLC comparison with authentic rutin from the aerial parts of *Osyris alba* (Iwashina *et al.*, 2008).

UV absorption maxima of the aglycone which was obtained by acid hydrolysis of **1**, **6** and **9** have λmax 253 and 374 nm, showing that they are myricetin type flavonols. Since the absorption peaks in the NaOMe spectrum degenerated, it was shown that free 3- and 4′-hydroxyl groups are present in flavonol. The presence of an *ortho*-dihydroxyl group in the B-ring and free hydroxyl group in 5-position was detected by a comparison of the spectrum of the flavonoid in the presence of $AlCl_3$ with that obtained in $AlCl_3$/HCl, i.e. the bathochromic shift in Band I, and then the hypsochromic shift of 26 nm of the $AlCl_3$ spectrum on the addition of HCl. Moreover, the presence of free 7-hydroxyl group was shown by the bathochromic shift of 16 nm of Band II. Thus, the presence of free 3-, 5-, 7-, 3′- and 4′-hydroxyl groups was proved. However, since molecular ion peak, *m/z* 333 $[M+H]^+$ appeared, the compound is pentahydroxy-monomethoxyflavone. The position of an additional methoxyl group was determined to be 5′-position, because this aglycone is myricetin type. Thus, the aglycone from **1**, **6** and **9** was identified as 3,5,7,3′,4′-pentahydroxy-5′-methoxyflavone, i.e., laricitrin (Fig. 9). The position of glycosidic sugars was shown to be 3-position by UV spectral properties of the original glycosides, **1**, **6** and **9**.

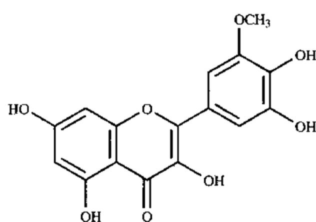

Fig. 9. Laricitrin.

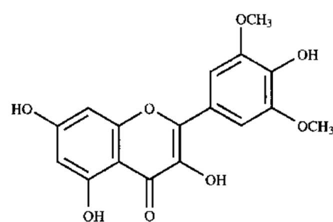

Fig. 10. Syringetin.

Glucose, and rhamnose and glucose were liberated by acid hydrolysis of **1** and **6**. Moreover, since molecular ion peaks, *m/z* 657 $[M+H]^+$ and 641 $[M+H]^+$, appeared on LC-MS of **1** and **6**, it was shown that 2 mol glucose, and each 1 mol of glucose and rhamnose attached to laricitrin, respectively. Thus, **1** and **6** were characterized as laricitrin 3-*O*-diglucoside and laricitrin 3-*O*-rhamnosylglucoside, respectively. Although the attachment of each 1 mol of rhamnose and hexose was shown by LC-MS of **9**, hexose could not characterize for small amount of the original glycoside.

LC-MS survey of the aglycone of **12** proved that the compound is tetrahydroxy-dimethoxyflavone. The presence of free 3-, 5-, 7- and 4′-hydroxyl groups was shown by UV spectral survey in addition to NaOMe, $AlCl_3$, $AlCl_3$/HCl, NaOAc or NaOAc/H_3BO_3 (Mabry *et al.*, 1970). Since UV spectral property in MeOH was that of myricetin type flavonol, it was clear that two additional methoxyl groups are attached to 3′- and 5′-positions. Thus, aglycone of **12** was characterized as 3,5,7,4′-tetrahydroxy-3′,5′-dimethoxyflavone, i.e. syringetin (Fig. 10). The attachment of each 1 mol of rhamnose and hexose to 3-position of aglycone was determined by UV spectra (Mabry *et al.*, 1970) and LC-MS survey. However, identification of glycosidic sugars could not be performed for small amount of the original glycoside.

Flavonoids **7**, **10** and **15** were kaempferol 3-*O*-glycosides which were shown by acid hydrolysis and UV spectral survey. In LC-MS, they were characterized as kaempferol 3-*O*-pentosylhexoside (**7**), 3-*O*-rhamnosylrhamnosylhexoside (**10**) and 3-*O*-rhamnosylhexoside (**15**). Glucose (**7**), and rhamnose and glucose (**10** and **15**) were detected by PC survey of the glycosidic sugars, respectively. However, other sugars were could not be found for small amount of the original glycosides. Since fragment ion peak, *m/z* 449 $[M-146+H]^+$ did not appear on LC-MS of **15**, the glycoside may be kaempferol 3-*O*-glucosylrhamnoside, i.e. (1→3) or (1→4) but not rhamnosylglucoside. Other **7** and **10** were characterized as kaempferol 3-*O*-pentosylglucoside and kaempferol 3-*O*-rhamnosylrhamnosylglucoside.

Of 15 flavonols which were obtained in this survey, **4**, **5** and **11** have already been reported from the flowers of *Gladiolus* × *grandiflora* cultivar “Ariake” (Takemura *et al.*, 2005). However, other 12 flavonols were found in *Gladiolus* species for the first time.

Distribution of flavonol glycosides among the perianths of Gladiolus *cultivars*

The perianths of eighty-nine *Gladiolus* × *grandiflora* cultivars including 12 purple, 13 reddish purple, 13 pink, 19 red, 2 orange, 16 white, 11 yellow and 3 green cultivars were surveyed for flavonoid compounds by HPLC (Table 1). Of their flavonoids, distribution of anthocyanins has already been reported (Takemura *et al.*, 2008). Major flavonol of many *Gladiulus* cultivars (60 cultivars) was kaempferol 3-*O*-rutinoside (**11**) in all flower colors. Kaempferol 3-*O*-sophoroside (**4**) and kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside (**14**) were also found in many *Gladiolus* cultivars as major flavonols. Relatively many kinds of flavonols were found in purple and reddish purple cultivars. Of their compounds, laricitrin 3-*O*-rhamnosylglucoside (**6**) was especially occurred in purple cultivars, together with minor

quercetin 3-*O*-rutinoside (**5**). On the other hand, a few kinds of the flavonols, e.g., **4**, **11** and **14**, occurred in pink, red, orange, white and green cultivars as major compounds. Kaempferol 3-*O*-pentosylglucoside (**7**), myricetin 3-*O*-rhamnoside (**8**), laricitrin 3-*O*-rhamnosylhexoside (**9**), kaempferol 3-*O*-rhamnosylrhamnosylglucoside (**10**) and kaempferol 3-*O*-glucosylrhamnoside (**15**) were the scattered occurrence during all flower colors. In this survey, almost major flavonols were kaempferol glycosides, especially **11**, **4** and **14**. Although it was suggested that kaempferol glycosides contribute to creamy tone of white flowers in carnation (Iwashina *et al.*, 2010), in general they do not act as yellow pigments, even if they were abundantly accumulated (Hashimoto *et al.*, 2008).

In also yellow (and orange) cultivars, kaempferol glycosides were major flavonols in all cultivars (Table 1). The presence of carotenoids was presumed in yellow and orange perianths, because the diagnostic spectral curves of carotenoids appear in intact UV-visible spectral survey of the yellow and orange perianths of *Gladiolus* cultivars (data are not shown). However, in purple *Gladiolus* cultivars, flavonols may be represent the more purplish color as copigments. Copigment substances do not contribute to flower colors by themselves, but they cause a bathochromic shift of the visible λmax of natural anthocyanins, which makes them appear bluer and increase absorptivity.

Thus, various copigment substances, e.g. isovitexin in *Iris ensata* Thunb. (Yabuya *et al.*, 1997), quercetin 4′-*O*-glucoside, quercetin 3-*O*-rhamnoside and quercetin 3-*O*-glucoside in *Fuchsia hybrida* Voss (Yazaki, 1976), swertiajaponin in *Iris* ×*hollandica* cultivar “Tiger’s Eye” (Mizuno *et al.*, 2015), swertisin 2″-*O*-(4‴-acetylrhamnoside) in *I.* ×*hollandica* cultivar “Blue Diamond” (Mizuno *et al.*, 2013), have been reported from the purple to blue flowers. In *Gladiolus* cultivars, we presume that purple colors are due to copigmentation, and now survey in progress.

Acknowledgments

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