Floral Morphology and MADS Gene Expression in Double-flowered Japanese Evergreen Azalea

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Double flower form is an important trait of ornamental flower plants such as Japanese azalea because of its high visual appeal. We investigated floral phenotypes and a MADS-box C class gene to characterize floral morphology and this gene in single- and double-flowered cultivars of Japanese azaleas. Normal floral organs (sepals, petals, stamens, and carpels) were observed from each whorl of single flowers, whereas in double flowers, petaloid stamens or petals were observed from the third and fourth whorls, and there were no normal stamens or carpels in any flowers. Molecular analysis revealed that expression of the azalea AGAMOUS/PLENA (AG/PLE) homolog was lower in the inner two whorls of double flowers than in inner two whorls of single flowers, and expression of the AG/PLE homolog was higher in the double-flowered cultivars ‘Surugaman-yo’ and ‘Yodogawa’ than in other double-flowered cultivars. Moreover, sequence analysis of AG/PLE mRNA revealed deletion mutations in the coding regions of the AG/PLE homolog in ‘Surugaman-yo’ and ‘Yodogawa’. These results suggest that the double flowers of Japanese azalea cultivars are formed as the down-regulated expression and deleted sequence mutation of the azalea AG/PLE homolog.

Key Words: homeotic mutant, Japanese azalea, MADS-box gene, petaloid stamens.

Introduction

Japanese azaleas (Rhododendron spp.) are among the most popular garden plants, and are a globally important genetic resource as ornamental shrubs. Many wild individuals and hundreds of azalea cultivars have been selected by horticulturists from natural populations of indigenous evergreen azalea species and their hybrids since the Edo era (1603–1867) in Japan (Kunishige and Kobayashi, 1980; Kurashige and Kobayashi, 2008). A monograph on azaleas, “Kinshu-makura” (the English translation of which is “A Brocade Pillow: Azaleas of Old Japan”), which was edited in 1692, describes more than 300 azalea cultivars. In this monograph, leaf and flower characteristics of Japanese azalea cultivars, such as shape, color, and size, were recorded, and notably, several double-flowered cultivars mentioned in this monograph are still cultivated today (Ito and Creech, 1984). For example, ‘Shiroman-e’ and ‘Surugaman-e’, which are currently called ‘Shiroman-yo’ and ‘Surugaman-yo’, respectively (Fig. 1), have a number of petals in the double flower form. Although several double-flowered cultivars have been cultivated for more than 300 years in Japan and their flowers have visual appeal as ornamental plants, there have been no reports regarding morphological and molecular analyses of double flowers in Japanese azalea cultivars.

Generally, the single flowers of dicotyledonous plants are composed of four types of organs: sepals in the first whorl, petals in the second whorl, stamens in the third whorl, and a carpel in the fourth whorl. In eudicots, floral organ identities are explained by the ABC model, which has been established from studies on two model plants, Arabidopsis thaliana and Antirrhinum majus (Coen and Meyerowitz, 1991). The ABC model includes many genes encoding MADS-box transcription factors that have a conserved MADS domain, A-region, K-box, and C-terminal region (Münster et al., 1997). According to this model, genes are classified into three functional types. A-function genes, APETALA1 and SQUAMOSA in A. thaliana and A. majus, respectively, are expressed in the first and second whorls. The B-function genes, APETALA3/DEFICIENS and PISTILLATA/GLOBOSA in
A. thaliana/A. majus, are expressed in the second and third whorls, and their encoded proteins gain their B-function when they form heterodimers (Krizek and Meyerowitz, 1996). The C-function genes are expressed in the third and fourth whorls, and play an important role in stamen and carpel formation. Stamen and carpel organ identities are specified by a single C-function gene, AGAMOUS (AG), in A. thaliana, but by two C-function genes, PLENA (PLE) and FARINELLI, in A. majus. In these model plants, the repression of C-function genes results in the conversion of stamens to petals in the third whorl, and carpels to sepals and/or petals in the fourth whorl (Bowman et al., 1989; Bradley et al., 1993). In addition to its role in determining floral organ identity, AG also plays a role in terminating flower development (Lenhard et al., 2001). Thus, attenuated C-class function could increase petal development, inhibit stamen development, and also increase the floral organ number, which is consistent with the formation of double flowers. Double-flowered phenotypes result from C-function repression in a number of horticultural plants, including Gentiana scabra (Nakatsuka et al., 2015), Camellia japonica (Sun et al., 2014), Petunia hybrida (Noor et al., 2014), and Cyclamen persicum (Tanaka et al., 2013). Therefore, it is likely that the double flowers of Japanese azalea cultivars result from lost or impaired C-function genes. In previous analysis of azalea MADS C-class genes, azalea AG/PLE homologs named RkAG1 and RmAG1 were isolated from R. kaempferi and R. macrosepalum, respectively. Natural staminoid-petaled and narrow-petal mutants in the second whorl may be caused by the expression of azalea AG/PLE homologs (Tasaki et al., 2012; 2014). However, although azalea AG/PLE homologs have been characterized in natural staminoid-petaled and narrow-petal mutants of Japanese azalea cultivars, the gene has not been characterized in natural double flowers in Japanese azalea cultivars.

In this study, we attempted to characterize natural double flowers in several Japanese azalea cultivars. We performed expression analysis of AG/PLE homologs in each floral organ and partially sequenced the mRNA of several double-flowered cultivars, focusing on the C-class AG/PLE homolog. To our knowledge, this is the first report of the characterization of an azalea AG/PLE homolog involved in the floral morphogenesis of double-flowered azaleas.

**Materials and Methods**

**Sampling and morphological investigation**

Single-flowered azaleas, R. ripense, R. mucronatum, R. macrosepalum, R. yedoense f. poukhanense, and Kurume azalea (R. obtusum) ‘Wakakaede’, and their respective double-flowered cultivars, ‘Fujiman-yo’, ‘Shiroman-yo’, ‘Surugaman-yo’, ‘Yodogawa’, ‘Fukuyu’, and R. sataense ‘Full-double flower form’, were obtained from the azalea resources collection of the Plant Breeding Laboratory of the Faculty of Life and Environmental Sciences, Shimane University (Fig. 1). To investigate flower morphology, abaxial or adaxial epidermal cells of each floral organ in single and double flowers were analyzed by scanning electron microscopy (SEM: TM300, HITACHI, Tokyo, Japan). For total RNA extraction, the floral organs of each whorl from 10–20 flowers and two floral buds (just before flowering) were sampled from each plant. In particular, the first whorl and inner three whorls were sampled from flowers of Kurume azalea ‘Fukujyu’ and R. sataense ‘Full-double flower form’, because these cultivars have petals instead of stamens and carpels in the inner two whorls. These samples were immediately frozen in liquid nitrogen and stored at −80°C until used for extraction.

**Expression analysis of the azalea AG/PLE homolog**

Total RNA was extracted from each floral organ and floral bud of all plant materials using an RNaseasy plant mini kit (Qiagen, Hilden, Germany). cDNAs were then synthesized from total RNA (1 μg) using ReverTra Ace®qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). To investigate RmAG1 expres-
separation in floral buds and floral organs, reverse transcription polymerase chain reaction (RT-qPCR) was performed in a 20-μL reaction mix containing 1 μL of 10× diluted cDNA templates, 10 μL of SYBR Premix Ex-Taq II (TaKaRa Bio, Shiga, Japan), and 0.25 μM of each primer (RmAG1; forward 5′-ACTACTCTGCCACGACCAA-3′ and reverse 5′-CCAAAAAGAACAACAAAAAG-3′ and azalea Histone H3; forward 5′-GAAACTCCCCATTCCAGA GGCT-3′ and reverse 5′-GCTGGATGCGACAGA GTTT-3′; Tasaki et al., 2012). Each RT-qPCR assay was conducted using a Thermal Cycler Dice Real-Time System (TaKaRa Bio) with an initial denaturation of 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. Three technical replicates were analyzed for each cDNA sample. Quantitation was performed by using the difference in the threshold cycle values (ΔCt) between the two samples (the target gene and the reference gene, Histone H3) to calculate the relative amounts of the template present. Relative expression was calculated for the RmAG1 gene, and the mean and standard error were calculated for the value of relative expression.

cDNA sequence analysis of the azalea AG/PLE homolog in double flowers

To isolate an abnormal AG/PLE homolog from the double-flowered cultivars, amplifications were carried out with flower bud-derived cDNA and primers, which were designed using sequences containing part of the MADS domain to 3′ untranslated region (UTR) from RmAG1-1 (AB754499), -2 (AB754500), and -3 (AB754501). In the PCR assay, each 20-μL reaction mixture contained 2 μL of 10× diluted cDNA templates, 1× Ex-Taq buffer, 200 μM dNTPs, and 0.2 μM of each primer (RmAG1; forward 5′-GAAAGACATGGCCT TCCCTAG-3′ and reverse 5′-CTCTACTAGCTATT TGTTCTTGATTCATAA-3′ and azalea ACTIN: forward 5′-AGCAATGTATGTTGCTATCC-3′ and reverse 5′-GCATGGATGGCACAGAGTCTAG-3′). The reaction conditions of the PCR were as follows: preheating at 94°C for 1 min; 32 cycles (RmAG1)/30 cycles (ACTIN) of denaturation at 94°C for 30 s, annealing at 60°C (RmAG1)/53°C (ACTIN) for 30 s, and extension at 72°C for 1 min (RmAG1)/20 s (ACTIN); and final extension at 72°C for 2 min. Azalea ACTIN was used as a positive control for cDNA templates in the PCR analysis. The amplified PCR products were separated by electrophoresis on 1% agarose gels (Nippon Genetics Co., Tokyo, Japan) in 0.5× TBE buffer at 100 V for 35 min. The amplified fragments were cloned into a pGEM-T easy vector (Promega, Madison, WI, USA) and Escherichia coli DH5α competent cells (Nippon Genetics Co., Japan) and sequenced using a BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The mRNA nucleotide and deduced amino acid sequences of the iso-

lated AG/PLE homolog were aligned using Genetyx ver. 12 (Software Development Co., Tokyo, Japan).

Results

Floral morphology of single and double flowers

In order to characterize the double flowers from Japanese azalea cultivars (Fig. 1), we compared each floral organ from single and double flowers (Fig. 2). The single-flowered ‘Shiroryukyu’, floral organs, namely, sepals, petals, stamens, and carpels, were observed from the first to fourth whorl, respectively (Fig. 2A). The floral pattern of other single flowers was similar to that observed in the flowers of ‘Shiroryukyu’ (Fig. 1).

Similar to single flowers, normal sepals and petals were observed in the first and second whorls of all double flowers; however, the pattern in the third and fourth whorls differed. In ‘Shiroman-yo’, ‘Fujiman-yo’, and ‘Yodogawa’, petals with anthers were observed in the third whorl, whereas petals without anthers were observed from the Kurume azalea ‘Fukujyu’ (Fig. 2B–E). In the fourth whorl, a new flower, which had a sepal-petal-petal pattern, was observed from ‘Shiroman-yo’ and ‘Fujiman-yo’ (Figs. 2B1 and C1), whereas sepal-like organs were observed from ‘Yodogawa’ (Fig. 2D1). The floral organ patterns of ‘Surugaman-yo’ and R. sataense ‘Full-double flower form’ were similar to those in the flowers of ‘Fujiman-yo’ and ‘Fukujyu’, respectively (Figs. 1 and 2).

In the SEM analysis of double flowers, epidermal cells on the abaxial surface of sepal and adaxial surface of petals in the first and second whorls were of the same pattern as in the sepals and petals of single flowers, respectively (Figs. 2A2–E2 and A3–E3). In the third whorl, epidermal cells of the adaxial surface in all double flowers were similar to those in the petals of single and double flowers (Fig. 2B4–E4). Furthermore, the sepals of new flowers and sepal-like organs in the fourth whorl resembled in arrangement and size the epidermal cells in sepals of the first whorl (Fig. 2A5–D5). In the inner two whorls of ‘Fukujyu’, epidermal cells of the adaxial surface had a similar pattern to those of petals in single flowers (Fig. 2E4). In none of the double flowers were normal stamens and carpels observed.

Expression analysis of azalea AG/PLE homolog in single and double flowers

To investigate the relationship between the double flower phenotype and azalea AG/PLE homologs, RT-qPCR was performed on cDNA derived each floral organ and flower bud using RmAG1 primers, which have been used in previous studies (Tasaki et al., 2014). In single flowers, RmAG1 was more highly expressed in floral buds and the inner two whorls than in the outer two whorls, and more highly expressed in the third whorl than in the fourth whorl. In double flowers, there was very low expression of RmAG1 in flower buds and all whorls of ‘Fujiman-yo’, ‘Shiroman-yo’, ‘Fukujyu’,
and *R. sataense* ‘Full-double flower form’; however, compared with these four double-flowered cultivars, *RmAG1* showed higher expression in the flower buds and inner two whorls of ‘Surugaman-yo’ and ‘Yodogawa’ (Fig. 3).

Sequence analysis of abnormal mRNA in the azalea AG/PLE homolog in double flowers

To investigate the mRNA structure of the *AG/PLE* homolog expressed in the third and fourth whorls of ‘Surugaman-yo’ and ‘Yodogawa’, we carried out PCR amplification and sequence analysis of flower bud-derived cDNA (Fig. 4). For PCR amplification of the mutated region in the *AG/PLE* homolog, we designed a primer pair that can amplify a region encompassing part of the MADS domain to the C-terminal region in the deduced amino acid sequence of *RmAG1* (Fig. 4A). A band of approximately 1 kb was detected from all single flowers, whereas a band of smaller size was detected in ‘Surugaman-yo’ and ‘Yodogawa’, but not from other double-flowered cultivars (Fig. 4B).

After cloning the amplified PCR products from the floral buds of double flowers, we identified three sequences in *R. yedoense* f. *poukhanense*, *R. macrospalum* ‘Surugaman-yo’, and *R. yedoense* ‘Yodogawa’. In *R. yedoense* f. *poukhanense*, three sequences of approximately 1004, 1013, and 1007-bp were identified to compare sequences between wild type and double-flowered cultivars of *R. yedoense*, and named *RyAG1*-1 (DDBJ accession number LC169777), -2 (DDBJ accession number LC169778), and -3 (DDBJ accession number LC169779), respectively. These sequences showed over 95% identity with *RmAG1* sequences. In addition, three mutated sequences in *RmAG1* (*RmAG1* mutation-1, -2, and -3) of ‘Surugaman-yo’ (approximately 922, 869, and 860-bp, respectively) and three mutated sequences in *RyAG1* (*RyAG1* mutation-1, -2, and -3) of ‘Yodogawa’ (approximately 962, 971, and 1120-bp, respectively) were identified. Subsequently, when we compared the mutant sequences with *RmAG1*
and RyAG1 sequences, we detected deleted sequences of 82 and 144-bp in the I-region or/and K-box of the RmAG1 mutation-1 and mutation-2/-3, respectively. Further, a deleted sequence of 42 bp was identified in all RyAG1 mutant sequences, and insertion of an unknown sequence of 158 bp was found in the K-box of RyAG1 mutation-3. Sequences of the putative C-terminal region were conserved in all the identified mutants (Fig. 4C). Moreover, in a comparison of the deduce amino acid sequences of AG/PLE homologs, partial deletion mutations of the putative I-region or K-box were found in all the identified mutants, and stop codons were detected in frameshifted sequences in both the RmAG1 mutation-1 and RyAG1 mutation-3 (Fig. 4D).

**Discussion**

In the present study, in order to investigate the relationship between morphological and molecular characteristics in double flowers of Japanese azalea cultivars, we carried out microscopic and expression analyses of the floral organs of each flower whorl.

Morphological investigation revealed a single whorl of petals in single flowers, whereas double flowers had multiple whorls of petals and vestigial or missing reproductive organs (Fig. 2). In the multiple whorls, petals, or petal-like organs, some of which were mosaic organs of petals and stamens, morphologically resembled petals of the second whorl in single and double flowers (Fig. 2B–E). These gradual changes from stamen and carpel to petaloid stamens and new flower (or inner petals) suggest that inner petals may partially acquire petal
Fig. 4. Sequence structure analyses of mRNA and deduced amino acids in the azalea AG/PLE homolog. A: Schematic diagram showing primers and the MIKC regions in RmAG1 (Tasaki et al., 2014). Horizontal lines indicate 5' and 3' untranslated regions (UTRs), and closed and hatched boxes indicate AG motifs I and II, respectively. M, asterisk, and closed arrows indicate start codon, stop codon, and primer region, respectively. B: PCR amplification of azalea AG/PLE homolog mRNA from partial MADS domain to 3' UTR. Numbers indicate (1) Rhododendron ripense, (2) R. ripense 'Fujiman-yo', (3) R. mucronatum 'Shiroryukyu', (4) R. mucronatum 'Shiroman-yo', (5) R. macrosepallum, (6) R. macrosepallum 'Surugaman-yo', (7) R. yedoense f. poukhanense, (8) R. yedoense 'Yodogawa', (9) Kurume azalea 'Wakakaede', (10) Kurume azalea 'Fukuiju', and (11) R. sataense 'Full-double flower form'. M indicates a 100-bp ladder (Nippon Genetic Co.) and 1 kb indicates the band size. Azalea ACTIN was used as a template positive control. C: cDNA sequence comparison with RmAG1-1/-2/-3 of R. macrosepallum (Tasaki et al., 2014) and isolated RyAG1-1/-2/-3 (R. yedoense f. poukhanense), RmAG1 mutation-1/-2/-3 (R. macrosepallum 'Surugaman-yo'), and RyAG1 mutation-1/-2/-3 (R. yedoense 'Yodogawa'). M and asterisk indicate start codon and stop codon, respectively. D: Comparisons of deduced amino acid sequences in RmAG1-1/-2/-3, RyAG1-1/-2/-3, RmAG1 mutation-1/-2/-3, and RyAG1 mutation-1/-2/-3. Opened boxes indicate deleted regions.
identity through the conversion of stamens and carpels. The morphological changes in the third and fourth whorls observed in double-flowered Japanese azalea cultivars closely resemble the homeotic ag mutant flowers seen in both model plants and horticultural plants.

In *A. thaliana*, ag mutant flowers consist of many sepals and petals, and also chimeric organs consisting partly of sepal and partly of petal tissue. Furthermore, the central cells of the second flower also develop as a new flower, which in turn produces another new flower within itself, and so on, until mature flowers with more than 70 organs develop (Bowman et al., 1989). In *A. majus*, recessive mutations of *PLE* result in a loss of C function, which gives rise to petals in place of stamens and petaloid organs in place of carpels (Bradley et al., 1993). In the fourth whorl of flowers inoculated with *PETUNIA MADS-BOX GENE3/FLORAL BINDING PROTEIN6-VIGS*, which represses the expression of the *Petunia AG/PLE* homolog, the cultivar-dependent conversion of carpels into a new flower was observed in *Petunia* ‘Mambo Purple’ (Noor et al., 2014). In *Cyclamen persicum*, *35S:CpAG1-SRDX* and *35S:CpAG2-SRDX* cyclamen, which lack both *CpAG1* and *CpAG2* expression, produced multi-petal flowers with a repeated structure of tens of petals instead of stamens and carpels (Tanaka et al., 2013). In addition to transgenic flowers, the double-flowered cultivar ‘Shibaxueshi’ (*Camellia japonica*) is a typical formal double type with petals from the outer layer to the inside. Furthermore, the stamens and carpels were missing and the expression levels of *CjAG* in floral buds were markedly reduced, suggesting that the loss of *CjAG* expression is involved in double flower development (Sun et al., 2014). In *Cyclamen* double-flowered cultivars, the lack of *CpAG1* expression causes the homeotic conversion of stamens and carpels into petals (Tanaka et al., 2013). In azalea double-flowered cultivars, *RmAG1* expression in the third and fourth whorls was lower than that in the third and fourth whorls of single-flowered cultivars (Fig. 3). These results suggest that the transition of stamens and carpels to petals or petaloid stamens in the third and fourth whorls of double-flowered Japanese azalea cultivars is mediated by down-regulation of *AG/PLE* homolog expression.

Interestingly, even though the expression of the azalea *AG/PLE* homolog was detected in ‘Surugaman-yō’ and ‘Yodogawa’ (Figs. 3 and 4B), double flowers had a different floral phenotype compared to single flowers (Figs. 1 and 2). When we carried out sequence analysis to investigate the partial mRNA structure of the *AG/PLE* homolog in floral bud-derived cDNA of ‘Surugaman-yō’ and ‘Yodogawa’, deletion of exons and insertion of unknown sequences were identified in double flowers (Fig. 4C). Furthermore, stop codons in a frameshifted mutation that had deleted part of the I-region or K-box was detected in the deduced amino acid sequences of *RmAG1* mutation-1 and *RyAG1* mutation-3. In *A. thaliana*, floral MADS proteins conserve the K-box that mediates protein–protein interaction, and the subdomains of the K-box are important for protein–protein interactions of plant MADS proteins (Yang and Jack, 2004). In *Thalictrum thalictroides* ‘Double White’, molecular analysis of *ThAG1*, which is an AG homolog of *T. thalictroides*, alleles revealed the insertion of a retrotransposon causing either nonsense-mediated decay of transcripts or alternative splicing that resulted in mutant proteins with K-box deletions (Galimba et al., 2012). These features of mRNA structure and deduced amino acid sequences suggest that in the azalea *AG/PLE* homolog, which contributes to specifying stamen and carpel identity, the K-box deletion mutation and frameshift mutation may cause either partial or complete loss of activity.

Moreover, in the *ple* mutant flowers of *A. majus*, *PLE* expression was reduced in the inner two whorls of the flower by insertion of a transposon in the second intron of the *PLE* gene (Bradley et al., 1993). In a double-flowered mutant of Japanese gentian (*G. scabra*), in which the stamens are substituted with petals, *GsAG1* expression was down-regulated by insertion of a *Tgs1* (transposable element of *G. scabra*) in the intron of the *GsAG1* gene (Nakatsuka et al., 2015). Similar to these reports, it is highly possible that the *AG/PLE* homolog of double-flowered azaleas carries a transposon insertion mutation on account of the very low expression of the *AG/PLE* homolog in all floral organs of azalea double flowers (Fig. 3) and the presence an unknown sequence in *RyAG1* mutation-3 (Fig. 4C). However, because we doubt whether the unknown sequence is a transposon sequence, gDNA analysis will be needed in single and double flowers.

In conclusion, the findings of this study suggest that the double flower phenomenon in azaleas may be explained by ABC model genes, and it is evident that the double flower phenotype in azaleas is caused by down-regulated expression and abnormal mRNA of the *AG/PLE* homolog in the third and fourth whorls. However, how the expression of the *AG/PLE* homolog is down-regulated in double-flowered cultivars remains to be clarified. Therefore, the relationship between expression level and gDNA structure of *AG/PLE* homologs in the double flower form of Japanese azalea cultivars should be investigated.

**Literature Cited**


