Comparison of Expression *CCD4* Gene Levels in Petals of Evergreen Azalea Species

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Intersubgeneric crosses between white-flowered evergreen azalea species and a yellow-flowered deciduous azalea (*Rhododendron japonicum* f. *flavum*) can produce yellow-flowered evergreen azaleas. The petal color of F₁ progenies from these crosses is known to fade during petal development due to a high expression of the carotenoid cleavage dioxygenase 4 (*CCD4*) gene inherited from the evergreen species. In this study, to facilitate the selection of evergreen azalea species as cross parents that have low expression levels of *CCD4* in developing petals, *CCD4* expression levels were compared among 16 evergreen azalea species belonging to the subgenus *Tsutsusi*. The identity values of the deduced amino acid sequences of *CCD4* were high (from 97% to 100%) among evergreen azalea species. The expression levels of *CCD4* genes in petals of evergreen azalea species at the day of anthesis varied widely from 2.04 to 20.47 times relative to that in *R. japonicum* f. *flavum*. Among these species, *R. amanoi* maintained low *CCD4* expression levels throughout all petal development stages that were not significantly different from those of *R. japonicum* f. *flavum* (JPN). This species is therefore considered a promising breeding material for producing yellow-flowered evergreen azaleas.

Key Words: *CCD4*, fading of petal color, *Rhododendron*.

Introduction

Evergreen azaleas (subgenus *Tsutsusi* of the genus *Rhododendron*) are highly popular ornamental woody plants. Cultivated varieties have been developed by natural and artificial hybridization and selection among evergreen species belonging to the subgenus *Tsutsusi* in the genus *Rhododendron*. The flower colors of cultivated varieties are mainly variations of white, pink, red, or purple. However, no yellow-flowered cultivar of evergreen azaleas has been produced to date.

Creating yellow-flowered cultivars is one of the important breeding objectives for evergreen azaleas. *R. japonicum* f. *flavum* is a yellow-flowered deciduous species belonging to the subgenus *Pentanthera* (Yamazaki, 1996). Its yellow petals contain carotenoid compounds such as β -carotene and lutein as the main pigments (Ureshino et al., 2016). To create yellow-

flowered evergreen azaleas, we conducted intersubgeneric crosses between evergreen azalea species and *R. japonicum* f. *flavum*, and many progenies were obtained (Ureshino et al., 1998). Although these progenies were semi-deciduous, the petal color of the buds on the progenies was light yellow just before anthesis, and these petals contained carotenoid pigments (Miyajima et al., 2000). However, the light-yellow color of the petals was not stable, and it faded as the flower developed.

In a previous report, we compared the concentration and composition of carotenoids and the expression patterns of carotenoid biosynthesis and cleavage genes during petal development in a yellow-flowered deciduous azalea (*Rhododendron japonicum* f. *flavum*), a white-flowered evergreen azalea ('Miyamasatsuki'), and their progeny, to determine the factors that cause discoloration as the petals of the progeny develop (Ureshino et al., 2016). In petals of *R. japonicum* f. *flavum*, a high expression of carotenoid biosynthetic genes and low expression of *CCD4* were observed, and total carotenoid concentrations were maintained during petal development. On the contrary, in white-flowered evergreen azalea, low expression levels of carotenoid biosynthetic genes and a high expression of *CCD4* were observed during petal development, and as a result,

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carotenoids barely accumulated in the petals. The progeny has a relatively high expression of carotenoid biosynthetic genes inherited from *R. japonicum* f. *flavum*. Carotenoid degradation in the petals of the progeny was mediated by the high *CCD4* gene expression inherited from white-flowered evergreen azalea. Thus, selecting evergreen azaleas that show low *CCD4* expression during petal development as breeding material is useful to avoid discoloration of petals in the progenies.

Many evergreen azalea species belong to the section Tsutsusi of the subgenus *Tsutsusi*; the latter includes two subsections—subsect. Scabra and subsect. Tsutsusi (Yamazaki, 1996). There are 22 species in this section, and morphological traits and growth habitats vary widely among them. Therefore, there may also be variation in *CCD4* expression among these species. The objective of this study was to identify the species that maintained low expression levels of *CCD4* in petals during flower

development in 16 evergreen azalea species.

Materials and Methods

CCD4 gene isolation and sequence analysis

Plant materials are listed in Table 1. As evergreen azalea species, 26 plants of the subgenus *Tsutsusi* were used, in which six species belonged to the ser. Scabra subsect. Scabra, four species belonged to ser. Tsutsusi of subsect. Tsutsusi, and seven species belonged to ser. *Kaempferia* of subsect. Tsutsusi. For *R. transiens*, *R. kaempferi*, *R. sataense*, *R. tosaense*, and *R. kiusianum*, several different clones were used as plant material. *Rhododendron japonicum* f. *flavum* belonging to the subgenus *Pentanthera* was also used.

Total RNA was extracted from petals on the day of anthesis from all plants using a Nucleospin RNA Plant kit (Takara Bio Inc., Shiga, Japan), and cDNA was synthesized using a PrimeScript II Hi Fidelity RT-PCR kit

Table 1. Species and accession numbers of the *CCD4* genes used in this study.

Taxon	Collection site	Abbreviation	Accession No.
Subgen. <i>Pentanthera</i>			
<i>R. japonicum</i> f. <i>flavum</i>	Oita pref.	JPN	LC053677
Subgen. <i>Tsutsusi</i>			
Subsect. Scabra			
Ser. Scabra			
<i>R. scabrum</i>	Iriomote Is., Okinawa pref.	SCA	LC440313
<i>R. oldhamii</i>	Taiwan	OLD	LC440317
<i>R. scabrum</i> var. <i>yakuinsuriae</i>	Yakushima Is., Kagoshima pref.	YAK	LC440314
<i>R. amanoi</i>	Iriomote Is., Okinawa pref.	AMA	LC440315
<i>R. ripens</i>	Unknown	RIP	LC440318
<i>R. yedoense</i> var. <i>yedoense</i> f. <i>poukhanense</i>	Tsushima Is., Nagasaki pref.	YED	LC440316
Subsect. Tsutsusi			
Ser. Tsutsusi			
<i>R. indicum</i>	Yakushima Is., Kagoshima pref.	IND	LC440308
<i>R. eiocarpum</i> #1	Nakanoshima Is., Kagoshima pref.	ERI1	LC440311
<i>R. eiocarpum</i> #2	Yakushima Is., Kagoshima pref.	ERI2	LC440312
<i>R. eiocarpum</i> var. <i>tawadae</i>	Okinawa pref.	TAW	LC440309
<i>R. nakaharae</i>	Taiwan	NAK	LC440310
Ser. <i>Kaempferia</i>			
<i>R. simsii</i>	Okinawa pref.	SIM	LC440304
<i>R. transiens</i> #1	Unknown	TRA1	LC440306
<i>R. transiens</i> #2	Unknown	TRA2	LC440307
<i>R. transiens</i> #3	Unknown	TRA3	LC440305
<i>R. kaempferi</i> #1	Kagoshima pref.	KAE1	LC440296
<i>R. kaempferi</i> #2	Saga pref.	KAE2	LC440297
<i>R. kaempferi</i> #3	Kagoshima pref.	KAE3	LC440295
<i>R. sataense</i> #1	Kagoshima pref.	SAT1	LC440298
<i>R. sataense</i> #2	Kagoshima pref.	SAT2	LC440299
<i>R. sataense</i> #3	Kagoshima pref.	SAT3	LC440300
<i>R. kaempferi</i> var. <i>macrogonema</i>	Tokyo pref.	MAC	LC440301
<i>R. tosaense</i> #1	Miyazaki pref.	TOS1	LC440303
<i>R. tosaense</i> #2	Miyazaki pref.	TOS2	LC440302
<i>R. kiusianum</i> #1	Oita pref.	KIU1	LC440293
<i>R. kiusianum</i> #2	Kagoshima pref.	KIU2	LC440294

(Takara Bio). To isolate coding sequences (CDS) of the carotenoid cleavage dioxygenases gene (*CCD4*), RT-PCR was conducted using gene-specific primers (forward; 5'GGTATCAACGCAGAGTACATGG3', reverse; 5'GCCGGGACTACAAGTTTACAAC3') and cDNA from petals at stage 2. PCR amplifications were carried out in a total volume of 50 μ l containing 25 ng of template cDNA, 0.5 μ M of each primer, 50 mM of dNTPs, 5 μ l of 10 $\times$ reaction buffer and 2.5 unit of TaKaRa *Taq* DNA HS (Takara Bio). Amplification was carried out using Program Temp Control System TP-350 (Takara Bio) with 35 cycles at 10 sec at 98°C, 30 sec at 58 and 2.5 min at 72°C and one cycle at 72°C for 5 min. The amplified PCR products were cloned into the pMD20-T vector (Mighty TA Cloning Kit; Takara Bio), and were sequenced with a Big Dye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The deduced amino acid sequences were aligned with the ClustalW program (<http://www.clustal.org>).

Interspecific comparison of CCD4 expression levels in petals at the day of anthesis using real-time PCR

Species listed in Table 1 were used except for two species, *R. transiens* #1 (TRA 1) and *R. ripens* (RIP). These two species were excluded because their nucleotide sequences did not match the annealing sites of the designed primer (Fig. S1). Three flowers were harvested from each plant at the day of anthesis (stage 2). Total RNA was extracted from the petals of each flower as mentioned above, and cDNA was synthesized using PrimeScript RT Master Mix (Perfect Real Time) (Takara Bio). The transcript levels of the *CCD4* gene were analyzed by quantitative real-time PCR (RT-qPCR) using SYBR Premix Ex *Taq* II polymerase (Takara Bio) and a Thermal Cycler Dice Real Time System II MRQ (Takara Bio), according to the manufacturers' instructions. The primer pair for the *CCD4* gene was designed based on the consensus sequences from all plants used in this study (forward; 5'GCCAAGGAGCCCAATAACC3', reverse; 5'CGATTGTCATC CATCACC3'). cDNA was synthesized using PrimeScriptTM RT Master Mix (Perfect Real Time) (Takara Bio). The reaction mixture (25 μ l) consisted of 12.5 μ l of 2 $\times$ SYBR Premix Ex *Taq* II (Takara Bio), 0.4 μ M each of the forward and reverse primers, 2 μ l of the cDNA template (corresponding to 8 ng of total RNA), and 8.5 μ l of dH₂O. PCR was performed using the following program: an initial denaturation of 30 s at 95°C, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. Amplification of the ACTIN (Nakatsuka et al., 2008), pyruvate dehydrogenase, and histone H3 genes (De Keyser et al., 2007) under identical conditions was conducted as an internal control to normalize the cDNA levels. Real-time PCR data were analyzed using Multiplate RQ software (Takara Bio), which can analyze multiple reference genes. Calculations by the soft-

ware were based on the GeNorm manual (Vandesompele et al., 2002). From the data for each PCR, the relative expression values of *CCD4* were calculated and normalized against the reference genes using standard curve methods. These experiments were conducted with three biological replicates.

Observation of CCD4 expression levels during petal development

We chose six species belonging to the subgenus *Tsutsusi* that included *R. amanoi*, *R. scabrum* var. *yakuinsurae*, *R. indicum*, *R. eiocarpum* var. *tawadae*, *R. simsii*, and *R. kaempferi* var. *macrogemma*, and *R. japonicum* f. *flavum* belonging to the subgenus *Pentanthera*, as plant materials. Three flowers were harvested from each plant at different stages of development. The stages were divided into 1, 2, 3, and 4, which referred to petals at one day before flowering, at the day of anthesis, at two days after anthesis, and at five days after anthesis, respectively (Fig. 1). The petals from each flower were used for RT-qPCR using the

Fig. 1. Flowers of seven species at different stages. Stage1, one day before flowering; Stage 2, the day of anthesis; Stage 3, two days after anthesis; Stage 4, five days after anthesis.

aforementioned procedures. Three biological replicates were conducted in this experiment.

Results

Sequence analysis of *CCD4*

The CDS of *CCD4* in evergreen azalea species, except *R. nakaharae* and *R. eiocarpum* var. *tawadae*, was 1803 bp long and encoded a protein of 600 amino acids. The CDS of *R. nakaharae* was 1788 bp long and encoded a protein of 595 amino acids, and the CDS of *R. eiocarpum* var. *tawadae* was 1800 bp long and encoded a protein of 599 amino acids. *CCD4* genes in all species had four highly conserved histidine residues that have been previously described as typical ligands of a non-heme iron cofactor required for (di)-oxygenase activity, conserved glutamates or aspartate that are used to fix iron-ligating histidines (Kloer and Schulz, 2006), had a predicted plastid-targeting sequence in the N-terminal region (1–35) (Fig. S2). The percent identity values of the deduced amino acid sequences of *CCD4* were from 95% to 96% between *R. japonicum* f. *flavum*

and evergreen azaleas, and from 97% to 100% among evergreen azalea species. These sequences are available in the DNA Data Bank of Japan (DDBJ). The accession numbers are listed in Table 1.

Interspecific comparison of the *CCD4* gene expression levels in petals at the day of anthesis

The *CCD4* gene expression levels in petals at the day of anthesis in all evergreen azalea species were higher than in *R. japonicum* f. *flavum* (JPN), which varied widely from 2.04 to 20.47 times relative to that of *R. japonicum* f. *flavum* (Fig. 2). The lowest expression level values among evergreen azaleas were 2.04 in *R. amanoi* (AMA), 2.31 in *R. yedoense* var. *yedoense* f. *poukhanense* (YED), 2.63 in *R. indicum* (IND), and 3.00 in *R. scabrum* var. *yakuinsuriae* (YAK), respectively.

Expression of *CCD4* during petal development

The expression pattern of *CCD4* during petal development differed among species (Fig. 3). In *R. amanoi*

Fig. 2. Comparison of the relative expression levels of *CCD4* in the petals of *Rhododendron* spp. on the day of anthesis. Error bars indicate SE. Abbreviations of azalea species are given in Table 1.

Fig. 3. Changes in the expression pattern of *CCD4* during petal development. Values marked with different letters indicate statistically significant difference (Tukey's test, 5% significance). Error bars indicate SE. Abbreviations of azalea species are given in Table 1.

(AMA), *R. eriocarpum* var. *tawadae* (TAW), *R. simsii* (SIM), and *R. kaempferi* var. *macrogemma* (MAC), *CCD4* expression levels increased from stage 1 to stage 2, and then decreased. In *R. japonicum* f. *flavum* (JPN), *R. indicum* (IND) and *R. scabrum* var. *yakuinsuriae* (YAK), *CCD4* expression levels increased from stage 1 to stage 3, and then decreased. Compared with the low *CCD4* expression levels of *R. japonicum* f. *flavum* (JPN), significantly high expression levels were observed for all stages in *R. eriocarpum* var. *tawadae* (TAW) and *R. simsii* (SIM), after stage 2 in *R. kaempferi* var. *macrogemma* (MAC), and at stage 4 in *R. indicum* (IND). In contrast, *R. amanoi* (AMA) and *R. scabrum* var. *yakuinsuriae* (YAK) had low *CCD4* expression levels throughout all stages; these were not significantly different from those of *R. japonicum* f. *flavum* (JPN). In particular, *CCD4* expression levels in *R. amanoi* (AMA) were the lowest after stage 2 among six evergreen species and were lower than those of *R. japonicum* f. *flavum* (JPN) after stage 3.

Discussion

The contribution of *CCD4* to white color formation in petals by cleaving carotenoids into colorless compounds has been reported for *Chrysanthemum* species (Ohmiya et al., 2006) and *Lilium brownii* var. *colchesteri* (Hai et al., 2012). Carotenoids are metabolized to apocarotenoids through reactions catalyzed by *CCD* (Kato et al., 2006). *CCD4* also played a major role in causing discoloration during petal development of the progeny of the intersubgeneric cross between evergreen azalea species and *R. japonicum* f. *flavum* (Ureshino et al., 2016). In the present study, *CCD4* was detected in various evergreen azalea species belonging to the subgenus *Tsutsusi*. The deduced amino acid sequences among them showed high similarity and included all amino acid residues necessary for binding to a non-heme iron cofactor (Kloer and Schulz, 2006). Based on these findings, it is considered that all *CCD4* genes are functional.

It was reported that *Chrysanthemum CCD4* (*CmCCD4a*) comprises a small gene family in the genome, and several homologs are expressed in ray petals (Yoshioka et al., 2012). In a previous report, we cloned of *CCD4* at least five times to isolate the homologs in *R. japonicum* f. *flavum* and the white-flowered evergreen azalea ‘Miyamasatsuki’ (Ureshino et al., 2016). In *R. japonicum* f. *flavum*, only one sequence was obtained. In ‘Miyamasatsuki’, two sequences were obtained. Since ‘Miyamasatsuki’ is an interspecific F_1 hybrid between *R. kiusianum* and *R. eriocarpum*, the two sequences are considered to be allelic variations. Thus, it was considered that *CCD4* did not construct a gene family in azalea.

In contrast, the expression levels of *CCD4* genes varied widely among the evergreen azalea species. Among them, it was noteworthy that *R. amanoi* and

R. scabrum var. *yakuinsuriae*, which belong to subsect. *Scabra*, maintained low *CCD4* expression levels throughout all petal development stages, and were not significantly different to those of *R. japonicum* f. *flavum*. It is known that the structural properties of the promoter play a crucial role in gene expression (Das and Bansal, 2019). Proximal and distal regions upstream of the core promoter contain different regulatory sequences such as enhancers, silencers, insulators, and cis-elements that contribute to the fine regulation of gene expression at the transcriptional level (Hernandez-Garcia and Finer, 2014). In *Oryza sativa*, mutations such as insertions, deletions and substitutions in the promoter region caused the down-regulation of a disease resistance gene (*Xa13*). (Chu et al., 2006). Differences in the gene expression levels of the *CCD4* gene among evergreen azalea species may be affected by such mutations in the promoter region. To clarify this point, we will isolate the promoter region of *CCD4* and compare the sequences among evergreen azalea species.

In the petals of F_1 progenies of the intersubgeneric crosses between evergreen azalea species and *R. japonicum* f. *flavum*, carotenoid degradation was mediated by a high *CCD4* gene expression inherited from evergreen azalea species (Ureshino et al., 2016). Selection of evergreen species as cross parents that have low expression levels of the *CCD4* gene throughout petal development is the key to overcoming carotenoid degradation in the petals of progenies. *R. amanoi* is, therefore, considered to be a promising breeding material for producing yellow-flowered evergreen azaleas.

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