Expression of MADS-box Genes in Narrow-petaled Cultivars of *Rhododendron macrosepalum* Maxim.

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Japanese old azalea cultivars have various floral mutations. We isolated MADS-box class C homologous genes from wild-type *Rhododendron macrosepalum* to analyze the expression patterns in the floral organs of the narrow-petal mutational cultivars ‘Hanaguruma’, ‘Gin-no-zai’, and ‘Seigaiha’. The *AG* homologous genes *RmAG1-1/-2/-3* were 99–100% identical to *RkAG1-1/-2*, which was isolated from *R. kaempferi*. In ‘Hanaguruma’, ‘Seigaiha’, and the wild type, the relative expression level of *RmAG* in whorl 2 was much lower than that in whorl 3. In contrast, the relative expression level of *RmAG* in whorl 2 of ‘Gin-no-zai’ was approximately 15.5% of that in whorl 3. In ‘Gin-no-zai’, the petals with mutations were categorized as 1 of these 3 types: type 1, consisting of narrow petals with traces of anthers; type 2, consisting of narrow petals only; and type 3, consisting of petals that coalesced with the neighboring petals. The relative expression levels of *RmAG* gradually increased from type 3 to type 1 petals. These results suggest that the degree of staminoidy for the petals in whorl 2 is attributable to the expression levels of *RmAG*.

Key Words: AGAMOUS, azalea, choripetalous flower, sai-zaki form, staminoid petal.

**Introduction**

In Japan, old azalea cultivars have been developed since the Edo era (i.e., 1603–1867) from the natural populations of a variety of species of the *Rhododendron* subgenus *Tsutsusi*, section *Tsutsusi* (Ericaceae), for example, *R. kaempferi*, *R. macrosepalum*, *R. indicum*, and *R. ripense* (Kobayashi et al., 2000; Kunishige and Kobayashi, 1980; Kurashige and Kobayashi, 2008). Japanese people appreciate singular morphological mutations. For example, the “sai-zaki” form of choripetalous corollas, which consist of 5 independent narrow petals, and the “mise-mo-sho”, which is long-lasting corolla mutation with temporal change of colour, were valued for their ornamental value and have been preserved to date (Ito and Creech, 1984). In our previous study, the ‘Hanaguruma’, ‘Gin-no-zai’, and ‘Seigaiha’ cultivars of *R. macrosepalum*, which are often called “sai-zaki”, were investigated for their morphological features and fertility. The results suggested that their characteristically narrow organs were due to a decrease in the cell counts caused by weak development in the transverse plane (Tasaki et al., 2012a). Our future studies will examine factors related to the formation of narrow petals that compose the choripetalous corolla in “sai-zaki”. Following our previous study, several narrow petals with traces of anthers, suspected to be attributable to a floral homeotic mutation, were observed in the flowers of ‘Gin-no-zai’.

The identities of 4 floral organs have been specified by the classic ABC model, which consists of floral homeotic MADS-box genes from classes A, B, and C (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). The expression of the class A genes alone determines sepal development, whereas the coexpression of class A and B genes or class B and C genes specifies the formation of petals or stamens, respectively. The expression of class C genes alone results in carpel development. In azalea, the presumed class B MADS-box genes, *RpPI* from *R. ripense* and *RpAP3* from *R. × pulchrum*, were isolated (Cheon et al., 2011; Nakatsuka et al., 2009). These genes were similar to *DEFICIENS* (DEF) and *GLOBOSA* (GLO) in *Antirrhinum majus* and *APETALA3* (AP3) and...
Isolation and sequence analysis of MADS-box class C genes from wild-type *R. macrosepalum*

The extraction and reverse transcription of total RNA from wild-type *R. macrosepalum*, the cloning of *AG*-like genes using the primer set for *RkAG*, and the sequencing of target fragments were performed as described previously (Tasaki et al., 2012b), with the exception that cloning was performed using *Escherichia coli* DH5α-competent cells (Nippon Gene, Tokyo, Japan). The amplification conditions were as follows: 30 s at 98°C, followed by 30 cycles each of 98°C for 10 s, 59°C for 30 s, and 72°C for 1 min 30 s. The DNA sequences were translated into amino acid sequences by using the program GENETYX WIN, version 11.0 (Software Development). Multiple sequences of the azalea clones were aligned using ClustalW, version 2.0 (Thompson et al., 1994).

Expression analysis by semiquantitative RT-PCR and RT-quantitative PCR

Semiquantitative RT-PCR and/or RT-quantitative PCR were performed for expression analyses of MADS-box class B and C genes in the floral organs of the wild type and cultivars (i.e., ‘Hanaguruma’, ‘Gin-no-zai’, and ‘Seigaiha’) in *R. macrosepalum*. The total RNA from each floral organ, which was a mix obtained from several flowers just after opening, was extracted. Reverse transcription, semiquantitative RT-PCR, RT-qPCR, and their analyses were performed as previously described (Tasaki et al., 2012b). The expression analyses by RT-PCR were conducted in monoplicate and RT-qPCR in technical triplicates for each organ. Primers were designed for *RpAP3* (DDBJ accession no. AB598826; Choen et al., 2011), *RrPI* (DDBJ accession no. AB639033; Nakatsuka et al., 2009), and *RkAG1-1* and *RkAG1-2* (DDBJ accession nos. AB639031 and AB639032, respectively). *ACTIN*

Materials and Methods

Plant materials

The wild-type *R. macrosepalum* Maxim. and its cultivars, ‘Hanaguruma’, ‘Gin-no-zai’, and ‘Seigaiha’ (Wilson and Rehder, 1921; Yamazaki, 1996) used in this study were obtained from the azalea resources collection of the Plant Breeding Laboratory of the Faculty of Life and Environmental Science of Shimane University (Fig. 1).

**Fig. 1.** The flower and petal shapes of plant materials. A and E, *R. macrosepalum*. Five fused petals composed a sympetalous flower. B and F, ‘Hanaguruma’; C and G, ‘Gin-no-zai’; D and H, ‘Seigaiha’. G1–G3 shows the 3 types of petals, as classified by morphological differences. G1 (type 1): the narrowest petals, which are recognized by traces of anthers. G2 (type 2): narrow petals, which are separated from neighboring petals. G3 (type 3): petals coalescent with neighboring petals.
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RmAG1-2 (DDBJ accession no. AB754500), and RmAG1-3 (DDBJ accession no. AB754501). The base sequence of each RmAG varied by 11 bases in the protein-coding region. In addition, RmAG1-2 possessed a 9-base insertion in the K-box, which was translated as three serines (S121–123). Similar base substitutions in the same positions were confirmed in whorls 3 and 4 of ‘Hanaguruma’, ‘Gin-no-zai’, and ‘Seigaiha’.

Otherwise, the 3 genes differed by only 1 amino acid, which was a phenylalanine (F229) or a leucine (L229) in the AG motif I. The deduced amino acid sequences of the isolated genes are shown in Figure 2. The homology of the deduced amino acid sequences between RmAG1-1/-2/-3 of R. macrosepalum and RkAG1-1/-2 of R. kaempferi was 99–100% (Fig. 2).

Expression analysis of class B and C genes in the floral organs
To analyze the expression patterns of the homologous genes of RrPI, RpAP3, and RmAG in the floral organs of ‘Hanaguruma’, ‘Gin-no-zai’, and ‘Seigaiha’, semiquantitative RT-PCR was performed. The primer set for RrPI amplified multiple homologous genes from whorls 2 and 3 of the wild-type R. macrosepalum and in ‘Hanaguruma’ and ‘Gin-no-zai’ (Fig. 3). In ‘Seigaiha’, 1 band was obtained for whorls 2 and 3. The primer set for RpAP3 produced an amplicon for samples from all the whorls of all plants (Fig. 3). RmAG expression was detected in whorls 3 and 4 of all plants. In addition, ‘Gin-no-zai’ exhibited strong expression of RmAG in whorls 1 and 2, although similar expression levels were not detected in those whorls in the wild-type R. macrosepalum, ‘Hanaguruma’, and ‘Seigaiha’.

To analyze the relative expression levels of class B and C homologous genes in floral organs within a plant, [Nakatsuka et al., 2008; DDBJ accession no. AB610421] and Histone H3 (De Keyser et al., 2007) genes were used as internal controls. RT-PCR and RTq-PCR were performed using the same primers that were used in our previous study (Tasaki et al., 2012b) because the homology of the amino acids of AG-like genes between R. macrosepalum and R. kaempferi was 99–100%.

Results
Morphological traits
The corollas of ‘Hanaguruma’, ‘Gin-no-zai’, and ‘Seigaiha’ were choripetalous and consisted of 5 independent petals (Fig. 1B–D). Wild-type R. macrosepalum had a gamopetalous corolla (Fig. 1A, E). In ‘Gin-no-zai’, 3 types of petal forms were observed: type 1, which had the most narrow petals, which were recognized by the presence of traces of anthers (Fig. 1G1); type 2, in which a narrow petal was separated from neighboring petals (Fig. 1G2); and type 3, wherein a petal coalenced with the neighboring petals (Fig. 1G3). Almost all petals of ‘Gin-no-zai’ were categorized as type 2. In ‘Hanaguruma’ and ‘Seigaiha’, petals with traces of anthers were not observed (Fig. 1F, H).

cDNA cloning and sequence analysis of C-class genes from R. macrosepalum
cDNA clones of the AG homologue genes were isolated from stamens in whorl 3 and pistils in whorl 4 of R. macrosepalum plants. Eleven fragments were sequenced and 3 types of sequences with homology to RkAG1-1/-2 of R. kaempferi were identified. The full-length sequences of the 3 AG homologue genes contained 39 bp of 5′ untranslated regions (UTRs), 759 bp of a coding sequence, and 238 bp of 3′ UTRs. These genes were named RmAG1-1 (DDBJ accession no. AB754499), RmAG1-2, and RmAG1-3 (DDBJ accession no. AB754500).
Discussion

In recent years, morphological and molecular biological analyses of mutational traits in azalea cultivars have been undertaken by our research group. ‘Hanaguruma’, ‘Gin-no-zai’, and ‘Seigaiha’ were characterized in a previous study, and it was clarified that their choripetalous corollas, often called “sai-zaki”, were due to weak development in the transverse plane (Tasaki et al., 2012a). To understand the genetic causes of this morphological mutation, we focused on MADS-box genes in the current study. Interestingly, narrow petals with traces of anthers were found in some flowers of ‘Gin-no-zai’. Additionally, gradual morphological mutations in the floral organ were often found in the double flower of azalea cultivars, which is a stamen mutated into a petal in whorl 3, and seems to be caused by growth conditions and/or environmental factors. We collected 3 types of definitive petals of ‘Gin-no-zai’ for genetic analyses.

To analyze the expression of AG-like genes in the floral organs of “sai-zaki” cultivars, RmAG1-1/-2/-3 were isolated from R. macrosepalum. RmAG1-1/-2/-3 has 99–100% homology with the putative MADS-box class C genes RkAG1-1/-2, which were isolated from wild-type
R. kaempferi in a previous study (Tasaki et al., 2012b). RkAG have highly conserved MADS domains and AG motifs, which are characteristic of MADS-box genes and AG homologues, and showed 73–75% amino acid sequence similarities with MADS-box class C genes, including PN in Ipomoea nil, FBP6 in Petunia × hybrida, and PLE in A. majus (Bradley et al., 1993; Davies et al., 1999; Kramer et al., 2004; Nitasaka, 2003). RmAG expression was mainly detected in whorls 3 and 4 of ‘Hanaguruma’, ‘Gin-no-zai’, ‘Seigaiha’, and wild-type R. macrosepalum. In ‘Gin-no-zai’, RmAG expression in whorl 2 was definitively confirmed by RT-PCR and RT-qPCR. ‘Kagaribi’ and ‘Kinshibe’ of R. kaempferi showed staminoidy and staminoid petals in whorl 2. The RkAG expression level in whorl 2 of ‘Kagaribi’ was approximately 27% relative to that in whorl 3, while the RkAG expression level in whorl 2 of ‘Kinshibe’ was nearly equal to that in whorl 3. In ‘Gin-no-zai’, AG genes expression in whorl 2 was approximately 15.5%, relative to that in whorl 3. This whorl 2 expression level in ‘Gin-no-zai’ was stronger than that of the wild type, suggesting the possibility of staminoidy of the petals. However, the degree of morphological mutation was lower than that of ‘Kagaribi’ and ‘Kinshibe’. RmAG expression in whorl 1 of ‘Hanaguruma’ was also confirmed in wild-type R. kaempferi. However, the expression in whorl 2 also was not detected in such cases. These results suggest that the identity of petals in whorl 2 is regulated by some factors suppressing the expression of RmAG in whorl 2. For example, microRNA, FISTULATA in A. majus and BLIND in P. hybrida, control the spatial restrictions of homeotic class C genes to the inner floral whorls (Cartolano et al., 2007). A similar mechanism might be related to the staminoidy and/or staminoid petals in ‘Gin-no-zai’ of R. macrosepalum and ‘Kagaribi’ and ‘Kinshibe’ of R. kaempferi.

The morphological evidence of homeotic mutation into stamens from petals in whorl 2 of ‘Gin-no-zai’ was the presence of traces of anthers. The expansion of the petal lamina of ‘Gin-no-zai’ was not uniform. Narrow long epidermal cells were observed on the surface of staminoid petals in whorl 2 of ‘Kagaribi’ and ‘Kinshibe’, as well as that of the stamen in whorl 3 of the wild type of R. kaempferi (Tasaki et al., 2012b; Fig. 2). In addition, trichomes were observed on the surface of the staminoid petals of ‘Kinshibe’. Continuous morphology between wide petal-like and narrow stamen-like organs has been reported in the CPPU-treated paracorollas of Torenia fournieri (Niki et al., 2012). In the epidermis of narrow paracorollas, petal-like conical cells or stamen-like slender cells were arranged. In this case, it is hypothesized that the expression patterns of MADS-box class A genes reflect the unstable floral organ identity of narrow paracorollas. In contrast, the morphological transformation of the whorl 2 organs in azalea seems to be reflected by the expression levels of the predicted AG-like class C genes, which generate morphological differences between ‘Kagaribi’, ‘Kinshibe’, and ‘Gin-no-zai’.

The RT-qPCR analysis of type 1–3 petals in ‘Gin-no-zai’ showed that RmAG expression levels differed significantly among the 3 types. The highest expression levels of this gene were observed in type 1, consisting of the narrowest petals with traces of anthers. In the lily, the petaloid stamens in whorl 3 of ‘Elodie’ varied in their morphological mutation level with the level of expression of the AG-like gene LeLAG1 (Akiti et al., 2011). It has been suggested that the expression level of this AG-like gene is correlated with the degree of petaloidy of the stamens in ‘Elodie’. Likewise, in whorl 2 of ‘Gin-no-zai’, it is thought that the expression levels of RmAG and the phased morphological mutations are related.

In all plants, the deduced class B gene-expression patterns were similar to those of the wild type. In ‘Seigaiha’, amplified PI-like gene fragments in whorls 2 and 3 were confirmed as 2 different sequences. AP3-like genes were expressed in all whorls of three cultivars, as well as the wild type. Such class B gene-expression patterns have also been confirmed in Kurume azalea, Edo-kirishima azalea, and several wild species (Koga et al., 2012; Ohtani et al., 2007). In the mature flowers of kiwifruit, the orthologues of AP3 were expressed in all floral organs, with higher accumulation detected in the petal and stamen tissues. Furthermore, the orthologue of PI was exclusively expressed in the petals and stamens (Varkonyi-Gasic et al., 2011). In such cases, whorl specification of B functionality seems to be dependent on PI-like gene expression. The expression patterns of class B genes in this study do not contradict the suggestion that phased RmAG expression in whorl 2 of ‘Gin-no-zai’ is related to staminoidy of the petal. However, our research group performed expression analyses of MADS-box genes using the floral organs of azalea just after flower opening. In Vitis vinifera and Sophora tetraptera, the expression levels of the orthologues of AP3 and PI during floral organ differentiation and/or development significantly increased (Poupin et al., 2007; Song et al., 2008). Therefore, for detailed analysis of the MADS-box gene-expression levels and patterns in azalea, we will need to examine the appropriate stage for expression analysis.

It is difficult to conclude that ‘Hanaguruma’ and ‘Seigaiha’ have mutations related to the expression level of RmAG, judging from these results. In ‘Hanaguruma’, the candidate gene responsible for the mutant phenotype is not yet known. On the other hand, ‘Seigaiha’ is similar to ‘Kin-kujyaku’, in which the MADS-box gene-expression pattern was similar to that of the wild type (Tasaki et al., 2012b). These 2 cultivars apparently have a narrow mutation in the lateral organs. To investigate the cause, seedlings obtained from various cross combinations were grown as candidates for new cultivars. To understand the various mutations within azalea cultivars, it is necessary to consider other homeotic genes. WOX family genes operate in regions such as the shoot apical meristem, the root apical meristem, and the
embryo for the development of the plant body. For example, PRESSED FLOWER in Arabidopsis and MAEWEST in Petunia regulate lateral axis-dependent development of the lateral organs (Matsumoto and Okada, 2001; Vandenbussche et al., 2009). Its orthologs in azalea may have functions related to narrow mutations in organs, such as that found in ‘Seigaiha’.

Based on the results of the current study, we suggest that the narrow petal form of ‘Gin-no-zai’ is determined by $RmAG$ expression levels in whorl 2. In addition, “sai-zaki” cultivars in azalea will be subdivided into several groups by its related genes. Several additional factors, as mentioned above, need to be considered with respect to the gene responsible for phenotypic mutations in ‘Hanaguruma’ and ‘Seigaiha’. To clarify the cause, we investigated progenies from various cross combinations by using currently used “sai-zaki” cultivars. The expansion of genetic information for flower development will be important for applications in azalea breeding (e.g., as DNA markers). Furthermore, we propose that Japanese old azalea cultivars, which have various mutations, are valuable as plant materials to clarify the mechanisms responsible for morphological formation in flowers.

**Literature Cited**


