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Plastid DNA Inheritance of Progenies from Diallel Cross among Related Evergreen Azalea Species Belonging to Series Kaempferia

Takeru Itabashi[1], Kenji Ureshino[1,2]* and Akira Suzuki[1,2]

[1]*The United Graduate School of Agricultural Sciences, Iwate University, Iwate 020–8550, Japan*
[2]*Faculty of Agriculture, Iwate University, Iwate 020–8550, Japan*

To clarify the main causes affecting the mode of plastid DNA (ptDNA) inheritance in *Rhododendron* spp., ptDNA inheritance of progenies from diallel crosses among six evergreen azalea species belonging to series Kaempferia was investigated with PCR-SSCP analysis. Polymorphism was detected among parental species, except one combination between *R. kiusianum* and *R. tosaense*. Maternal ptDNA inheritance was observed in all crosses, whereas paternal inheritance was found in 16/28 crosses. High frequency (33.3 to 87.5%) of paternal ptDNA inheritance was observed in all crosses with *R. transiens* as a seed parent, whereas the frequency was relatively low (less than 8.3%) in the reverse cross, except *R. tosaense* × *R. transiens*. In contrast, no or slight (less than 12.5%) paternal ptDNA inheritance was observed in the cross with *R. kaempferi* var. *macrogemma*, *R. kiusianum* and *R. simsii* as a seed parent. When these species were used as pollen parents in the cross with other three species, i.e. *R. transiens*, *R. kaempferi* and *R. tosaense*, the frequency was usually high (45.0 to 90.9%, except *R. kaempferi* × *R. kaempferi* var. *macrogemma*).

Key Words: diallel cross, paternal ptDNA inheritance, PCR-SSCP, *Rhododendron*, universal primers.

Introduction

Cytoplasmic DNA has been used for phylogenic study because of several advantages over nuclear DNA: (1) it is generally inherited from a uniparent, and (2) hardly undergoes recombination during reproduction (Birky, 1995). In most angiosperm genera, plastid DNA (ptDNA) is generally inherited from the mother; however, it is not strict in some genera, and biparental inheritance has been reported (Corriveau and Coleman, 1988; Harris and Ingram, 1991; Kirk and Tilney-Bassett, 1978; Kuroiwa, 1991; Nagata et al., 1999). In *Rhododendron*, it has been reported that the mode of ptDNA inheritance was biparental with several inter-specific crosses (Kobayashi, 1996; Michishita et al., 2002; Ureshino et al., 1999). The frequency of paternal ptDNA is, however, variable among the cross combinations.

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* Corresponding author (E-mail: ureshino@iwate-u.ac.jp).

The mode of ptDNA inheritance is an important factor for wide crosses of azalea, because albinism of the progenies is caused by plastome-genome incompatibility (Ureshino et al., 1999). Ureshino et al. (1998, 1999) conducted wide crosses between evergreen azaleas and *R. japonicum* f. *flavum*, and reported as follows: (1) Seeds could be obtained only when evergreen azaleas were used as seed parents; (2) Many progenies were albino because of plastome-genome incompatibility between the plastome from evergreen azaleas (maternal ptDNA inheritance) and nuclear genome from *R. japonicum* f. *flavum*; (3) The green plant could be obtained only when ptDNA was inherited from *R. japonicum* f. *flavum* (paternal ptDNA inheritance); (4) Paternal ptDNA inheritance widely varied among evergreen species used as seed parents.

Clarifying the factors for ptDNA inheritance is therefore considered very important for the better design of a breeding program for *Rhododendron*. To evaluate ptDNA transmission of progenies accurately, ptDNA polymorphism and high cross ability among cross parents are required.

Series Kaempferia is a representative evergreen azalea series, in which many horticulturally important species are included. Furthermore, high cross compatibility exists among species, and albino progenies do not occur

in these interspecific crosses (Michishita et al., 2003). These species, therefore, are considered to be valuable materials for evaluating ptDNA transmission.

For investigation of ptDNA transmission, a molecular marker technique such as RFLP and PCR-RFLP has been used (Chat et al., 1999; Yao et al., 1994). These methods are very powerful tools to detect polymorphisms among species; however, to obtain a polymorphism with these methods, mutation of the restriction site is required. Therefore, it is difficult to detect a polymorphism among closely related species. As a more sensitive polymorphic marker, the PCR-SSCP method has been used to detect a polymorphism in some closely inter- or intra-species and investigated for the inheritance pattern of ptDNA (Chen et al., 2002; Shiraishi et al., 2001).

In this study, we investigated the ptDNA inheritance of progenies from diallel crosses among series Kaempferia's species using the PCR-SSCP method.

Materials and Methods

Plant materials

Progenies from diallel crosses among six evergreen azalea species (Table 1) belonging to series Kaempferia were used. One individual was used for each parental species. Most of these progenies were provided by the Faculty of Agriculture, Kyushu University, Japan. For some combinations, crosses with the same parental stocks were conducted to obtain a sufficient number of progenies; however, they could not be crossed in some combinations, because the flowering time of *R. kaempferi* was later than other species, and, only a few flowers were obtained in *R. simsii*. Therefore, progenies from *R. kiusianum* (KIU) × *R. kaempferi* (KAE), *R. simsii* (SIM) × *R. kaempferi* (KAE) and *R. simsii* (SIM) × *R. kaempferi* var. *macrogemma* (MCR) were investigated only 7, 1, and 7 individual(s), respectively.

DNA isolation

Total genomic DNA was extracted from 70 mg of frozen leaves by a modified CTAB method (Kobayashi et al., 1998).

PtDNA amplification

For detecting polymorphisms among parental species, 12 regions, including a non-coding region in ptDNA, were amplified via polymerase chain reaction (PCR). The primer sets of *trn*L and *trn*L-*trn*F were designed by Taberlet et al. (1991), and those of *trn*G, *trn*G-*trn*M, *trn*V-*trn*M, *trn*W-*trn*P, *rpl*16, *rps*16, *pet*B, *pet*D-*rpo*A, *psb*C-*trn*S, and *atp*F were designed by Nishizawa and Watano (2000) (Table 2). PCR amplifications were carried out in a total volume of 25 μL containing 25 ng of template DNA, 0.5 μM of each primer, 2.0 mM of $MgCl_2$, 0.1 mM of dNTPs, 2.5 μL of 10 × reaction buffer and 0.5 unit of *Taq* DNA polymerase (Roche Diagnostics, Germany). Amplification was carried out using a Program Temp Control System TP-240 (TaKaRa Bio, Japan) with one cycle at 94°C for 3 min and 35 cycles for 1 min at 94°C, 1 min annealing and 1 min at 72°C and one cycle at 72°C for 10 min. The annealing temperature of each primer set was 43°C in *trn*G, 45°C in *trn*V-*trn*M, *pet*B and *psb*C-*trn*S, 47°C in *trn*G-*trn*M, *rpl*16, *rps*16, *pet*D-*rpo*A, and *atp*F, 49°C in *trn*L and *trn*L-*trn*F, and 51°C in *trn*W-*trn*P, respectively. PCR products were checked by electrophoresis in 1.5% agarose (SIGMA, Germany) gels containing 0.005% ethidium bromide at 100 V for 40 min, and then the gels were photographed under ultraviolet illumination.

PCR-SSCP analysis

Two μL of PCR products were mixed with 8 μL of formamide dye consisting of 90% (v/v) formamide, 0.5% (v/v) bromophenol-blue and 8% (v/v) glycerin. The mixture was denatured by heating at 95°C for 3 min, and immediately cooled on ice. The mixture was then electrophoresed (6 μL per lane) in non-denatured polyacrylamide 0.5 × TBE gel containing 25% (v/v) MDE gel solution (TaKaRa Bio) and 10% (v/v) glycerol. Electrophoresis was performed in 0.5 × TBE buffer at 100 V for 8 h. The temperature was kept at 4°C by a Cooling Water Circulator LTP-125 (AS ONE, Japan) during electrophoresis. After electrophoresis, the gel was stained with Silver Stain Kit (ATTO, Japan) following the manufacture's instructions.

Investigation of ptDNA inheritance

Using the ptDNA polymorphic region among parental species, ptDNA inheritance of the progenies was investigated. After the investigation, the paternal ptDNA inheritance ratio was calculated in each cross.

Table 1. *Rhododendron* species used in this study.

Species	Code	Collection site
Series Kaempferia		
R. transiens	TRA	Kanto area, unknown
R. tosaense	TOS	Hyuga City, Miyazaki Pref.
R. kaempferi	KAE	Mt. Kurokami, Saga Pref.
R. kaempferi var. *macrogemma*	MCR	Osima Is., Tokyo.
R. kiusianum	KIU	Sensuikyo, Oita Pref.
R. simsii	SIM	Okinawa Is., Okinawa Pref.

Table 2. Universal primers in this study.

Amplification regions	Primer name	Primer sequence	Amplified length (bp)
*trn*L(UAA) intron	*trn*L	5'-CGAAATCGGTAGACGCTACG-3' 5'-GGGGATAGAGGGACTTGAA-3'	630
*trn*L(UAA)-*trn*F(GAA) intergenic region	*trn*L-*trn*F	5'-GGTTCAAGTCCCTCTATCCC-3' 5'-ATTTGAACTGGTGACACGAG-3'	520
*trn*G(UCC) intron	*trn*G	5'-GGTAAAAGTGTGATTCGTTC-3' 5'-ATCTTCATCCATGGATCCT-3'	290
*trn*G(GCC)-*trn*M(GAU) intergenic region	*trn*G-*trn*M	5'-TCTCTTTGCCAAGGAGAAGA-3' 5'-ATAACCTTGAGGGTTCGAAT-3'	270
*trn*V(UAG)-*trn*M(GAU) intergenic region	*trn*V-*trn*M	5'-TGTAAACGAGTTGCTCTACC-3' 5'-CTTACCACTGATTAAGTAG-3'	Could not amplify
*trn*W(GCA)-*trn*P(UGG) intergenic region	*trn*W-*trn*P	5'-GATTTGAACCTACGACATCG-3' 5'-GATGTGGCGCAGCTTGGTAG-3'	360
*rpl*16 intron	*rpl*16	5'-GTTTCTTCTCATCCAGCTCC-3' 5'-GAAAGAGTCAATATTCGCCC-3'	400
*rps*16 intron	*rps*16	5'-CCCCCTAGAAACGTATAGGA-3' 5'-CGAAGTAATGTCTAAACCCA-3'	440
*pet*B intron	*pet*B	5'-AGAGATGGTTCTACTTCGTC-3' 5'-TTCATACTAGAACCACGATG-3'	360
*pet*D-*rpo*A intergenic region	*pet*D-*rpo*A	5'-GGGCATTGGTGCAACATTAC-3' 5'-CAGCCAAGAAGATCTTATGA-3'	Could not amplify
*psb*C-*trn*S(UGA) intergenic region	*psb*C-*trn*S	5'-TGAACCTGTTCTTTCCATGA-3' 5'-GAACTATCGAGGGTTCGAAT-3'	310
*atp*F intron	*atp*F	5'-TTCATTTGGCTCTCACGCTC-3' 5'-AATGCTGAATCGACGACCTA-3'	280

Results

DNA amplification and detection of polymorphism by PCR-SSCP

DNA amplification succeeded in 10 of 12 primer sets excluding *trn*V-*trn*M and *pet*D-*rpo*A. The fragment lengths were 270 bp to 630 bp depending on each primer set (Fig. 1 and Table 2). The fragments were the same length among species when the same primer set was used; therefore, these primer sets could not be detected for polymorphism among species with agarose gel electrophoresis. Using PCR-SSCP methods, polymorphisms were detected in seven of ten primer sets excluding *trn*G, *rps*16, and *psb*C-*trn*S (Table 3). With these seven primers, five species could be discriminated from other species, except from the combination of *R. kiusianum* (KIU) and *R. tosaense* (TOS).

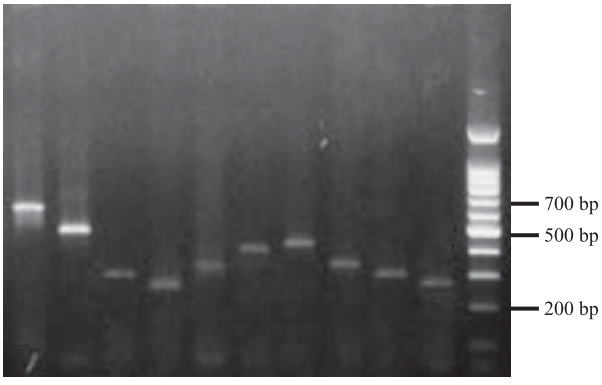

Fig. 1. Fragment length of PCR products derived from total genomic DNA of *R. transiens* using each primer set. Lane 1: *trn*L. Lane 2: *trn*L-*trn*F. Lane 3: *trn*G. Lane 4: *trn*G-*trn*M. Lane 5: *trn*W-*trn*P. Lane 6: *rpl*16. Lane 7: *rps*16. Lane 8: *pet*B. Lane 9: *psb*C-*trn*S. Lane 10: *atp*F. Lane 11: 100 bp ladder marker.

Table 3. Banded phenotype at 10 regions in *Rhododendron* species belonging to series Kaempferia.

Species	Primer pairs									
	*trn*L	*trn*L-*trn*F	*trn*G	*trn*G-*trn*M	*trn*W-*trn*P	*rpl*16	*rps*16	*pet*B	*psb*C-*trn*S	*atp*F
TRA	B[z]	B	A	B	B	B	A	B	A	B
TOS	A	A	A	A	A	A	A	A	A	A
KAE	A	A	A	A	A	A	A	A	A	C
MCR	A	C	A	A	A	A	A	A	A	A
KIU	A	A	A	A	A	A	A	A	A	A
SIM	A	A	A	C	C	A	A	C	A	A

[z] Different letters are the polymorphic pattern.

PtDNA inheritance of progenies from diallel crosses

From the results of PCR-SSCP analysis, three primer sets were used in ptDNA inheritance investigation (Fig. 2). Progenies from 28 cross combinations, except for the reciprocal cross between *R. kiusianum* (KIU) and *R. tosaense* (TOS), were investigated for the inheritance of ptDNA. Maternal ptDNA inheritance was observed in all cross combinations. Paternal inheritance was observed in 16 cross combinations, whereas the frequency varied among cross combinations (1.6 to 90.9%) (Table 4).

In all crosses with *R. transiens* (TRA) as a seed parent, the frequency of paternal ptDNA inheritance was relatively high (33.3 to 87.5%), whereas the frequency was low in the reverse cross (less than 8.3%), except for one cross of *R. tosaense* (TOS) × *R. transiens* (TRA) (Fig. 3).

When using *R. kaempferi* var. *macrogemma* (MCR), *R. kiusianum* (KIU) and *R. simsii* (SIM) as a seed parent, paternal ptDNA inheritance was observed in 2/5, 1/4, and 1/5 crosses, respectively, although the percentage

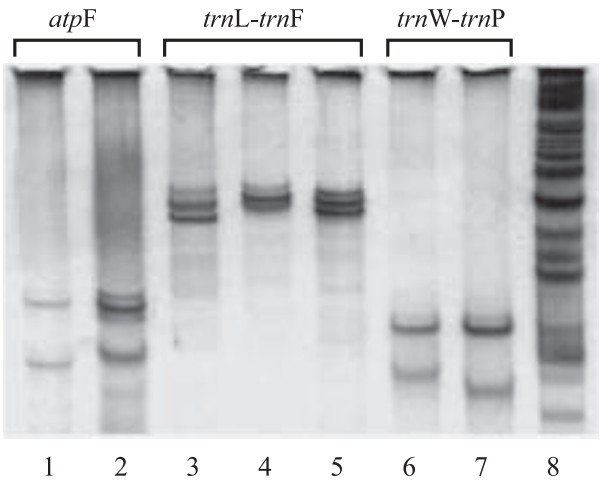

Fig. 2. Polymorphic PCR-SSCP banding patterns of PCR products amplified for *atp*F, *trn*L-*trn*F and *trn*W-*trn*P that used investigation of ptDNA inheritance in diallel cross. Lanes 1 and 3: KAE, Lanes 2 and 4: MCR, Lane 5: TRA, Lane 6: TOS, Lane 7: SIM, Lane 8: 100 bp ladder marker.

Table 4. Frequency of paternal ptDNA inheritance of progenies from diallel crosses among six evergreen azalea species belonging to series Kaempferia.

Female \ Male	TRA	TOS	KAE	MCR	KIU	SIM
TRA	—	14/42 (33.3)	18/37 (48.6)	12/19 (63.2)	14/16 (87.5)	52/69 (75.4)
TOS	4/12 (33.3)[z]	—	0/12 (0)	18/40 (45.0)	—[y]	10/11 (90.9)
KAE	1/12 (8.3)	15/25 (60.0)	—	0/13 (0)	30/54 (55.6)	11/20 (55.0)
MCR	0/31 (0)	1/62 (1.6)	0/16 (0)	—	0/17 (0)	4/40 (10.0)
KIU	0/15 (0)	—[y]	0/7 (0)	0/26 (0)	—	2/16 (12.5)
SIM	0/12 (0)	0/27 (0)	0/1 (0)	0/7 (0)	1/30 (3.3)	—

[z] Number of paternal ptDNA inheritances of progenies/number of progenies tested (%).
[y] Non-polymorphism between parents.

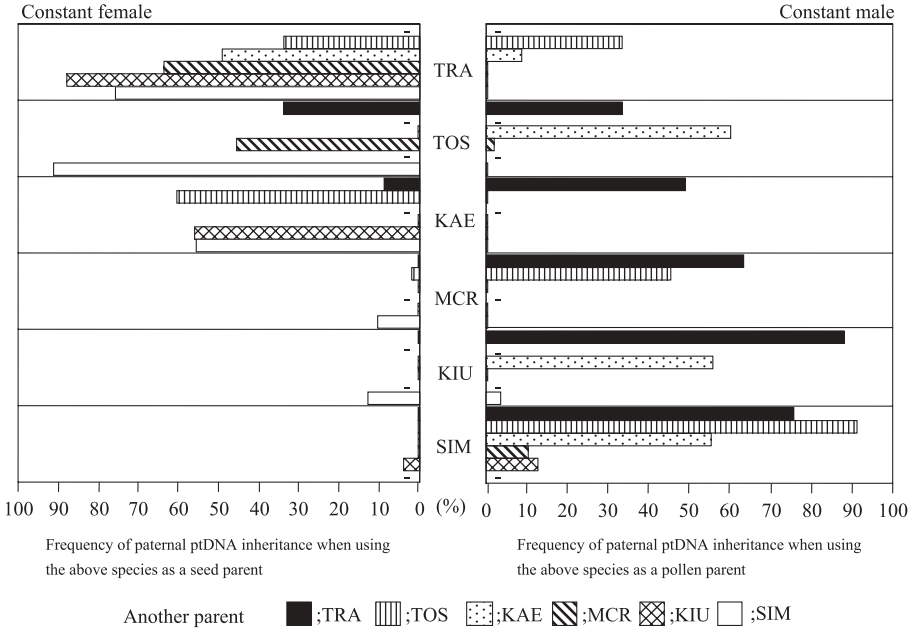

Fig. 3. Comparing percentage of paternal ptDNA inheritance between seed parents and pollen parents in each reciprocal cross. Bar=not investigated.

was very low (less than 12.5%). When these species were crossed with three other species, i.e. *R. transiens* (TRA), *R. tosaense* (TOS) and *R. kaempferi* (KAE), as a pollen parent, the frequency of paternal ptDNA was relatively high (45.0 to 90.9%), excluding *R. kaempferi* (KAE) × *R. kaempferi* var. *macrogemma* (MCR) (Fig. 3).

In crosses with *R. tosaense* (TOS) and *R. kaempferi* (KAE) as the seed parent, the frequency pattern of paternal ptDNA inheritance was segregated into two types. For *R. tosaense* (TOS), no paternal ptDNA inheritance was observed in the cross with *R. kaempferi* (KAE), whereas there was a relatively high frequency in other species. For *R. kaempferi* (KAE), no and low paternal ptDNA inheritance was observed in the cross with *R. kaempferi* var. *macrogemma* (MCR) and *R. transiens* (TRA), whereas these was a relatively high frequency in other crosses (Fig. 3).

Discussion

It is known that ptDNA is usually inherited from the mother in angiosperm genera, whereas biparental ptDNA inheritance is also reported in some species (Corriveau and Coleman, 1988; Harris and Ingram, 1991). Our study showed that ptDNA of progenies from interspecific crosses of *Rhododendron* was inherited from both parents. This result indicated that the mode of ptDNA inheritance in *Rhododendron* was biparental.

However, the frequency of paternal ptDNA inheritance varied widely among cross combinations. As a determination factor for the frequency of paternal ptDNA inheritance, two main factors were reported by previous researchers. One was controlled by the nuclear genotype of either parent. This type is reported in *Pelargonium* (Tilney-Bassett et al., 1992) and *Petunia* (Derepas and Dulieu, 1992). In *Pelargonium*, the frequency of paternal ptDNA inheritance was controlled by the genotype of a seed parent, and the segregation pattern was repeatable with a constant female and varying males. In *Petunia*, the frequency was controlled by the genotype of a pollen parent, and the pattern was repeatable with a constant male and varying females. In this study, the frequency of paternal ptDNA inheritance was repeatable in the cross with four species, i.e. *R. transiens*, *R. kaempferi* var. *macrogemma*, *R. kiusianum*, and *R. simsii*, as constant females, whereas it was variable in the cross with two other species, i.e. *R. tosaense* and *R. kaempferi*, as constant females. This result indicates that although the pattern of paternal ptDNA inheritance in *Rhododendron* is mainly affected by a seed parent, it is not directly controlled by the seed parent genotype.

The other factor was controlled by the plastome genotype. This type is reported in *Oenothera* (Tilney-Bassett, 1991), in which each species had their own rate of multiplication, and competition of the multiplication rate among parental plastids in zygotes resulted in the pattern of ptDNA inheritance of progenies (Schötz, 1954). Schötz (1968) classified the plastid into three category, i.e. the fastest-multiplying plastid (F type), the slowest-multiplying plastid (S type) and the average-multiplying plastid (M type), in *Oenothera*. When crosses were made between females with S type plastids and males with F type plastids, the male plastid transmission was high. On the other hand, it was very low in reverse crosses. In the present study, high frequency of paternal ptDNA inheritance (more than 33.3%) was observed in the cross with *R. transiens* as a seed parent, whereas it was relatively low (less than 8.3%) in species as a pollen parent, except one cross of *R. tosaense* × *R. transiens*. Furthermore, in *R. kaempferi* var. *macrogemma*, *R. kusianum* and *R. simsii*, paternal ptDNA inheritance was low (less than 12.5%) in crosses of these species as seed parents to other three species (*R. transiens*, *R. tosaense* and *R. kaempferi*), and was high (more than 45.0%) in reverse crosses, except *R. kaempferi* × *R. kaempferi* var. *macrogemma*. These results were very similar to the reports in *Oenothera*, even excluding combinations with few investigated plants, i.e. *R. kusianum* × *R. kaempferi*, *R. simsii* × *R. kaempferi* and *R. simsii* × *R. kaempferi* var. *macrogemma*. Therefore, we conclude that the transmission pattern of ptDNA is determined by the parental plastome in *Rhododendron*.

As described above, the ptDNA transmission pattern in *Rhododendron* could be classified into two major types. In particular, *R. transiens* has high potential for paternal ptDNA transmission in species as a seed parent without distinction of pollen parents. This species has the largest number of polymorphisms among species (seven of ten regions) (Table 3), and is therefore a phylogenetically distant species. In fact, Ureshino et al. (2006) reported that the plastid of *R. transiens* was related to series Scabra (subsection Scabra) rather than series Kaempferia (subsection Tsutsusi). Okamoto and Suto (2000) also suggested that *R. transiens* had undergone a hereditary effect from subsection Scabra. For this reason, it is probable that the ptDNA inheritance pattern of *R. transiens* differs from others.

Some results differed between the present and previous studies. Ureshino et al. (1998) reported that when *R. kiusianum* was used as a seed parent, a relatively high frequency of green progenies (resulting from paternal ptDNA inheritance) was obtained in the cross with *R. japonicum* f. *flavum*, whereas the frequency of paternal ptDNA inheritance was relatively low in our study. In *Rhododendron*, geographic differentiation has been recognized among the same species (Ueno et al., 2004); therefore, the existence of local or individual variation in the frequency of paternal ptDNA inheritance is also considered in the same species. However, since we used one individual for each parental species, this could not be detected. To clarify this, other individuals sampled from different areas should be used for each species. In future, differentiation of the ptDNA transmission pattern in *Rhododendron* should be clarified

from investigation of the relationship between ptDNA inheritance and phylogeny.

Clarification of the mode of ptDNA inheritance is very important for wide crosses of *Rhododendron* spp., because the appearance of albino progeny is determined by plastome-genome incompatibility (Kita et al., 2005; Michishita et al., 2002; Ureshino et al., 1999). We have been conducted many wide crosses between evergreen azalea species and deciduous yellow-flowered *R. japonicum* f. *flavum* to produce yellow-flowered evergreen azaleas (Ureshino et al., 1998, 1999). From those studies, we suggested that the three-way cross between a F_1 hybrid (produced by crossing a species with high cross compatibility to *R. japonicum* f. *flavum* and a species with a high potential paternal ptDNA transmission rate, resulting in a green plant appearance) and *R. japonicum* f. *flavum* was a useful method to achieve that (Ureshino et al., 1998). In the present study, we clarified that *R. transiens* had high potential for paternal ptDNA transmission in species as a seed parent without the distinction of pollen parents. In fact, in our previous study, when these species was used as a seed parent, the highest frequency of green progenies (about 30%) was observed in the cross between several evergreen azalea species and *R. japonicum* f. *flavum* (Ureshino et al., 2002). From these, this species might also be useful breeding material in a three-way cross.

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