

Review

Study of Cross Incompatibility between Evergreen and Deciduous Azaleas

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A brief account of research in our laboratory on the mechanism of the cross incompatibility between evergreen azalea species and yellow flowered deciduous azalea, *Rhododendron japonicum* form *flavum*, i.e. mechanism of unilateral cross incompatibility and albino progenies appearance, is given. Furthermore, the strategy for obtaining vigorous green progenies effectively was also summarized.

Key Words: albino, plastome-genome incompatibility, ptDNA inheritance, *Rhododendron japonicum* form *flavum*, three-way cross.

Introduction

The genus *Rhododendron* is representative of the genera which contain a large number of horticulturally important ornamental plants (Galle, 1985a). The domestication of azalea in Japan is first described in “Meigetsuki”, which was written in the Kamakura era (1192–1333) (Kunishige, 1983; Yamasaki and Yamasaki, 1976). Cultivated varieties have been developed by natural hybridization and selection among evergreen species such as *Rhododendron obtusum*, *R. kaempferi*, *R. kiusianum*, *R. sataense*, *R. eriocarpum*, *R. simsii*, *R. indicum*, *R. ripense*, and *R. scabrum* for the past 400 years. To date, they consist of several horticultural groups, such as “Kurume tsutsuji”, “Hirado tsutsuji”, “Edokirishima”, “Satsuki”, and “Ryukyutsutsuji”. The flower colors of cultivated varieties vary from red to purple, and this variation is considered to be the result of the recombination of genes controlling flavonoid biosynthesis through natural or artificial hybridization among evergreen species (Kunishige and Kobayashi, 1980; Miyajima, 1988); however, there is no yellow-flowered cultivar among evergreen species.

Raising yellow-flowered cultivars is an important breeding objective for evergreen azaleas. Several subgenera contain yellow-flowered species. The subgen. *Pentanthera* is a subgenus belonging to the deciduous azalea series, and contains several yellow-flowered species, e.g., *R. japonicum* f. *flavum* Suringar, *R. molle* G. Don, and *R. luteum* Sweet (Galle, 1985b). The major petal pigments in these species are carotenoid com-

pounds (Santamour Jr. and Dumuth, 1978; Spathmann, 1980). Among them, *R. japonicum* f. *flavum* is native in Japan and is mainly distributed in low mountainous areas of Kyushu (Kunishige, 1984).

Many breeders have tried to raise yellow-flowered evergreen azaleas using intersubgeneric crosses between evergreen species and yellow-flowered deciduous species belonging to subgen. *Pentanthera* (Akabane et al., 1971; Heursel, 1981; Noguchi, 1932; Yamaguchi et al., 1985). Most of these crosses, however, failed to obtain progenies because of the low frequency of capsule set and viable seeds. Almost all of the progenies from the crosses, if obtained, were albino plants, which withered away within a year (Heursel, 1981; Yamaguchi, 1986). An effective breeding method to obtain many viable seeds and to reduce albino plants is desired.

Interspecific cross incompatibilities are recognized in many cross combinations of *Rhododendron*, and it is known that those barriers operate at many different stages both before and after fertilization by the degree of relatedness of parental species (Rouse et al., 1993; Williams et al., 1982). Although such information would considerably contribute to success in interspecific breeding, there is poor information on the mechanism of the cross incompatibility between evergreen species and *R. japonicum* f. *flavum*.

The purpose of this study is to establish an effective breeding system to obtain green leaf-colored and viable interspecific F₁ hybrids between evergreen species and *R. japonicum* f. *flavum*.

1. Crossability of Intersubgeneric Crosses between Evergreen Azaleas and *R. japonicum* f. *flavum*

In a large genus such as *Rhododendron*, with several levels in the taxonomic hierarchy, it is not surprising to find the complete spectrum from full compatibility to total incompatibility in interspecific crosses (Williams et al., 1990). Although interspecific crosses between evergreen species and *R. japonicum* f. *flavum* have been conducted by several researchers (Akabane et al., 1971; Heursel, 1981; Noguchi, 1932; Yamaguchi et al., 1985), most of the crosses were unsuccessful because of their cross incompatibilities, with the following results.

1) Unilateral cross incompatibility

In reciprocal crosses between evergreen azalea species and *R. japonicum* f. *flavum*, the capsule sets only when an evergreen species was used as the seed parent. To clarify the mechanism of the unilateral cross incompatibility, pollen tube behavior in the style was compared among reciprocal crosses (Fig. 1). Germination of pollens on the stigma surface was observed in both crossings. In the cross using the evergreen species as the seed parent, pollen tubes penetrated the lower style until 96 h after pollination with normal morphological character. Pollen tubes also penetrated the upper style in the cross with *R. japonicum* f. *flavum* as the seed parent, whereas most showed abnormal morphological character, e.g., wide diameter, spiraling, and heavy callose deposition, and immediately arrested at this point.

2) Crossability in a cross with an evergreen species as the seed parent

As mentioned above, the capsule sets in crosses with an evergreen species as the seed parent; however, the frequency of germinable seeds varied among the species

used as the seed parent. The level of cross compatibility was mainly classified into three types, i.e. no capsule set, set capsule with non-viable seeds or with viable seeds (Table 1). The number of viable seeds per capsule also varied among species. In these evergreen azalea species, *R. eriocarpum* had the highest cross compatibility to *R. japonicum* f. *flavum*. On the other hand, the effect of pollen parents, i.e. the genotype of *R. japonicum*

Table 1. Difference of cross compatibility in the cross of evergreen azalea × *R. japonicum* f. *flavum*.

Levels of cross compatibility	Species
No capsule set	Subsect. Scabra Ser. Scabra <i>R. ripense</i>
Capsule sets with ungerminant seeds	Subsect. Tsutsusi Ser. Seryllifolia <i>R. serpyllifolium</i>
Germinant seeds per capsule are below 20.	Subsect. Tsutsusi Ser. Kaempferia <i>R. kaempferi</i> <i>R. kiusianum</i> <i>R. tosaense</i>
Germinant seeds per capsule are from 20 to 100.	Subsect. Tsutsusi Ser. Kaempferia <i>R. sataense</i> <i>R. simsii</i> <i>R. transiens</i>
Germinant seeds per capsule are above 100.	Subsect. Tsutsusi Ser. Tsutsusi <i>R. indicum</i> <i>R. eriocarpum</i> Ser. kaempferia <i>R. kaempferi</i> var. <i>macrogamma</i>

Evaluation was conducted for each species with at least two individuals.

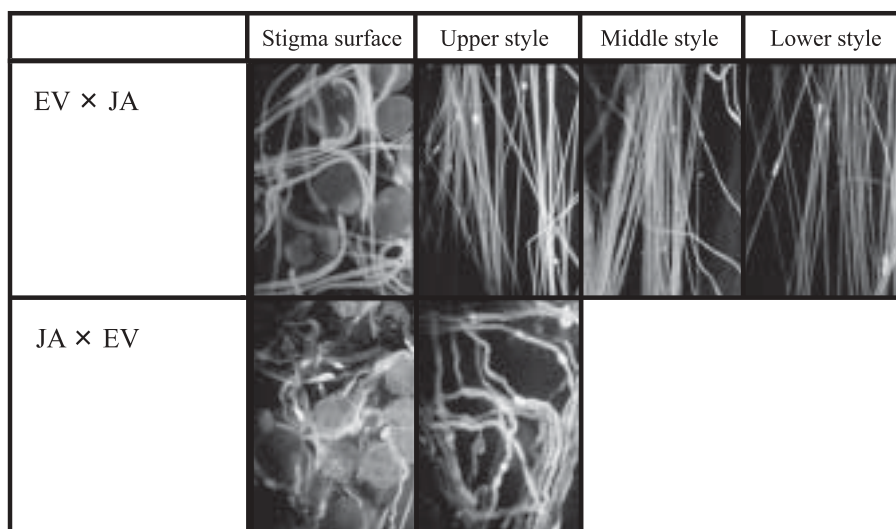


Fig. 1. Comparison of pollen tube growth into the style of the reciprocal cross between F₁ hybrids from (*R. kiusianum* #2 × *R. eriocarpum* #4) (EV) and *R. japonicum* f. *flavum* #13 (JA) (Ureshino et al., 2000). Observation was conducted 96 h after pollination.

f. flavum, on crossability was reported by Okamoto et al. (2006). It was considered that the selection of the pollen donor was also important.

3) Appearance of albino seedlings

The appearance of albinos has been observed in several angiosperm genera and is considered to be caused by plastome-genome incompatibility (Hagemann, 1992; Kirk and Tilney-Bassett, 1978). In crosses with an evergreen species as the seed parent, most progenies were albino, which was caused by plastome-genome incompatibility between the plastome from evergreen species and the nuclear genome from *R. japonicum f. flavum*, and green progenies could be obtained only when the plastome was inherited from *R. japonicum f. flavum* (paternal inheritance) (Fig. 2).

The plastid DNA (ptDNA) is predominantly inherited from the maternal parent in most angiosperms (Corriveau and Coleman, 1988; Harris and Ingram, 1991). In angiosperm genera, molecular and cellular mechanisms generally exclude organelles of the pollen parent during pollen development (Birky Jr., 1995; Rebound and Zeyl, 1994). Most such controls are, however, not stringent (Birky Jr., 1983) so that biparental inheritance in the post-zygotic stage has been reported, or at least postulated, in many different taxa, including *Rhododendron* species (Corriveau and Coleman, 1988; Harris and Ingram, 1991; Kirk and Tilney-Basset, 1978; Kuroiwa, 1991; Sodmergen et al., 1998). The relaxed control of cytoplasmic DNA inheritance (especially in ptDNA) results in the same progeny of individuals carrying plastid genes either exclusively from the mother or exclusively from the father, or else from both parents. The frequency of paternal ptDNA inheritance, resulting

from green progenies therefore varied from 0% to about 30% among the crosses using various evergreen azalea species (Table 2). A relatively high frequency of green progenies was obtained in the cross with *R. kiusianum* and *R. transiens*.

2. Strategy for effective obtainment of green progenies

1) Three-way cross

As mentioned above, cross incompatibility between evergreen azaleas and *R. japonicum f. flavum* resulted in two factors, i.e. low frequency of viable seeds and high frequency of albinos. Furthermore, no species had potential for a high frequency of viable seeds and green progenies in a single cross. For example, although *R. kiusianum* had high ability to increase the frequency of green plants among seedlings, the number of green seedlings obtained from one capsule became very low because of pollination cross incompatibility with *R. japonicum f. flavum*. However, when *R. kiusianum* was crossed with *R. eriocarpum*, their F₁ progenies indicated high cross compatibility and high ability to produce green plants in the crossing with *R. japonicum* as pollen parents. In the cross, the number of green progenies per capsule was relatively increased when compared to that observed in a single cross (Table 3). This result indicates that two factors related to cross incompatibility between evergreen species and *R. japonicum f. flavum* could be overcome by sexual hybridization between species having high pollination cross compatibility and those having high ability to produce green plants among seedlings.

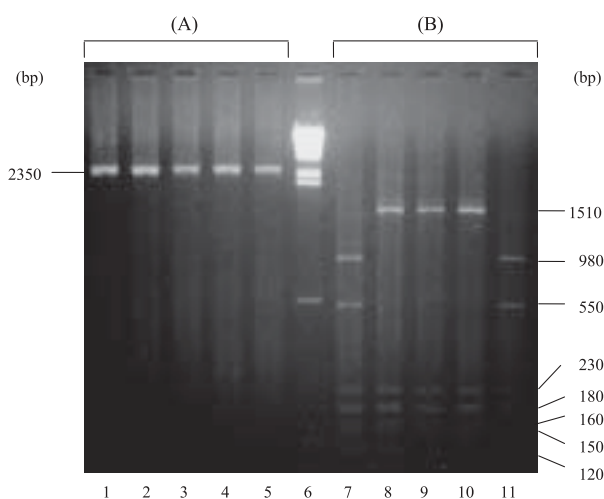


Fig. 2. Profiles of PCR products (A) at *Matk* region and restriction fragment of the products digested with *TaqI* (B) (Ureshino et al., 1999). Lane 1 and 7: F₁ from *R. kiusianum* #2 × *R. eriocarpum* #3. Lane 2 and 8: *R. japonicum f. flavum* #2. Lane 3 and 9: Green progeny #1. Lane 4 and 10: Pale green progeny #1. Lane 5 and 11: Albino progeny #1. Lane 6: λ/*HindIII*.

Table 2. Difference of green progenies appearance in the cross of evergreen azalea × *R. japonicum f. flavum*.

Frequency of green progenies	Species
X < 1%	Subsect. Tsutsusi
	Ser. Tsutsusi
	<i>R. indicum</i>
	<i>R. eriocarpum</i>
	Ser. kaempferia
	<i>R. kaempferi</i>
	<i>R. tosaense</i>
1 ≤ X < 10%	<i>R. kaempferi</i> var. <i>macrogamma</i>
	Subsect. Tsutsusi
	Ser. kaempferia
10 ≤ X < 20%	<i>R. sataense</i>
	<i>R. simsii</i>
20 ≤ X < 30%	Subsect. Tsutsusi
	Ser. kaempferia
	<i>R. kiusianum</i>
	<i>R. transiens</i>

Evaluation was conducted for each species with at least two individuals.

Table 3. Comparison of cross compatibility and green plant appearance in single and three-way crosses.

Cross	Number of germinant seeds per a capsule (A)	Frequency of green progenies (%) (B)	Number of green seeds per a capsule (A) × (B)/100
Single cross			
<i>R. eriocarpum</i> #1 × <i>R. japonicum</i> #1	98.8	0	0
<i>R. eriocarpum</i> #1 × <i>R. japonicum</i> #2	135.0	0	0
<i>R. kiusianum</i> #1 × <i>R. japonicum</i> #1	1.7	16.7	0.2
<i>R. kiusianum</i> #1 × <i>R. japonicum</i> #2	15.7	9.4	1.4
Three-way cross			
(<i>R. kiusianum</i> #2 × <i>R. eriocarpum</i> #2) × <i>R. japonicum</i> #1	226.4	20.2	45.7
(<i>R. kiusianum</i> #2 × <i>R. eriocarpum</i> #2) × <i>R. japonicum</i> #2	75.6	15.3	11.5
(<i>R. kiusianum</i> #2 × <i>R. eriocarpum</i> #3) × <i>R. japonicum</i> #1	129.3	11.9	15.3
(<i>R. kiusianum</i> #2 × <i>R. eriocarpum</i> #3) × <i>R. japonicum</i> #2	333.0	12.4	41.2

Data were collected at 2 months after seed sowing.

Table 4. Correlation between leaf color and ptDNA inheritance of progenies from *R. kiusianum* × *R. japonicum* f. *flavum*. (Ureshino and Miyajima, 2002)

Cross	Leaf color ^z	Number of progenies	ptDNA	
			M	P
<i>R. kiusianum</i> #1 × <i>R. japonicum</i> #1	G	5	0	5
	PG	3	0	3
	AL	25	25	0
<i>R. kiusianum</i> #1 × <i>R. japonicum</i> #2	G	2	1	1
	PG	3	0	3
	AL	7	7	0

^z G, green; PG, pale green; AL, albino.

2) Inter-ploid cross

Albino progenies are a serious problem in remote crossings because of their inability to achieve sound growth during acclimation. Albino progenies were reported widely in cross combinations of intergeneric cross between *Menziesia* and *Rhododendron* to intraseries cross between *R. serpyllifolium* and other species belonging to the series *kaempferia* (Kita et al., 2005a, b; Michishita et al., 2002). As mentioned above, it was revealed that albino seedlings were the result of the plastome-genome incompatibility between the plastome from evergreen species 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*; however, one green progeny from *R. kiusianum* × *R. japonicum* f. *flavum* had maternal ptDNA (from *R. kiusianum*) (Table 4). This green progeny was triploid with a 2x nuclear genome from *R. kiusianum* and 1x from *R. japonicum* f. *flavum* (Figs. 3, 4). From the results, we presumed that the incompatibility between the plastome from evergreen species and the nuclear genome from *R. japonicum* f. *flavum* was overcome with the existence of the 2x nuclear genome from evergreen species and

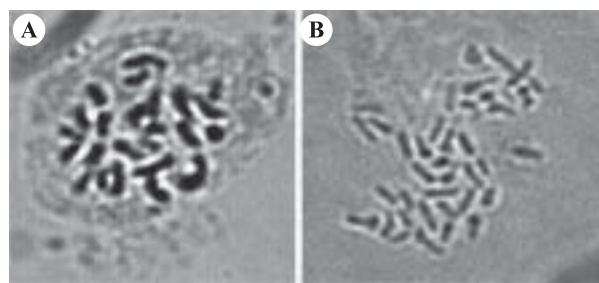


Fig. 3. Somatic chromosome from shoot tip cell of green progenies in *R. kiusianum* × *R. japonicum* f. *flavum* (Ureshino and Miyajima, 2002). A: Diploid progeny with paternal ptDNA ($2n = 2x = 26$). B: Triploid progeny with maternal ptDNA ($2n = 3x = 39$).

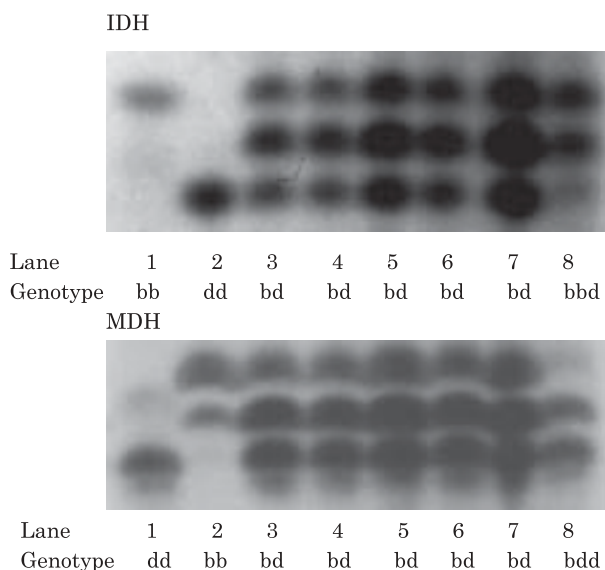


Fig. 4. Gel showing banding patterns of progenies from *R. kiusianum* × *R. japonicum* f. *flavum* at two isozymes (Ureshino and Miyajima, 2002). Lane 1: *R. kiusianum*. Lane 2: *R. japonicum* f. *flavum*. Lane 3 and 4: albino progeny. Lane 5 and 6: pale green progeny. Lane 7: green progeny (diploid). Lane 8: green progeny (triploid).

Table 5. Comparison of green progeny appearance between intra- and inter-ploid crosses of *R. indicum* × *R. japonicum* f. *flavum*.

Seed parent	Number of seeds per a capsule ^z	Number of seed cultured	% of seed germination	Leaf color of progenies (ptDNA inheritance) ^y	
				Green	Albino
[4x × 2x]					
<i>R. indicum</i> #1	185.7	238	47.5	188 (m)	0
<i>R. indicum</i> #2	169.0	292	16.9	49 (m)	0
[2x × 2x]					
<i>R. indicum</i> #3	543.3	543	79.0	0	190 (m)
<i>R. indicum</i> #4	206.5	206	16.8	0	45 (m)

^z Average of at least four capsules.

^y m, maternal inheritance.

1x from *R. japonicum* f. *flavum*.

To clarify this, we crossed of tetraploid evergreen species × diploid *R. japonicum* f. *flavum* (Table 5). In these crossings, the progenies had the 2x nuclear genome from evergreen species and 1x nuclear genome from *R. japonicum* f. *flavum* and became green leaf-colored plants with maternal ptDNA.

3. Future study

From the above study, we clarified that two strategies were useful for obtaining green progenies efficiently, and many green progenies have been obtained. Among them, the progenies from three-way cross entered anthesis and set pale-yellow flowers with carotenoids. These progenies, however, were semi-deciduous and highly sterile. To improve the semi-deciduous traits, chromosome doubling of the progenies was conducted, and the derived plants were back-crossed to evergreen azaleas. These BC₁ plants were vigorously developed with evergreen traits (Ureshino et al., 2006). In future, we will investigate their flower colors.

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