

Identification of Flavonoids in Leaves of a Labile Bicolor Flowering Dahlia (*Dahlia variabilis*) ‘Yuino’

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Bicolor flowering dahlias are a group of cultivars that produce inflorescences with bicolored petals characterized by a colored basal part and a white tip. However, they frequently produce single-colored petals, even if they are vegetatively propagated. In a previous study, strong relationships between inflorescence color and leaf phenotype were observed in a red–white bicolor flowering dahlia ‘Yuino’; red petal-producing individuals accumulate flavonoids in leaves, whereas only bicolor petal-producing individuals tend not to accumulate them in leaves. Flavonoids in leaves are assumed to be chalcones. In this study, we investigated flavonoids in the leaves of ‘Yuino’ by nuclear magnetic resonance analysis and identified six caffeoyl esters, four flavonol derivatives, and three novel butein derivatives in the flavonoid-rich leaves of ‘Yuino’. The three novel compounds were butein 4',4-*O*-di-[2-*O*-(β-glucopyranosyl)-β-glucopyranoside], butein 4'-*O*-[2-*O*-(β-glucopyranosyl)-β-glucopyranoside]-4-*O*-β-glucopyranoside, and butein 4'-[6-*O*-(3-hydroxy-3-methylglutaryl)-β-glucopyranoside]-4-*O*-β-glucopyranoside. On the other hand, only caffeoyl esters were detected in flavonoid-poor leaves. These data demonstrated that flavonoid-rich leaves accumulated the flavonoids of butein and flavonol derivatives. The common enzyme for the biosynthesis of butein and flavonol derivatives is chalcone synthase; thus, the importance of chalcone synthase for phenotypic lability in ‘Yuino’ was confirmed.

Key Words: butein, chalcone, flavonol.

Introduction

Dahlia (*Dahlia variabilis*) is popular cut flower or garden plant because of the wide variation in its flower traits, such as shapes, sizes, and colors. In particular, the flower colors of cultivars of this species are diversified, and several cultivars have red, purple, pink, yellow, ivory white, black, variegated, and bicolor petals. Bicolor dahlia cultivars are a group of cultivars that produce inflorescences with petals exhibiting a colored base and a white tip. The prominent common characteristic of bicolor dahlia cultivars is the lability or instability of petal colors; in addition to original bicolor petals, they frequently produce single-colored petals without a white tip regardless of whether they are vegetatively propagated. This lability of petal color prevents uniform

production of cut or pot flowers of bicolor dahlias.

In our previous study (Ohno et al., 2016), strong relationships between inflorescence colors and leaf flavonoid accumulation were evident, namely, red petal-producing individuals accumulated flavonoids in leaves, whereas only bicolor petal-producing individuals tended not to accumulate them in leaves. This suggested that the phenotypic lability of bicolor dahlias was not only limited in petal colors, but also in leaves. At that time, the flavonoids were presumed to be chalcone derivatives based on the absorption spectra of each peak. However, the flavonoids accumulating in flavonoid-rich leaves can provide information to identify the gene or pathway that is important for the lability of petal color. Thus, in the present study, to confirm that leaves accumulated flavonoids and to reveal that types of compounds that are accumulated in leaves, we identified flavonoids and caffeoyl esters accumulated in leaves by nuclear magnetic resonance (NMR) analysis.

Materials and Methods

Plant materials

The red–white bicolor flowering dahlia cultivar

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‘Yuino’ was used for the present experiment. The cuttings of ‘Yuino’ were grown in the experimental field or greenhouse at Kyoto University (Kyoto, Japan). For NMR analysis, the leaves of the ‘Yuino’ R-line, which tend to produce red petals and to accumulate flavonoids in leaves with high frequency, retaining this characteristic through vegetative propagation (Ohno et al., 2016), were used.

Isolation and purification of A–L

The freeze-dried leaves of ‘Yuino’ R-line (Ohno et al., 2016) (80 g) were immersed in methanol–water (1:1, v/v) (5 L) at room temperature for 1 day and extracted. A–L shown in Figure 1A were isolated and purified by paper chromatography (PC) and a Sephadex™ LH-20 (GE Healthcare Japan, Tokyo, Japan) column (35 × 300 mm) chromatography (CC). The amounts of purified A–L obtained from the leaves were as follows: A (7 mg), mixture of B-1 and B-2 (9 mg), C (0.5 mg), D (0.5 mg), E (9 mg), F (6 mg), G (12 mg), H (0.5 mg), I (18 mg), J (2 mg), K (3 mg), and L (7 mg).

Thin-layer chromatography (TLC) analysis

TLC was performed on plastic-coated cellulose sheets (Merck, Darmstadt, Germany) using seven mobile phases: 1) BAW (n-BuOH-HOAc-H₂O, 4:1:2, v/v/v), 2) BuHCl (n-BuOH-2N HCl, 1:1, v/v, upper

layer), 3) AHW (HOAc-HCl-H₂O, 15:3:82, v/v/v), 4) 1% HCl for polyphenols with ultraviolet light and BAW, 5) EAA (EtOAc-HOAc-H₂O, 3:1:1, v/v/v), 6) ETN (EtOH-NH₄OH-H₂O, 16:1:3, v/v/v), and 7) EFW (EtOAc-HCOOH-H₂O, 5:2:1, v/v/v) for sugars with aniline hydrogen phthalate spray reagent (Harborne, 1984; Tatsuzawa and Hosokawa, 2016).

High-performance liquid chromatography (HPLC) analysis

Analytical HPLC was performed on an LC 10A system (Shimadzu, Kyoto, Japan) using a Waters C18 (4.6 × 250 mm, Nihon Waters K. K., Tokyo, Japan) column at 40°C with a flow rate of 1 mL·min⁻¹ and monitoring at 350 nm for polyphenols. The eluant was applied as a linear gradient elution for 40 min from 20% to 85% solvent B (1.5% H₃PO₄, 20% HOAc, 25% MeCN in H₂O) in solvent A (1.5% H₃PO₄ in H₂O) with 5 min of re-equilibration at 20% solvent B (method 1). The other eluant for 3-hydroxy-3-methylglutaric acid was applied as an isocratic elution of solvent A for 10 min and monitoring at 210 nm (Tatsuzawa and Hosokawa, 2016) (method 2).

Spectroscopic analyses

Ultraviolet–visible spectra of flavonoids were recorded in MeOH, MeOH + NaOMe, MeOH + AlCl₃, MeOH + AlCl₃ + HCl, MeOH + NaOAc, and MeOH + NaOAc + H₃BO₃ (from 200 to 500 nm) using a UV-Vis Multi-Purpose Spectrophotometer (MPS-2450; Shimadzu). High-resolution fast-atom bombardment mass (FAB-MS) spectra were determined on a JEOL JMS-700 Mass spectrometer (JEOL, Ltd., Tokyo, Japan) operating in the positive ion mode using a 1:1 mixture of dithiothreitol and 3-nitrobenzyl alcohol as a matrix. ¹H (400 MHz) and ¹³C (100 MHz) NMR spectra were measured on a JEOL AL-400 NMR spectrometer (JEOL, Ltd.) using CD₃OD-*d*₄ and/or dimethyl sulfoxide (DMSO)-*d*₆ as a solvent. Chemical shifts are reported on the δ -scale from tetramethylsilane as the internal standard, and coupling constants (*J*) are in Hz.

Results

Over 30 peaks at 350 nm were obtained in the methanol–water (1:1, v/v) extracts from the freeze-dried flavonoid-rich leaves of ‘Yuino’ by HPLC analysis (Fig. 1A). However, most peaks after 15 min of retention time were not detected from freeze-dried flavonoid-poor leaves (Fig. 1B). We eventually identified 13 of these peaks from freeze-dried flavonoid-rich leaves, and the percentages of the identified peaks calculated as the total contents were 8.3% (A), 15.7% (B-1), 7.8% (B-2), 0.6% (C), 1.2% (D), 6.8% (E), 1.8% (F), 8.4% (G), 1.6% (H), 8.0% (I), 1.3% (J), 9.8% (K), and 3.1% (L) (Fig. 1A). Compounds were extracted from 80-g freeze-dried flavonoid-rich leaves of ‘Yuino’ in 5-L methanol–water, followed by isolation using PC

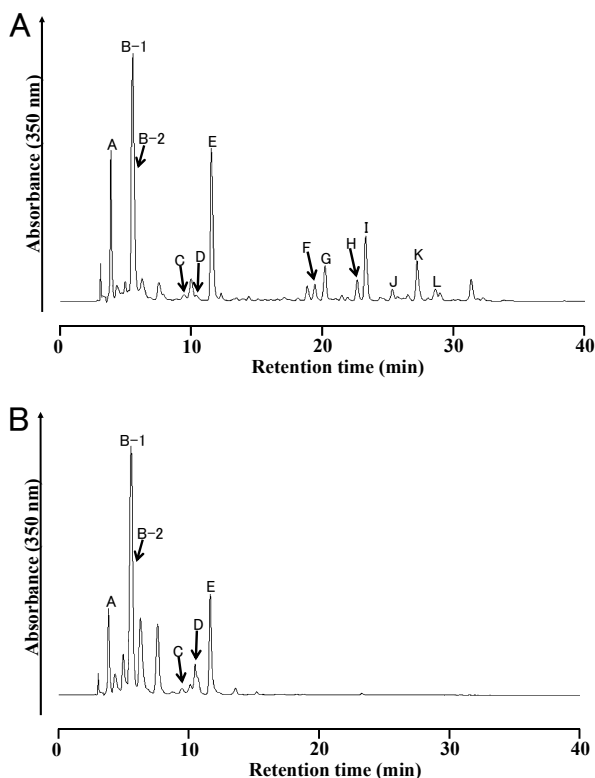


Fig. 1. High-performance liquid chromatography profile of leaf extracts in (A) flavonoid-rich leaves and (B) flavonoid-poor leaves. Each compound was identified using nuclear magnetic resonance analyses.

and Sephadex™ LH-20 CC, and then analyzed by NMR. The chromatographic and spectroscopic properties of **A–L** are summarized below.

Known compounds

3-*O*-[(*trans*)-Caffeoyl]-glucaric acid (*A*)

Pale Brown powder: TLC: UV; blue, UV+NH₃, bright blue, (*R_f*-values) BAW 0.53, BuHCl 0.48, 1% HCl 0.60, AHW 0.76; HPLC; *t_R*(min) 3.8 (method 1), PDA [$\lambda_{\max}$ (nm)] 328, 301sh, FAB-MS *m/z* 373 [M+H]⁺ (calc. for C₁₅H₁₇O₁₁), HR-FAB-MS (calc. 373.0771, found: 373.0782); ¹H-NMR(CD₃OD-*d*₄, int TMS, 400 MHz): **Caffeic acid**, δ 7.04(*d*, *J*=1.7 Hz, H-2), 6.77(*d*, *J*=8.2 Hz, H-5), 6.94(*dd*, *J*=1.7, 8.2 Hz, H-6), 6.28(*d*, *J*=15.9 Hz, H-7(α)), 7.60(*d*, *J*=15.9 Hz, H-8(β)), **Glucaric acid**, 4.53(*d*, *J*=1.7 Hz, H-2'(5')), 5.64(*dd*, *J*=1.7, 8.5 Hz, H-3'(4')), 4.33(*dd*, *J*=1.4, 8.5 Hz, H-4'(3')), 4.41(*brs*, H-5'(2')); ¹³C-NMR(CD₃OD-*d*₄, int TMS, 100 MHz): **Caffeic acid**, δ 128.2(C-1), 116.4(C-2), 147.2(C-3), 149.9(C-4), 117.2(C-5), 123.9(C-6), 114.9(C-7(α)), 148.4(C-8(β)), 168.9(C-9), **Glucaric acid**, 175.3(C-1'(6')), 71.8(C-2'(5')), 77.2(C-3'(4')), 74.0(C-4'(3')), 73.1(C-5'(2')), 176.0(C-6'(1')).

2-*O*-[(*trans*)-Caffeoyl]-glucaric acid (*B-1*)

Pale brown powder: TLC: UV; blue, UV+NH₃, bright blue, (*R_f*-values) BAW 0.52, BuHCl 0.53, 1% HCl 0.57, AHW 0.77; HPLC; *t_R*(min) 5.5 (method 1), PDA [$\lambda_{\max}$ (nm)] 328, 301sh, FAB-MS *m/z* 373 [M+H]⁺ (calc. for C₁₅H₁₇O₁₁), HR-FAB-MS (calc. for C₁₅H₁₇O₁₁: 373.0771, found: 373.0784); ¹H-NMR(CD₃OD-*d*₄, int TMS, 400 MHz): **Caffeic acid**, δ 7.04(*d*, *J*=2.0 Hz, H-2), 6.77(*d*, *J*=8.3 Hz, H-5), 6.94(*dd*, *J*=2.0, 8.3 Hz, H-6), 6.31(*d*, *J*=15.9 Hz, H-7(α)), 7.58(*d*, *J*=15.9 Hz, H-8(β)), **Glucaric acid**, 5.37(*d*, *J*=2.2 Hz, H-2'(5')), 4.56(*dd*, *J*=2.2, 7.7 Hz, H-3'(4')), 4.06(*brd*, *J*=7.7 Hz, H-4'(3')), 4.10(*brs*, H-5'(2')); ¹³C-NMR(CD₃OD-*d*₄, int TMS, 100 MHz): **Caffeic acid**, δ 128.0(C-1), 115.1(C-2), 146.8(C-3), 149.8(C-4), 116.5(C-5), 122.9(C-6), 115.8(C-7(α)), 146.9(C-8(β)), 169.1(C-9), **Glucaric acid**, 171.8(C-1'(6')), 74.0(C-2'(5')), 72.9(C-3'(4')), 74.0(C-4'(3')), 72.2(C-5'(2')), 175.9(C-6'(1')).

5-*O*-[(*trans*)-Caffeoyl]-quinic acid (*B-2*) (*neochlorogenic acid*)

Pale brown powder: TLC: UV; blue, UV+NH₃, bright blue, (*R_f*-values) BAW 0.58, BuHCl 0.62, 1% HCl 0.57, AHW 0.77; HPLC; *t_R*(min) 5.6 (method 1), PDA [$\lambda_{\max}$ (nm)] 326, 300sh, FAB-MS *m/z* 355 [M+H]⁺ (calc. for C₁₆H₁₉O₉), HR-FAB-MS (calc. for C₁₆H₁₉O₉: 355.1029, found: 355.1015); ¹H-NMR(CD₃OD-*d*₄, int TMS, 400 MHz): **Caffeic acid**, δ 7.09(*d*, *J*=2.0 Hz, H-2), 6.79(*d*, *J*=8.3 Hz, H-5), 6.98(*dd*, *J*=2.0, 8.3 Hz, H-6), 6.38(*d*, *J*=15.9 Hz, H-7(α)), 7.70(*d*, *J*=15.9 Hz, H-8(β)), **Quinic acid**, 2.13(*brd*, *J*=13.1 Hz, H-2'a), 1.96(*dd*, *J*=9.5, 13.1 Hz, H-2'b), 4.14(*ddd*, *J*=4.1, 8.9, 8.9 Hz, H-3'), 3.66(*dd*,

J=3.2, 8.3 Hz, H-4'), 5.36(*m*, H-5'), 2.20(*m*, H-6'a), 2.17(*m*, H-6'b); ¹³C-NMR(CD₃OD-*d*₄, int TMS, 100 MHz): **Caffeic acid**, δ 127.8(C-1), 115.2(C-2), 146.9(C-3), 149.5(C-4), 116.5(C-5), 123.3(C-6), 114.3(C-7(α)), 148.2(C-8(β)), 168.5(C-9), **Quinic acid**, 74.0(C-1'), 41.4(C-2'), 68.5(C-3'), 74.7(C-4'), 73.0(C-5'), 36.8(C-6'), 171.8(C-7').

4-*O*-[(*trans*)-Caffeoyl]-quinic acid (*C*) (*cryptochlorogenic acid*)

HPLC; *t_R*(min) 9.5 (method 1), PDA [$\lambda_{\max}$ (nm)] 325, 300sh. [This compound was identified by direct comparison with a commercial standard (SIGMA-ALDRICH Co., St. Louis, MO, USA)].

3-*O*-[(*trans*)-Caffeoyl]-quinic acid (*D*) (*chlorogenic acid*)

HPLC; *t_R*(min) 10.5 (method 1), PDA [$\lambda_{\max}$ (nm)] 325, 300sh. [This compound was identified by direct comparison with a commercial standard (Wako Pure Chemical Industries, Ltd, Osaka, Japan)].

2-*O*-[(*trans*)-Caffeoyl]-glyceric acid (*E*)

Brown powder: TLC: UV; blue, UV+NH₃, bright blue, (*R_f*-values) BAW 0.76, BuHCl 0.75, 1% HCl 0.38, AHW 0.66; HPLC; *t_R*(min) 11.7 (method 1), PDA [$\lambda_{\max}$ (nm)] 327, 301sh, HR-FAB-MS; [M+H]⁺ (calc. for C₁₂H₁₃O₇: 269.0661, found: 269.0653); FAB-MS *m/z* 269 [M+H]⁺ (calc. for C₁₂H₁₃O₇), HR-FAB-MS (calc. 269.0661, found: 269.0653); ¹H-NMR(CD₃OD-*d*₄, int TMS, 400 MHz): **Caffeic acid**, δ 7.07(*d*, *J*=2.0 Hz, H-2), 6.78(*d*, *J*=8.0 Hz, H-5), 6.97(*dd*, *J*=2.0, 8.0 Hz, H-6), 6.37(*d*, *J*=15.9 Hz, H-7(α)), 7.66(*d*, *J*=15.9 Hz, H-8(β)), **Glyceric acid**, 5.15(*t*, *J*=4.3 Hz, H-2'), 3.98(*d*, *J*=4.3 Hz, H-3'); ¹H-NMR(DMSO-*d*₆, int TMS, 400 MHz): **Caffeic acid**, δ 7.08(*d*, *J*=2.0 Hz, H-2), 6.79(*d*, *J*=8.0 Hz, H-5), 7.02(*dd*, *J*=2.0, 8.0 Hz, H-6), 6.32(*d*, *J*=15.8 Hz, H-7(α)), 7.56(*d*, *J*=15.8 Hz, H-8(β)), **Glyceric acid**, 4.79(*t*, *J*=4.4 Hz, H-2'), 3.80(*d*, *J*=4.4 Hz, H-3'); ¹³C-NMR(DMSO-*d*₆, int TMS, 100 MHz): **Caffeic acid**, δ 125.4(C-1), 114.7(C-2), 145.5(C-3), 148.5(C-4), 115.7(C-5), 121.4(C-6), 113.5(C-7(α)), 145.7(C-8(β)), 166.1(C-9), **Glyceric acid**, 169.8(C-1'), 74.1(C-2'), 60.9(C-3').

Quercetin 3-*O*-[(6-*O*-rhamnosyl)-galactoside] (*H*)

HPLC; *t_R*(min) 22.6 (method 1), PDA [$\lambda_{\max}$ (nm)] 354, 307sh, 266. [This compound was identified by direct comparison with an authentic standard (Tatsuzawa et al., 2015)].

Quercetin 3-*O*-[(6-*O*-rhamnosyl)-glucoside] (*I*) (*rutin*) (*I*)

Pale yellow powder: UV: $\lambda_{\max}$ (nm) in MeOH 356, 286sh, 266sh, 255; +NaOMe 373, 322, 270; +AlCl₃ 416, 273; +AlCl₃/HCl 398, 358, 298sh, 268; +NaOAc 356, 265sh, 257; +NaOAc/H₃BO₃ 375, 261. TLC: UV; dark brown, UV+NH₃, yellow, (*R_f*-values) BAW 0.58, BuHCl 0.52, 1% HCl 0.18, AHW 0.54; HPLC (method 1); *t_R*(min) 23.2, PDA [$\lambda_{\max}$ (nm)] 354, 300sh, 263, HR-FAB-MS; [M+H]⁺ calc. for C₂₇H₃₁O₁₆: 611.1612, found: 611.1605. FAB-MS *m/z* 611 [M+H]⁺ (calc. for C₂₇H₃₁O₁₆), HR-FAB-MS (calc. 611.1612, found:

611.1605); $^1\text{H-NMR}(\text{CD}_3\text{OD}-d_4$, int TMS, 400 MHz): **Quercetin**, δ 6.20(*d*, $J=2.2$ Hz, H-6), 6.39(*d*, $J=2.2$ Hz, H-8), 7.67(*d*, $J=2.2$, 8.0 Hz, H-2'), 6.87(*d*, $J=8.6$ Hz, H-5'), 7.63(*dd*, $J=2.2$, 8.6 Hz, H-6'), **Glucose**, 5.11(*d*, $J=7.6$ Hz, H-1), 3.45(*t*, $J=7.8$ Hz, H-2), 3.42(*t*, $J=8.3$ Hz, H-3), 3.27(*t*, $J=8.3$ Hz, H-4), 3.44(*m*, H-5), 3.37(*m*, H-6a), 3.80(*dd*, $J=1.0$, 11.0 Hz, H-6b), **Rhamnose**, 4.52(*d*, $J=1.5$ Hz, H-1), 3.64(*dd*, $J=1.7$, 3.4 Hz, H-2), 3.54(*dd*, $J=3.4$, 9.5 Hz, H-3), 3.28(*t*, $J=9.5$ Hz, H-4), 3.44(*m*, H-5), 1.12(*d*, $J=6.1$ Hz, $-\text{CH}_3$); $^1\text{H-NMR}(\text{DMSO}-d_6$, int TMS, 400 MHz): **Quercetin**, δ 6.20(*d*, $J=2.0$ Hz, H-6), 6.40(*d*, $J=2.0$ Hz, H-8), 7.54(*s*, 8.0 Hz, H-2'), 6.86(*d*, $J=8.7$ Hz, H-5'), 7.55(*brd*, $J=8.7$ Hz, H-6'), **Glucose**, 5.35(*d*, $J=7.8$ Hz, H-1), 3.25(*t*, $J=8.8$ Hz, H-2), 3.28(*t*, $J=9.0$ Hz, H-3), 3.10(*m*, H-4), 3.24(*m*, H-5), 3.30(*m*, H-6a), 3.71(*brd*, $J=10.5$ Hz, H-6b), **Rhamnose**, 4.40(*brs*, H-1), 3.41(*brs*, H-2), 3.25(*m*, H-3), 3.09(*t*, $J=9.3$ Hz, H-4), 3.26(*m*, H-5), 1.00(*d*, $J=6.1$ Hz, $-\text{CH}_3$); $^{13}\text{C-NMR}(\text{DMSO}-d_6$, int TMS, 100 MHz): **Quercetin**, δ 156.8(C-2), 133.5(C-3), 177.5(C-4), 161.4(C-5), 99.0(C-6), 164.5(C-7), 93.9(C-8), 156.7(C-9), 104.1(C-10), 121.3(C-1'), 116.4(C-2'), 148.6(C-3'), 144.9(C-4'), 115.4(C-5'), 121.8(C-6'), **Glucose**, 101.4(C-1), 76.6(C-2), 76.0(C-3), 70.2(C-4), 74.3(C-5), 67.2(C-6), **Rhamnose**, 100.9(C-1), 70.5(C-2), 70.7(C-3), 72.0(C-4), 68.4(C-5), 17.9($-\text{CH}_3$).

Kaempferol 3-O-[(6-O-rhamnosyl)-galactoside] (J)

Pale yellow powder: UV: $\lambda_{\text{max}}(\text{nm})$ in MeOH 342, 289, 266; +NaOMe 363, 305, 273; +AlCl₃ 388, 353, 305, 273; +AlCl₃/HCl 392, 344, 302, 274; +NaOAc 345, 319, 299, 268; +NaOAc/H₃BO₃ 349, 294, 266. TLC: UV; brown, UV+NH₃, yellow, (R_f -values) BAW 0.68, BuHCl 0.60, 1% HCl 0.30, AHW 0.66; HPLC (method 1); $t_R(\text{min})$ 25.2, PDA [$\lambda_{\text{max}}(\text{nm})$] 347, 297sh, 265, FAB-MS m/z 595 [$\text{M}+\text{H}]^+$ (calc. for C₂₇H₃₁O₁₅), HR-FAB-MS (calc. 595.1663, found: 595.1656); $^1\text{H-NMR}(\text{CD}_3\text{OD}-d_4$, int TMS, 400 MHz): **Kaempferol**, δ 6.21(*d*, $J=2.0$ Hz, H-6), 6.40(*d*, $J=2.0$ Hz, H-8), 8.10(*d*, $J=9.0$ Hz, H-2', 6'), 6.88(*d*, $J=9.0$ Hz, H-3', 5'), **Galactose**, 5.04(*d*, $J=7.8$ Hz, H-1), 3.79(*dd*, $J=7.8$, 9.6 Hz, H-2), 3.49(*dd*, $J=3.4$, 9.6 Hz, H-3), 3.76(*d*, $J=3.4$ Hz, H-4), 3.53(*m*, H-5), 3.62(*m*, H-6a), 3.72(*dd*, $J=5.6$, 10.3 Hz, H-6b), **Rhamnose**, 4.51(*brs*, H-1), 3.58(*dd*, $J=2.0$, 3.4 Hz, H-2), 3.53(*m*, H-3), 3.28(*t*, $J=9.5$ Hz, H-4), 3.38(*dd*, $J=6.6$, 9.6 Hz, H-5), 1.18(*d*, $J=6.1$ Hz, $-\text{CH}_3$); $^{13}\text{C-NMR}(\text{CD}_3\text{OD}-d_4$, int TMS, 100 MHz): **Kaempferol**, δ 161.7(C-2), 135.7(C-3), 179.6(C-4), 163.1(C-5), 100.2(C-6), 166.6(C-7), 95.0(C-8), 158.6(C-9), 105.2(C-10), 122.7(C-1'), 132.5(C-2', 6'), 116.2(C-3', 5'), 159.3(C-4'), **Galactose**, 105.6(C-1), 73.0(C-2), 75.1(C-3), 70.2(C-4), 75.4(C-5), 67.4(C-6), **Rhamnose**, 102.0(C-1), 72.1(C-2), 72.3(C-3), 73.9(C-4), 69.8(C-5), 18.0($-\text{CH}_3$).

Kaempferol 3-O-[(6-O-rhamnosyl)-glucoside] (K)

Pale yellow powder: UV: $\lambda_{\text{max}}(\text{nm})$ in MeOH 345, 292, 265; +NaOMe 368, 300, 273; +AlCl₃ 392, 351,

305, 273; +AlCl₃/HCl 389, 345, 301, 273; +NaOAc 348, 266; +NaOAc/H₃BO₃ 348, 266. TLC: UV; brown, UV+NH₃, yellow, (R_f -values) BAW 0.66, BuHCl 0.62, 1% HCl 0.24, AHW 0.63; HPLC (method 1); $t_R(\text{min})$ 27.1, PDA [$\lambda_{\text{max}}(\text{nm})$] 347, 297sh, 265, HR-FAB-MS (calc. 595.1663, found: 595.1665); $^1\text{H-NMR}(\text{CD}_3\text{OD}-d_4$, int TMS, 400 MHz): **Kaempferol**, δ 6.21(*d*, $J=2.0$ Hz, H-6), 6.40(*d*, $J=2.0$ Hz, H-8), 8.06(*d*, $J=9.0$ Hz, H-2', 6'), 6.89(*d*, $J=9.0$ Hz, H-3', 5'), **Glucose**, 5.12(*d*, $J=7.3$ Hz, H-1), 3.42(*t*, $J=8.0$ Hz, H-2), 3.40(*t*, $J=8.0$ Hz, H-3), 3.23(*m*, H-4), 3.44(*m*, H-5), 3.36(*m*, H-6a), 3.80(*brd*, $J=10.5$ Hz, H-6b), **Rhamnose**, 4.51(*brs*, H-1), 3.63(*dd*, $J=1.5$, 3.4 Hz, H-2), 3.52(*dd*, $J=3.4$, 9.6 Hz, H-3), 3.27(*m*, H-4), 3.46(*m*, H-5), 1.12(*d*, $J=6.4$ Hz, $-\text{CH}_3$); $^{13}\text{C-NMR}(\text{CD}_3\text{OD}-d_4$, int TMS, 100 MHz): **Kaempferol**, δ 161.6(C-2), 135.5(C-3), 179.4(C-4), 163.1(C-5), 100.2(C-6), 166.6(C-7), 95.1(C-8), 158.7(C-9), 105.6(C-10), 122.8(C-1'), 132.4(C-2', 6'), 116.2(C-3', 5'), 159.4(C-4'), **Glucose**, 104.7(C-1), 75.8(C-2), 78.2(C-3), 71.5(C-4), 77.3(C-5), 68.6(C-6), **Rhamnose**, 102.5(C-1), 72.1(C-2), 72.3(C-3), 73.9(C-4), 69.8(C-5), 18.0($-\text{CH}_3$).

Novel compounds

The acid hydrolysis of **F**, **G**, and **L** yielded butein [Rt (min) 36.0, method 1] as their aglycone and glucose (R_f -values: BAW 0.32, EAA 0.22, ETN 0.50, EFW 0.19) as their sugar (Harborne, 1984). Moreover, 3-hydroxy-3-methylglutaric acid [Rt (min) 6.8, method 2] was detected in the hydrolysate of **L**. The aglycone, sugar, and acid were identified by direct comparison with commercial standards.

The structures of **F**, **G**, and **L** were confirmed based on the analyses of their ^1H (400 MHz), ^{13}C (100 MHz), and two-dimensional [correlation spectroscopy (COSY), nuclear Overhauser spectroscopy (NOESY), ^1H - ^{13}C heteronuclear multiple-quantum correlation spectroscopy (HMQC), and ^1H - ^{13}C heteronuclear multiple-bond correlation (HMBC)] NMR spectra in CD₃OD-*d*₄ and DMSO-*d*₆, as well as their high-resolution FAB-MS (HR-FAB-MS) (LMS-700; JEOL Ltd.).

Butein 4'-O-[2-O-(β-glucopyranosyl)-β-glucopyranoside]-4-O-β-glucopyranoside (G)

Bright yellow powder: UV: $\lambda_{\text{max}}(\text{nm})$ in MeOH 365, 318, 260; +NaOMe 365, 318sh, 260; +AlCl₃ 424, 382, 319, 269; +AlCl₃/HCl 422, 382, 319, 269; +NaOAc 365, 318, 260; +NaOAc/H₃BO₃ 365, 318, 260. TLC: UV; brown, UV+NH₃, orange, (R_f -values) BAW 0.37, BuHCl 0.17, 1% HCl 0.12, AHW 0.50; HPLC (method 1); $t_R(\text{min})$ 20.1, PDA [$\lambda_{\text{max}}(\text{nm})$] 369, 316sh, HR-FAB-MS; [$\text{M}+\text{H}]^+$ calc. for C₃₃H₄₃O₂₀: 759.2348, found: 759.2349; $^1\text{H-NMR}(\text{CD}_3\text{OD}-d_4$, int TMS, 400 MHz): **Butein**, δ 7.28(*brs*, H-2), 7.24(*d*, $J=8.2$ Hz, H-5), 7.21(*dd*, $J=2.0$, 8.2 Hz, H-6), 7.66(*d*, $J=15.4$ Hz, H-7(α)), 7.77(*d*, $J=15.4$ Hz, H-8(β)), 6.62(*d*, $J=2.2$ Hz,

H-3'), 6.70(*dd*, $J=2.2$, 9.0 Hz, H-5'), 8.06(*d*, $J=9.0$ Hz, H-6'), **Glucose A**, 5.24(*d*, $J=7.0$ Hz, H-1), 3.69(*t*, $J=9.0$ Hz, H-2), 3.44(*m*, H-3), 3.39(*m*, H-4), 3.50(*m*, H-5), 3.71(*m*, H-6a), 3.91(*dd*, $J=4.6$, 12.0 Hz, H-6b), **Glucose B**, 4.89(*d*, $J=7.6$ Hz, H-1), 3.50(*t*, $J=8.0$ Hz, H-2), 3.49(*t*, $J=8.3$ Hz, H-3), 3.47(*m*, H-4), 3.61(*m*, H-5), 3.69(*m*, H-6a), 3.73(*m*, H-6b), **Glucose C**, 4.66(*d*, $J=7.8$ Hz, H-1), 3.23(*t*, $J=8.3$ Hz, H-2), 3.29(*m*, H-3), 3.38(*m*, H-4), 3.47(*m*, H-5), 3.60(*m*, H-6a), 3.73(*m*, H-6b); $^1\text{H-NMR}$ (DMSO- d_6 , int TMS, 400 MHz): **Butein**, δ 7.45(*d*, $J=1.7$ Hz, H-2), 7.16(*d*, $J=8.5$ Hz, H-5), 7.30(*dd*, $J=1.7$, 8.5 Hz, H-6), 7.73(*d*, $J=15.4$ Hz, H-7(α)), 7.87(*d*, $J=15.4$ Hz, H-8(β)), 6.60(*d*, $J=2.2$ Hz, H-3'), 6.66(*dd*, $J=2.2$, 8.8 Hz, H-5'), 8.29(*d*, $J=8.8$ Hz, H-6'), **Glucose A**, 5.19(*d*, $J=7.2$ Hz, H-1), 3.52(*t*, $J=8.0$ Hz, H-2), 3.48(*m*, H-3), 3.32(*t*, $J=9.0$ Hz, H-4), 3.36(*m*, H-5), 3.47(*m*, H-6a), 3.73(*brd*, $J=11.0$ Hz, H-6b), **Glucose B**, 4.82(*d*, $J=7.1$ Hz, H-1), 3.34(*t*, $J=8.0$ Hz, H-2), 3.31(*t*, $J=9.0$ Hz, H-3), 3.25(*t*, $J=8.5$ Hz, H-4), 3.20(*m*, H-5), 3.38(*m*, H-6a), 3.70(*m*, H-6b), **Glucose C**, 4.48(*d*, $J=8.0$ Hz, H-1), 3.00(*t*, $J=8.3$ Hz, H-2), 3.18(*m*, H-3), 3.15(*m*, H-4), 3.34(*m*, H-4), 3.34(*m*, H-5), 3.42(*m*, H-6a), 3.50(*m*, H-6b); $^{13}\text{C-NMR}$ (DMSO- d_6 , int TMS, 100 MHz): **Butein**, δ 129.1(C-1), 115.6(C-2), 147.0(C-3), 147.9(C-4), 115.9(C-5), 119.1(C-6), 144.6(C-7), 122.0(C-8), 192.0(C-9), 114.7(C-1'), 163.3(C-2'), 103.4(C-3'), 165.1(C-4'), 108.3(C-5'), 132.5(C-6'), **Glucose A**, 101.5(C-1), 82.6(C-2), 75.6(C-3), 69.1(C-4), 76.8(C-5), 60.6(C-6), **Glucose B**, 104.5(C-1), 73.1(C-2), 75.7(C-3), 69.4(C-4), 77.2(C-5), 60.4(C-6), **Glucose C**, 97.5(C-1), 74.6(C-2), 76.1(C-3), 69.7(C-4), 76.7(C-5), 60.4(C-6).

The molecular ion $[\text{M}+\text{H}]^+$ of **G** was observed at m/z 759 ($\text{C}_{33}\text{H}_{43}\text{O}_{20}$), indicating that **G** comprised butein with three molecules of glucose. The elemental components were confirmed by measuring its HR-FAB-MS. The structure was elucidated based on the analysis of its NMR spectra. The chemical shifts of six aromatic protons and two *trans*-olefinic protons of the butein moiety with their coupling constants were analyzed. Chemical shifts in the sugar moieties were observed in the region of δ 5.19–3.00 (DMSO- d_6) and δ 5.24–3.23 (CD₃OD- d_4), where three anomeric protons were present at δ 5.19 (d , $J=7.2$ Hz, glucose A), δ 4.48 (d , $J=8.0$ Hz, glucose C), and δ 4.82 (d , $J=7.1$ Hz, glucose B) (DMSO- d_6) and at δ 5.24 (d , $J=7.0$ Hz, glucose A), δ 4.66 (d , $J=7.8$ Hz, glucose C), and δ 4.89 (d , $J=7.6$ Hz, glucose B) (CD₃OD- d_4). Based on the observed coupling constants, glucoses were assumed to be in the β -pyranose form. Based on the results analyses of their COSY spectra analysis, the characteristic methine proton signal [δ 3.52 (DMSO- d_6) and δ 3.69 (CD₃OD- d_4)] shifted to a lower magnetic field was assigned to the H-2 proton of glucose A (Fossen and Andersen, 2006). This result indicated that the OH-2 of glucose A must be glycosylated with a sugar. The NOESY spectra

(DMSO- d_6 and CD₃OD- d_4) were used to determine the sites of attachment of the sugars and butein moieties. The signal of the H-1 (δ 5.19: DMSO- d_6 and δ 5.24: CD₃OD- d_4) proton of glucose A, H-1 (δ 4.48: DMSO- d_6 and δ 4.66: CD₃OD- d_4) proton of glucose C, and H-1 (δ 4.82: DMSO- d_6 and δ 4.89: CD₃OD- d_4) proton of glucose B were correlated with the signals of the H-3' and H-5' (δ 6.60 and 6.66: DMSO- d_6 and δ 6.62 and 6.70: CD₃OD- d_4) protons of butein, H-2 (δ 3.52: DMSO- d_6 and δ 3.69: CD₃OD- d_4) proton of glucose A and H-5 (δ 7.16: DMSO- d_6 and δ 7.24: CD₃OD- d_4) proton of butein in the NOESY spectrum, respectively. These characteristic features revealed that the OH-4' position of butein, OH-2 position of glucose A and OH-4 position of butein are bonded to glucose A, glucose C, and glucose B, respectively. Consequently, the structure of **G** was determined to be butein 4'-*O*-[2-*O*-(β -glucopyranosyl)- β -glucopyranoside]-4-*O*- β -glucopyranoside (Fig. 2), which is a newly identified flavonoid in plants (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Veitch and Grayer, 2008, 2011). This structure was also confirmed by the analysis of its ^{13}C , including the ^1H - ^{13}C HMQC and ^1H - ^{13}C HMBC spectra.

Butein 4',4'-*O*-di-[2-*O*-(β -glucopyranosyl)- β -glucopyranoside] (**F**)

Bright yellow powder: UV: λ_{max} (nm) in MeOH 360, 311, 283sh, 259sh; +NaOMe 360, 278sh, 259sh; +AlCl₃ 416, 368, 315, 267sh; +AlCl₃/HCl 416, 364, 312, 265sh; +NaOAc 361, 313, 280sh, 259sh; +NaOAc/H₃BO₃ 361, 313, 280sh, 259sh. TLC: UV; brown, UV +NH₃, orange, (R_f -values) BAW 0.29, BuHCl 0.10, 1% HCl 0.33, AHW 0.71; HPLC (method 1); t_R (min), 19.4, PDA [λ_{max} (nm)] 369, 316sh, HR-FAB-MS; $[\text{M}+\text{H}]^+$ calc. for $\text{C}_{39}\text{H}_{53}\text{O}_{25}$: 921.2876. found: 921.2887; $^1\text{H-NMR}$ (CD₃OD- d_4 , int TMS, 400 MHz): **Butein**, δ 7.31(*d*, $J=2.2$ Hz, H-2), 7.29(*d*, $J=8.4$ Hz, H-5), 7.22(*dd*, $J=2.2$, 8.4 Hz, H-6), 7.69(*d*, $J=15.4$ Hz, H-7(α)), 7.79(*d*, $J=15.4$ Hz, H-8(β)), 6.62(*d*, $J=2.2$ Hz, H-3'), 6.70(*dd*, $J=2.2$, 9.0 Hz, H-5'), 8.08(*d*, $J=9.0$ Hz, H-6'), **Glucose A**, 5.25(*d*, $J=7.3$ Hz, H-1), 3.72(*t*, $J=9.0$ Hz, H-2), 3.47(*m*, H-3), 3.40(*m*, H-4), 3.48(*m*, H-5), 3.75(*m*, H-6a), 3.93(*brd*, $J=12.0$ Hz, H-6b), **Glucose B**, 4.93(*d*, $J=7.8$ Hz, H-1), 3.80(*t*, $J=8.7$ Hz, H-2), 3.70(*m*, H-3), 3.47(*m*, H-4), 3.60(*m*, H-5), 3.69(*m*, H-6a), 3.90(*brd*, $J=12.0$ Hz, H-6b), **Glucose C**, 4.66(*d*, $J=7.8$ Hz, H-1), 3.22(*t*, $J=8.4$ Hz, H-2), 3.30–3.90(H-3, H-4, H-5, H-6a, H-6b), **Glucose D**, 4.76(*d*, $J=7.8$ Hz, H-1), 3.27(*t*, $J=8.4$ Hz, H-2), 3.30–3.90(H-3, H-4, H-5, H-6a, H-6b); $^1\text{H-NMR}$ (DMSO- d_6 , int TMS, 400 MHz): **Butein**, δ 7.48(*d*, $J=1.7$ Hz, H-2), 7.18(*d*, $J=8.6$ Hz, H-5), 7.33(*dd*, $J=1.7$, 8.6 Hz, H-6), 7.74(*d*, $J=15.4$ Hz, H-7(α)), 7.88(*d*, $J=15.4$ Hz, H-8(β)), 6.61(*d*, $J=2.2$ Hz, H-3'), 6.67(*dd*, $J=2.2$, 9.3 Hz, H-5'), 8.30(*d*, $J=9.3$ Hz, H-6'), **Glucose A**, 5.19(*d*, $J=7.1$ Hz, H-1), 3.51(*m*, H-2), 3.10–3.80(H-3, H-4, H-5, H-6a, H-6b), **Glucose B**, 4.99(*d*, $J=7.3$ Hz, H-1), 3.62(*t*, $J=8.9$ Hz,

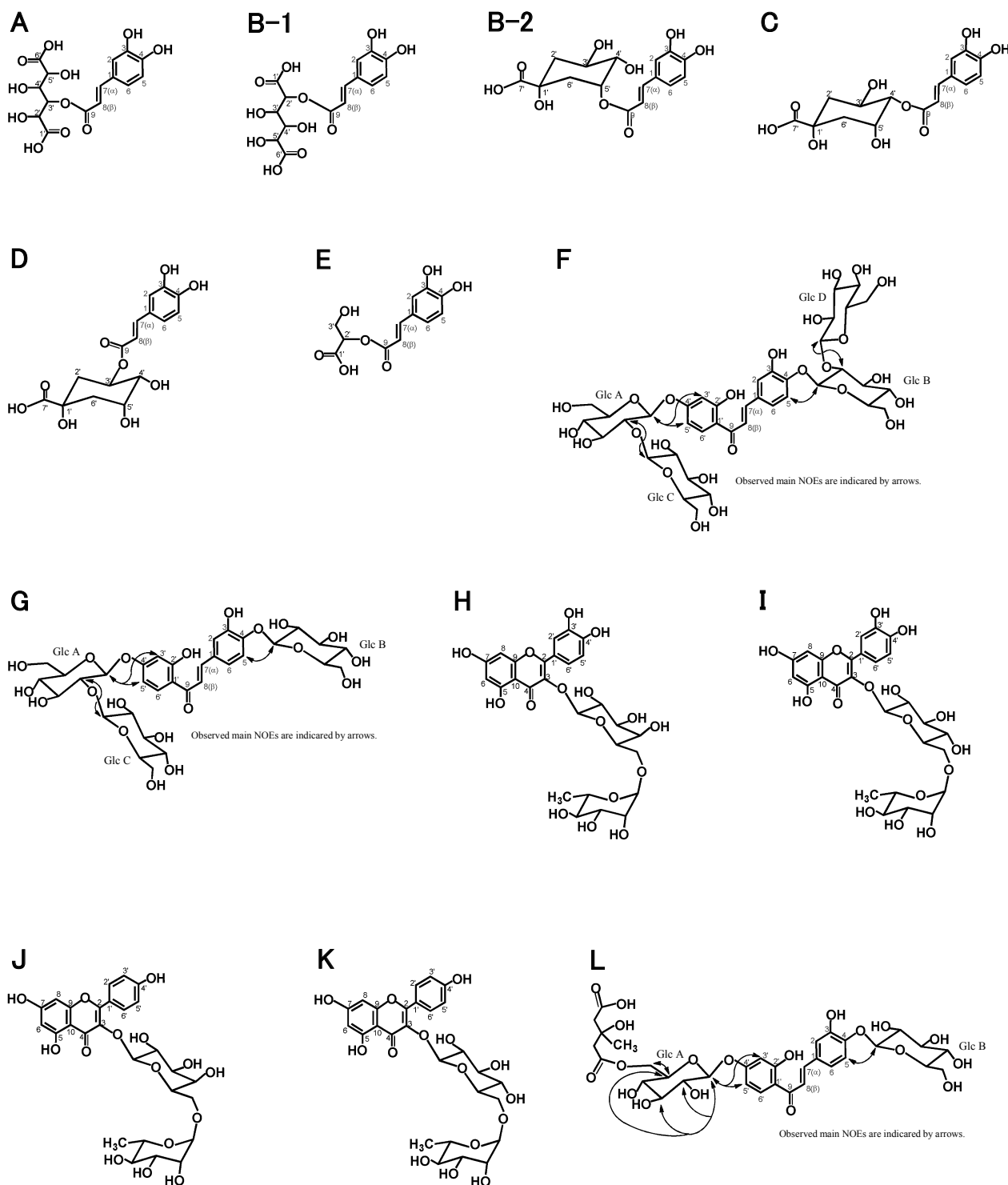


Fig. 2. Structures of flavonoids and caffeoyl esters in leaves. **A**, 3-*O*-[(*trans*)-caffeoyl]-glucaric acid; **B-1**, 2-*O*-[(*trans*)-caffeoyl]-glucaric acid; **B-2**, 5-*O*-[(*trans*)-caffeoyl]-quinic acid (neochlorogenic acid); **C**, 4-*O*-[(*trans*)-caffeoyl]-quinic acid (cryptochlorogenic acid); **D**, 3-*O*-[(*trans*)-caffeoyl]-quinic acid (chlorogenic acid); **E**, 2-*O*-[(*trans*)-caffeoyl]-glyceric acid; **F**, butein 4',4'-di-[2-*O*-(β-glucopyranosyl)-β-glucopyranoside]; **G**, butein 4'-*O*-[2-*O*-(β-glucopyranosyl)-β-glucopyranoside]-4-*O*-β-glucopyranoside; **H**, quercetin 3-*O*-[6-*O*-(rhamnosyl)-glucoside]; **I**, quercetin 3-*O*-[6-*O*-(rhamnosyl)-galactoside]; **J**, kaempferol 3-*O*-[6-*O*-(rhamnosyl)-glucoside]; **K**, kaempferol 3-*O*-[6-*O*-(rhamnosyl)-galactoside]; **L**, butein 4'-[6-*O*-(3-hydroxy-3-methylglutaryl)-β-glucopyranoside]-4-*O*-β-glucopyranoside.

H-2), 3.10–3.80(H-3, H-4, H-5, H-6a, H-6b), **Glucose C**, 4.48(*d*, $J=7.8$ Hz, H-1), 3.00(*t*, $J=8.4$ Hz, H-2), 3.13(*m*, H-3), 3.10–3.80(H-4, H-5, H-6a, H-6b),

Glucose D, 4.62(*d*, $J=7.8$ Hz, H-1), 3.02(*t*, $J=8.7$ Hz, H-2), 3.17(*m*, H-3), 3.10–3.80(H-4, H-5, H-6a, H-6b); ^{13}C -NMR(DMSO- d_6 , int TMS, 100 MHz): **Butein**,

8129.6 (C-1), 115.4(C-2), 147.1(C-3), 147.9(C-4), 116.2(C-5), 119.4(C-6), 144.8(C-7), 122.5(C-8), 192.3(C-9), 114.9(C-1'), 163.6(C-2'), 103.6(C-3'), 165.3(C-4'), 108.6(C-5'), 132.7(C-6'), **Glucose A**, 100.0(C-1), 82.8(C-2), 76.3(C-3), 69.5(C-4), 77.0(C-5), 60.6(C-6), **Glucose B**, 104.7(C-1), 81.8(C-2), 76.3(C-3), 69.3(C-4), 77.0(C-5), 60.6(C-6), **Glucose C**, 98.2(C-1), 74.8(C-2), 75.9(C-3), 69.6(C-4), 77.0(C-5), 60.6(C-6), **Glucose D**, 104.0(C-1), 74.5(C-2), 75.9(C-3), 69.6(C-4), 77.0(C-5), 60.6(C-6).

The molecular ion $[M+H]^+$ of **F** was observed at m/z 921 ($C_{39}H_{53}O_{25}$), indicating that **F** comprised butein with four molecules of glucose. The elemental components were confirmed by measuring its HR-FAB-MS. The structure was elucidated based on the analysis of its NMR spectra according to the same process as described above for the structure determination of **G**. The 1H NMR spectra of **F** was similar to that of **G**, except for the additional signals of glucose D and OH-2 of glucose B. The chemical shift (δ 3.62; DMSO- d_6 and δ 3.80; CD_3OD-d_4) of the methine proton of glucose B was shifted to a magnetic field lower than those of **G**, indicating that OH-2 of glucose B was glycosylated with glucose D in **G**. Moreover, the signal of the H-1 (δ 5.19: DMSO- d_6 and δ 5.25: CD_3OD-d_4) proton of glucose A, H-1 (δ 4.48: DMSO- d_6 and δ 4.66: CD_3OD-d_4) proton of glucose C, H-1 (δ 4.99: DMSO- d_6 and δ 4.93: CD_3OD-d_4) proton of glucose B, and H-1 (δ 4.62: DMSO- d_6 and δ 4.76: CD_3OD-d_4) proton of glucose D were correlated with the signals of the H-3' and H-5' (δ 6.61 and 6.67: DMSO- d_6 and δ 6.62 and 6.70: CD_3OD-d_4) protons of butein, H-2 (δ 3.51: DMSO- d_6 and δ 3.72: CD_3OD-d_4) proton of glucose A, H-5 (δ 7.18: DMSO- d_6 and δ 7.29: CD_3OD-d_4) proton of butein, and H-2 (δ 3.62: DMSO- d_6 and δ 3.80: CD_3OD-d_4) proton of glucose B in the NOESY spectrum, respectively. Therefore, the structure of **F** was determined to be butein 4'-*O*-di-[2-*O*-(β -glucopyranosyl)- β -glucopyranoside] (Fig. 2), which is a newly identified flavonoid in plants (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Veitch and Grayer, 2008, 2011). This structure was also confirmed by the analysis of its ^{13}C NMR spectra.

Butein 4'-[6-*O*-(3-hydroxy-3-methylglutaryl)- β -glucopyranoside]-4-*O*- β -glucopyranoside (**L**)

Yellow powder: UV: $\lambda_{max}(nm)$ in MeOH 364, 317, 259; +NaOMe 364, 317sh, 259; +AlCl₃ 422, 377, 320, 266; +AlCl₃/HCl 421, 378, 320, 266; +NaOAc 364, 317, 259; +NaOAc/H₃BO₃ 364, 317, 259. TLC: UV; brown, UV+NH₃, orange, (R_f -values) BAW 0.50, BuHCl 0.37, 1% HCl 0.04, AHW 0.37; HPLC (method 1); $t_R(min)$ 28.4, PDA [$\lambda_{max}(nm)$] 368, 318sh, HR-FAB-MS; $[M+H]^+$ calc. for $C_{33}H_{41}O_{19}$: 741.2242. found: 741.2246; 1H -NMR(CD_3OD-d_4 , int TMS, 400 MHz): **Butein**, δ 7.46(*d*, $J=2.0$ Hz, H-2), 7.23(*d*, $J=8.8$ Hz, H-5), 7.19(*dd*, $J=2.0$, 8.8 Hz, H-6), 7.73(*d*, $J=15.4$ Hz, H-7(α)), 7.78(*d*, $J=15.4$ Hz, H-8(β)), 6.30(*d*, $J=2.4$ Hz,

H-3'), 6.74(*dd*, $J=2.4$, 8.8 Hz, H-5'), 8.14(*d*, $J=8.8$ Hz, H-6'), **Glucose A**, 5.02(*d*, $J=7.2$ Hz, H-1), 3.49(*t*, $J=8.1$ Hz, H-2), 3.47(*t*, $J=8.1$, H-3), 3.41(*t*, $J=8.3$, H-4), 3.74(*m*, H-5), 4.19(*dd*, $J=6.8$, 11.9 Hz, H-6a), 4.48(*dd*, $J=2.0$, 11.9 Hz, H-6b), **Glucose B**, 4.92(*d*, $J=7.2$ Hz, H-1), 3.44(*t*, $J=8.3$ Hz, H-2), 3.35(*m*, H-3), 3.27(*m*, H-4), 3.35(*m*, H-5), 3.72(*dd*, $J=5.4$, 12.0 Hz, H-6a), 3.91(*dd*, $J=2.0$, 12.0 Hz, H-6b), **3-hydroxy-3-methylglutaric acid**, 2.54(*d*, $J=15.5$ Hz, H-2a), 2.37(*d*, $J=15.5$ Hz, H-2b), 2.64(*brs*, H-4a and b), 1.30(*s*, -CH₃); 1H -NMR(DMSO- d_6 , int TMS, 400 MHz): **Butein**, δ 7.07(*m*, H-2), 7.08(*brs*, H-5), 7.06(*brd*, $J=8.3$ Hz, H-6), 7.67(*d*, $J=15.4$ Hz, H-7(α)), 7.90(*d*, $J=15.4$ Hz, H-8(β)), 6.70(*d*, $J=2.2$ Hz, H-3'), 6.74(*dd*, $J=2.2$, 9.0 Hz, H-5'), 8.17(*d*, $J=9.0$ Hz, H-6'), **Glucose A**, 4.92(*d*, $J=7.3$ Hz, H-1), 3.28(*t*, $J=8.8$ Hz, H-2), 3.20(*m*, H-3), 3.45(*m*, H-4), 3.70(*m*, H-5), 3.89(*dd*, $J=8.7$, 11.9 Hz, H-6a), 4.48(*brd*, $J=11.9$ Hz, H-6b), **Glucose B**, 4.89(*d*, $J=7.6$ Hz, H-1), 3.31(*t*, $J=8.1$ Hz, H-2), 3.29(*m*, H-3), 3.10(*t*, $J=9.1$ Hz, H-4), 3.14(*m*, H-5), 3.46(*m*, H-6a), 3.71(*brd*, $J=11.9$ Hz, H-6b), **3-hydroxy-3-methylglutaric acid**, 2.26(*d*, $J=15.6$ Hz, H-2a), 2.18(*d*, $J=15.6$ Hz, H-2b), 2.51(*brs*, H-4a and b), 1.21(*s*, -CH₃); ^{13}C -NMR(DMSO- d_6 , int TMS, 100 MHz): **Butein**, δ 129.1(C-1), 115.2(C-2), 144.7(C-3), 148.1(C-4), 115.2(C-5), 119.4(C-6), 143.3(C-7), 120.9(C-8), 191.5(C-9), 115.2(C-1'), 163.4(C-2'), 104.6(C-3'), 164.2(C-4'), 108.3(C-5'), 131.9(C-6'), **Glucose A**, 101.1(C-1), 73.0(C-2), 75.0(C-3), 69.2(C-4), 76.1(C-5), 63.9(C-6), **Glucose B**, 100.9(C-1), 73.0(C-2), 75.0(C-3), 69.2(C-4), 76.1(C-5), 61.3(C-6), **3-hydroxy-3-methylglutaric acid**, 176.0(C-1), 48.3(C-2), 69.7(C-3), 46.8(C-4), 171.0(C-5), 27.9(-CH₃).

The molecular ion $[M+H]^+$ of **L** was observed at m/z 741 ($C_{33}H_{41}O_{19}$), indicating that **L** comprised butein with two molecules of glucose and one molecule of 3-hydroxy-3-methylglutaric acid. The elemental components were confirmed by measuring its HR-FAB-MS, and the mass data are summarized in this section. The structure was elucidated based on the analysis of the NMR spectra according to the same process as described above for the structural determination of **G**. The 1H NMR spectra of **L** was similar to those of **G**, except for the additional signals of 3-hydroxy-3-methylglutaric acid and without the glucose C moieties. The chemical shift (δ 3.89 H-6a and 4.48 H-6b; DMSO- d_6 and δ 4.19 H-6a and 4.48 H-6b; CD_3OD-d_4) of methylene protons of glucose A were shifted to a magnetic field lower than those of **G**, indicating that the OH-6 of glucose A was acylated with 3-hydroxy-3-methylglutaric acid in **L**. Therefore, the structure of **L** was determined to be butein 4'-[6-*O*-(3-hydroxy-3-methylglutaryl)- β -glucopyranoside]-4-*O*- β -glucopyranoside (Fig. 2), which is a newly identified flavonoid in plants (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Veitch and Grayer, 2008, 2011). This structure

was also confirmed by the analysis of its ^{13}C NMR spectra.

Discussion

In our previous report (Ohno et al., 2016), ‘Yuino’ leaves were classified into two types: flavonoid-rich leaves and flavonoid-poor leaves, and a relationship between petal colors and leaf flavonoid accumulation was evident, namely red petal-producing individuals accumulated flavonoids in leaves, whereas only bicolor petal-producing individuals tended not to accumulate them in leaves. At that time, the flavonoids in the leaves of ‘Yuino’ were presumed to be chalcone-like compounds using photodiode array analysis. Thus, in this study, to confirm this assumption and to identify each flavonoid compound in leaves of ‘Yuino’, we performed NMR analysis.

By analysis at 350 nm using HPLC, more than 30 peaks were detected from flavonoid-rich leaves (Fig. 1A), whereas only peaks before 15 min of retention time were detected from flavonoid-poor leaves (Fig. 1B). The structures of known compounds were determined by comparing their spectroscopic and/or chromatographic data with values in the literature and were identified as 3-*O*-[(*trans*)-caffeoyl]-glucaric acid (**A**) (Maas et al., 2009; Nagels et al., 1982), 2-*O*-[(*trans*)-caffeoyl]-glucaric acid (**B-1**) (Strack et al., 1987), 5-*O*-[(*trans*)-caffeoyl]-quinic acid (neo-chlorogenic acid) (**B-2**) (Liu et al., 2013), 4-*O*-[(*trans*)-caffeoyl]-quinic acid (crypto-chlorogenic acid) (**C**) (Tatsuzawa et al., 2015), 3-*O*-[(*trans*)-caffeoyl]-quinic acid (chlorogenic acid) (**D**) (Tatsuzawa et al., 2015), 2-*O*-[(*trans*)-caffeoyl]-glyceric acid (**E**) (Hahn and Nahrstedt, 1993), quercetin 3-*O*-[6-*O*-(rhamnosyl)-galactoside] (**H**) (Tatsuzawa et al., 2015), quercetin 3-*O*-[6-*O*-(rhamnosyl)-glucoside] (rutin) (**I**) (Tatsuzawa et al., 2015), kaempferol 3-*O*-[6-*O*-(rhamnosyl)-galactoside] (**J**) (Tatsuzawa et al., 2015), and kaempferol 3-*O*-[6-*O*-(rhamnosyl)-glucoside] (**K**) (El-Sherbeni et al., 2014) (Fig. 2). The structures of novel compounds were determined to be butein 4',4-*O*-di-[2-*O*-(β -glucopyranosyl)- β -glucopyranoside] (**F**), butein 4'-*O*-[2-*O*-(β -glucopyranosyl)- β -glucopyranoside]-4-*O*- β -glucopyranoside (**G**), and butein 4'-[6-*O*-(3-hydroxy-3-methylglutaryl)- β -glucopyranoside]-4-*O*- β -gluco-pyranoside (**L**) (Fig. 2). These flavonoids were different from those isolated from the leaves of *Dahlia tenuicaulis* (5,7,4'-trimethoxyflavanone, 5-hydroxy-7,4'-dimethoxyflavanone, 2'-hydroxy-4,4,6'-trimethoxy-chalcone, and 5,7,4'-trimethoxyflavan-4-ol) (Lam and Wrang, 1975). These data demonstrated that flavonoid-rich leaves, but not flavonoid-poor leaves, have the potential to synthesize butein and flavonol derivatives.

Butein is a type of bright yellow chalcone. Both butein and flavonol are formed by the condensation of three molecules of malonyl-coenzyme A (CoA) with one molecule of 4-coumaroyl-CoA. The enzymes pre-

sumably involved in butein synthesis are chalcone synthase (CHS), chalcone reductase, and chalcone 3-hydroxylase (Ohno et al., 2011a; Schlangen et al., 2010), whereas those involved in flavonol synthesis are CHS, chalcone isomerase, flavanone 3-hydroxylase, and flavonol synthase (Winkel-Shirley, 2001). Thus, common steps to biosynthesis butein and flavonol are chalcone biosynthesis and its upstream. Therefore, it was implied that CHS and/or upstream phenylpropanoid pathway enzyme activity was lost in flavonoid-poor leaves. In fact, in flavonoid-poor leaves, the expression of *DvCHS2*, which encodes the abundantly expressed CHS in leaves, was silenced by post-transcriptional gene silencing (PTGS) (Ohno et al., submitted). This suggested that the loss of buteins and flavonols in flavonoid-poor leaves resulted from the PTGS of *DvCHS2*. The PTGS of *DvCHS2* is also important for bicolor formation and lability of petal colors in bicolor flowering cultivars (Ohno et al., 2011b; Ohno et al., submitted). Therefore, the data obtained in the present study supports the importance of PTGS of *DvCHS2* in the phenotypic lability of the bicolor flowering dahlia ‘Yuino’.

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