Analyses of Pigment Compositions and Anthocyanin Biosynthesis Gene Expression in Hirado Azalea Cultivars

Sunisa Meanchaipiboon¹, Nobuo Kobayashi² and Akira Nakatsuka²*

¹The United Graduate School of Agricultural Sciences, Tottori University, Tottori 680-8853, Japan
²Faculty of Life and Environmental Sciences, Shimane University, Matsue 690-8504, Japan

The Hirado azalea is a large flowering plant and Rhododendron scabrum, R. ripense, R. × mucronatum, and other related cultivars are considered to be its parents. In this study, we investigated the correlation of Hirado azalea cultivars with wild species and old cultivars by analyzing anthocyanidin composition patterns and the expression of anthocyanin biosynthesis genes. Hirado azalea cultivars were divided into four groups according to their pigment compositions. Hirado azalea cultivars with only cyanidin derivatives had red colored flowers similar to those of R. scabrum. Hirado azalea cultivars with both cyanidin and delphinidin derivatives as well as flavonol produced similar flower colors to those of R. ripense and R. macrosepulam. Hirado azalea cultivars with only flavonol had white colored flowers similar to those of R. mucronatum ‘Shiro-ryūkyū’. Hirado azalea cultivars with cyanidin derivatives and flavonol exhibited a wider range of flower colors compared to their parents. All samples expressed F3'H, DFR, and ANS genes, as determined by real-time quantitative RT-PCR. However, the F3'5'H gene was expressed only in samples containing delphinidin derivatives. Moreover, ‘Shiro-ryūkyū’ also expressed all four genes, as did cultivars with colored flowers, even though its flowers are white. These results suggested that the hybridization of Hirado azalea using R. scabrum as the base may produce a wide range of flower colors besides red owing to the presence of the F3'5'H gene from R. ripense, R. macrosepulam, and ‘Shiro-ryūkyū’.

Key Words: flavonoid pigment, flower color, hydroxylation, Rhododendron scabrum.

Introduction

There are about 50 Rhododendron species endemic to Japan. Some of them belong to the subgenus Tsutsusi, section Tsutsusi, and are considered to be important genetic resources for breeding ornamental evergreen azaleas (Kobayashi, 2013; Kobayashi and Kurashige, 2018). Since the Edo era (1603–1868), horticulturalists have selected cultivars and hybrids of the evergreen azaleas from natural population species such as Rhododendron kaempferi, R. macrosepulam, R. indicum, R. ripense, and R. obtusum (Kobayashi et al., 1995, 2000). This floral mutant selection contributed to the development of flower colors and flower shapes of various azalea cultivar groups such as Ōkirishima, Ryūkyū, Edo Kirishima, Kurume, Hirado, and Satsuki (Kobayashi, 2013).

Anthocyanin and flavonol are the main pigments of evergreen azalea flowers. Anthocyanins are stable and water-soluble pigments (Asen and Budin, 1966; De Loose, 1969; Mizuta et al., 2009). Anthocyanins are modified anthocyanidins; major anthocyanidins in azaleas are cyanidin and delphinidin derivatives. Flavonol has two major derivatives, myricetin and quercetin. Cyanidin derivatives are cyanidin (Cy) and peonidin (Pn), while delphinidin derivatives are delphinidin (Dp), petunidin (Pt), and malvidin (Mv). Previous studies have reported that purple-colored flowers of R. kiusianum contain both cyanidin and delphinidin derivatives, whereas red-colored flowers of R. kaempferi contains only cyanidin derivatives (Sakata et al., 1991, 1993). Natural hybrids, intermediates of the above two Rhododendron species, display a range of flower colors.

The Hirado azalea cultivar belongs to a group of evergreen azaleas bred in Hirado city, Nagasaki Prefecture...
in Japan (Galle, 1987; Kobayashi, 2016). They are large flowered cultivars with various flower colors (Tamura, 1963). Tamura (1963) investigated Hirado azalea cultivars and their putative parents, R. scabrum, R. ripense, R. × mucronatum, and other cultivars based on their floral characteristics such as color, shape, and number of pistils and stamens. R. scabrum is widely considered to be one of the founding parents for the hybridization of Hirado azalea cultivars as the morphologies they exhibit are similar. The red-colored flowers of R. scabrum contain only cyanidin derivatives (Mizuta et al., 2009). Therefore, it is speculated that the hybridization of R. scabrum with other wild species, or old cultivars containing delphinidin derivatives and flavonol, may have contributed to the wide variety of flower colors observed in Hirado azalea. At present, there are about 300 Hirado azalea cultivars in Japan (Galle, 1987; Kobayashi, 2016).

Currently, Hirado azalea cultivars have only been studied in terms of their similar morphology with their putative parents. There has been no report on their pigment composition or gene expression analysis. In order to develop a new flower color breeding program for large flowered hybrids, it is necessary to understand the lineage from which Hirado azalea cultivars developed. Therefore, we investigated Hirado azalea cultivars and their related parents based on flower color, pigment composition, and flavonoid-related biosynthesis gene expression and identified the correlations among them.

Materials and Methods

Plant materials

Hirado azalea cultivars were collected as pot plants. These were cutting clones from authorized collections in Hirado city, Nagasaki Prefecture. Fresh petals of fully open flowers of evergreen azaleas (without blotches), including six samples of three wild species and 25 cultivars of the subgenus Tsutsusi, were collected from an experimental field of the Faculty of Life and Environmental Science, Shimane University, Shimane Prefecture from the middle of April to the end of June in two years, 2018 and 2019 (Fig. 1; Table 1).

The fresh petals were boiled in water at 100°C for 10 s and dried for 20 h at 40°C. They were then stored in a desiccator at 4°C until HPLC analysis was performed. For molecular analysis, fresh petals of six wild species and 18 cultivars were collected at stage 3 (candle stage, closed flower buds). Petals were frozen in liquid nitrogen and stored at −80°C until RNA extraction was performed.

Flower color measurement

The flower color of each sample was recorded by photograph (Fig. 1). Flower color was measured using the Royal Horticultural Society Color Chart (RHSCC 6th edition) and a Color reader (CR-10; Konica Minolta Sensing Inc., Tokyo, Japan) for lightness \( L^* \) and two chromatic components \( a^* \) and \( b^* \) (Mizuta et al., 2009).

HPLC analysis of anthocyanidin and flavonol

The procedures used for the pigment extraction were performed according to Mizuta et al. (2009) with minor modifications. Dried petals (ca. 50 mg) of each sample were extracted for 24 h at 4°C in the absence of light with 4 mL of 50% CH$_3$COOH in H$_2$O. The crude extracts were concentrated to small amounts and hydrolyzed with 4 mL of 2N hydrochloric acid at 100°C for 1 h. The hydrolysates were absorbed on a Sep-pak C$_{18}$ cartridge. The cartridge was washed to eliminate any water soluble or hydrophilic contaminants and the anthocyanidins and flavonols were eluted by 50% CH$_3$COOH in H$_2$O. The HPLC system used LC solution (Shimadzu Corp., Kyoto, Japan), an SPD-M20A UV-Vis photodiode array detector, a LC-20AD liquid
chromatograph and a CTO-20A column oven with a Poroshell 120 SB-C18 (2.1 mm i.d. × 50 mm; Agilent Technologies, USA). The HPLC analytical conditions used to investigate the anthocyanidin composition were a ratio of 20% solvent A [MeOH] to 80% solvent B [HCOOH-H₂O (1:99, v/v)]. The samples were run for 40 minutes at 40°C with a flow rate of 0.5 mL/min and monitored at 530 nm. The same HPLC system was used to investigate the presence of flavonols and monitoring was done at 360 nm (Mizuta et al., 2014).

RNA extraction and cDNA synthesis

Total RNA was extracted from the petals using the Hot-borate method (Wan and Wilkins, 1994). To avoid DNA contamination, DNA digestion was performed according to Mizuta et al. (2010). Total RNA (5 μg) treated with DNase I was reverse-transcribed by oligo (dT) and ReverTra Ace reverse transcriptase (TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturer’s instructions. The first strand of synthesized cDNA was used for gene expression analysis.

Gene expression analysis by qRT-PCR

The investigation of gene expression involved anthocyanin biosynthesis was carried out according to Mizuta et al. (2014) with minor modifications. The gene-specific primers for F3’H (flavonoid 3’ hydroxylase) (GenBank/EMBL/DDBJ Accession no: AB289597), F3’5’H (flavonoid 3’, 5’ hydroxylase) (AB289598), DFR (dihydroflavonol reductase) (AB289995), ANS (anthocyanidin synthase) (AB289596), and Histone H3 (AM932886) were used in this study (De Keyser et al., 2009; Nakatsu et al., 2008).

Table 1. The evergreen azaleas used in this study.

| Section or cultivar group | Sample no. | Materials | RHSCC* | L* | a* | b* | h*
|---------------------------|------------|-----------|--------|----|----|----|----|
| Wild species              | Sec. Tsutsusi | 1        | R. scabrum 1 | 47D | 57.6 | 49.3 | 12.4 | 14.1 |
|                           |            | 2        | R. scabrum 2 | 50B | 47.1 | 52.5 | 18.5 | 19.4 |
|                           |            | 3        | R. ripense 1 | 75A | 55.1 | 43.2 | −22.3 | 328.2 |
|                           |            | 4        | R. ripense 2 | N80D | 60.1 | 31.0 | −19.2 | 332.7 |
|                           |            | 5        | R. macrosepalum 1 | 76B | 68.6 | 24.1 | −12.6 | 332.4 |
|                           |            | 6        | R. macrosepalum 2 | 84C | 65.2 | 28.4 | −14.2 | 333.4 |
| Cultivars                 | <Ōkirishima group> | 7        | R. × pulchrum ‘Ōmurasaki’ | N74B | 45.0 | 51.5 | −18 | 340.7 |
|                           |            | 8        | ‘Hinomoto’ | 47C | 47.7 | 55.2 | 12.7 | 13.0 |
|                           |            | 9        | ’Raijin’ | 47D | 56.3 | 49.6 | 13.4 | 15.1 |
|                           |            | 10       | ‘Rashōmon’ | 51B | 50.8 | 51.2 | 15.3 | 16.6 |
|                           |            | 11       | ‘Hiōgi’ | 52C | 50.1 | 50.8 | 9.9 | 11.0 |
|                           |            | 12       | ‘Kumo-no-ue’ | 52C | 62.0 | 46.4 | 11.3 | 13.7 |
|                           |            | 13       | ‘Miyo-no-haru’ | 55B | 57.8 | 48.6 | 10.2 | 11.8 |
|                           |            | 14       | ‘Heiwa-no-hikari’ | 55C | 67.9 | 42.8 | 8.0 | 10.6 |
|                           |            | 15       | ‘Shinsō’ | N57C | 57.6 | 50.0 | −2.5 | 357.1 |
|                           |            | 16       | ‘Wakakoma’ | N57C | 51.2 | 55.4 | −3.1 | 356.8 |
|                           |            | 17       | ‘Zanshō’ | N57D | 50.8 | 56.2 | −4.1 | 355.8 |
|                           | <Hirado azalea group> | 18       | ‘Hinode’ | 63B | 48.4 | 55.2 | 1.3 | 1.3 |
|                           |            | 19       | ‘Shō-no-shin’ | N66C | 52.8 | 55.0 | −3.0 | 356.9 |
|                           |            | 20       | ‘Seibo’ | N66C | 57.7 | 49.1 | −8.7 | 350.0 |
|                           |            | 21       | ‘Saotome’ | 67C | 49.0 | 57.7 | −1.6 | 358.4 |
|                           |            | 22       | ‘Banzairaku’ | 68B | 54.9 | 53.2 | −5.6 | 354.0 |
|                           |            | 23       | ‘Ademurasaki’ | 72C | 47.5 | 50.5 | −16.2 | 342.2 |
|                           |            | 24       | ‘Momoyama’ | 73B | 63.9 | 43.6 | −7.0 | 350.9 |
|                           |            | 25       | ‘Taihō’ | N74D | 52.6 | 46.6 | −15.6 | 341.5 |
|                           |            | 26       | ‘Hirado-no-homare’ | 77D | 64.4 | 34.4 | −12.1 | 340.6 |
|                           |            | 27       | ‘Hakuhō’ | NN155C | 92.8 | 1.1 | 6.5 | 80.4 |
|                           |            | 28       | ‘Shiro-ryūkyū’ | NN155D | 92.4 | 1.4 | 6.0 | 76.6 |
|                           |            | 29       | ‘Tanima-no-yuki’ | NN155D | 93.6 | 1.1 | 4.8 | 77.6 |
|                           |            | 30       | ‘Hatsuyuki’ | NN155D | 93.4 | 1.4 | 4.2 | 71.8 |
|                           | <Ryūkyū azalea group> | 31       | R. × mucronatum ‘Shiro-ryūkyū’ | NN155D | 92.9 | 1.5 | 5.8 | 75.2 |

x Refer to the Royal Horticultural Society Colour Chart.
y L*, lightness; a* and b*, chromatic components.
x h, hue angle (degree) = arctan (b*/a*).

RNA extraction and cDNA synthesis

Total RNA was extracted from the petals using the Hot-borate method (Wan and Wilkins, 1994). To avoid DNA contamination, DNA digestion was performed according to Mizuta et al. (2010). Total RNA (5 μg) treated with DNase I was reverse-transcribed by oligo (dT) and ReverTra Ace reverse transcriptase (TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturer’s instructions. The first strand of synthesized cDNA was used for gene expression analysis.

Gene expression analysis by qRT-PCR

The investigation of gene expression involved anthocyanin biosynthesis was carried out according to Mizuta et al. (2014) with minor modifications. The gene-specific primers for F3’H (flavonoid 3’ hydroxylase) (GenBank/EMBL/DDBJ Accession no: AB289597), F3’5’H (flavonoid 3’, 5’ hydroxylase) (AB289598), DFR (dihydroflavonol reductase) (AB289995), ANS (anthocyanidin synthase) (AB289596), and Histone H3 (AM932886) were used in this study (De Keyser et al., 2009; Nakatsu et al., 2008).
The cDNA was amplified using TB Green II (Takara Bio Inc., Shiga, Japan) with a Thermal Cycler Dice Real Time System. Amplification of histone cDNA was used as an internal control and performed under identical conditions to normalize the levels of cDNA. The thermal cycling conditions were 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and finally 30 s at 60°C. Three replications were done for each cDNA sample. Quantitation was performed by using the difference in the cycle threshold values between the target genes and reference gene (Histone H3) to calculate the relative amounts of the template presence (Cheon et al., 2017).

The mean and standard error were calculated for the relative expression value.

Results

Flower color measurement analysis

The RHSCC numbers of *R. scabrum* 1 and 2 were 47D and 50B, both of which belonged to a red group. For *R. ripense* and *R. macrosepalum*, the RHSCC numbers were 75A and 76B, respectively and they belonged to a purple group. The RHSCC numbers of *R. ripense* 2 and *R. macrosepalum* were N80D and 84C, and they belonged to a purple-violet group. The RHSCC numbers of ‘Shiro-ryūkyū’ and ‘Ōmurasaki’ were NN155D and N74B, and they belonged to a white and red-purple group, respectively. The Hirado azalea cultivars had RHSCC in a range from 47C (red group) to 84C (purple-violet group), and from NN155C to NN155D (white). This shows that Hirado azalea cultivars had a wide range of flower colors (Fig. 1).

In the red group, the $a^*$ and $b^*$ values were in the range of 42.8 to 55.2 and 8.0 to 18.5, respectively. In the red-purple group, the $a^*$ and $b^*$ values were in the range of 43.6 to 57.7 and −18.0 to 1.3, respectively, while these values in the purple and purple-violet groups were in the range of 24.1 to 43.2 and −22.3 to −12.1, respectively. For the white group, the $a^*$ and $b^*$ values varied from 1.1 to 1.5 and 4.2 to 6.5, respectively (Table 1.). The chromatic components $a^*$ and $b^*$ of Hirado azalea cultivars showed that they were gently distributed into four clusters according to their flower color group. Flower colors of Hirado cultivars were more diverse than their related parents (Fig. 2). The hue angle ($h$) of the red flower group varied from 14.1° to 19.4°, and the red-purple group varied from 340.7° to 1.3°. The purple and purple-violet flower group varied from 328.2° to 340.6°, and the white flower group varied from 71.8° to 80.4°.

The lightness ($L^*$ value) of the red flower group was in the range of 47.1 to 67.9, whereas the red-purple flower group ranged from 45.0 to 63.9. The $L^*$ values of the purple and purple-violet flower group varied from 55.1 to 68.6. In the white flower group, the $L^*$ values varied from 92.4 to 93.6 (Table 1).

Pigment composition analyses

The HPLC analysis showed that only cyanidin derivatives (Cy and Pn) were found in *R. scabrum* 1 and 2, while Cy-, Dp-derivatives (Dp, Pt, and Mv) and flavonol were found in wild species (*R. ripense* 1 and 2, *R. macrosepalum* 1 and 2) and ‘Ōmurasaki’ (Table 2). HPLC analysis ‘Shiro-ryūkyū’ revealed the presence of small anthocyanidin peaks of both cyanidin and delphinidin derivatives; however, anthocyanin was not present (data not shown).

Hirado azalea cultivars were divided into four groups according to their pigment compositions. The first group was composed of four red flowered cultivars, ‘Hinomoto’, ‘Rajin’, ‘Rashōmon’, and ‘Hiōgi’, pigmented with Cy-derivatives and with no flavonol. The second group was composed of three red flowered cultivars, ‘Kumo-no-ue’, ‘Heiwa-no-hikari’, and ‘Miyo-no-harai’, and nine red-purple flowers pigmented with Cy-derivatives and flavonol (Table 2). The third group included two red-purple flowers, ‘Ademurasaki’ and ‘Taihō’, and one purple flower, ‘Hirado-no-homare’ contained both Cy- and Dp-derivatives as well as flavonol. The fourth group included four white flowered Hirado azalea cultivars containing only flavonol.

Anthocyanin synthesis-related gene expression analyses

The four genes, F3’H, F3’5’H, DFR, and ANS, extracted from petals were investigated using qRT-PCR. F3’H, the synthesis gene for the Cy-derivative, was expressed in all samples. F3’5’H, the synthesis gene for the Dp-derivative, was expressed only in the samples containing Dp-derivatives. DFR and ANS were expressed in all the samples, similar to F3’H (Fig. 3). All white flowers also expressed all four genes similarly to colored flowers, and this indicates the potential to produce anthocyanidin pigments.

Discussion

In order to understand pigment composition and gene
expression, we investigated the relationships between Hirado azalea cultivars and their related wild species based on their pigment composition using HPLC. We also analyzed their gene expression using qRT-PCR. An earlier study analyzed the pigment composition of *R. scabrum*, *R. ripense*, *R. macrosepalum*, and ‘Shiro-ryūkyū’ by HPLC (Mizuta et al., 2009). *R. scabrum* had large red flowers pigmented with Cy-derivatives; *R. ripense* and *R. macrosepalum* had large purple and purple-violet flowers, all of which were pigmented with Cy- and Dp-derivatives and flavonol (Fig. 1; Table 2). ‘Shiro-ryūkyū’ contained only flavonol. Recent research also confirmed that no anthocyanins were detected in white flowers of five wild species, including *R. mucronatum* (Du et al., 2018).

Hirado cultivar groups were divided into four pigment groups: 1) Cy-derivatives only without flavonol, 2) Cy-derivatives and flavonol, 3) Cy- and Dp-derivatives and flavonol, and 4) flavonol only. The distribution of the chromatic components $a^*$ and $b^*$ were diversely distributed. Moreover, the $b^*$ distribution could be divided into two major groups; positive and negative $b^*$ (Fig. 2; Table 1) and a correlation between flower color and pigment was shown. Negative $b^*$ values indicate that flowers tended to have a blueish tone, that is, flowers with Cy-derivatives and flavonol, while flowers with both Cy- and Dp-derivatives and flavonol tended to have bluer tone.

The correlation of flower colors and pigment compositions between the Hirado azalea cultivars and their parents was investigated. The RHSCC of Hirado azalea cultivars varied from 47C to 52C and only Cy-derivatives contributed to their red flower colors (Fig. 2; Tables 1 and 2). De Loose (1970b) investigated

| Flower color group | Sample no. | Materials | Anthocyanidin (%)$^z$ | Flavonol$^y$ |
|--------------------|------------|-----------|----------------------|-------------|
| Red                | 1          | *R. scabrum* 1 | 100 0 0 0 0   | −           |
|                    | 2          | *R. scabrum* 2 | 100 0 0 0 0   | −           |
|                    | 8          | ‘Hinomoto’     | 100 0 0 0 0   | −           |
|                    | 9          | ‘Raijin’       | 100 0 0 0 0   | −           |
|                    | 10         | ‘Rashōmon’     | 100 0 0 0 0   | −           |
|                    | 11         | ‘Hiōgi’        | 65.4 34.6 0 0 0 | −           |
|                    | 12         | ‘Kumo-no-ue’   | 100 0 0 0 0   | +           |
|                    | 13         | ‘Heiwa-no-hikari’ | 100 0 0 0 0   | +           |
|                    | 14         | ‘Miyō-no-haru’ | 72.7 27.3 0 0 0 | +           |
| Red-purple         | 15         | ‘Shinshō’      | 100 0 0 0 0   | +           |
|                    | 16         | ‘Wakakoma’     | 100 0 0 0 0   | +           |
|                    | 17         | ‘Zanshō’       | 100 0 0 0 0   | +           |
|                    | 18         | ‘Hinode’       | 100 0 0 0 0   | +           |
|                    | 19         | ‘Shō-no-shin’  | 100 0 0 0 0   | +           |
|                    | 22         | ‘Banzairaku’   | 100 0 0 0 0   | +           |
|                    | 24         | ‘Momoyama’     | 100 0 0 0 0   | +           |
|                    | 20         | ‘Seibo’        | 46.4 53.6 0 0 0 | +           |
|                    | 21         | ‘Saotome’      | 61.6 38.4 0 0 0 | +           |
|                    | 7          | ‘Ōmurasaki’    | 24 21 11 4 40 | +           |
|                    | 23         | ‘Ademurasaki’  | 42.5 3.5 24.7 4.3 25.1 | + |
|                    | 25         | ‘Taihō’        | 47.4 2.3 27.3 5.9 15.9 | + |
| Purple             | 3          | *R. ripense* 1 | 35 14 7 0 45   | +           |
|                    | 5          | *R. macrosepalum* 1 | 56.6 5.1 21.1 0 17.3 | + |
|                    | 26         | ‘Hirado-no-homare’ | 39.5 0 60.5 0 0   | +           |
| Purple-violet      | 4          | *R. ripense* 2 | 42.9 6.7 9.0 0 41.4 | + |
|                    | 6          | *R. macrosepalum* 2 | 58.9 7.7 11.4 1.1 20.9 | + |
| White$^x$          | 27         | ‘Hakuhō’       | 73.8 0 23.2 0 0   | +           |
|                    | 28         | ‘Shiro-kyū’    | 72.4 0 27.6 0 0   | +           |
|                    | 29         | ‘Tanima-no-yuki’ | 83.8 0 16.2 0 0   | +           |
|                    | 30         | ‘Hatsu-yuki’   | 68.5 0 31.5 0 0   | +           |
|                    | 31         | ‘Shiro-ryūkyū’ | 63.0 0 37.0 0 0   | +           |

$^z$ Cy: Cyanidin, Pn: Peonidin, Dp: Delphinidin, Pt: Petunidin, Mv: Malvidin.
$^y$ (−): absent, (+): present.
$^x$ White flowers contained only little amount of anthocyanidins.
the flower color and pigment compositions of natural bud-variants of *R. simsii*. The RHSCC of the cultivar ‘Mme Petrick’ was 57D, with high levels of both Cy-derivatives and flavonol, while that of its orange sports was 50B, with low levels of flavonol. This corresponds with our result in that Hirado azalea cultivars contained only cyanidin derivatives and the RHSCC was less than or equal to 52. This suggests that the pigments had the same effects on flower color in Hirado azalea cultivars as those observed in small flowered *R. simsii*.

Another group of Hirado azaleas contained Cy-derivatives and flavonol. Their RHSCC varied from 52C to 73B and had wider range of purplish red color flowers (Fig. 2; Tables 1 and 2). Moreover, co-pigmentation was observed in the red-purple group. The co-pigmentation between anthocyanin and flavonol is known to contribute to a bluing effect, as previously reported in *R. simsii* (Asen et al., 1971, 1972; De Loose, 1970a; Huyen et al., 2016).

The Hirado cultivar group pigmented with Cy- and Dp-derivatives and flavonol, with RHSCC ranging from 72C to 77D, had reddish purple and purple flowers (Fig. 1; Tables 1 and 2). They were more bluish than the second group of Hirado azalea cultivars that contained Cy-derivatives and flavonol. This may be due to the presence of Dp-derivatives that contribute to a bluish coloration in various plants. Normally, *Rosa hybrida* contains cyanidin derivatives and flavonol; therefore, it lacks blue flower hues such as purple and blue. In the genetically engineered rose cultivar ‘Lavande’ that carries F3’5’H, the production of delphinidin derivatives was triggered and this resulted in bluer flower hues. The greater the percentage of delphinidin derivatives, the higher the bathochromic shifts towards bluer color were observed (Katsumoto et al., 2007). Similarly, Hirado azalea cultivars that contained delphinidin derivatives showed a bathochromic shift towards a bluer tone to a greater extent than Hirado azalea cultivars containing only cyanidin derivatives and flavonol (Fig. 4). The last group was Hirado azalea containing only flavonol without any anthocyanin. They
were white flowered Hirado azalea with an RHSCC range of NN155C to NN155D, similar to ‘Shiro-ryūkyū’.

The gene expression of four genes was investigated. All samples were shown to express F3’H, DFR, and ANS, while F3’5’H was expressed only in samples containing delphinidin derivatives. Moreover, ‘Shiro-ryūkyū’ normally expressed F3’H, F3’5’H, DFR, and ANS like colored flowers, despite the absence of anthocyanin (data not shown). White flowered Hirado azalea cultivars also expressed all four genes the same as ‘Shiro-ryūkyū’. Mizuta et al. (2009) also reported that no anthocyanins were detected, but anthocyanidins were detected, in white flowers of R. ripense and ‘Shiro-ryūkyū’. This implies that ‘Shiro-ryūkyū’ and the white flowered Hirado azaleas ‘Hakuhō’, ‘Shiro-kyaku’, ‘Tanima-no-yuki’, and ‘Hatsuyuki’ are able to synthesize the anthocyanin precursor as demonstrated by peaks detected in the anthocyanidin analysis. The absence of anthocyanin may result from a malfunction in the downstream step of anthocyanin biosynthesis. It is also known that white is homozygote recessive in R. × mucronatum (Heursel and Horn, 1977). We assume that old white flower cultivars such as ‘Shiro-ryūkyū’ express F3’5’H, but R. scabrum with no F3’5’H expression was used for development of white Hirado cultivars.

Delphinium flowers have white, red, and blue flowers which contain flavonol, pelargonidin (Pg), and Dp, respectively. However, they do not contain any Cy, which is synthesized by F3’H. Delphinium zalii had white flowers and contained only flavonol. However, it had functional F3’H and lacked ANS, resulting in an absence of anthocyanins. Delphinium cardinal has red flowers pigmented with Pg and lacks F3’H, so it did not contain any Cy. The hybridization of these two cultivars resulted in purple flowered progenies which had hydroxylation ability, thus could synthesize Cy. This suggests that D. zalii has functional F3’H which can be passed down to its progenies (Sakaguchi et al., 2019).

Similarly, ‘Shiro-ryūkyū’ was able to synthesize Dp-derivatives. This suggests that the hydroxylation ability of ‘Shiro-ryūkyū’ may be passed down to progenies like delphinium. Red flowered Hirado azalea cultivars exhibited similar patterns to R. scabrum as they contained only Cy-derivatives and showed no expression of F3’5’H. On the hand, non-red colored flowers of Hirado cultivars also contained either flavonol, or both Dp-derivatives and flavonol. This indicates that the cross combination of R. scabrum with other wild species or cultivars may have contributed to the wider flower color variation in Hirado azalea cultivars.

In this study, we found a correlation between Hirado azalea cultivars and their related parents. This knowledge will be useful to develop a breeding program for large flowered azalea cultivars. Currently, we are investigating the genomic DNA of large flowered wild species for F3’5’H sequences. This may lead to the development of an F3’5’H marker to determine the origin of cultivars containing Dp in the future.

Acknowledgements

The authors thank the Faculty of Life and Environmental Sciences at Shimane University for financial support in publishing this report.

Literature Cited

Asen, S. and P. S. Budin. 1966. Cyanidin 3-arabinoside-5-glucoside, an anthocyanin with a new glycosidic pattern, from flowers of “Red Wing” azaleas. Phytochemistry 5: 1257–1261.

Asen, S., R. N. Stewart and K. H. Norris. 1971. Co-pigmentation effect of quercetin glycosides on absorption characteristics of cyanidin glycosides and color of red wing azalea. Phytochemistry 10: 171–175.

Asen, S., R. N. Stewart and K. H. Norris. 1972. Co-pigmentation of anthocyanins in plant tissues and its effect on color. Phytochemistry 11: 1139–1144.

Cheon, K. S., A. Nakatsuka, T. Kasaki and N. Kobayashi. 2017. Floral morphology and MADS gene expression in double-flowered Japanese evergreen azalea. Hort. J. 86: 269–276.

De Keyser, E., J. De Riek and E. Van Bockstaele. 2009. Discovery of species-wide EST-derived markers in Rhododendron by intron-flanking primer design. Mol. Breeding. 23: 171–178.

De Loose, R. 1969. The flower pigments of the belgian hybrids of Rhododendron simsii and other species and varieties from Rhododendron subseries obtusum. Phytochemistry 8: 253–259.

De Loose, R. 1970a. Flavonoid glycosides in the petals of some Rhododendron species and hybrids. Phytochemistry 9: 875–879.

De Loose, R. 1970b. Flower pigment composition of natural bud-variants among hybrid chinese azaleas, Rhododendron simsii (Planch.). J. Hortic. Sci. 45: 265–274.

Du, H., L. Lai, F. Wang, W. Sun, L. Zhang, X. Li, L. Wang, L. Jiang and Y. Zheng. 2018. Characterization of flower colouration in 30 Rhododendron species via anthocyanin and flavonol identification and quantitative traits. Plant Biol. 20: 121–129.

Galle, F. C. 1987. Azaleas. Revised and enlarged Ed. Portland, Oregon: Timber Press Inc.

Heursel, J. and W. Horn. 1977. A hypothesis on the inheritance of flower colors and flavonoids in Rhododendron simsii Planch. Z. Pflanzenzüchtung 79: 238–249.

Huyen, D., K. Ureshino, D. Thanh Van and I. Miyajima. 2016. Co-pigmentation of anthocyanin-flavonol in the blotch area of Rhododendron simsii Planch. flowers. Hort. J. 85: 232–237.

Katsumoto, Y., M. Fukuchi-Mizutani, Y. Fukui, F. Brugliera, T. A. Holton, M. Karon, N. Nakamura, K. Yonekura-Sakakibara, J. Tobgami, A. Pigire, G. Q. Tao, N. S. Nehra, C. Y. Lu, B. K. Dyson, S. Tsuda, T. Ashikari, T. Kusumi, J. G. Mason and Y. Tanaka. 2007. Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating delphinidin. Plant Cell Physiol. 48: 1589–1600.

Kobayashi, N. 2013. Evaluation and application of evergreen azalea resources of Japan. Acta Hortic. 990: 213–219.

Kobayashi, N. 2016. Tsutsuji. p. 151–180. In: M. Shibata (ed.).
Hana no hinshu kairyou no nihonshi, Bunkyō, Tokyo (in Japanese).

Kobayashi, N. and Y. Kurashige. 2018. Noto Kirishima Azalea Guidebook. Matsue: Laboratory of Plant Breeding, Faculty of Life and Environmental Science, Shimane University.

Kobayashi, N., T. Handa, K. Yoshimura, Y. Tsumura, K. Arisumi and K. Takayanagi. 2000. Evidence for introgressive hybridization based on chloroplast DNA polymorphism and morphological variation in wild evergreen azalea populations of the Kirishima mountains, Japan. Edinb. J. Bot. 57: 209–219.

Kobayashi, N., R. Takeuchi, T. Handa and K. Takayanagi. 1995. Cultivar identification of evergreen azalea with RAPD method. J. Japan. Soc. Hort. Sci. 64: 611–616.

Mizuta, D., T. Ban, I. Miyajima, A. Nakatsuka and N. Kobayashi. 2009. Comparison of flower color with anthocyanin composition patterns in evergreen azalea. Sci. Hortic. 122: 594–602.

Mizuta, D., A. Nakatsuka, T. Ban, I. Miyajima and N. Kobayashi. 2014. Pigment composition patterns and expression of anthocyanin biosynthesis genes in Rhododendron kiusianum, R. kaempferi, and their natural hybrids on Kirishima Mountain Mass, Japan. J. Japan. Soc. Hort. Sci. 83: 156–162.

Mizuta, D., A. Nakatsuka, I. Miyajima, T. Ban and N. Kobayashi. 2010. Pigment composition patterns and expression analysis of flavonoid biosynthesis genes in the petals of evergreen azalea ‘Oomurasaki’ and its red flower sport. Plant Breeding 129: 558–562.

Nakatsuka, A., D. Mizuta, Y. Kii, I. Miyajima and N. Kobayashi. 2008. Isolation and expression analysis of flavonoid biosynthesis genes in evergreen azalea. Sci. Hortic. 118: 314–320.

Sakata, Y., K. Arisumi and I. Miyajima. 1991. Some morphological and pigmental characteristics in Rhododendron kaempferi Planch., R. kiusianum Makino and R. eriocarpum Nakai in southern Kyushu. J. Japan. Soc. Hort. Sci. 60: 669–675.

Sakata, Y., I. Miyajima and K. Arisumi. 1993. Variations in some morphological and pigmental characteristics in Rhododendron kaempferi Planch., R. kiusianum Makino and their natural hybrids on Kirishima mountain mass. J. Japan. Soc. Hort. Sci. 61: 925–932.

Sakaguchi, K., C. Isobe, K. Fujita, Y. Ozeki and T. Miyahara. 2019. Production of novel red-purple delphinium flowers containing cyanidin-based anthocyanin using hybridization breeding. Hort. J. 88: 514–520.

Tamura, T. 1963. Studies on the Hirado-azaleas, with special reference to their formation. Bulletin of the Horticultural Research Station D: 155–185 (in Japanese with English abstract).

Wan, C. Y. and T. A. Wilkins. 1994. A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (Gossypium hirsutum L.). Anal. Biochem. 223: 7–12.