Maternal Nuclear Genome Controls Paternal ptDNA Inheritance in Progeny from Interspecific Crosses of *Rhododendron* spp.

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The genetic factor controlling the mode of plastid DNA (ptDNA) inheritance in *Rhododendron* was investigated using polymerase chain reaction-single-stranded conformation polymorphism (PCR-SSCP) analysis at the *trnL-trnF* intergeneric region. In the cross of *R. kaempferi* var. *macrogemma* × *R. simsii*, a low frequency of paternal ptDNA inheritance was observed in the progeny. In the cross of *R. transiens* × *R. simsii*, a high frequency of paternal ptDNA was observed in the progeny. When the F₁ plants from *R. kaempferi* var. *macrogemma* × *R. transiens* were used as seed parents and were crossed with *R. simsii*, the pattern of ptDNA inheritance was segregated into 2 types among cross combinations at a ratio of 1:1, namely, low (2.0–11.1%) and high (32.6–66.6%) frequencies of paternal ptDNA inheritance, although the F₁ plants had identical ptDNA from *R. kaempferi* var. *macrogemma*. From these results, we conclude that the mode of ptDNA inheritance is controlled by at least one major gene in the maternal nucleus.

Key Words: biparental inheritance, paternal inheritance, PCR-SSCP, *Rhododendron transiens*.

Introduction

Plastid DNA (ptDNA) variation has been valuable as a genetic marker in phylogenetic studies because recombination during reproduction is limited (Birky, 1995). In the majority of angiosperms, the inheritance of ptDNA is purely maternal (Kirk and Tilney-Bassett, 1978). In these genera, since paternal ptDNA is excluded during pollen mitosis or during sperm cell formation or development, ptDNA inheritance is largely regulated from the male lineage (Hagemann and Schröder, 1989).

In several angiosperm genera, however, paternal ptDNA is not excluded until fertilization, which suggests the possibility of biparental ptDNA inheritance (Hagemann and Schröder, 1989). Corriveau and Coleman (1988) suggested that about 14% of 235 angiosperms have the potential for biparental ptDNA inheritance. Regardless of the ptDNA transmission

potential of pollen, ptDNA in progeny was inherited from both parents (biparental) as well as from each parent (uniparental) (Snijder et al., 2007). This indicates that alternative replication or degradation of ptDNA occurs during zygotic embryo development. Furthermore, the occurrence of each ptDNA type varied from infrequently to frequently among not only different genera but also different species within the same genus (Tilney-Bassett and Birky, 1981).

Genetic control of ptDNA inheritance in progeny was reported in several biparental-type genera. In an intraspecific cross of *Pelargonium zonale*, the mode of ptDNA inheritance was controlled by 2 nuclear genes of the female parent (Tilney-Bassett and Birky, 1981; Tilney-Bassett et al., 1992). Conversely, in *Oenothera*, the effect of the maternal plastid genome on ptDNA inheritance was exhibited in plants with a constant nuclear background (from *O. hookeri*) and various plastomes (from different species) as cross parents (Chiu et al., 1988). Furthermore, the partial contribution of the maternal nuclear genome to the mode of ptDNA inheritance was also reported in *Oenothera* (Schötz, 1974, 1975). Thus, the genetic factor controlling ptDNA inheritance varies among genera.

The genus *Rhododendron* is representative of a large

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number of horticulturally important ornamental plants (Galle, 1985). PtDNA inheritance is an important factor for wide crosses of azaleas because albinism of the progeny is caused by plastome-genome incompatibility (Ureshino et al., 1999). Clarification of the factor determining ptDNA inheritance is thus considered to be very important for the better design of breeding programs in *Rhododendron*. The mode of ptDNA inheritance in this genus is also biparental, which was confirmed by observation of fluorescently stained generative cells (Kaul et al., 1987; Kuroiwa, 1991). Although progeny from interspecific crosses had maternal, paternal, or biparental ptDNA, frequencies of occurrence differed among cross combinations (Michishita et al., 2002; Ureshino et al., 1999). In a previous study, we investigated ptDNA inheritance in progeny from diallel crosses among 6 evergreen azalea species belonging to subsection *Kaempferia*. Our results showed the following: (1) the frequency of paternal ptDNA inheritance in progeny was repeatable among crosses with a constant female; (2) species were classified into 3 types, F (fast), M (medium), and S (slow), by the multiplication rate of ptDNA; and (3) paternal ptDNA inheritance occurred frequently in the cross of S (as a seed parent) $\times$ F or M (as a pollen parent), and occurred infrequently in the reverse cross (Itabashi et al., 2008). From these results, we previously concluded that the transmission pattern of ptDNA is determined by competition of the parental plastomes. We also suggested that the seed parent genotype, namely, either the maternal nuclear or the maternal plastid genome, also affects ptDNA inheritance in progeny. However, it is not clear which genome is closely related to the control of ptDNA inheritance.

The objective of this study is to clarify the genetic factor that controls ptDNA inheritance in progeny. To achieve this goal, F_1 plants from the interspecific cross between F- and S-type species were used as seed parents and were crossed with other F-type species. Derived progeny were investigated for ptDNA inheritance using polymerase chain reaction-single-stranded conformation polymorphism (PCR-SSCP) analysis.

Materials and Methods

(1) Plant materials

Two species, *Rhododendron kaempferi* var. *macrogemma* (MAC) and *R. transiens* (TRA), and their F_1 plants were used as seed parents, and crossed with *R. simsii* (SIM). *R. kaempferi* var. *macrogemma* and *R. simsii* are F-type plants, and *R. transiens* is an S-type plant; these classifications were clarified in a previous report (Itabashi et al., 2008). These 3 species belonging to the section *Kaempferia* are easily crossed with each other (Michishita et al., 2003). As F_1 plants, 21 plants with ptDNA of *R. kaempferi* var. *macrogemma* were derived from *R. kaempferi* var. *macrogemma* $\times$ *R. transiens*, and four plants with ptDNA of *R. transiens* were from the reverse cross. Progeny from

crosses with *R. simsii* as a pollen parent were used for segregation analysis of ptDNA.

(2) Segregation analysis of ptDNA of progeny

The *trnL*(UAA)–*trnF*(GAA) intergenic region in ptDNA was amplified by polymerase chain reaction (PCR). The primer sets were 5'-GGTTCAAGTCCCTC TATCCC-3' and 5'-ATTTGAACTGGTGACACGAG-3' designed by Taberlet et al. (1991). PCR amplifications were carried out according to a previous report (Itabashi et al., 2008). PCR products were checked by electrophoresis in 1.5% agarose (Sigma-Aldrich Japan, Tokyo, Japan) gels containing 0.005% ethidium bromide at 100 V for 40 min, and then the gels were photographed under ultraviolet illumination and used for PCR-SSCP analysis. The PCR-SSCP analysis was conducted following the procedure described in a previous report (Itabashi et al., 2008). Furthermore, to confirm that the polymorphism among cross parents in PCR-SSCP was based on the nucleotide difference, amplified PCR products were cloned into the pMD20-T vector (Mighty TA Cloning Kit; TaKaRa, Otsu, Japan) and sequenced with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Results

(1) Detection of polymorphism among cross parents by PCR-SSCP analysis

Although PCR products of three species, namely, *Rhododendron kaempferi* var. *macrogemma* (MAC), *R. transiens* (TRA) and *R. simsii* (SIM), at the *trnL*–*trnF* region indicated the same single-banded phenotype (459 bp) on an agarose gel (Fig. 1A), different-banded phenotypes were detected using PCR-SSCP analysis (Fig. 1B). Alignment of the sequences of this region revealed the presence of four substitution points among these three species (Fig. 2). From this, it was clear that the polymorphism among cross parents in PCR-SSCP was based on the nucleotide difference.

(2) ptDNA inheritance of progeny in crosses with *R. simsii* (SIM) as a pollen parent

The frequency of paternal ptDNA inheritance of progeny was 2.2% in the cross with *R. kaempferi* var. *macrogemma* (MAC, F type) as a seed parent, and was 33.3% in the cross with *R. transiens* (TRA, S type) as a seed parent (Table 1).

When 21 F_1 individuals [with ptDNA of *R. kaempferi* var. *macrogemma* (MAC)] from *R. kaempferi* var. *macrogemma* (MAC) $\times$ *R. transiens* (TRA) were used as seed parents, ptDNA of progeny was inherited from a seed parent, a pollen parent, or both parents (Table 2). PtDNA inheritance from a seed parent was observed in all crosses, the inheritance from a pollen parent in 19 crosses, and the inheritance from both parents in 14 crosses. The frequency of paternal ptDNA inheritance in progeny ranged from 2.2 to 66.6% among the cross

combinations. Two peaks of the segregation patterns by the frequency were recognized the peak with the frequency range of 2.0–11.1% including 11 cross combinations and that of 32.6–66.6% including 10 combinations (Table 2; Fig. 3). The chi-square test of goodness-of-fit showed that the ratio agreed with a 1:1 ratio at the 5% probability level.

When four F_1 individuals [with ptDNA of *R. transiens* (TRA)] from *R. transiens* (TRA) $\times$ *R. kaempferi* var. *macrogemma* (MAC) were used as

Fig. 1. Banding patterns of PCR products (A) amplified for *trnL*–*trnF* region and the SSCP polymorphism (B) of cross parents. Lanes 1 and 5, *R. kaempferi* var. *macrogemma* (MAC); Lanes 2 and 6, *R. transiens* (TRA); Lanes 3 and 7, *R. simsii* (SIM); Lane 4, λ HindIII marker; Lane 8, 100 bp ladder marker.

seed parents, the frequency of paternal ptDNA inheritance in progeny was also segregated into two types (low frequency from 0 to 12.9% and high frequency from 62.9 to 88.2%) among the cross combinations (Table 3).

Discussion

The segregation pattern of ptDNA in F_1 progeny was almost the same as that described in a previous study (Itabashi et al., 2008), namely, the frequency of paternal inheritance is high in *Rhododendron transiens* $\times$ *R. simsii* (S type $\times$ F type) and low in *R. kaempferi* var. *macrogemma* $\times$ *R. simsii* (F type $\times$ F type). This result indicates that the mode of ptDNA inheritance is genetically stable and strongly affected by maternal nuclear or plastid genotype in *Rhododendron* spp.

In crosses of their F_1 plants $\times$ *R. simsii*, although the

Fig. 2. Sequence comparison of *trnL*(UAA)–*trnF*(GAA) intergenic region among parental species. Different nucleotides are indicated with a black background. MCR, *R. kaempferi* var. *macrogemma*; SIM, *R. simsii*; TRA, *R. transiens*.

Table 1. ptDNA inheritance of F_1 progeny from interspecific crosses of *Rhododendron*.

Cross combination	ptDNA type of seed parent	No. of progeny tested	ptDNA inheritance in progeny ^z			Frequency of paternal ptDNA ^y (%)
			M	P	B	
MCR1 ^x $\times$ SIM1	MCR	45	44	1	0	2.2
TRA1 $\times$ SIM1	TRA	33	22	11	0	33.3

^z M, maternal ptDNA; P, paternal ptDNA; B, biparental ptDNA.

^y Including progeny with both paternal and biparental ptDNA.

^x MCR, *R. kaempferi* var. *macrogemma*; SIM, *R. simsii*; TRA, *R. transiens*.

Table 2. ptDNA inheritance in progeny from crosses between F₁ plants of *R. kaempferi* var. *macrogemma* × *R. transiens* and *R. simsii*.

Cross combination	ptDNA type of seed parent	No. of progeny tested	ptDNA inheritance in progeny ^z			Frequency of paternal ptDNA ^y (%)
			M	P	B	
F ₁ -1 × SIM1 ^x	MCR	50	49	0	1	2.0
F ₁ -2 × SIM1	MCR	34	33	0	1	2.9
F ₁ -3 × SIM1	MCR	51	49	1	1	4.0
F ₁ -4 × SIM1	MCR	45	42	0	3	6.7
F ₁ -5 × SIM1	MCR	44	41	3	0	6.8
F ₁ -6 × SIM1	MCR	39	36	2	1	7.7
F ₁ -7 × SIM1	MCR	52	48	4	0	7.7
F ₁ -8 × SIM1	MCR	38	35	1	2	7.9
F ₁ -9 × SIM1	MCR	52	47	4	1	9.6
F ₁ -10 × SIM1	MCR	47	42	5	0	10.6
F ₁ -11 × SIM1	MCR	45	40	4	1	11.1
F ₁ -12 × SIM1	MCR	43	29	14	0	32.6
F ₁ -13 × SIM1	MCR	52	35	13	4	32.6
F ₁ -14 × SIM1	MCR	50	33	17	0	34.0
F ₁ -15 × SIM1	MCR	53	34	18	1	35.8
F ₁ -16 × SIM1	MCR	50	27	23	0	46.0
F ₁ -17 × SIM1	MCR	45	22	22	1	51.1
F ₁ -18 × SIM1	MCR	35	16	14	5	54.3
F ₁ -19 × SIM1	MCR	44	20	22	2	54.5
F ₁ -20 × SIM1	MCR	27	10	17	0	63.0
F ₁ -21 × SIM1	MCR	51	17	33	1	66.6

^z M, maternal ptDNA; P, paternal ptDNA; B, biparental ptDNA.^y Including progeny with both paternal and biparental ptDNA.^x SIM, *R. simsii*; MCR, *R. kaempferi* var. *macrogemma*.**Table 3.** ptDNA inheritance in progeny from crosses between F₁ plants of *R. transiens* × *R. kaempferi* var. *macrogemma* and *R. simsii*.

Cross combination	ptDNA type of seed parent	No. of progeny tested	ptDNA inheritance in progeny ^z			Frequency of paternal ptDNA ^y (%)
			M	P	B	
F ₁ -1 × SIM1 ^x	TRA	18	18	0	0	0
F ₁ -2 × SIM1	TRA	31	27	4	0	12.9
F ₁ -3 × SIM1	TRA	35	13	18	4	62.9
F ₁ -4 × SIM1	TRA	51	6	45	0	88.2

^z M, maternal ptDNA; P, paternal ptDNA; B, biparental ptDNA.^y Including progeny with both paternal and biparental ptDNA.^x SIM, *R. simsii*; TRA, *R. transiens*. **Fig. 3.** Distribution of F₁ plants from *R. kaempferi* var. *macrogemma* × *R. transiens* based on the frequency of progeny with paternal ptDNA in the cross of F₁ × *R. simsii*.

ptDNA of the F₁ plants was identical, the frequency of paternal ptDNA was segregated among cross combinations. If the mode of ptDNA inheritance is controlled by the maternal plastid genome alone, the frequency of paternal ptDNA should not be segregated among the cross combinations. This finding clearly indicates that a gene(s) in the maternal nuclear genome controls the mode of ptDNA inheritance.

The contribution of the maternal nuclear genome to the mode of ptDNA inheritance was reported in several plants, such as *Pelargonium* and alfalfa (Masoud et al., 1990; Tilney-Bassett and Birky, 1981; Tilney-Bassett et al., 1992). In alfalfa, maternal nuclear control of plastid transmission was clarified by a three-plant reciprocal crossing scheme (Masoud et al., 1990). A genetic

factor involved in the mode of ptDNA inheritance was reported in *Pelargonium*. In this genus, the mode of ptDNA inheritance was found to be determined by a pair of complementary genes (*Pr1* and *Pr2*) in the maternal nuclear genome, and was segregated into 2 types depending on the genotype of the female parent (Tilney-Bassett and Birky, 1981; Tilney-Bassett et al., 1992). In the present study, the distribution of F_1 plants from *R. kaempferi* var. *macrogemma* $\times$ *R. transiens* based on the frequency of progeny with paternal ptDNA in the cross of $F_1 \times R. simsii$ segregated into two groups, namely, high- and low-frequency groups, at a the ratio of 1:1. This segregation ratio indicates that the inheritance of ptDNA is controlled by one dominant gene (A), and the F_1 plants used as seed parents are obtained in crosses with a recessive homozygous (aa) genotype and a heterozygous (Aa) genotype parent (Fig. 4).

Rhododendron transiens is classified into subsection Kaempferia by its morphological traits (Yamazaki, 1996). Previous studies, however, reported that this species had undergone a hereditary effect from subsection Scabra (Miyano et al., 2013; Okamoto and Suto, 2000; Ureshino et al., 2006). Therefore, it is considered that *R. transiens* is a hybrid originating from subsection Kaempferia and subsection Scabra species. This species may thus be the heterozygous parent with a dominant allele (A) given the high frequency of paternal ptDNA inheritance and the recessive allele (a) (Fig. 4).

As mentioned above, although the effect of nuclear gene(s) of the seed parent on the inheritance of ptDNA in progeny has been reported in *Pelargonium* and alfalfa, the function of this gene(s) on the inheritance is not clear. On the other hand, a cytological approach was conducted to clarify the mechanism of involvement of the nuclear gene(s) of the seed parent in the inheritance of ptDNA. In alfalfa, the position of maternal plastids in unfertilized mature egg cells was the key to paternal ptDNA inheritance (Rusche et al., 1995). Although there is no direct evidence, the position of maternal

plastids might be governed by a maternal nuclear gene(s) and might determine the ptDNA inheritance of progeny. In order to clarify the function of the maternal nuclear gene that is identified in the present study, cytological observation of mature egg cells may be effective.

Several results that cannot be explained by only a maternal nuclear gene were also obtained in this study. One is the appearance of progeny with ptDNA of both parents in crosses with F_1 plants as the seed parent. Biparental ptDNA inheritance in an interspecific cross of *Rhododendron* was reported previously (Kobayashi et al., 2013; Ureshino et al., 1999). In some plants that have ptDNA transmission potential from pollen, it is considered that alternative replication or degradation of ptDNA occurs at various stages during and after the zygote stage of the embryo (Birky, 1995; Snijder et al., 2007). In this study, although 5-year-old F_1 plants were used for SSCP analysis in the cross with *R. transiens* or *R. kaempferi* var. *macrogemma* as the seed parent, young seedlings (2 months old) were used for the analysis in the cross with F_1 plants as the seed parent. As such, the timing of alternative sorting out of ptDNA might be relatively late in *Rhododendron*, which allows the existence of biparental ptDNA in young plants.

Another such result is the difference in the degree of the high frequency of paternal ptDNA among cross combinations. The degree in the cross with F_1 plants having ptDNA from *R. kaempferi* var. *macrogemma* was lower (32.5–66.6%) than that from *R. transiens* (62.9–88.2%). In a previous study, we reported that parental plastome competition was one of the main factors determining ptDNA inheritance, and categorized ptDNA of *R. kaempferi* var. *macrogemma* and *R. simsii* as the fast migrating type (F type) and ptDNA of *R. transiens* as the slow migrating type (S type) (Itabashi et al., 2008). Thus, the difference of the degree of the high frequency of paternal ptDNA among two F_1 groups is considered to be the result of plastome competition. Furthermore, although seed parents have the

Fig. 4. Schematic diagram of the mode of inheritance of chloroplast DNA considered from the results of this experiment. A, a dominant allele; a, a recessive allele.

same ptDNA (from *R. transiens*), a difference of the degree of the high frequency of paternal ptDNA was also observed between *R. transiens* × *R. simsii* (33.3%) and (*R. transiens* × *R. kaempferi* var. *macrogemma*) × *R. simsii* (62.9–88.2%). The reason for this the difference cannot be explained by the present study, but other factors, for example, alternative replication or degradation of ptDNA after the zygote stage, may affect ptDNA inheritance.

In this study, although the seed parents had identical ptDNA, the frequency of paternal ptDNA was segregated among cross combinations. We therefore concluded that the mode of ptDNA inheritance is mainly controlled by one major gene in the maternal nucleus in *Rhododendron* spp. Clarification of the mode of ptDNA inheritance is very important for the wide cross of *Rhododendron* spp. because the appearance of albino progeny is determined by plastome-genome incompatibility (Michishita et al., 2002; Ureshino et al., 1999). For example, in the cross between evergreen azalea species and *R. japonicum* f. *flavum*, seedlings were obtained only when the evergreen azaleas were used as seed parents, and seedlings with green-colored leaves were obtained only when ptDNA of progeny was inherited from *R. japonicum* f. *flavum*, that is, paternal ptDNA inheritance (Ureshino et al., 1999). In the present study, we clarified that one major gene of the maternal nucleus in *R. transiens* influenced the high frequency of paternal ptDNA inheritance of progeny. This species is thus considered to be useful as a bridge plant for obtaining seedlings with green-colored leaves effectively in wide crosses of various evergreen azalea species × *R. japonicum* f. *flavum*. Thus, the results of the present experiment are considered to be useful for conducting wide cross breeding of azaleas effectively.

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