J. Japan. Soc. Hort. Sci. 79 (2): 215–220. 2010.
Available online at www.jstage.jst.go.jp/browse/jjshs1


# An Acylated Cyanidin 3,7-diglucoside in the Bluish Flowers of *Bletilla striata* 'Murasaki Shikibu' (Orchidaceae)

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**A novel acylated anthocyanin was identified from the violet flowers of *Bletilla striata* 'Murasaki Shikibu' as a major floral anthocyanin. This pigment was based on cyanidin 3,7-diglucoside as its deacylanthocyanin, and was acylated with two molecules of caffeic acid. The structure was unambiguously elucidated by chemical and spectroscopic methods to be cyanidin 3-*O*-(β-glucopyranoside)-7-*O*-[6-*O*-(4-*O*-(6-*O*-(4-*O*-(β-glucopyranosyl)-*trans*-caffeoyl)-β-glucopyranosyl)-*trans*-caffeoyl)-β-glucopyranoside]. Based on the results of the present study, the bluing effect was discussed for the violet flowers of a given cultivar.**

**Key Words:** acylated cyanidin 3,7-diglucoside, *Bletilla striata* (Thunberg) Reichenbach f., bluish flower color, Orchidaceae.

## Introduction

As a part of our continuing work on flower color variation due to acylated anthocyanins in *Bletilla striata* (Thunberg) Reichenbach f., we have previously reported the isolation of eight polyacylated cyanidin 3,7,3'-triglucosides from red–purple, purple, purple–violet, and white flower cultivars of *B. striata* (Saito et al., 1995a). To the best of our knowledge, 41 acylated anthocyanins have been isolated and determined from various flowers of the plants in the Orchidaceae (Fossen and Øvstedal, 2003; Saito et al., 1994, 1995a; Strack et al., 1986, 1989; Tatsuzawa et al., 1994, 1996a, b, 1997, 1998, 2004, 2005, 2006; Williams et al., 2002). On the basis of these results, hydroxycinnamic acids of acylated anthocyanins were revealed to be responsible for the stabilization and bluing of the flower color. In particular, remarkable bathochromic shifts were observed depending on increasing numbers of hydroxycinnamic acids in acylated anthocyanins of these flowers (Honda and Saito, 2002). In 1980's, a mutant plant with bluish flower color (violet) was collected from a natural habitat of *B. striata* in Japan. This plant was vegetatively propagated and released to the market. Plants were raised *in vitro* from seeds which were produced by self-pollination of this mutant plant. The phenotype of those plants in respect to flower color was almost identical. These *Bletilla* plants with bluish flower color (violet) were designated as 'Murasaki Shikibu'.

Though the breeding of the genus *Bletilla* has been conducted recently by intra- as well as inter-specific hybridization, 'Murasaki Shikibu' plants has never been utilized as a plant material for the improvement of flower color in this genus. There is no information on the relationship between flower color and pigments in 'Murasaki Shikibu' plants. Further information on this noble flower color would accelerate the breeding program for the selection of flower color efficiently in the genus *Bletilla* in the future. In the present study, we investigated floral anthocyanin pigments in the new violet flower of *B. striata*, and found a novel acylated anthocyanin, cyanidin 3-glucoside-7-di-(glucosylcaffeoyl)-glucoside, as a major pigment. Furthermore, the information on the breeding of new flower colors in orchids was discussed based on these results.

Received; September 14, 2009. Accepted; December 7, 2009.
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## Materials and Methods

*General procedures*

Thin layer chromatography (TLC) was conducted on cellulose-coated plastic sheets (Merck, Darmstadt, Germany) using six mobile phases: BAW (*n*-BuOH/HOAc/$H_2O$, 4 : 1 : 2, v/v/v), BuHCl (*n*-BuOH/2N HCl, 1 : 1, v/v, upper layer), AHW (HOAc/HCl/$H_2O$, 15 : 3 : 82, v/v/v), 1% HCl for anthocyanins and organic acid, and BAW and ETN ($EtOH/NH_4OH/H_2O$, 16 : 1 : 3, v/v/v) for sugars with detection using UV light and the aniline hydrogen phthalate spray reagent (Harborne, 1984).

Analytical high performance liquid chromatography (HPLC) was performed with a LC 10A system (Shimadzu Co. Ltd., Kyoto, Japan) using a Waters C18 (4.6 ϕ × 250 mm, Waters, Massachusetts, USA) column at 40°C with a flow rate of 1 mL·min$^{-1}$ and monitoring at 530 nm. The eluant was applied as a linear gradient elution for 40 min from 20 to 85% solvent B (1.5% $H_3PO_4$, 20% HOAc, 25% MeCN in $H_2O$) in solvent A (1.5% $H_3PO_4$ in $H_2O$). UV-Vis spectra were recorded on an MPS-2450 (Shimadzu) in 0.1% HCl-MeOH (from 200 to 700 nm). Spectral absorption of the fresh flower was directly measured on the intact petals using a recording spectrophotometer operated as a double-beam instrument (MPS-2450; Shimadzu) (Saito, 1967; Yokoi and Saito, 1973). Fast atom bombardment mass spectra (FABMS) were obtained in the positive ion mode using the magic bullet (5 : 1 mixture of dithiothreitol and dithioerythritol) as a matrix with JMS SX-102 (JEOL Ltd., Tokyo, Japan). NMR spectra were recorded on JMN GX-500 (JEOL) at 500 MHz for $^1$H spectra, and at 125.78 MHz for $^{13}$C spectra in $CD_3OD$-$CF_3COOD$ (9 : 1). Chemical shifts are reported relative to a tetra methyl silane (TMS) internal standard (δ), and coupling constants (*J*) are in Hz.

*Plant materials*

Mature seeds were obtained by self-pollination from an original division plant of a bluish flower color mutant of *Bletilla striata* 'Murasaki Shikibu'. The seeds were sown asymbiotically *in vitro* on agar-solidified New Dogashima (ND) medium (Yamazaki and Miyoshi, 2006). After two times of subcultures *in vitro*, plantlets were acclimatized and cultivated in a greenhouse for 7 months. Approximately 400 acclimatized plants were transferred to the field of Akita Pref. University and grown for 3 years. A voucher specimen of this plant was deposited in Tsukuba Botanical Garden, National Science Museum (TBG). The violet flower color [Violet 87D by the Royal Horticultural Society Colour Chart and b*(−19.04)/a*(19.87) = −0.96 by a SE-2000 Spectro Color Meter (Nippon Denshoku Industries Co., Ltd., Tokyo, Japan)] was almost identical in respect to intensity and distribution in the perianths in the plant population. From *ca.* 800 inflorescences, 5,806 flowers were harvested from late May to late June in 2007 and 2008 on the day of bloom. Perianths were isolated by hand, air dried for 2–3 days at 38°C, and kept at −10°C until used.

*Isolation and purification of anthocyanins*

Dried perianths (*ca.* 180 g) of *B. striata* 'Murasaki Shikibu' plants were immersed in 5% HOAc-$H_2O$ (5 L) at room temperature for 12 h and extracted. The extract was passed through a Diaion HP-20 (Nippon Rensui Co., Tokyo, Japan) column (90 × 150 mm), on which anthocyanins were absorbed. The column was then thoroughly washed with 5% HOAc-$H_2O$ (20 L) and eluted with 5% HOAc-MeOH (500 mL) to recover the anthocyanins. After concentration, the eluates were separated and purified with paper chromatography using BAW. The separated pigments were further purified with preparative HPLC, which was performed on a Waters C18 (19 ϕ × 150 mm, Waters) column at 40°C with a flow rate of 1 mL·min$^{-1}$ and monitoring at 530 nm. The solvent used was as follows: a linear gradient elution for 20 min from 55 to 60% solvent B in solvent A. As a major anthocyanin pigment, a concentrated pigment **1** (Fig. 1) fraction was dissolved in a small volume of 5% HOAc-EtOH, followed by the addition of excess $Et_2O$ to give precipitated pigment **1** (*ca.* 30 mg).

*Pigment 1*

Dark red–purple powder: UV-VIS (in 0.1% HCl-MeOH): λmax 533, (318), 285 nm, $E_{acyl}/E_{max}$ = 145, $E_{440}/E_{max}$ = 22, $AlCl_3$ shift +; TLC: ($R_f$-values) BAW 0.15, BuHCl 0.03, 1% HCl 0.04, AHW 0.17; HPLC: *R*t (min) 21.9.; high-resolution FAB mass spectra (HR-FABMS) calc. for $C_{57}H_{63}O_{32}$: 1259.3302. found: 1259.3337; $^1$H NMR δ cyanidin: 8.70 (s, H-4), 6.74 (brd, J = 2.1 Hz, H-6), 7.02 (brd, J = 2.1 Hz, H-8), 7.98 (brd, J = 2.2 Hz, H-2'), 6.95 (d, J = 8.9 Hz, H-5'), 8.23 (dd, J = 2.2, 8.9 Hz, H-6'). Caffeic acid I: 6.98 (brd, J = 1.9 Hz, H-2), 6.71 (d, J = 8.2 Hz, H-5), 6.47 (dd, J = 1.9, 8.2 Hz, H-6), 6.14 (d, J = 15.9 Hz, H-7(α)), 7.02 (d, J = 15.9 Hz, H-8(β)). Caffeic acid II: 6.83 (brd, J = 1.9 Hz, H-2), 6.74 (d, J = 8.3 Hz, H-5), 6.66 (dd, J = 1.9, 8.3 Hz, H-6), 6.39 (d, J = 15.9 Hz, H-7(α)), 7.47 (d, J = 15.9 Hz, H-8(β)). Glucose A: 5.29 (d, J = 8.0 Hz, H-1), 3.79 (t, J = 8.3 Hz, H-2), 3.66 (t, J = 9.0 Hz, H-3), 3.58 (m, H-4), 3.45 (m, H-5), 3.74 (m, H-6a), 3.78 (m, H-6b). Glucose B: 5.22 (d, J = 8.0 Hz, H-1), 3.69 (t, J = 7.9 Hz, H-2), 3.60 (t, J = 8.0 Hz, H-3), 3.4 (m, H-4), 4.00 (t, J = 8.6 Hz, H-5), 4.31 (dd, J = 9.8, 11.9 Hz, H-6a), 4.77 (brd, J = 11.9 Hz, H-6b). Glucose C: 4.57 (d, J = 7.7 Hz, H-1), 3.45 (m, H-2), 3.37 (t, J = 9.0 Hz, H-3), 3.62 (m, H-4), 3.69 (m, H-5), 3.95 (m, H-6a), 4.10 (brd, 10.4 Hz, H-6b). Glucose D: 4.88 (d, J = 8.0 Hz, H-1), 3.60 (t, J = 8.0 Hz, H-2), 3.28 (m, H-3), 3.45 (m, H-4), 3.56 (m, H-5), 3.69 (m, H-6a), 3.93 (m, H-6b). $^{13}$C NMR δ cyanidin: 164.0 (C-2), 147.0 (C-3), 134.1 (C-4), 156.0 (C-5), 105.4 (C-6), 167.1 (C-7), 94.6 (C-8), 157.6 (C-9), 113.7 (C-10), 121.1 (C-1'), 118.7 (C-2'), 147.5 (C-3'), 156.8 (C-4'), 117.8 (C-

5'), 129.3 (C-6'). Caffeic acid I: 130.1 (C-1), 113.7 (C-2), 147.9 (C-3), 148.4 (C-4), 116.9 (C-5), 122.2 (C-6), 117.7 (C-7(α)), 145.1 (C-8(β)), 168.3 (C-9(COOH)). Caffeic acid II: 130.1 (C-1), 117.1 (C-2), 147.9 (C-3), 148.6 (C-4), 116.7 (C-5), 120.2 (C-6), 115.2 (C-7(α)), 146.4 (C-8(β)), 168.6 (C-9(COOH)). Glucose A: 103.4 (C-1), 74.7 (C-2), 77.2 (C-3), 74.3 (C-4), 77.6 (C-5), 62.1 (C-6). Glucose B: 101.5 (C-1), 74.5 (C-2), 77.6 (C-3), 74.7 (C-4), 74.8 (C-5), 65.6 (C-6). Glucose C: 102.5 (C-1), 71.1 (C-2), 77.8 (C-3), 75.1 (C-4), 74.5 (C-5), 62.7 (C-6). Glucose D: 102.3 (C-1), 71.1 (C-2), 77.8 (C-3), 72.1 (C-4), 73.5 (C-5), 62.6 (C-6).

*Anthocyanidin (cyanidin), glucose and caffeic acid*

Acid hydrolysis of pigment **1** (*ca.* 0.5 mg) was achieved by 2N HCl (1 mL) at 90°C for 2 h and resulted in the isolation of cyanidin, glucose and caffeic acid.

1. *Cyanidin*. UV-VIS (in 0.1% HCl-MeOH): λmax 536, 273 nm, $E_{440}/E_{max} = 44\%$, $AlCl_3$ shift +; TLC: ($R_f$-values) Forestal (HOAc/HCl/$H_2O$, 30 : 3 : 10, v/v/v) 0.42; HPLC: *R*t (min) 22.4
2. *Glucose*. TLC: ($R_f$-values) BAW 0.23, ETN 0.34.
3. *Caffeic acid*. TLC: ($R_f$-values) BAW 0.61, ETN 0.31; HPLC: *R*t (min) 10.4.

*Deacylanthocyanin (cyanidin 3,7-diglucoside) and 4-O-Glucosyl-caffeic acid*

Pigment **1** (*ca.* 0.5 mg) was dissolved in 2N NaOH (1 mL) using a degassed syringe to stir for 15 min. The solution was then acidified with 2N HCl (1.1 mL). The solution was used for TLC and HPLC with authentic cyanidin 3,7-diglucoside (seranin) and 4-*O*-glucosyl-caffeic acid obtained from *Serapias lingua* (Strack et al., 1989) and *Leschenaultia* 'Violet Lena' (Saito et al., 2007), respectively.

1. *Deacylanthocyanin (cyanidin 3,7-diglucoside)* UV-VIS (in 0.1% HCl-MeOH): λmax 525, 281 nm, $E_{440}/E_{max} = 25\%$, $AlCl_3$ shift +; TLC: ($R_f$ values) BAW 0.10, BuHCl 0.03, 1% HCl 0.13, AHW 0.34; HPLC: *R*t (min) 8.4.
2. *4-O-Glucosyl-caffeic acid* UV (in MeOH): λmax 316, 288 nm TLC: ($R_f$ values) BAW 0.52, BuHCl 0.46, 1% HCl 0.33 and 0.72, AHW 0.55 and 0.99; HPLC: *R*t (min) 7.4.

## Results and Discussion

*Anthocyanins of violet flowers of Bletilla striata 'Murasaki Shikibu' plants*

By HPLC analysis of the extract from violet flowers of *Bletilla striata* 'Murasaki Shikibu' plants, over 20 anthocyanin peaks were observed. From these peaks, a major anthocyanin peak (pigment **1**: 56.0% of the total anthocyanin contents calculated from the HPLC peak area at 530 nm) was detected in 5% HOAc extract from fresh violet flowers in *B. striata* 'Murasaki Shikibu' plants. This major anthocyanin pigment (pigment **1**) was isolated from dried flowers and purified according to the procedure described previously (Tatsuzawa et al., 2006). The chromatographic and spectroscopic properties of the pigment are summarized in the section on pigment **1** in materials and methods. Cyanidin, glucose and caffeic acid were compared by TLC and HPLC behaviors with those of authentic samples. Alkaline hydrolysis of pigment **1** (*ca.* 0.5 mg) yielded a deacylated anthocyanin and glucosyl-caffeic acid. These structures were identified by the analysis of TLC and HPLC in comparison with authentic cyanidin 3,7-diglucoside (seranin) and 4-*O*-glucosyl-caffeic acid obtained from acylated anthocyanins of *Serapias lingua* (Strack et al., 1989) and *Leschenaultia* 'Violet Lena' (Saito et al., 2007), respectively.

*Pigment 1*

The molecular ion $[M]^+$ of pigment **1** was observed at *m/z* 1259.33 ($C_{57}H_{63}O_{32}$) using FABMS, indicating that pigment **1** is composed of cyanidin with four molecules of glucose and two molecules of caffeic acid. The elemental components of pigments **1** were confirmed by measuring its high resolution FABMS.

The structure of pigment **1** was further elucidated by investigation of its $^1H$ and $^{13}C$ NMR spectra, including 2D COSY, 2D NOESY, HMQC and HMBC spectra (Fig. 1).

The chemical shifts of twelve aromatic protons of cyanidin and caffeic acid moieties with their coupling constants were assigned by analysis of its 2D COSY spectrum, as shown in section pigment **1** in materials and methods. The $^1H$ NMR spectrum exhibited four olefinic proton signals of caffeic acid with large coupling constants ($J = 15.9$ Hz and 15.9 Hz). These two caffeic acids were determined to be in *trans* configuration. Chemical shifts of the sugar moieties were observed in the region of δ3.28–5.29, where the four anomeric protons resonated at δ5.29 (*d*, $J = 8.0$ Hz, Glc A-H1), δ5.22 (*d*, $J = 8.0$ Hz, Glc B-H1), δ4.57 (*d*, $J = 7.7$ Hz, Glc C-H1), and δ4.88 (*d*, $J = 8.0$ Hz, Glc D-H1), respectively. Based on the observed coupling constants, these four sugars were assumed to be in the β-pyranose form (see section *pigment **1*** in materials and methods). By analysis of the 2D COSY spectrum, four characteristic proton signals shifted to a lower magnetic field were assigned as methylene protons of Glc B (δ4.31 and 4.77, H-6a and -6b) and Glc C (δ3.95 and 4.10, H-6a and -6b), respectively. These results indicated that two OH-6 groups of Glc B and Glc C were acylated with caffeic acids I and II, respectively. NOESY and HMBC spectra were analyzed to distinguish the attachment positions of caffeic acid and glucose with cyanidin aglycone (Fig. 1). The signals of the anomeric protons of Glc A and Glc B were correlated to the signals of the C-3 (δ147.0) and C-7 (δ167.1) carbon of cyanidin, respectively, in the HMBC spectrum, and to the signals of the proton H-4 (δ8.70) and H-8 and -6 (δ7.02 and 6.74), respectively, in the NOESY spectrum of cyanidin. These characteristic

features revealed that the OH-3 and OH-7 positions of cyanidin are both bound to glucose molecules. The signals of the anomeric protons of Glc C and Glc D were correlated to the signals of the H-5 (δ6.71) proton of caffeic acid I and H-5 (δ6.74) proton of caffeic acid II in the NOESY spectrum, respectively, while the OH-4s of caffeic acids I and II were glycosylated with Glc C and Glc D, respectively. Furthermore, H-6b (δ4.77) of Glc B was correlated to the signal of the -CO- (δ168.3) carbon of caffeic acid I in the HMBC spectra. This result supported that the OH-6 of Glc B was esterified with caffeic acid I (Fig. 1). Consequently, the structure of pigment **1** was elucidated to be cyanidin 3-*O*-(β-glucopyranoside)-7-*O*-[6-*O*-(4-*O*-(6-*O*-(4-*O*-(β-glucopyranosyl)-*trans*-caffeoyl)-β-glucopyranosyl)-*trans*-caffeoyl)-β-glucopyranoside] (Fig. 1), which is a new anthocyanin in plants (Andersen and Jordheim, 2006; Harborne and Baxter, 1999).

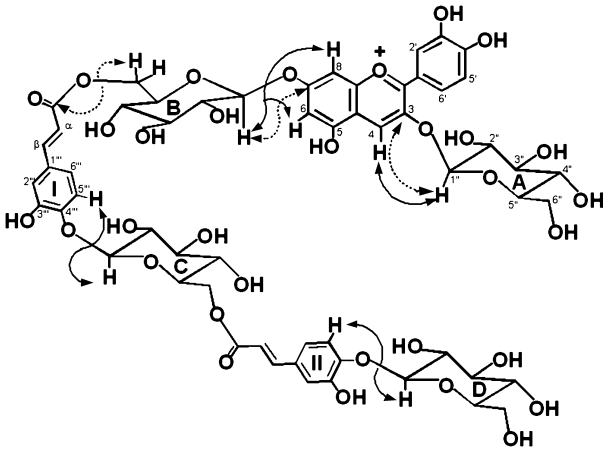

**Fig. 1.** Pigment **1** from the violet flowers of *Bletilla striata*. Observed main NOEs are indicated by arrows. Observed HMBC correlations are indicated by dotted arrows.

*Flower color and anthocyanins of 'Murasaki Shikibu' plant*

The distribution of 7-polyacylated anthocyanins, which are acylated with more than two molecules of aromatic acids at the 7-glycosyl residue, has been reported previously in the four families, Ranunculaceae, Campanulaceae, Compositae, and Goodeniaceae (Honda and Saito, 2002; Saito et al., 2007). In the present study, pigment **1** was found to be a 7-polyacylated anthocyanin. Thus, the family Orchidaceae should be considered as the fifth family possessing 7-polyacylated anthocyanin.

In *Bletilla striata* and other members in some genera of the subfamily Epidendroideae in the Orchidaceae, the flowers contained polyacylated anthocyanidin 3,7,3'-triglucosides and showed colors from red–purple to purple (Tatsuzawa et al., 2004). Thus, we presume that the bluish flowers of 'Murasaki Shikibu' plants may be due to the absence or partial inhibition of the enzyme reaction of anthocyanin 3'-glucosyltrasferase based on the structural analysis of anthocyanins with normal red–purple flowers (Saito et al., 1995a). Fresh perianthes of

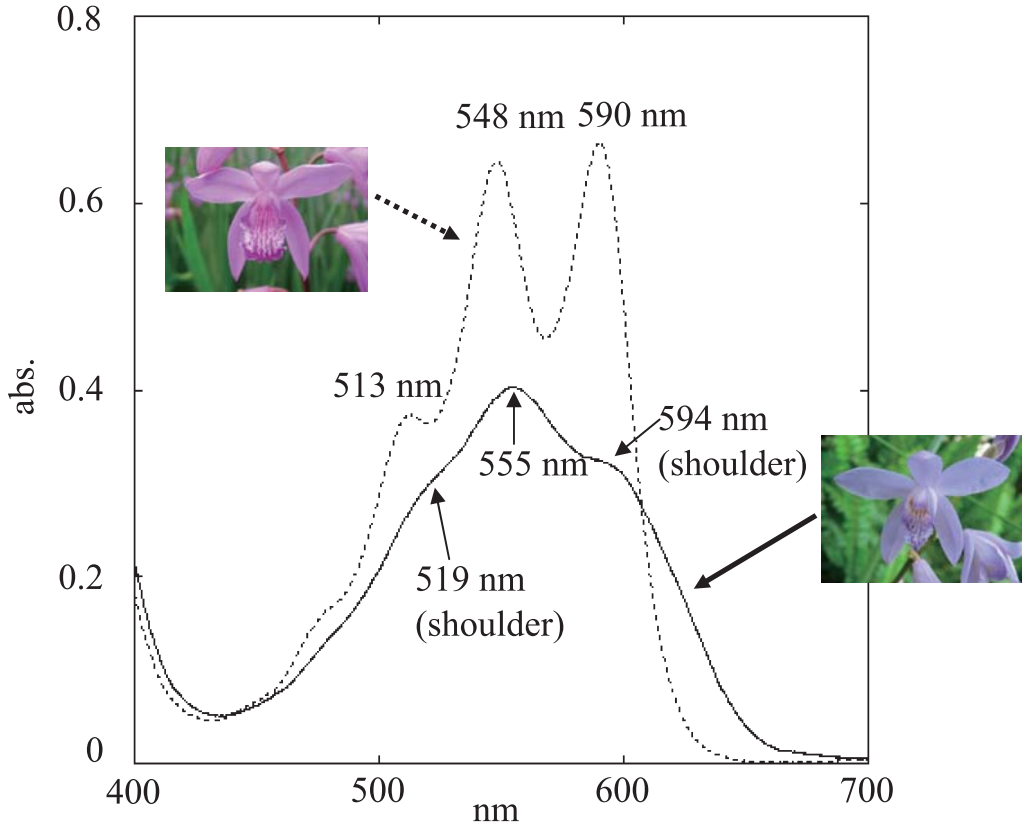


**Fig. 2.** Visible absorption spectra of fresh perianthes of *B. striata* 'Murasaki Shikibu' (—) and fresh normal red–purple perianthes of *B. striata* (---).

'Murasaki Shikibu' plant exhibited a violet color with a chromaticity value of −0.96. The visible absorption curve of intact perianthes (Fig. 2) gave $\lambda_{max}$ at 519sh, 555, 594sh nm and was higher than that of normal red–purple flowers of *B. striata* (chromaticity value = −0.65) (Saito et al., 1995a) of 513, 548, 590 nm (Fig. 2).

As well-recognized previously, 3'-*O*-glycosylation of anthocyanin produces a small color shift towards a shorter wavelength (Saito and Harborne, 1983); for example, cyanidin 3,7-diglucoside (λmax 525, 280 nm in 0.1% HCl-MeOH) and cyanidin 3,7,3'-triglucoside (λmax 513, 281 nm in 0.1% HCl-MeOH). In the present study, it was revealed that the violet flowers of 'Murasaki Shikibu' plant contained cyanidin 3,7-diglucoside as the major deacylanthocyanin, but the normal red–purple flowers of *B. striata* contained cyanidin 3,7,3'-triglucoside as the deacylanthocyanin (Saito et al., 1995a). Thus, the flowers of 'Murasaki Shikibu' plants exhibit a violet color in contrast to the red–purple color of the normal flowers of this plant. On the acylation of anthocyanin, the normal red–purple flowers of this plant contained 7,3'-dipolyacylated anthocyanins as the main anthocyanins. Polyacylated anthocyanins with aromatic acids at the 7- and 3'-positions are considered to make relatively stable and rigid stacking sandwich structures between anthocyanidin and aromatic acids with intermolecular copigmentation, and show characteristic strong and sharp three λmax *in vivo* (Fig. 2) (Figueiredo et al., 1999; Honda and Saito, 2002).

On the other hand, 'Murasaki Shikibu' plant anthocyanin, 7-polyacylated anthocyanin, exhibits rather a dull and broad absorption band, with a λmax (555 nm) in the region of 470–650 nm (Fig. 2), indicating the formation of weak and imperfect sandwich structures being the mixture of open, half-sandwich, and sandwich forms as for the cases of *Platycodon grandiflorium* and *Leschenaultia* 'Violet Lena' (Honda and Saito, 2002; Saito et al., 2007). Therefore, it is presumed that there are two characteristic features of polyacylated cyanidin glycosides for producing the violet color of the flowers of 'Murasaki Shikibu' plant as follows: (1): the 3'-OH of B-ring is free from glycosylation and (2): acylation with hydroxycinnamic acids is restricted only to the 7-*O*-glucose residue.

A similar effect could be expected in other orchids which are glycosylated in the OH-3' position of anthocyanidins, such as *Cattleya*, *Dendrobium*, *Laelia*, ×*Laeliocattleya*, *Phalaenopsis*, *Sophronitis*, and *Vanda* (Saito et al., 1994, 1995a; Tatsuzawa et al., 1994, 1996a, b, 1997, 1998, 2004, 2005, 2006; Williams et al., 2002). In these genera, we are able to postulate that the bluing effect, which is similar to that of 'Murasaki Shikibu' plant, is induced when anthocyanin 3'-glucosyltransferase is obstructed by the recombination of genes by crossbreeding or spontaneous as well as artificial mutation. In general, orchids have a long juvenile phase that requires several years of growth before they flower (Duan and Yazawa, 1994). Thus, our findings would contribute to the efficient improvement of bluish flower breeding for orchids. DNA marker(s) of anthocyanin 3'-glucosyltransferase will be developed for the efficient selection of plants with bluish flowers in breeding populations in their juvenile phase.

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