Application of Mutant *ap3* Allele-based Markers for the Selection of the Long-lasting Flower Phenotype (Misome-shō) in Evergreen Azalea Cultivars

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There is a long-lasting flower trait with a temporal color change, known as “misome-shō”, in Japanese evergreen azalea. This trait has been found in several wild Japanese evergreen azalea species, such as *Rhododendron kaempferi* ‘Nikkō-misome’, *R. macrosepalum* ‘Kochō-zoroi’, *R. indicum* ‘Chōjyu-hō’, and *R. × hannoense* ‘Amagi-beni-chōjyu’. The corollas of long-lasting flower cultivars undergo a conversion of normal corollas to sepaloid corollas due to loss of function of the MADS-box B class gene, *APETALA3* (*AP3*) homolog. Also, the long-lasting flower trait was shown to be recessive to normal flowers and controlled by a single gene. To develop a DNA marker for selection of the long-lasting flower phenotype, we carried out a multiplex-PCR approach to detect the *ap3* mutant allele related to long-lasting flower traits, and investigated the flower phenotypes in crossed progenies of 23 cross combinations and 245 individuals. The normal flower phenotype individuals in the crossed progenies were homozygous for the normal allele or heterozygous for the normal allele and the *ap3* mutant allele. On the other hand, the long-lasting flower phenotype individuals in the crossed progenies were homozygous for the *ap3* mutant allele of long-lasting cultivars. These results support the idea that the long-lasting flower trait is caused by a mutation in the *AP3* homolog, and it has been newly clarified that any combination of these mutant alleles in long-lasting flower cultivars has the long-lasting flower phenotype. In conclusion, our data indicate that efficient selection of individuals with long-lasting flowers will be possible by using selection DNA markers linked to the long-lasting flower trait.

Key Words: *AP3*, breeding, MADS-box gene, marker assisted selection, mutant allele.

Introduction

Among the 50 *Rhododendron* species native to Japan (Kurashige et al., 1998), species within the subgenus *Tsutsusi*, section *Tsutsusi*, such as *R. kaempferi*, *R. indicum*, *R. kiusianum*, *R. ripense*, and *R. macrosepalum*, are important genetic resources for the evergreen azaleas used as ornamental shrubs or pot azaleas (Kobayashi, 2020). Each cultivar group, such as Edo Kirishima, Kurume, Ryūkyū, Hirado, and Satsuki, has been developed since the Edo era (1603–1867) by selection and crossing based on these wild species as they have high ornamental value, are easy to cultivate,

and exhibit interspecific hybridization (Kobayashi et al., 2000; Kurashige and Kobayashi, 2008; Kobayashi, 2016). During the azalea boom in the middle of the 17th century, cultivars with flower color variations and shape mutants, such as white flowers, double flowers, and hose-in-hose flowers, were selected and collected from wild species all over the country. Many of these cultivars were described in the first monograph on azaleas, “Kinshū - makura”, edited in 1692 (Ito and Creech, 1984; Kobayashi, 2016). This monograph provided the first description of ‘Misome-guruma’, a cultivar with small light-red flowers, long stamens, and a long blooming season, with the flowers turning greenish in June (Ito and Creech, 1984). The long-lasting flower trait, known as “misome-shō”, has been found in several wild species, such as *R. kaempferi* ‘Nikkō-misome’, *R. macrosepalum* ‘Kochō-zoroi’, *R. indicum* ‘Chōjyu-hō’, and *R. × hannoense* ‘Amagi-beni-chōjyu’.

In order to select breeding material for long-lasting flower cultivars, we have conducted various studies on

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floral morphology, inheritance, and a MADS-box B class gene related to floral morphology. In a previous morphological study, we indicated that the long-lasting flower trait is a floral homeotic mutant with a sepaloid corolla (Kobayashi et al., 2010; Gobara et al., 2017). In addition, the long-lasting flower trait was shown to be a recessive trait of the normal flower phenotype and is controlled by a single gene (Gobara et al., 2017). Therefore, it takes a long-term breeding cycle to develop long-lasting flowers with new flower traits, such as double flowers and hose-in-hose flowers.

A marker-associated selection (MAS) (Xu and Crouch, 2008) system is important for rapid selection of the long-lasting flower phenotype, which is a recessive trait, during the juvenile period. In long-lasting cultivars, transcription of the *AP3* homolog was reduced, and a long terminal repeat retrotransposon was independently inserted into exons 1, 2, and 7 or an unknown sequence in exon 1 of the gDNA of each cultivar. This insertion apparently abolished the normal mRNA sequence of the *AP3* homolog (Cheon et al., 2018). Based on this insertion, DNA markers for the detection of the *ap3* mutant allele were developed for each long-lasting cultivar. In a previous study (Cheon et al., 2018), normal flower phenotypes of the F₁ progenies between normal and long-lasting cultivars were heterozygous for the normal and *ap3* mutant alleles. In addition, it was shown that long-lasting flowers in crossed progenies between normal and long-lasting cultivars were homozygous for the *ap3* mutant allele in one cross combination.

In this study, to develop a rapid selection marker for the long-lasting flower phenotype in evergreen azaleas, we carried out a multiplex-PCR approach to detect the *ap3* mutant allele related to the long-lasting flower trait in crossed progenies of various cross combinations using the DNA markers described by Cheon et al. (2018).

Materials and Methods

Plant materials

The normal flower azaleas *R. kaempferi*, *R. macrosepalum*, *R. indicum* ‘Ōsakazuki’, *R. ripense*, *R. × pulchrum* ‘Ōmurasaki’, and Kurume azalea ‘Wakakaede’, and the long-lasting flower cultivars *R. kaempferi* ‘Nikkō-misome’, *R. macrosepalum* ‘Kochō-zoroi’, *R. indicum* ‘Chōjyu-hō’, and *R. × hannoense* ‘Amagi-beni-chōjyu’ were used. A total of 69 F₁ progenies from 11 cross combinations between normal and long-lasting cultivars and a total of 25 F₁ progenies of six cross combinations between long-lasting cultivars were used (Table 1). In addition, a total of 151 crossed progenies of six cross combinations between the F₁ hybrids of normal and long-lasting cultivars were used (Table 2). These plants were obtained from the azalea resource collection at the Plant Breeding Laboratory of the Faculty of Life and

Environmental Sciences, Shimane University.

DNA marker analysis

Genomic DNA was extracted from the leaves of each plant using the modified CTAB method (Kobayashi et al., 1998). The multiplex-PCR primers described by Cheon et al. (2018) that can distinguish each *ap3* mutant allele in long-lasting cultivars are shown in Table 3 and Figure 1A. The Pr2 and Pr8 primers, which were newly designed from the *AP3* homolog sequences of *R. kaempferi* ‘Nikkō-misome’ (*rkap3-NM*, DDBJ Acc. No. AB861605) and *R. × hannoense* ‘Amagi-beni-chōjyu’ (*rhap3-AC*, DDBJ Acc. No. AB861608), were used in this study. The three PCR primers Pr8, Pr9, and Pr10 were designed to amplify approximately 425 bp fragments in ‘Nikkō-misome’, and approximately 180 bp fragments in cultivars other than ‘Nikkō-misome’. The three PCR primers Pr1, Pr3, and Pr4 were designed to amplify approximately 315 bp fragments in ‘Kochō-zoroi’ (*rmap3-KZ*, DDBJ Acc. No. AB861606), and approximately 180 bp fragments in cultivars other than ‘Kochō-zoroi’. The three PCR primers Pr5, Pr6, and Pr7 were designed to amplify approximately 600 bp fragments in ‘Chōjyu-hō’ (*riap3-CH*, DDBJ Acc. No. AB861607), and approximately 220 bp fragments in cultivars other than ‘Chōjyu-hō’. The three PCR primers Pr1, Pr2, and Pr3 were designed to amplify approximately 500 bp fragments in ‘Amagi-beni-chōjyu’, and approximately 180 bp fragment in cultivars other than ‘Amagi-beni-chōjyu’. A multiplex-PCR was performed with the primer set in Table 3 to detect the *ap3* mutant alleles of each long-lasting cultivar and the normal alleles of normal flowers as the insets in *rmap3-KZ* and *rhap3-AC* were located in exon 1. The normal alleles, *rmap3-KZ*, and *rhap3-AC* were detected using four primers, Pr1, Pr2, Pr3, and Pr4. The multiplex-PCR was performed in 10 µL reaction mixtures containing 10 ng DNA template, 1 × Ex-Taq buffer, 200 µM dNTPs, 0.25 U Ex-Taq (TaKaRa Bio Inc., Shiga, Japan), and 0.2 µM of each primer. The reaction conditions were as follows: denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 2 min. The amplified PCR products were analyzed by electrophoresis in a 1.5% agarose gel (Nippon Genetics Co., Ltd., Tokyo, Japan) using 0.5× TBE buffer and run at 100 V for 30 min. When using the Pr2 and Pr9 primers, the annealing temperature was changed to 56°C. The crossed progenies between the F₁ hybrids of normal and long-lasting cultivars had their genotypes determined using the DNA markers before blooming. We investigated the flower phenotypes of the crossed progenies that bloomed from 2013 to 2020 and identified genotypes of the *AP3* homolog through the multiplex-PCR.

Table 1. PCR amplification profiles for the *ap3* mutant alleles and the flower phenotypes in F₁ progenies.

Line	Parents			Phenotype ^z	Genotype ^y
	Seed	×	Pollen		
	<i>R. macrosepalum</i> ‘Kochō-zoroi’			L	<i>rmap3-KZ/rmap3-KZ</i>
	<i>R. indicum</i> ‘Chōjyu-hō’			L	<i>riap3-CH/riap3-CH</i>
	<i>R. × hannoense</i> ‘Amagi-beni-chōjyu’			L	<i>rhap3-AC/rhap3-AC</i>
	Hybrid ‘Tenkō’ (<i>R. × hannoense</i> ‘Amagi-beni-chōjyu’ × <i>R. kaempferi</i> ‘Nikkō-misome’)			L	<i>rkap3-NM/rhap3-AC</i>
	Kurume azalea ‘Wakakaede’			N	<i>AP3/AP3</i>
	Kurume azalea ‘Kirin’			N	<i>AP3/AP3</i>
	<i>R. oldhamii</i>			N	<i>AP3/AP3</i>
	<i>R. × pulchrum</i> ‘Ōmurasaki’			N	<i>AP3/AP3</i>
	<i>R. ripense</i>			N	<i>AP3/AP3</i>
	<i>R. kiusianum</i>			N	<i>AP3/AP3</i>
	Hybrid ‘Tō-sei’ (<i>R. transiens</i> ‘Hatsusimo’ × pot azalea ‘Star light’)			N	<i>AP3/AP3</i>
	Long-lasting flower-type		Normal flower-type		
06012	<i>R. kaempferi</i> ‘Nikkō-misome’	×	Kurume azalea ‘Wakakaede’	N (14)	<i>AP3/rkap3-NM</i>
07097	<i>R. indicum</i> ‘Chōjyu-hō’	×	Kurume azalea ‘Kirin’	N (4)	<i>AP3/riap3-CH</i>
06025	<i>R. indicum</i> ‘Chōjyu-hō’	×	<i>R. oldhamii</i>	N (3)	<i>AP3/riap3-CH</i>
99088	<i>R. × hannoense</i> ‘Amagi-beni-chōjyu’	×	<i>R. × pulchrum</i> ‘Ōmurasaki’	N (5)	<i>AP3/rhap3-AC</i>
05216	<i>R. × hannoense</i> ‘Amagi-beni-chōjyu’	×	Kurume azalea ‘Kirin’	N (4)	<i>AP3/rhap3-AC</i>
99094	<i>R. × hannoense</i> ‘Amagi-beni-chōjyu’	×	<i>R. ripense</i>	N (4)	<i>AP3/rhap3-AC</i>
04093	Hybrid ‘Tenkō’	×	<i>R. × pulchrum</i> ‘Ōmurasaki’	N (6)	<i>AP3/rhap3-AC</i>
				N (6)	<i>AP3/rkap3-NM</i>
	Normal flower-type		Long-lasting flower-type		
05063	Kurume azalea ‘Wakakaede’	×	<i>R. indicum</i> ‘Chōjyu-hō’	N (10)	<i>AP3/riap3-CH</i>
04171	<i>R. oldhamii</i>	×	<i>R. macrosepalum</i> ‘Kochō-zoroi’	N (3)	<i>AP3/rmap3-KZ</i>
99090	<i>R. kiusianum</i>	×	<i>R. × hannoense</i> ‘Amagi-beni-chōjyu’	N (2)	<i>AP3/rhap3-AC</i>
04083	Hybrid ‘Tō-sei’	×	Hybrid ‘Tenkō’	N (2)	<i>AP3/rhap3-AC</i>
				N (6)	<i>AP3/rkap3-NM</i>
	Long-lasting flower-type		Long-lasting flower-type		
06032	<i>R. kaempferi</i> ‘Nikkō-misome’	×	<i>R. indicum</i> ‘Chōjyu-hō’	L (3)	<i>rkap3-NM/riap3-CH</i>
06021	<i>R. indicum</i> ‘Chōjyu-hō’	×	<i>R. kaempferi</i> ‘Nikkō-misome’	L (2)	<i>rkap3-NM/riap3-CH</i>
06027	<i>R. macrosepalum</i> ‘Kochō-zoroi’	×	<i>R. kaempferi</i> ‘Nikkō-misome’	L (2)	<i>rkap3-NM/rmap3-KZ</i>
06019	<i>R. × hannoense</i> ‘Amagi-beni-chōjyu’	×	<i>R. kaempferi</i> ‘Nikkō-misome’	L (9)	<i>rkap3-NM/rhap3-AC</i>
05067	<i>R. × hannoense</i> ‘Amagi-beni-chōjyu’	×	<i>R. indicum</i> ‘Chōjyu-hō’	L (7)	<i>riap3-CH/rhap3-AC</i>
98051	<i>R. × hannoense</i> ‘Amagi-beni-chōjyu’	×	<i>R. macrosepalum</i> ‘Kochō-zoroi’	L (2)	<i>rmap3-KZ/rhap3-AC</i>

^z N: normal flower phenotype. L: long-lasting flower phenotype. Parentheses show the individual number.

^y Genotype was based on the multiplex-PCR products, which were *rkap3-NM* (425 bp); *rmap3-KZ* (315 bp); *riap3-CH* (600 bp); and *rhap3-AC* (500 bp) as shown in the mutant allele. *AP3* shows the normal allele.

Results

Detection of the *ap3* mutant allele in long-lasting cultivars

The PCR products obtained using the three primers (Pr8, Pr9, and Pr10) amplified approximately 425 bp fragments in ‘Nikkō-misome’, and approximately 180 bp fragments in the other cultivars (Fig. 1B). The PCR products obtained using the primers Pr5, Pr6, and Pr7 amplified approximately 600 bp fragments in ‘Chōjyu-hō’, and approximately 220 bp fragments in cultivars other than ‘Chōjyu-hō’ (Fig. 1B). The PCR products obtained using the four primers Pr1, Pr2, Pr3, and Pr4 amplified approximately 315 bp fragments in

‘Kochō-zoroi’, 500 bp fragments in ‘Amagi-beni-chōjyu’, and 180 bp fragments in cultivars other than these (Fig. 1B).

Detection of the *ap3* mutant allele in crossed progenies

All analyzed F₁ progenies between the normal and long-lasting cultivars had normal flower phenotypes and were heterozygous for the *ap3* mutant allele and the normal allele (Table 1; Fig. 1C). On the other hand, all analyzed F₁ progenies between the long-lasting cultivars had long-lasting flower phenotypes and were homozygous for the *ap3* mutant alleles (Table 1; Fig. 1D).

In the crossed progenies between the F₁ hybrids of

Table 2. PCR amplification profiles for the *ap3* mutant alleles and the flower phenotypes in the crossed progenies between F₁ hybrids of normal and long-lasting cultivars.

Line	Parents (allele/allele)			Phenotype ^z	Genotype ^y
	Seed	×	Pollen		
13003	04093-5 (<i>AP3/rkap3-NM</i>)	×	05216-1 (<i>AP3/rhap3-AC</i>)	N (6)	<i>AP3/AP3</i>
				N (3)	<i>AP3/rkap3-NM</i>
				N (3)	<i>AP3/rhap3-AC</i>
				L (3)	<i>rkap3-NM/rhap3-AC</i>
13006	04093-5 (<i>AP3/rkap3-NM</i>)	×	07097-1 (<i>AP3/riap3-CH</i>)	N (9)	<i>AP3/AP3</i>
				N (9)	<i>AP3/rkap3-NM</i>
				N (9)	<i>AP3/riap3-CH</i>
				L (7)	<i>rkap3-NM/riap3-CH</i>
13013	04093-5 (<i>AP3/rkap3-NM</i>)	×	06025-1 (<i>AP3/riap3-CH</i>)	N (2)	<i>AP3/AP3</i>
				N (7)	<i>AP3/rkap3-NM</i>
				N (7)	<i>AP3/riap3-CH</i>
				L (3)	<i>rkap3-NM/riap3-CH</i>
13016	04171-1 (<i>AP3/rmap3-KZ</i>)	×	07097-1 (<i>AP3/riap3-CH</i>)	N (10)	<i>AP3/AP3</i>
				N (9)	<i>AP3/rmap3-KZ</i>
				N (7)	<i>AP3/riap3-CH</i>
				L (7)	<i>rmap3-KZ/riap3-CH</i>
13018	04171-1 (<i>AP3/rmap3-KZ</i>)	×	05216-1 (<i>AP3/rhap3-AC</i>)	N (8)	<i>AP3/AP3</i>
				N (8)	<i>AP3/rmap3-KZ</i>
				N (5)	<i>AP3/rhap3-AC</i>
				L (6)	<i>rmap3-KZ/rhap3-AC</i>
13020	06025-1 (<i>AP3/riap3-CH</i>)	×	05216-1 (<i>AP3/rhap3-AC</i>)	N (5)	<i>AP3/AP3</i>
				N (8)	<i>AP3/riap3-CH</i>
				N (5)	<i>AP3/rhap3-AC</i>
				L (5)	<i>riap3-CH/rhap3-AC</i>

^z N: normal flower phenotype. L: long-lasting flower phenotype. Parentheses show the individual number.

^y Genotype was based on multiplex-PCR products, which were *rkap3-NM* (425 bp); *rmap3-KZ* (315 bp); *riap3-CH* (600 bp); and *rhap3-AC* (500 bp) as shown in the mutant allele. *AP3* shows the normal allele.

Table 3. Primers used for the multiplex-PCR analysis.

Detecting gene	No.	Name	Sequence (5'-3')	Reference
<i>rhap3-AC</i> ('Amagi-beni-chōjyu')	Pr1	AP3-AMAGI (MAR) F1	GAGAGTGAAGAAATGGCGAG	Cheon et al., 2018
	Pr2	AP3 AMA-R1	CTTCAACTTTAAGTATTATAGGC	developed in this study
	Pr3	5RACE-AP3-2	GTATTCATGGAGCTTCTCGG	Cheon et al., 2018
<i>rmap3-KZ</i> ('Kochō-zoroi')	Pr1	AP3-AMAGI (MAR) F1	GAGAGTGAAGAAATGGCGAG	Cheon et al., 2018
	Pr3	5RACE-AP3-2	GTATTCATGGAGCTTCTCGG	Cheon et al., 2018
	Pr4	AP3-KOCHO (MAR) R	GGATAACGCCTCTAGAAGGG	Cheon et al., 2018
<i>riap3-CH</i> ('Chōjyu-hō')	Pr5	AP3-CHOJYU (MAR) F1	GCGAATACCTAGAACGAAGCA	Cheon et al., 2018
	Pr6	AP3-CHOJYU (MAR) R1	ATGGAGGAGCGTGGATTAAA	Cheon et al., 2018
	Pr7	AP3-CHOJYU (MAR) R2	GGGACCGATACCGCTATTTA	Cheon et al., 2018
<i>rkap3-NM</i> ('Nikkō-misome')	Pr8	AP3-NIKKO (MAR) F1	ATAGAACGCAATTGGAGAGGA	developed in this study
	Pr9	AP3 AMA-R1	CTTCAACTTTAAGTATTATAGGC	same with primer 2
	Pr10	AP3-NIKKO (MAR) R1	AAGCAAATGTCGTGAGATCG	Cheon et al., 2018

normal and long-lasting cultivars, normal and long-lasting flower phenotypes exhibited an approximately 3:1 split (Table 2; Fig. 3A). In all combinations of the crossed progenies between the F₁ hybrids of normal and long-lasting cultivars, normal alleles were detected in normal flower phenotypes, and homozygous *ap3*

mutant alleles were detected in the long-lasting flower phenotypes (Table 2; Fig. 2). The genotype segregation pattern in the crossed progenies between the F₁ hybrids of the normal and long-lasting cultivars, identified by the multiplex-PCR, corresponded to the expected ratio (Table 4).

Fig. 1. Multiplex-PCR to detect the normal *AP3* allele linked to the normal flower phenotype and the *ap3* mutant allele linked to the long-lasting flower phenotype. A: Schematic diagram showing the primer regions that distinguish normal and *ap3* mutant alleles (Cheon et al., 2018). E1–7 and lines indicate each exon and each intron, respectively. Horizontal arrows indicate regions and direction of primers. B: Results of multiplex-PCR amplification of the normal allele and *rkcap3-NM*, *riap3-CH*, *rmap3-KZ*, and *rhap3-AC*. Numbers indicate (1) *Rhododendron kaempferi*, (2) *R. macrosepalum*, (3) *R. indicum* ‘Ōsakazuki’, (4) *R. ripense*, (5) *R. × pulchrum* ‘Ōmurasaki’, (6) Kurume azalea ‘Wakakaede’, (7) *R. kaempferi* ‘Nikkō-misome’, (8) *R. macrosepalum* ‘Kochō-zoroi’, (9) *R. indicum* ‘Chōjyu-hō’, and (10) *R. × hancei* ‘Amagi-beni-chōjyu’. C: Results of multiplex-PCR amplifications in 99088 line (P1: *R. × hancei* ‘Amagi-beni-chōjyu’ × P2: *R. × pulchrum* ‘Ōmurasaki’). D: Results of the multiplex-PCR amplifications in 06032 line (P3: *R. kaempferi* ‘Nikkō-misome’ × P4: *R. indicum* ‘Chōjyu-hō’). N and L indicate the normal flower phenotype and the long-lasting flower phenotype, respectively. M indicates a 100 bp ladder (Nippon Genetics).

Discussion

The vase life of flowers is one of the most important characteristics required by consumers (Imanishi et al., 1992). Normal flowering azalea plants finish flowering in about two weeks. In contrast, long-lasting flower cultivars keep their corollas for more than 100 days, with the flowers then turning greenish (Kobayashi, 2013).

This is similar to the “autumn-like color” of hydrangeas, in which flowering continues with changes in the flower color (Yoshida et al., 2008).

The corollas of long-lasting flower cultivars involves the conversion of normal corollas to sepaloid corollas due to loss of function of the *AP3* homolog (Cheon et al., 2018). In addition, the long-lasting flower trait was shown to be recessive to normal flowers and con-

Fig. 2. Results of the multiplex-PCR amplifications in the 13006 line (04093 × 07097). N and L indicate the normal flower phenotype and the long-lasting flower phenotype, respectively. PC indicates the positive control (Pr8 + Pr9 + Pr10: ‘Nikkō-misome’; Pr5 + Pr6 + Pr7: ‘Chōjyuhō’). M indicates a 100 bp ladder (Nippon Genetics).

Fig. 3. The relationships between flower phenotypes and genotypes in crossed progenies of long-lasting cultivars derived from the four different species. A: The inheritance model of the long-lasting flower trait. Parentheses show the segregation ratio. B: The inheritance model of the flower phenotypes based on the *AP3* genotype combinations.

Table 4. Segregation data for the *ap3* mutant alleles in the crossed progenies between F₁ hybrids of normal and long-lasting cultivars.

Line	Parents			No. of progenies	Genotypic segregation			Expected ratio			χ^2	P
	Seed	×	Pollen		Homozygous normal allele	Normal allele and mutant allele	Homozygous mutant allele	Homozygous normal allele	Normal allele and mutant allele	Homozygous mutant allele		
13003	04093	×	05216	15	6	6	3	1	2	1	1.800	0.407
13006	04093	×	07097	34	9	18	7	1	2	1	0.353	0.838
13013	04093	×	06025	19	2	14	3	1	2	1	4.368	0.113
13016	04171	×	07097	33	10	16	7	1	2	1	0.576	0.750
13018	04171	×	05216	27	8	13	6	1	2	1	0.333	0.846
13020	06025	×	05216	23	5	13	5	1	2	1	0.391	0.822

trolled by a single gene (Gobara et al., 2017). Similar mutations of sepaloid petals have been found in other plants, and Singh et al. (2014) suggested that the development of sepaloid petals in *Papaver somniferum* was caused by the reduced expression of *PapsAP3-I*; this trait is a recessive trait controlled by a single gene. In herbaceous peony (*Paeonia lactiflora*), a spontaneous corolla mutant under natural conditions was closely associated with selective expression alterations of duplicated *AP3* and *PI* genes; down-regulation of dupli-

cated B class genes may result in a reduction in the *AP3-PI* heterodimer in sepaloid corollas (Gong et al., 2017). Additionally, Lange et al. (2013) suggested that sepaloid petal development in *Eschscholzia californica* was caused by a lack of function of the MADS-box B class gene, *EScaGLO*. DNA markers related to flower shape mutation have been developed for double-flowered cultivars caused by mutations of the MADS-box C class gene *AGAMOUS* in gentians (*Gentiana scabra*) (Tasaki et al., 2017) and *Matthiola incana*

(Nakatsuka and Koishi, 2018). However, there are no reports of any DNA markers related to an improvement in the ornamental value and vase life of flowers caused by B class gene mutations such as the long-lasting flower trait in azalea.

The long-lasting flower trait in azalea is commercially important, and we have been conducting studies on breeding materials (Kobayashi, 2020). In a previous study, DNA markers to detect the *ap3* mutant allele were developed in long-lasting cultivars (Cheon et al., 2018). In this study, we used a multiplex-PCR approach to detect the *ap3* mutant allele related to the long-lasting flower trait in normal flowers and long-lasting flower cultivars, and crossed progenies of 23 cross-combinations and 245 individuals using the DNA markers described by Cheon et al. (2018). As a result of the multiplex-PCR, we found normal flowers were homozygous for the *AP3* gene, and each long-lasting cultivar had a homozygous *ap3* mutant allele (Fig. 1B). In all cross combinations, individuals with the normal flower phenotype were homozygous for the normal allele or heterozygous for the normal allele and the *ap3* mutant allele (Tables 1 and 2). On the other hand, the F_1 progenies between long-lasting cultivars and long-lasting flower phenotype individuals in the cross progenies between F_1 hybrids of normal and long-lasting flower cultivars were homozygous for the *ap3* mutant allele derived from different species of the long-lasting cultivars (Tables 1 and 2).

The genotype that was detected before blooming by using the DNA markers was linked to the flower phenotype in all crossed progenies. The reliability of the DNA markers for long-lasting flower trait selection was confirmed in this study. Figure 3 shows the relationship between flower phenotypes and genotypes in crossed progenies of long-lasting cultivars derived from the four different species clarified in this report. It was revealed that all of the *ap3* mutant alleles in each long-lasting flower cultivar were recessive to normal alleles (Fig. 3B). There was no dominant/recessive relationship between the *ap3* mutant alleles of each long-lasting flower cultivar derived from the four different species, and we confirmed that any combination of these mutant alleles had a long-lasting flower phenotype (Fig. 3B). These results strengthen the concept that the long-lasting flower trait is caused by a mutation in the *AP3* homolog. These results also suggest the possibility of breeding new azalea cultivars with the characteristics of multiple species by interspecific crossing using long-lasting cultivars, rather than simply through varietal crossing.

We have been conducting various studies on the morphological mutations found in evergreen azalea cultivars such as hose-in-hose, double-flowers, narrow-petals, and narrow-leaf mutations, to apply them in our breeding program (Kobayashi, 2016). In previous studies, we reported the development of DNA markers to

select hose-in-hose cultivars (Cheon et al., 2017a), and the genetic analysis of the double-flowered trait (Cheon et al., 2017b) and narrow-petal and -leaf mutations (Tasaki et al., 2019). We are selecting the long-lasting flower phenotype during the juvenile period using these MAS systems. Among the selected individuals, candidates for new long-lasting flower cultivars have been developed.

In conclusion, interspecific crossing using the long-lasting flower trait that is present across species can contribute to the development of new long-lasting cultivars with high ornamental value. In addition, it was shown that an efficient selection process for long-lasting individuals is possible by using selection DNA markers linked to the long-lasting flower trait.

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