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Cross Compatibility of Intersubgeneric Hybrids of Azaleas on Backcross with Several Evergreen Species

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For the reduction of several undesirable traits, such as deciduousness and weak heat tolerance, of intersubgeneric hybrids between interspecific F_1 hybrids of evergreen azaleas and a deciduous species (*Rhododendron japonicum* f. *flavum*), back cross with evergreen azaleas was conducted. Though pollen of the intersubgeneric hybrids was highly sterile, fertility was restored in a tetraploid plant (9027-5). Unilateral cross incompatibility was observed between the intersubgeneric tetraploid hybrid 9027-5 and evergreen azaleas, i.e., progenies obtained only in the cross with the tetraploid plant 9027-5 used as a pollen parent. Leaf color of most of the progenies was green, and hybridity was confirmed by isozyme analysis. PtDNA of the green progenies inherited from the seed parent (evergreen azaleas), and plastome-genome incompatibility between the plastid genome from evergreen azaleas and nuclear genome from *R. japonicum* f. *flavum* was not observed in the back cross.

Key Words: azalea, back cross, gene dosage, *Rhododendron japonicum* f. *flavum*, yellow flower.

Introduction

The creation of yellow flowered varieties is one of the important breeding objectives in evergreen azaleas. To achieve this, many intersubgeneric crosses between evergreen azaleas belonging to the subgenus *Tsutsusi* and a yellow flowered deciduous species, *Rhododendron japonicum* f. *flavum* belonging to the subgenus *Pentanthera*, have been conducted by previous workers (Akabane et al., 1971; Noguchi, 1932; Yamaguchi et al., 1985). However, most of these crosses failed because of cross incompatibility and/or albinism of progenies.

We previously reported that: 1, unilateral cross incompatibility existed in the cross, and seedlings were obtained only when *R. japonicum* was used as a pollen parent (Ureshino et al., 2000), 2, the albinism of seedlings was caused by plastome-genome incompatibility between the plastid genome from evergreen azaleas and nuclear genome from *R. japonicum* f. *flavum*, and green plants were obtained only when the plastid genome of seedlings inherited from *R. japonicum* f. *flavum* was used as a pollen parent (Ureshino et al., 1999). We also reported that three-way cross of interspecific F_1 hybrids among evergreen azaleas × *R. japonicum* f. *flavum* was useful for reducing the appearance of albino seedlings, and many viable green progenies were obtained (Ureshino et al., 1998). These green progenies had pale-yellow flowers containing β-carotene, which is the main pigment of *R. japonicum* f. *flavum* (Miyajima et al., 2000). But several undesirable traits such as deciduousness, loosely branched shrubs, and a weak heat tolerance inherited from *R. japonicum* f. *flavum* remained (Kobayashi et al., 1996). To eliminate such undesirable traits, a further cross as F_2 or back cross to evergreen azaleas was required. In this study, we conducted several back cross combinations of intersubgeneric hybrids between evergreen azaleas and *R. japonicum* f. *flavum* to evergreen azaleas, and their cross compatibility of them was evaluated.

Materials and Methods

Pollen fertility

Intersubgeneric hybrids from a three-way cross between evergreen F_1 hybrids and *Rhododendron japonicum* f. *flavum* were used (Table 1). Pollen tetrads were stained with acetocarmine solution. Microscopic evaluation was carried out by counting the number of stained (viable) and not stained (sterile) grains in each tetrad. The evaluation of pollen viability was based on the calculation of the percentage of viable pollen grains in the total pollen grains. From each flower, 300 tetrads, i. e., 1200 pollen grains, were examined with three replications.

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Table 1. Pollen fertility of cross parents and the F_1 plants.

Cross parent and combination	Individual number	Ploidy level	Pollen fertility (%)[z]	Fertile pollen (%)	
				Dyad	Tetrad
Seed parent					
(*R. kiusianum* #1 × *R. eriocarpum* #1)	1	2x	92.0 ± 1.4	0	100
(*R. kiusianum* #1 × *R. indicum* #2)	1	2x	90.7 ± 6.6	0	100
Pollen parent					
R. japonicum f. *flavum*	1	2x	98.0 ± 1.7	0	100
R. japonicum f. *flavum*	2	2x	98.4 ± 1.9	0	100
F_1					
(*R. kiusianum* #1 × *R. eriocarpum* #1) #1 × *R. japonicum* f. *flavum* #1	1	2x	0	0	0
	2	2x	0	0	0
	3	2x	0	0	0
	4	2x	0	0	0
	5	2x	0	0	0
	6	2x	0.1 ± 0.3	100	0
	7	2x	0.3 ± 0.3	100	0
	8	2x	0.3 ± 0.3	100	0
	9	2x	1.0 ± 0.7	100	0
	10 ('9027-5')	4x	75.0 ± 12.3	0	100
(*R. kiusianum* #1 × *R. indicum* #2) #1 × *R. japonicum* f. *flavum* #2	1	2x	0.2 ± 0.3	100	0
	2	2x	0.3 ± 0.6	100	0
	3	2x	0	0	0
	4	2x	0	0	0

[z] Mean ± SE (n = 3).

Table 2. Capsule set and number of seeds per capsule in the cross with intersubgeneric hybrid 9027-5 as a pollen parent.

Seed parent	Capsule set[z]	No. of seeds per capsule[y]
2x		
R. indicum #1	5/6	82.0 ± 45.7[x]
R. eriocarpum #1	3/3	244.0 ± 48.0
R. eriocarpum #2	7/7	174.9 ± 24.0
(*R. kiusianum* #1 × *R. indicum* #2) #1	4/4	99.5 ± 35.0
(*R. kiusianum* #1 × *R. eriocarpum* #1) #1	6/6	116.8 ± 14.7
3x		
R. indicum #3	3/4	180.0 ± 31.9
4x		
R. indicum #4	11/11	190.3 ± 37.0
R. indicum #5	1/1	66.0

[z] 90 days after crossing.
[y] 180 days after crossing.
[x] Mean ± SE (n = 3–11).

Cross compatibility

Among intersubgeneric hybrids, a tetraploid plant (9027-5) was used as a cross parent. 9027-5 was derived from the cross between a F_1 hybrid (diploid) of *R. kiusianum* #1 × *R. eriocarpum* #1 and *R. japonicum* #1 (diploid), which was considered to be the unreduced gamete origin of the parent. Reciprocal crosses between 9027-5 and diploid, triploid, or tetraploid evergreen azaleas were conducted respectively from May to June 2003. Cross combinations are listed in Table 2. Capsules were harvested at 180 days after crossing, and derived seeds were used for in vitro sowing.

In vitro seed sowing

Seeds were soaked in 50 mg·L^{-1} GA_3 solution for 24 h, and sterilized in 10% sodium hypochlorite solution for

15 min followed by three washes in sterile distilled water. Seeds were then sown on Anderson rhododendron medium (pH 5.0) (Anderson, 1984) with 30 g·L^{-1} sucrose and 3.5 g·L^{-1} gellangum. The cultures were incubated at 25°C in a growth chamber with light (ca. 44.73 μmol·s^{-1}·m^{-2}) at a 16 h day length. After one month from primary culture, the percentage of germination seeds germination and leaf color of the seedlings were observed.

Isozyme analysis

Isozyme analysis was conducted for clarifying the hybridity and nuclear genome construction of the seedlings. Isozymes of glucosephosphate isomerase (GPI, EC 5.3.1.9), phosphoglucomutase (PGM, EC 2.7.5.1), and isocitrate dehydrogense (IDH, EC 1.1.1.41) were analyzed using the procedures described in a previous report (Ureshino et al., 1998).

Analysis of ptDNA inheritance through PCR-SSCP

The rps16 intron region of ptDNA was amplified via the polymerase chain reaction (PCR). Primer sequences were 5'-CCCCCTAGAAACGTATAGGA-3' for rps16/1F and 5'-CGAAGTAATGTCTAAACCCA-3' for rps16/1R, as reported by Nishizawa and Watano (2000). 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 (Amersham Biosciences). Amplification was carried out using TaKaRa PCR Thermal Cycler TP240 (TaKaRa) with one cycle at 95°C for 3 min, 35 cycles of 1 min at 94°C, 1 min at 47°C, 1 min at 72°C, and one cycle at 72°C for 10 min. One μL of PCR product was mixed with 4 μL of formamide dye consisting of 90% formamide, 0.5% BPB and 8% glycerin. The mixture was heated to 95°C for 3 min, and then plunged into ice. The mixture was then electrophoresed in polyacrylamide TBE gel (25% MDE gel solution (TaKaRa), 10% glycerin and 1 × TBE) for 8 h at 100 V, 4°C. After electrophoresis, banding patterns were detected by silver staining.

Results

Pollen fertility

In diploid intersubgeneric hybrids, the stainability of

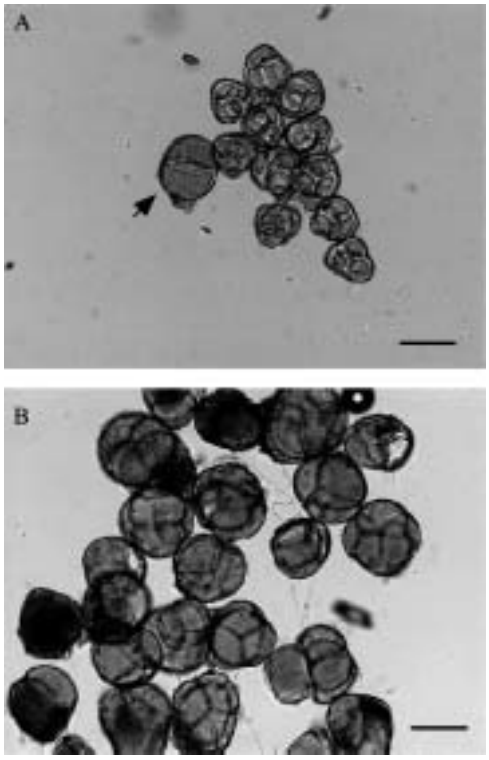

Fig. 1. Pollen tetrad of intersectional hybrids in (*R. kiusianum* #1 × *R. eriocarpum* #1) × *R. japonicum*. A, Diploid hybrid; B, Tetraploid hybrid. Arrow indicates a pollen dyad. Bars indicate 50 μm.

Table 3. Percentage of seeds germinating and the leaf color of seedlings in the cross with intersubgeneric hybrid 9027-5 as a pollen parent.

Seed parent	No. of seeds cultured	% of seeds germinating[z]	No. of seedlings		
			G[y]	AL[y]	EB[y]
2x					
R. indicum #1	300	8.0	20	0	4
R. eriocarpum #1	283	12.4	34	0	1
R. eriocarpum #2	300	4.3	13	0	0
(*R. kiusianum* #1 × *R. indicum* #2) #1	76	6.6	5	0	0
(*R. kiusianum* #1 × *R. eriocarpum* #1) #1	300	13.3	48	0	0
3x					
R. indicum #3	250	8.0	20	0	0
4x					
R. indicum #4	295	81.7	234	1	0
R. indicum #5	66	27.3	18	0	0

[z] 60 days after seed sowing.
[y] G: green, AL: albino, EB: adventitious embryo.

Table 4. Segregation of isozyme genotypes in progenies obtained from the cross with intersubgeneric hybrid 9027-5 as a pollen parent.

Seed parent	Locus	Parental genotype	Offspring genotype (Expected ratio)[z]		Observed offspring genotype (No.)
2x					
R. indicum #1	*Gpi-2*	ff × ddf'f'	ddf : ff'f' : dff'	(1 : 1 : 4)	dff'(3)
	Pgm-2	bb × bbdd	bbb : bdd : bbd	(1 : 1 : 4)	bbd(3)
	Idh-1	ee × ddee	dde : eee : dee	(1 : 1 : 4)	dee (3)
R. eriocarpum #1	*Gpi-2*	de × ddf'f'	ddd : dde : df'f : ef'f' : ddf' : def'	(1 : 1 : 1 : 1 : 4 : 4)	ddf'(11) : def'(4)
	Pgm-2	bb × bbdd	bbb : bdd : bbd	(1 : 1 : 4)	bbd(15)
	Idh-1	be × ddee	bdd : dde : bee : eee : bde : dee	(1 : 1 : 1 : 1 : 4 : 4)	bde(8) : dee(7)
R. eriocarpum #2	*Gpi-2*	de × ddf'f'	ddd : dde : df'f : ef'f' : ddf' : def'	(1 : 1 : 1 : 1 : 4 : 4)	ddf'(4) : def'(6)
	Pgm-2	bb × bbdd	bbb : bdd : bbd	(1 : 1 : 4)	bbd(10)
	Idh-1	be × ddee	bdd : dde : bee : eee : bde : dee	(1 : 1 : 1 : 1 : 4 : 4)	bde(3) : dee(7)
(*R. kiusianum* #1 × *R. eriocarpum* #1) #1	*Gpi-2*	df × ddf'f'	ddd : ddf : df'f : ff'f : ddf' : dff'	(1 : 1 : 1 : 1 : 4 : 4)	ddf'(15) : dff'(7)
	Pgm-2	ab × bbdd	abb : bbb : add : bdd : abd : bbd	(1 : 1 : 1 : 1 : 4 : 4)	abd(9) : bbd(13)
	Idh-1	be × ddee	bdd : dde : bee : eee : bde : dee	(1 : 1 : 1 : 1 : 4 : 4)	bde(10) : dee(12)
4x					
R. indicum #4	*Gpi-2*	ffff × ddf'f'	ddff : fff'f' : dfff'	(1 : 1 : 4)	dfff'(76)
	Pgm-2	aaab ×bbdd	aabb : abbb : aadd : abdd : aabd : abbd	(1 : 1 : 1 : 1 : 4 : 4)	aabd(38) : abbd(38)
	Idh-1	eeee × ddee	ddee : eeee : deee	(1 : 1 : 4)	deee(76)
R. indicum #5	*Gpi-2*	dffj × ddf'f'	dddf : dddj : ddff : ddfj : dff'f' : df'f'j : fff'f' : ff'f'j : ddff' : ddf'j : dfff' : dff'j	(2 : 1 : 1 : 2 : 2 : 1 : 1 : 2 : 8 : 4 : 4 : 8)	ddff'(1) : dfff'(2) : dff'j(1)
	Pgm-2	aabb × bbdd	aabb : abbb : bbbb : aadd : abdd : bbdd : aabd : abbd : bbbd	(1 : 4 : 1 : 1 : 4 : 1 : 4 : 16 : 4)	abbd(3)
	Idh-1	beee × ddee	bdde : ddee : beee : eeee : bdee : deee	(1 : 1 : 1 : 1 : 4 : 4)	bdee(2) : deee(1)

[z] Underlined genotypes theoretically occur only when gametes of intersubgeneric hybrid 9027-5 are produced allosyndetically.

Table 5. PtDNA inheritance of progenies from several evergreen azaleas × intersubgeneric hybrid 9027-5.

Seed parent	No. of progeny	PtDNA	
		Maternal	Paternal
2x			
R. eriocarpum #1	12	12	0
R. eriocarpum #2	6	6	0
(*R. kiusianum* #1 × *R. eriocarpum* #1) #1	11	10	1
4x			
R. indicum #4	68	68	0
R. indicum #5	3	3	0

pollen grains was quite low (≤ to 1.0%), and stained pollen formed a pollen dyad (Table 1, Fig. 1A), whereas that of tetraploid 9027-5 was high (75.0%) and formed a pollen tetrad (Table 1, Fig. 1B).

Cross compatibility

A capsule set was observed in the cross with 9027-5 as a pollen parent (Table 2), but not capsule set when 9027-5 was used as a seed parent (data not shown). The number of seeds per capsule was more than 50 in all crosses (Table 2). In crosses with diploids or triploids, the germination rate ranged from 4.3 to 13.3%, whereas it was 27.3–81.7% in crosses with tetraploids. Leaf color of most seedlings was green except for one albino seedling in *R. indicum* #4 × 9027-5 (Table 3).

Hybridity confirmation of progenies

Isozyme genotypes of (*R. kiusianum* #1 × *R. eriocarpum* #1) #1, which was the seed parent of 9027-5, were df at *Gpi-2*, ab at *Pgm-2*, and be at *Idh-1*. Genotypes of *R. japonicum* #1, which was the pollen parent of 9027-5, were f'f' at *Gpi-2*, dd at *Pgm-2*, and dd at *Idh-1* (data not shown). Genotypes of 9027-5 were ddf'f' at *Gpi-2*, bbdd at *Pgm-2* and ddee at *Idh-1*, which indicated that 9027-5 had two sets of the nuclear genome from (*R. kiusianum* #1 × *R. eriocarpum* #1) #1 and two sets from *R. japonicum* #1 (Table 4). The genotype of gametes in 9027-5 was classified into two types: allosyndetic and autosyndetic gametes. At *Gpi-2*, for example, the autosyndetic genotype was df' and allosyndetic ones were dd and f'f'. In the cross of *R. indicum* #1 × 9027-5, the offspring genotype at *Gpi-2*, therefore, was theoretically segregated into ddf, ff'f', and dff'. The former two genotypes were derived from allosyndetic gamete of 9027-5, and the latter was from autosyndetic gamete. In all crosses, progenies were confirmed to be hybrid in origin at three loci. Progenies from allosyndetic gametes were, however, not observed in all crosses.

PtDNA of progenies

From PCR-SSCP analysis, 9027-5 contained the ptDNA from *R. japonicum* f. *flavum*, and the banding pattern could be distinguished from that of evergreen azalea species. It was confirmed that the ptDNA of most progenies was inherited from the seed parent of evergreen azaleas (Table 5).

Discussion

Marked pollen sterility of intersubgeneric hybrids between evergreen and deciduous azaleas was found in the present study. However, in a tetraploid hybrid 9027-5, pollen fertility was restored. These results indicate that chromosome doubling to intersubgeneric hybrids is useful for conducting further crosses.

In the reciprocal cross between the intersubgeneric tetraploid hybrid 9027-5 and evergreen azaleas, unilateral cross incompatibility was observed. Capsules sets were observed only when the intersubgeneric tetraploid hybrid 9027-5 was used as a pollen parent. Similar phenomenon was reported in the cross between evergreen azaleas and *R. japonicum* f. *flavum*, and capsule sets failed in the cross with evergreen azaleas as a pollen parent because of the inhibition of pollen tube growth (Ureshino et al., 2000). Inhibition of the pollen tube growth of evergreen azaleas may be inherited by the tetraploid hybrid from its parent *R. japonicum* f. *flavum*.

The most serious problem in the cross between evergreen azaleas and *R. japonicum* f. *flavum* has been the albinism of seedlings caused by plastome-genome incompatibility between the plastome from evergreen azaleas and the nuclear genome from *R. japonicum* f. *flavum* (Ureshino et al., 1999). PtDNA of most of BC_1 seedlings were inherited from the seed parent, evergreen azaleas, whereas those seedlings had a green leaf color. Ureshino and Miyajima (2002) reported that plastome-genome incompatibility between the plastome from evergreen azaleas and the nuclear genome from *R. japonicum* could be overcome by gene dosage of the nuclear genome from evergreen azaleas, i. e., the nuclear genome construction of seedlings was 2x evergreen azaleas—1x *R. japonicum*. Sakai et al. (2004) also overcame hybrid albinism through an inter-ploidy cross of 4x evergreen azaleas × 2x *R. japonicum* f. *flavum*. From the results of isozyme analysis in this study, BC_1

progenies from allosyndetic gametes of 9027-5 were not observed. In the case of 4x evergreen azaleas as a seed parent, the ratio of the nuclear genome construction of BC_1 progenies was 3 (evergreen azaleas) : 1 (*R. japonicum* f. *flavum*). On the other hand, in the case of 2x evergreen azaleas as a seed parent, the ratio was 2 : 1. It was considered that gene dosage of the nuclear genome from evergreen azaleas results in progeny with green leaves.

The final aim of the intersubgeneric hybridization program between evergreen azaleas and *R. japonicum* f. *flavum* is the introduction of a yellow flower trait to evergreen azaleas. Chromosomal recombination between evergreen azaleas and *R. japonicum* f. *flavum*, however, seems to be unlikely from the results of isozyme analysis. The production of progeniy with alien additional chromosomes from *R. japonicum* f. *flavum* through the continuous backcrossing of evergreen azaleas, therefore, might be useful for achieving this.

In this study, we obtained various green progenies from crosses between intersubgeneric tetraploid hybrid 9027-5 and evergreen azaleas. Since the progenies are growing vigorously, fertility and several morphological traits such as flower color, deciduousness and heat tolerance will be investigated in a future study.

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ツツジ亜属間雑種を常緑性ツツジに戻し交雑したときの交雑和合性

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常緑性ツツジの種間雑種と落葉性ツツジ（キレンゲツツジ）との亜属間交配で得られた雑種個体について，落葉性や非耐暑性などの形質改善を目的として，常緑性ツツジへの戻し交雑を試みた．亜属間交雑で得られた雑種個体の花粉は高い不稔性を示したが，倍加した個体 9027-5 の稔性は回復していた．この 9027-5 に数種類の常緑性ツツジを正逆で戻し交雑したところ，9027-5 を花粉親にした場合にのみ実生が得られた．得られたほとんどすべての実生の葉色は緑色であり，アイソザイム分析によりこれらの雑種性が確認された．また，これら緑色実生の葉緑体 DNA は常緑性ツツジ由来であったことから，これまで常緑性ツツジとキレンゲツツジの交配でアルビノ実生出現の要因であった常緑性ツツジ由来の葉緑体ゲノムとキレンゲツツジ由来の核ゲノム間の不和合性は，BC_1 世代においては生じていないことが明らかとなった．