The Study on the Relationship between Leaf Colors and ptDNA Inheritance in Intersectional Cross of *Rhododendron kiusianum* × *R. japonicum* f. *flavum*, Resulting in an Unexpected Triploid Progeny

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Summary

The relationship between leaf color and plastid/nuclear genome construction of 45 seedlings from two crosses of *R. kiusianum* (2n=2x=26) × *R. japonicum* f. *flavum* (2n=2x=26) was examined to resolve the frequent appearances of albino progenies among crosses involving *Rhododendron* species. Leaf color of seedlings segregated into green, pale green, and albino at a ratio, ca. 1: 2: 7. An unexpected triploid green plant with 2x and 1x nuclear genome from *R. kiusianum* and *R. japonicum* f. *flavum*, respectively, was detected among the hybrids through isozyme analysis and chromosome observation. PCR–RFLP analysis of *matK* region revealed that plastid DNA (ptDNA) of all albino hybrids was inherited from *R. kiusianum*, whereas that of six of seven green hybrids inherited it from *R. japonicum* f. *flavum*. The triploid green progeny had ptDNA of *R. kiusianum*.

Key Words: albino, isozyme analysis, PCR–RFLP, plastome–genome incompatibility, *Rhododendron*.

Introduction

Albino seedlings, which often occur among interspecific progenies of *Rhododendron* species, have been deterrents to breeding because of their inability to reproduce (Akabane et al., 1973; Yamaguchi and Hirata, 1986). We made intersectional crossings between various evergreen azalea species and yellow-flowered deciduous species of *R. japonicum* f. *flavum* Suringar in an effort to produce yellow-flowered evergreen azalea cultivars (Ureshino et al., 1998). Seeds were obtained only when evergreen species was used as a seed parent; no capsule set in the reverse cross combinations. Among the crosses, the largest number of green progenies was obtained in a three-way cross between the interspecific evergreen F₁ hybrid of *R. kiusianum* × *R. eriocarpum* and *R. japonicum* f. *flavum*. Even in the three-way cross, however, most seedlings were albino which withered away within one year. Therefore, a better method for reducing the frequency of albino plants is desirable.

We previously pointed out that 1) albino progenies appear among *Rhododendron* hybrids because of incompatibility between the plastome from the evergreen species and the nuclear genome from *R. japonicum* f. *flavum* (deciduous species) and 2) green progenies could

be obtained only when plastid DNA (ptDNA) of the seedlings was inherited from *R. japonicum* f. *flavum* (paternal inheritance) (Ureshino et al., 1999).

To clarify the mechanism of albino plant appearance in detail, we first investigated the relationship between leaf color and ptDNA inheritance of progenies in various evergreen azalea species × *R. japonicum* f. *flavum*. Among the crosses, we found one green progeny with ptDNA from evergreen species (maternal inheritance) in the cross of *R. kiusianum* × *R. japonicum* f. *flavum*, which indicates that a more desirable breeding procedure for obtaining green progenies might exist. In this paper, we establish a relationship between leaf color of seedlings and its ptDNA inheritance among progenies from intersectional crosses of *Rhododendron*.

Materials and Methods

Plant materials

Mature seeds from two artificial crossings, *R. kiusianum* #1 (KIU1) × *R. japonicum* f. *flavum* #1 (JPN1) and *R. kiusianum* #1 (KIU1) × *R. japonicum* f. *flavum* #2 (JPN2), were cultured on Anderson rhododendron medium (pH 5.0) (Anderson, 1984) supplemented with 50 mg · liter⁻¹ of GA₃. JPN1 and JPN2 are different clones. All parental materials are maintained at Faculty of Agriculture of Kyushu University. Germinated seedlings were transferred into the medium with 10 mg · liter⁻¹ of 2ip for shoot multiplication of the seedlings. Multiple shoots were used in the following

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study.

Hybridity confirmation and ploidy analysis of seedlings

Isozyme analysis was conducted for hybridity confirmation of the seedlings, using three enzyme systems, phosphoglucumutase (PGM, EC 2. 7. 5. 1), isocitrate dehydrogenase (IDH, EC 1. 1. 1. 41) and malate dehydrogenase (MDH, EC 1. 1. 1. 37). A detailed procedure of electrophoresis was described previously (Ureshino et al., 1998). Shoot tips from two green seedlings were examined for chromosome counts as follows: they were pretreated with 0.002 M 8-hydroxyquinoline solution, macerated with 1 N HCl for 5 min at 60 °C, stained, and squashed according to the standard acetocarmine technique.

Analysis of ptDNA inheritance of progenies

PCR-RFLP analysis was used to resolve the mode of ptDNA inheritance by the progenies. Total DNA was extracted from 70 mg of frozen multiple shoots by modified CTAB method (Kobayashi et al., 1998).

MatK regions (Neuhaus and Link, 1987) found in ptDNA were amplified by PCR. Primer sequences were 5'-GGGGTTGCTAACTCAACGG for trnK-3914F and 5'-AACTAGTCGGATGGAGTAG for trnK-2R; both were reported by Johnson and Soltis (1994). PCR was 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 MgCl₂, 0.1 mM of dNTPs, 2.5 µg of 10× reaction buffer and 0.5 unit of *Taq* DNA polymerase (Boeringer Mannheim). Amplification was carried out with one cycle at 94 °C for 4 min and 35 cycles of 30 sec at 94 °C, 40 sec at 48 °C and 3 min at 72 °C and one cycle at 72 °C for 10 min.

PCR products were, then, digested by *Taq* I. The digests were electrophoresed in 1.5% agarose gel

(Routine use, Sigma) containing ethidium bromide.

Results

Ovule germination

Approximately 35% of the seeds germinated in both crosses (Table 1). Leaf color of the seedlings segregated into green, pale-green and albino at ratios of 5:9:39 for JPN1 and 3:3:11 for JPN2 as pollen parents.

Hybridity confirmation and ploidy analysis of seedlings

Well-defined banded patterns were obtained at three loci, *Pgm*-2, *Mdh*-2, and *Idh*-1 (Fig. 1). Parental species exhibited different single-banded phenotypes at these loci. The seedlings possessed the parental bands at *Pgm*-2 and both parental and intermediate bands at *Mdh*-2 and *Idh*-1. They were confirmed to be of hybrid origin except the one green seedling from KIU1 × JPN2, which had the same banded phenotypes as the seed parent at all loci. Since it was reported that MDH and IDH were dimeric and PGM was monomeric enzymes (Miyahara et al., 1993), the intermediate bands at *Mdh*-2 and *Idh*-1 were considered to be heterodimeric

Table 1. Seed germination and green plant appearance in *R. kiusianum* (KIU) × *R. japonicum* f. *flavum* (JPN).

Cross	No. of seeds cultured	% of germination ^z	Leaf color of seedlings ^y			% of green seedlings
			G	PG	AL	
KIU1 × JPN1	175	30.8	5	9	39	9.4
KIU1 × JPN2	46	37.0	3	3	11	17.6

^zGermination was observed 6 months after primary culture.

^yG; Green, PG; Pale-green, AL; Albino.

Fig. 1. Schematic zymograms for PGM, MDH and IDH isozymes of *R. kiusianum*, *R. japonicum* f. *flavum* and their offspring. ^zF, Female; M, Male. ^ySame genotype as the seed parent.

bands.

Band intensity from both parents in most of the hybrids was approximately the same at all loci (Fig. 2, lane 3–7). In the one green progeny from KIU1 $\times$ JPN2, however, the bands from the seed parent (KIU1) stained more densely than those from the pollen parent (JPN2) at all loci (Fig. 2, lane 8). The chromosome number from the shoot tip of this green progeny was 39, in contrast to 26 in another green progeny (Fig. 3). Since parental species in this study are diploids having $2n=2x=26$ chromosome, the green progeny with 39 chromosome is a triploid plant.

Fig. 2. Gel plates showing banding patterns of progenies from *R. kiusianum* #1 $\times$ *R. japonicum* f. *flavum* #2 at three isozymes. Lane 1; *R. kiusianum* #1, Lane 2; *R. japonicum* f. *flavum* #2, Lane 3 and 4; Albino progeny, Lane 5 and 6; Pale-green progeny, Lane 7 and 8; green progeny.

Fig. 3. Photomicrographs of Somatic chromosomes from shoot tip cell of green progenies in *R. kiusianum* #1 $\times$ *R. japonicum* f. *flavum* #2. A; Diploid progeny ($2n=2x=26$), B; Triploid progeny ($2n=3x=39$).

PtDNA inheritance of progenies

The parents of *R. kiusianum* and *R. japonicum* f. *flavum* were differentiated at the ptDNA matK gene by a restriction site recognized *Taq* I. The fragment pattern of KIU1 was 980/530/230/180/160/150 (bp), whereas that of JPN1 and JPN2 was 1510/230/180/160/150 (bp) (Fig. 4). PtDNA from albino hybrids showed only maternal (*R. kiusianum*) banding patterns, whereas that from pale-green hybrids showed paternal (*R. japonicum* f. *flavum*) bands in both crosses (Table 2). Six of seven green hybrids also contained paternal (*R. japonicum* f. *flavum*) ptDNA. The rest one was the triploid green progeny from KIU1 $\times$ JPN2, which possessed the

Table 2. PtDNA inheritance of seedlings from *R. kiusianum* $\times$ *R. japonicum* f. *flavum*.

Cross	Leaf color ^z	No. of hybrids tested	No. of hybrids with fragment pattern ^y	
			M	P
KIU1 $\times$ JPN1	G	5	0	3
	PG	3	0	5
	AL	25	25	0
KIU1 $\times$ JPN2	G	2	1 ^x	1
	PG	3	0	3
	AL	7	7	0

^zG; Green, PG; Pale-green, AL; Albino.

^yM; Maternal ptDNA, P; Paternal ptDNA.

^xThis green plant was the triploid progeny ($2n=3x=39$).

Fig. 4. Gel plates showing restriction fragments of PCR products digested with *Taq* I.

Lane 1; *R. kiusianum* #1, Lane 2; *R. japonicum* f. *flavum* #2, Lane 3; Albino progeny, Lane 4; Pale green progeny, Lane 5; Diploid green progeny, Lane 6; Triploid green progeny, Lane 7; λ /Hind III.

maternal (*R. kiusianum*) banding pattern (Table 2, Fig. 4).

Discussion

Isozyme markers generally indicate co-dominant inheritance, while some express gene dosages (Tsafaris et al., 1981). They were, therefore, used not only for hybridity confirmation of seedlings, but also for confirmation of the ploidy levels in some plants (Gottlieb, 1982; Birchler, 1983; Raelson and Grant, 1989). In this study, most seedlings had both parental bands with approximately the same intensity at three enzyme loci, thus confirming their hybrid origin, regardless of leaf colors. One green hybrid from KIUI1 × JPN2, however, had bands from *R. kiusianum* which stained more densely than did those of *R. japonicum* f. *flavum*. Our observations of the chromosomes established that this green progeny is a triploid plant. These results indicate that the nuclear genome construction of the green progeny is 2x from *R. kiusianum* (seed parent) and 1x from *R. japonicum* f. *flavum* (pollen parent).

The occurrence of triploid progenies from the cross between diploids was reported in some plants, e. g., apple (Chyi and Weeden, 1984), tomato (Kagan-Zur et al., 1991; Lapidot et al., 1994) and *Brachiaria setigera* (Muniyamma, 1977). These triploids were produced by the fertilization of 1x gamete and unreduced 2x gamete or by embryogenesis from 3n endosperm. In the cross with *R. kiusianum*, so far, there is no report on the appearance of unreduced gamete and embryogenesis from the endosperm. Thus, further studies are necessary to clarify the origin of the triploid progeny.

Albino progenies have been observed in interspecific crosses of several genera, e. g., *Oenothera* (Kirk and Tilney-Bassett, 1978), *Trifolium* (Pandey et al., 1987) and *Zantedeschia* (Yao et al., 1994). Hybrid albinism of these genera is considered to be caused by plastome-genome incompatibility (Kirk and Tilney-Bassett, 1978). In *Zantedeschia*, it was revealed that the hybrid albinism in an interspecific cross of *Z. odorata* × *Z. aethiopica* occurred when ptDNA of hybrids is inherited from *Z. odorata* (Yao et al., 1994). A similar phenomenon is seen in an interspecific cross of *Rhododendron*. We previously reported that albino progenies in the three-way cross between the evergreen F₁ hybrid of *R. kiusianum* × *R. eriocarpum* and *R. japonicum* f. *flavum* were caused by the plastome-genome incompatibility between the plastome from the evergreen F₁ hybrid and the nuclear genome from *R. japonicum* f. *flavum*, and that green progenies could be obtained only when ptDNA of seedlings was inherited from *R. japonicum* f. *flavum* (Ureshino et al., 1999). In this study, all albino progenies had maternal (*R. kiusianum*) ptDNA and six of seven of green progenies had paternal (*R. japonicum* f. *flavum*) ptDNA supporting our previous result.

However, the triploid progeny having 1x nuclear genome from *R. japonicum* f. *flavum* and 2x nuclear

genome from *R. kiusianum* contained maternal (*R. kiusianum*) ptDNA, whereas it vigorously developed after acclimatization with green leaf color. This result suggests that the plastome-genome incompatibility might be overcome on the occasion of the co-existence of 2x nuclear genome from evergreen species and 1x genome from *R. japonicum* f. *flavum* in one progeny.

Plastid DNA is usually inherited maternally in angiosperm genera (Corriveau and Coleman, 1988; Harris and Ingram, 1991). In azalea, however, paternal ptDNA inheritance was reported in some interspecific crosses (Kobayashi, 1996; Michishita et al., 1998; Ureshino et al., 1999). The frequency of paternal ptDNA inheritance in these crosses was lower than that of maternal inheritance. In this study, many seedlings had maternal ptDNA (about 65%), paternal ptDNA inheritance was also observed. In some *Rhododendron* species, the disappearance of paternal ptDNA does not occur during pollen and pollen tube development (Kuroiwa, 1991). Such phenomenon was observed in other angiosperm genera, e. g., *Oenothera* (Kirk and Tilney-Bassett, 1978) and *Campanula* (Harris and Ingraham, 1991). The elimination of paternal ptDNA is considered to occur late after fertilization and during embryo development (Birky, 1995). Transmission of ptDNA in *Rhododendron* may also occur late; such a relaxed mechanism might allow, to some extent, paternal ptDNA inheritance by progenies.

As mentioned above, plastid DNA is usually inherited from maternally in *Rhododendron* species (Corriveau and Coleman, 1988; Harris and Ingram, 1991), it is better to use *R. japonicum* f. *flavum* as the seed parent for obtaining more green plants in crosses with evergreen species. But such crosses, however, have met with failure because of the inhibition of pollen tube growth in styles (Ureshino et al., 2000). Alternatively, if a cross between a tetraploid evergreen species × diploid *R. japonicum* f. *flavum* is made, most progenies theoretically will have 2x nuclear genome from evergreen species, and 1x ones from *R. japonicum* f. *flavum* plus the ptDNA from evergreen species (maternal inheritance).

If such an interploidal plastome-genome incompatibility can be overcome through the appropriate selection of parents, the procedure might serve as a useful method for obtaining green progenies, effectively. In future study, we will attempt these crosses using various tetraploid evergreen species.

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ミヤマキリシマとキレンゲツツジとの節間交雑における三倍体実生の出現と
実生の葉色および葉緑体 DNA の遺伝性との関連

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摘 要

ツツジ属 (*Rhododendron*) の遠縁交雑におけるアルビノ実生の出現要因を明らかにするために、ミヤマキリシマ (*R. kiusianum*, $2n=2x=26$) × キレンゲツツジ (*R. japonicum* f. *flavum*, $2n=2x=26$) の 2 交配で得られた 45 個体の実生について、葉色と葉緑体 / 核ゲノム構成との関係について調査した。実生の葉色は緑色、淡緑色およびアルビノに 1:2:7 の割合で分離した。また、アイソザイム分析および染色体数の調査を行ったところ、緑色実生の中に二倍性のミヤマキリシマ由来の核ゲノムと一倍性のキレンゲツツジ由来のそれを併せ持つ三

倍体実生が 1 個体含まれていた。PCR-RFLP により実生の葉緑体 DNA を調査したところ、アルビノ実生はすべて種子親のミヤマキリシマ由来であったが、ほとんどの緑色実生では花粉親のキレンゲツツジ由来であった。しかし、前述の三倍体緑色実生は、種子親のミヤマキリシマ由来の葉緑体ゲノムを有していた。

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