Plastid Inheritance and Plastome–genome Incompatibility of Intergeneric Hybrids between *Menziesia* and *Rhododendron*

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Summary

Intergeneric hybridization between *Menziesia* and *Rhododendron* generates many albino or pale–green seedlings. To clarify the factors that cause albino phenotype, we analyzed the inheritance of plastid (pt) DNA by PCR–RFLP analysis on the intergeneric hybrids between two genera and an intraspecific hybrid within *Menziesia* by reciprocal cross–pollination, respectively. All albino and pale–green intergeneric progenies contained the ptDNA of *Rhododendron*, whereas green progenies contained that of *Menziesia*, indicating that *Rhododendron* plastome is incompatible with *Menziesia* nuclear genome. Moreover, when *M. multiflora* was used as a female parent, most progenies transmitted their plastids paternally. Plastome–genome incompatibility seems to be independent from ptDNA inheritance in *Menziesia*–*Rhododendron* hybrids.

Key Words: intergeneric hybrid, *Menziesia*, plastome–genome, ptDNA inheritance, *Rhododendron*.

Introduction

Menziesia J. E. Smith is a small genus in Ericaceae with seven endemic species in Japan and two in North America (Stevens, 1982; Tashiro and Hatta, 1986). The bell-shaped flowers are pink, purplish red, white or light yellow. These attractive characters, especially bell-shaped flower, are not present in Japanese evergreen azaleas. *Menziesia* has also some attractive breeding characters, i. e. clustering multi flowers (3–10) in one floral bud and red- or yellow-colored leaves in autumn. Recent molecular phylogeny with *matK* sequence analysis on the *Rhododendron* species and its related genera revealed that the genus *Menziesia* was nested in the genus *Rhododendron* (Kurashige et al., 2001). According to this result, we tried to obtain new azalea hybrids by reciprocal cross–pollination between *M. multiflora* and several species of *Rhododendron* (Kita et al., 2005). This hybridization resulted in intergeneric hybrid seedlings exhibiting pale–green or albino phenotypes, and they were unresponsive to be acclimatized. To obtain more vigorous green hybrid progenies for efficient breeding, characterization about the generation of albino phenotype is required.

Hybrid chlorophyll deficiency has been observed in

other genera and believed to be the results of plastome–genome incompatibility (Hagemann, 1992; Kirk and Tilney–Bassett, 1978). In interspecific hybrids of *Zantedeschia*, the plastome–genome incompatibility appears to be significant in controlling plastid (pt) DNA inheritance (Yao et al., 1994). In the genus *Rhododendron*, plastome–genome incompatibility in generating albino or variegated phenotype was described in some combination of interspecific hybridization (Akabane et al., 1971; Noguchi, 1932). Ureshino et al. (1999) obtained albino seedlings in interspecific three–way crosses, (*R. kiusianum* × *R. eriocarpum*) × *R. japonicum* f. *flavum*, that showed unilateral cross–compatibility. Based on the results of PCR–RFLP analysis, they supposed that albino progenies were caused by the incompatibility between plastome from F₁ hybrids of *R. kiusianum* × *R. eriocarpum* and nuclear genome of *R. japonicum* f. *flavum*.

To clarify plastome–genome incompatibility in intergeneric hybrids between *Menziesia* and *Rhododendron*, we analyzed ptDNA inheritance by using PCR–RFLP. We also applied this analysis on the intraspecific F₁ hybrid within the genus *Menziesia* by making reciprocal crosses between *M. multiflora* and *M. multiflora* var. *purpurea*. We discuss the interaction of ptDNA inheritance and leaf color in these intergeneric and intraspecific hybrids.

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Materials and Methods

Plant material and hybrid culture

Parental cross combinations of species used in this study are listed in Table 1. Capsules were collected 120 days after pollination and sterilized in 1% sodium hypochlorite solution for 10 min followed by thrice washes in sterile distilled water. Sterilized immature seeds were sowed and the resulting seedlings cultured on Anderson's rhododendron medium (Anderson, 1984) supplemented with $50 \text{ mg} \cdot \text{L}^{-1}$ gibberellic acid (GA_3), $30 \text{ g} \cdot \text{L}^{-1}$ sucrose, and solidified with $3 \text{ g} \cdot \text{L}^{-1}$ gellan gum after adjustment at pH 5.0. Germination ratio was calculated one month after sowing. Germinated seedlings were then transferred to a growth medium, containing $10 \text{ mg} \cdot \text{L}^{-1}$ N6-(2-Isopentenyl) adenine (2ip), $30 \text{ g} \cdot \text{L}^{-1}$ sucrose, and $3 \text{ g} \cdot \text{L}^{-1}$ gellan gum (pH 5.0) to induce multiple shoots for DNA analysis. The cultured seedlings were incubated at 25°C under a 16-h photoperiod, illuminated with fluorescent lights (ca. $60 \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$).

PCR-RFLP analysis of nuclear internal transcribed spacer (ITS) region was done to confirm their hybridity according to Kita et al. (2005).

DNA isolation and pt DNA analysis

Total genomic DNA was extracted from 70 mg of frozen, multiple shoots by a modified CTAB method (Kobayashi et al., 1995) or by using DNeasy Plant mini kit (QIAGEN).

The *matK-trnK* region of ptDNA was amplified via polymerase chain reaction (PCR). The location and sequence of primers (Kurashige et al., 2001), used in this study, were indicated in Fig.1. Most species could be combined with trnKn3914F-trnK2R as the primer combination to amplify this region, however *R. kiusianum* could not be treated with these primer sets. Hence, we designed another internal forward primer matK1MF, combined with trnK2R for PCR. The same primer combination, matK1MF-trnK2R, was also utilized for the following RFLP analysis of *M. multiflora* × *M. multiflora* var. *purpurea*. PCR amplifications were carried out in a total volume of $30 \mu\text{L}$ containing 10 ng of template DNA, $0.2 \mu\text{M}$ of each primer, 4 mM of dNTPs, $3 \mu\text{L}$ of $10 \times$ reaction buffer, and 1 unit of Ex-Taq DNA polymerase (TaKaRa) by using iCycler2400 (BIO-RAD U.S.A.) with one cycle at 94°C for 1 min followed by 30 cycles of 1 min at 94°C , 1 min at 58°C , and 1 min 30 s at 72°C .

Restriction sites of *M. multiflora*, *M. multiflora* var.

Table 1. Cross combination, germination rate of immature seeds and leaf colors of progenies.

Cross combination $\text{♀} \times \text{♂}$	No. of independent crossing	No. of immature seeds cultured	Percent of germination ^z	No. of seedlings (% of seedlings ^y)			
				G ^x	PG	Sec	Albino
<i>M. multiflora</i> (2) ^w × <i>R. kaempferi</i> (2) ^w	4	90	34.4	7 (17.1)	19 (46.3)	3 (7.3)	12 (29.3)
<i>M. multiflora</i> (2) × <i>R. kiusianum</i> (3)	4	250	47.2	10 (8.5)	8 (6.8)	0	100 (84.7)
<i>M. multiflora</i> (3) × <i>R. tashiroi</i> (3)	6	2073	33.6	96 (13.8)	77 (11.0)	107 (15.4)	417 (59.8)
<i>R. kiusianum</i> (2) × <i>M. multiflora</i> (2)	4	188	17.6	0	9 (27.3)	0	24 (72.7)
<i>M. multiflora</i> (2) × <i>M. multiflora</i> var. <i>purpurea</i> (1)	2	100	82.0	82 (100)	0	0	0
<i>M. multiflora</i> var. <i>purpurea</i> (1) × <i>M. multiflora</i> (2)	2	100	78.0	78 (100)	0	0	0

^z (No. of germinated seeds/No. of cultured seeds) × 100.

^y (No. of colored seedlings/total no. of seedlings) × 100.

^x Leaf pigmentation: G, green; PG, pale-green; Sec, sectional chimera of green and white.

^w No. of individuals used for crossing.

Fig. 1. Scheme showing the PCR primer locations and digestion sites in *matK-trnK* region. ①, trnKn3914F, 5'-GGGGTTGCTAACTCAAC-3'; ②, matK1MF, 5'-GATAAGTTTACCGAGGTAGC-3'; ③, trnK2R, 5'-AACTAGTCGGATGGAGTAG-3'. ▽: *Nsp V* digestion site of both *M. multiflora* and *Rhododendron* species, ▼: *Nsp V* digestion site of only *M. multiflora*. △: *Ear I* digestion site of both *M. multiflora* and *M. multiflora* var. *purpurea*, ▲: *Ear I* digestion site of only *M. multiflora*.

Table 2. PCR products and the fragment patterns restricted by *Nsp* V or *Ear* I in parents.

Parent	Primer combination	PCR products (bp)	Restriction enzyme	Fragment pattern (bp)
<i>M. multiflora</i>	trnKn3914F-trnK2R	2500	<i>Nsp</i> V	1200/1000/300
<i>M. multiflora</i>	matK1MF-trnK2R	1900	<i>Nsp</i> V	1200/400/300
<i>R. kaempferi</i>	trnKn3914F-trnK2R	2500	<i>Nsp</i> V	1500/1000
<i>R. tashiroi</i>	trnKn3914F-trnK2R	2500	<i>Nsp</i> V	1500/1000
<i>R. kiusianum</i>	matK1MF-trnK2R	1900	<i>Nsp</i> V	1500/400
<i>M. multiflora</i>	matK1MF-trnK2R	1900	<i>Ear</i> I	870/650/240/180/25
<i>M. multiflora</i> var. <i>purpurea</i>	matK1MF-trnK2R	1900	<i>Ear</i> I	870/650/420/25

purpurea and *Rhododendron* species were analyzed from *trnK*-*matK* sequences by using a software GENETYX-MAC Ver.12.0 (Software Development). The restriction endonuclease, *Nsp* V, which has a different digestion position between *Menziesia* species and *Rhododendron* species in the *matK*-*trnK* region was selected (Fig. 1). Likewise, *Ear* I was selected for a different digestion position between *M. multiflora* and *M. multiflora* var. *purpurea*. PCR products were digested by 5 units of *Nsp* V or 3 units of *Ear* I (Nippon Gene) at 37°C for 2 h. After digestion, the restriction fragments (Table 2) were separated by electrophoresis in 2% agarose gels at 50 V for 1 h. After electrophoresis, agarose gel was soaked in 0.01% ethidium bromide solution for 15 min and photographed under ultraviolet illumination.

Results

Immature seed germination, leaf colors and confirming hybridity

In all cross combinations, germination ratios of immature seeds in vitro varied from 17.6% to 82.0%. Intraspecific reciprocal crosses within *M. multiflora* had significantly higher germination ratios (82.0% and 78.0%). The lowest germination ratio (17.6%) was obtained in intergeneric crosses between *Menziesia* and

Rhododendron, when *R. kiusianum* was used as female parent. The leaf colors of the seedlings were classified into four classes: green, pale-green, sectionally variegated chimera and albino segments or albino (Table 1). When *M. multiflora* was used as a female parent in an intergeneric cross of *M. multiflora* × *Rhododendron* species, a majority of the progenies had pale-green or albino leaves in all crosses, whereas those of *R. kiusianum* × *M. multiflora* developed albino or pale-green phenotypes. On the contrary, all F₁ seedlings of intraspecific reciprocal crosses between *M. multiflora* and *M. multiflora* var. *purpurea* were green phenotypes.

Hybridity of *Menziesia*-*Rhododendron* intergeneric hybrid was confirmed in our previous study (Kita et al., 2005), whereas in reciprocal crosses between *M. multiflora* and *M. multiflora* var. *purpurea*, hybridity was also confirmed by PCR-RFLP of nrDNA ITS region (Fig. 2A). *M. multiflora* had two specific restricted fragments (500 and 400 bp), whereas *M. multiflora* var. *purpurea* had a different specific band (900 bp) that was not digested. All tested progenies had both specific restricted fragments, confirming that they were true hybrids between *M. multiflora* and *M. multiflora* var. *purpurea*.

ptDNA inheritance by PCR-RFLP analysis

For intergeneric crosses between *Menziesia* and *Rhododendron*, PCR products of the *matK*-*trnK* region that were amplified by primer sets of trnKn3914F and trnK2R indicated that the DNA fragments were approximately 2500 bp in *M. multiflora*, *R. kaempferi* and *R. tashiroi* as were those from their intergeneric hybrid seedlings. PCR products, amplified by primer sets of matK1MF and trnK2R, yielded DNA fragments of approximately 1900 bp in *M. multiflora* and *R. kiusianum*, and their intergeneric hybrid seedlings. After digestion of these PCR products by *Nsp* V, polymorphisms were observed between *M. multiflora* and *Rhododendron* species (Fig. 3); those of *M. multiflora* exhibited three restriction fragments of which two had specific sizes of 1200 and 300 bp, whereas the *Rhododendron* species yielded one specific fragment of 1500 bp.

The relationship between leaf colors and ptDNA inheritance of hybrids summarized Table 3 reveal that

Fig. 2. Gel plates showing products of nuclear ITS region digested by *Nsp* V confirmed hybridity of reciprocal cross of *M. multiflora* (M) and *M. multiflora* var. *purpurea* (P) (A), and plastid *trnK*-*matK* region digested by *Ear* I (B). Lane R, randomly mixed DNA of other 5 hybrids of [P] × [M]; Lane m, 100 bp ladder.

Fig. 3. Gel plates indicating plastid DNA inheritance in hybrids of *M. multiflora* (M) × *R. tashiroi* (T) crosses (A) and *M. multiflora* (M) × *R. kiusianum* (K) crosses (B). *Nsp* V – digested *matK* PCR products was separated on 2% agarose gel. g, Green hybrid; pg, Pale-green hybrid; a, Albino hybrid; sec g, Green part of sectional chimera; sec a, albino part of sectional chimera; m, 100 bp ladder.

Table 3. Hybridity and relationship between leaf color and ptDNA inheritance in seedlings.

Cross ♀ × ♂	Leaf pigmentation ^z	No. of offsprings tested	No. of seedlings confirmed their hybridity	No. of seedlings in which ptDNA inherited ^y		
				M	B	P
[intergeneric hybrid]						
<i>M. multiflora</i> × <i>R. kaempferi</i>	G	4	4	4	0	0
	PG	1	1	0	0	1
	Sec	3	3	0	3	0
	Albino	2	2	0	0	2
<i>M. multiflora</i> × <i>R. kiusianum</i>	G	1	1	1	0	0
	PG	1	1	0	0	1
	Albino	1	1	0	0	1
<i>M. multiflora</i> × <i>R. tashiroi</i>	G	3	3	3	0	0
	PG	2	2	0	0	2
	Sec	2	2	0	2	0
	Albino	5	5	0	0	5
<i>R. kiusianum</i> × <i>M. multiflora</i>	PG	2	2	2	0	0
	Albino	2	2	2	0	0
[intraspecific hybrid]						
<i>M. multiflora</i> × <i>M. multiflora</i> var. <i>purpurea</i>	G	9	9	3	0	6
<i>M. multiflora</i> var. <i>purpurea</i> × <i>M. multiflora</i>	G	8	8	8	0	0

^z Leaf pigmentation: G, green; PG, pale-green; Sec, sectional chimera of green and white.

^y M, maternal inheritance; B, biparental inheritance; P, paternal inheritance of ptDNA.

when *M. multiflora* was used as a female parent, all green hybrids had maternal *Menziesia*'s ptDNA, whereas, all pale-green or albino hybrids had paternal *Rhododendron*'s ptDNA. Sectional chimeric progenies contained biparental ptDNA, whereas albino parts of sectional chimeric progenies in cross of *M. multiflora* × *R. tashiroi* contained paternal ptDNA, while green parts contained both maternal and paternal ptDNA. All pale-green and albino hybrids derived from *R. kiusianum* × *M. multiflora* only had maternal *Rhododendron*'s ptDNA.

When the PCR product of *matK*-*trnK* region, resulting from a reciprocal intraspecific cross between *M. multiflora* × *M. multiflora* var. *purpurea*, was amplified by primer sets of *matK1MF* and *trnK2R*, it was found to

be approximately 1900 bp in these two species. Polymorphism of restriction fragments was observed between *M. multiflora* and *M. multiflora* var. *purpurea* after digestion with *Eae* I (Fig. 2B). *M. multiflora* disclosed four bands of which two were specific (160, 230 bp), whereas *M. multiflora* var. *purpurea* exhibited three bands with one specific fragment (390 bp).

When *M. multiflora* was used as a female parent, six out of nine hybrid seedlings that were investigated had paternal ptDNA, while three hybrids had maternal ptDNA. When *M. multiflora* var. *purpurea* was used as a female parent, all eight hybrids retained their maternal ptDNA.

Discussion

That all albino and pale-green seedlings of intergeneric hybrids between *Menziesia* and *Rhododendron* had ptDNA of *Rhododendron* while all green seedlings had maternal ptDNA of *Menziesia* indicate that albino and pale-green seedlings were the result of incompatibility between plastome from *Rhododendron* species and nuclear genome from *Menziesia*. It is known that pale-green or albino plants appear in crosses among distantly related species in *Rhododendron* (Akabane et al., 1971; Noguchi, 1932). Ureshino et al. (1999) also obtained albino seedlings in interspecific three-way crosses, (*R. kiusianum* × *R. eriocarpum*) × *R. japonicum* f. *flavum*, that demonstrate unilateral cross compatibility. The authors suggest that albinism is the result of incompatibility between plastome from the female parent which is F₁ hybrids of *R. kiusianum* × *R. eriocarpum* and nuclear genome from male parent which is *R. japonicum* f. *flavum*. ptDNA is generally inherited from the female in angiosperm (Corriveau and Coleman, 1988), just as Kobayashi (1996) reported that in *Rhododendron*, ptDNA was usually inherited from female parents, but occasionally from the male parents in interspecific crosses between closely related *R. kaempferi* and *R. kiusianum*. Our results (Table 1, 3) show that intergeneric hybridization between *Menziesia* and *Rhododendron*, when *M. multiflora* was used as a female parent, most of the progeny's ptDNA was inherited from paternal *Rhododendron* species. Gymnosperms are characterized by paternal plastid inheritance (Neale and Sederoff, 1988, 1989), but in angiosperms, plastids are predominantly transmitted paternally in few genera, such as *Medicago sativa* (Schumann and Hancock, 1989) and *Daucus* species (Boblenz et al., 1990). In Ericaceae, this is the first report of predominant transmission of male plastids in intergeneric hybrids.

In our reciprocal intergeneric crossing, a mode of a plastid transmission varied from mostly paternal to completely maternal. The varied transmitting mode of a plastid was also reported in *Zanthedeschia* (Yao et al., 1994). Their report disclosed that in progenies of *Z. odorata* (OD) × *Z. aethiopica* (AE), most ptDNA was inherited from maternal OD, while some biparental and paternal inheritance also appeared. However, in a cross of AE × OD, all hybrids had paternal ptDNA of OD. All hybrid progenies that possess ptDNA of OD were green, whereas those of ptDNA of AE yielded albino progenies. Thus, it seems that ptDNA inheritance changes to have green leaf color for seedlings; they suggested that plastome-genome incompatibility controls ptDNA inheritance. We propose that the variation of a transmitting mode of plastids seems to be the common phenomenon among hybridization of distantly related species. In our *Menziesia*-*Rhododendron* progenies, when *Menziesia* served the female parent, the paternal

Rhododendron's ptDNA was transmitted to the hybrid seedlings that became albino or pale-green. Consequently, we suggest that plastome-genome incompatibility is independent from ptDNA inheritance in *Menziesia*-*Rhododendron* hybrids.

In the intraspecific hybridization, *M. multiflora* and *M. multiflora* var. *purpurea*, when *M. multiflora* was used as a female parent, ptDNA was frequently inherited from the paternal parent (6/9), whereas, ptDNA was normally inherited from female parent when *M. multiflora* var. *purpurea* was used as female parent. These results indicate that ptDNA of the progenies was inherited from male parent when *M. multiflora* was used as a female parent. Additional analyses of ptDNA inheritance with more hybrid seedlings between these species are needed to confirm this tendency.

The results of this study show that if *M. multiflora* var. *purpurea* is used as the female parent for intergeneric hybridization with *Rhododendron*, more green hybrid seedlings would be obtained as a result of maternal inheritance of ptDNA from *Menziesia*. However, hybridization between *M. multiflora* var. *purpurea* as female parent and *R. tashiroi* as male parent, yielded mostly albino progenies as a result of paternal inheritance of *Rhododendron*'s ptDNA (data not shown). Consequently, the mode of ptDNA inheritance changes according to each cross combination. Hence, we need to search for an intergeneric cross combination from which we can obtain many green vigorous hybrids for breeding stocks.

Yao and Cohen (2000) suggested that a small number of genes play a major role in the plastome-genome incompatibility. In order to avoid albino appearance, we have to wait for genetically control technique by utilizing such genes.

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ヨウラクツツジ-ツツジ属間雑種におけるプラスチドDNAの遺伝性と 葉緑体ゲノム-ゲノム間の不調和について

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摘 要

ヨウラクツツジ-ツツジ属間雑種の多くはアルビノまたは淡緑色であった。アルビノが出現する原因を明らかにするために、属間雑種およびヨウラクツツジ属内の正逆交雑実生においてPCR-RFLP分析を行い、プラスチドDNAの遺伝性を調査した。その結果、すべてのアルビノと淡緑色個体はツツジ属のプラスチドDNAを有し、すべての緑色個体はヨウラクツツジ属のプラスチドDNAを有していた。したがっ

て、ツツジ属のプラスチドDNAとヨウラクツツジ属のゲノムとの間で不調和が起きていることが示唆された。さらに、ウラジロヨウラクを母親にした交配で得られた実生のほとんどがプラスチドDNAの父性遺伝を示したことから、葉緑体ゲノム-ゲノム間の不調和とプラスチドDNAの遺伝性とは互いに独立した関係にあることが示唆された。