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SUMMARY OF DOCTORAL THESIS

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Title: Flower color and anthocyanin biosynthesis genes analysis in Hirado azalea (*Rhododendron* x *pulchrum* Sweet)
（ヒラドツツジの花色およびアントシアニン合成遺伝子の解析）

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. Since the Edo era (1603-1868), the horticulturalists have selected cultivars and hybrids of the evergreen azaleas from natural population species. The 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. Hirado azalea (*R.* × *pulchrum* Sweet) is known for their large flower varying in colors including white to pink, red, and purple forms. The red-flowered *R. scabrum* G. Don, purple-flowered *R. ripense* Makino, and white-flowered *R.* × *mucronatum* (Blume) G. Don 'Shiro-ryūkyū' are the putative parents of the Hirado azalea cultivars based on their morphology including flower color, shape, and number of pistils and stamens. It is thought that *R. scabrum* is the main putative parent and almost all Hirado azalea cultivars are either similar to *R. scabrum* or *R.* × *pulchrum* 'Ōmurasaki'. However, the origin of this color variation is not well understood based on gene information. On the other hand, flower color is determined by combination of anthocyanins and flavonols as the major pigments in evergreen azalea. Cyanidin derivatives are cyanidin (Cy) and peonidin, whereas delphinidin derivatives are delphinidin (Dp), petunidin, and malvidin. The main anthocyanins in red flower are Cy derivatives, while those in purple flower are both Cy and Dp derivatives. Flavonols are colorless pigment, however its co-pigments with anthocyanin contribute to bluing effect. In this study, I investigated the correlation of Hirado azalea cultivars with their putative parents via genetic analyses and pigment compositions. This information would be helpful for a comprehensive understanding of the flower color characteristics of Hirado azalea cultivars, and it would be contributed to the breeding in respect of flower colors.

1. Analyses of pigment compositions and anthocyanin biosynthesis gene expressions in Hirado azalea cultivars.

Hirado azalea cultivars have only been studied for their similar morphology with their possible parents. There has been no report about their pigment composition and anthocyanin biosynthesis gene expression analysis. In order to develop a new flower color breeding program for large flowered hybrids, it is necessary to understand the pattern of how Hirado azalea cultivars were developed. Hence, I investigated Hirado azalea cultivars and their related parents based on the flower color, pigment composition, and anthocyanin-related biosynthesis gene (*F3'H*; flavonoid 3′ hydroxylase, *F3'5'H*; flavonoid 3′, 5' hydroxylase *DFR*; dihydroflavonol 4-reductase, and *ANS*; anthocyanin synthase) expression and identified the correlation amongst them. Hirado azalea cultivars were divided into four groups according to their pigment compositions. Hirado azalea cultivars ('Hinomoto', 'Raijin', 'Rashōmon' and

‘Hiōgi’) with only Cy derivatives had red colored flowers (Royal Horticultural Society Color Chart (RHSCC) 47C-52C) like those of *R. scabrum*. Hirado azalea cultivars (‘Ademurasaki’, ‘Taihō’ and ‘Hirado-no-homare’) with both Cy and Dp derivatives as well as flavonol exhibited purple flower colors (RHSCC 72C-77D) like those of *R. ripense* and *R. macrosepalum*. Hirado azalea cultivars (‘Hakuhō’, ‘Shiro-kujyaku’, ‘Tanima-no-yuki’, ‘Hatsuyuki’ and ‘Hakurakuten’) with only flavonol had white colored flowers (RHSCC NN155C-NN155D) like those of *R.* × *mucronatum* ‘Shiro-ryūkyū’. Hirado azalea cultivars (‘Kumo-no-ue’, ‘Heiwa-no-hikari’, ‘Miyo-no-haru’, ‘Shinshō’, ‘Zanshō’, ‘Hinode’, ‘Shō-no-shin’, ‘Banzairaku’, ‘Momoyama’, ‘Seibo’ and ‘Saotome’) with Cy derivatives and flavonol exhibited wider flower colors (RHSCC 52C-73B) compared with their putative parents. All Hirado azalea cultivars 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 wild species and azalea cultivars containing Dp derivatives. Moreover, *R.* × *mucronatum* ‘Shiro-ryūkyū’ and Hirado azalea cultivars (‘Hakuhō’, ‘Shiro-kujyaku’, and ‘Hakurakuten’) expressed all four genes (*F3′H*, *F3′5′H*, *DFR*, and *ANS*), as did cultivars with colored flowers; despite its flowers is white. These results suggested that the hybridization of Hirado azalea using *R. scabrum* as the base may produce wide range of flower colors besides red owing to the presence of the *F3′5′H* gene from *R. ripense*, *R. macrosepalum*, *R.* × *mucronatum* ‘Shiro-ryūkyū’.

2. Genetic relationship of Hirado azalea cultivars and their putative parents based on *F3′5′H*.

Hirado azalea cultivars show a wide range of flower color variation, and *F3′5′H* gene expression showed a correlation with Dp derivatives in Hirado azalea cultivars. However, the genetic basis of this color variation is not well understood. In this chapter, I investigated the anthocyanin pathway gene; *F3′5′H*, by genomic DNA analysis, cDNA sequence analysis, and deduction of amino acid sequences to assess the genetic relationships between these taxa and to investigate the genetic basis of color variation in this group. In *R. scabrum* and red and pink flowered Hirado azalea cultivars, in which Dp derivatives are absent, only the exon 2 region amplified using specific primers in coding region of *F3′5′H* gene, except exon 1, suggesting that DNA structure of exon 1 would be defective in these wild species and azalea cultivars lacked Dp derivatives. On the other hand, *R. ripense*, *R. macrosepalum*, and *R. yedoense* var. *poukhanense* and *R.* × *mucronatum* ‘Shiro-ryūkyū,’ with Dp derivatives have normal DNA structure of *F3′5′H* gene. The lengths of the *F3′5′H* cDNA nucleotide sequences of these wild species were 1533 bp (510 AA), whereas in *R.* × *mucronatum* ‘Shiro-ryūkyū,’ two different sequence lengths were observed—1533 and 1551 bp (510 and 516 AA). *R. ripense*, *R.* × *mucronatum* ‘Shiro-ryūkyū’, and purple and white flowered four Hirado azalea cultivars (‘Ademurasaki’, ‘Hirado-no-homare’, ‘Shirokujyaku’ and ‘Hakuhō’) were grouped in the same cluster in the *F3′5′H* gene phylogeny. Among the four Hirado azalea cultivars, the lengths of *F3′5′H* in coding region were 1551 bp, which had a 5 bp insertion in adjacent to the stop codon, in ‘Ademurasaki’ and ‘Hakuhō’. Whereas ‘Hirado-no-homare’ and ‘Shirokujyaku’ lacked this insertion and showed 1533 bp coding region. When PCR was performed to distinguish 5 bp insertion, the amplified product was found in some *R. ripense* individuals and *R.* × *mucronatum* ‘Shiro-ryūkyū’ but not in *R. scabrum* and *R. macrosepalum*. These results suggest that the wide range of flower color in Hirado azalea cultivars is caused by variation in the *F3′5′H* genotype derived from hybridization between *R. scabrum* and either *R. ripense* or *R.* × *mucronatum* ‘Shiro-ryūkyū.’

In this study, I analyzed Hirado cultivar and related wild species of evergreen azalea to assess the relationship between flower colors, anthocyanin composition and expression of anthocyanin biosynthesis genes, especially *F3′5′H* gene. The profiles of gene expression and pigment composition will progress understanding of the regulation of azalea pigmentation and it would be also useful for breeding new azalea cultivars with novel flower colors.