Plastome–genome Incompatibility of *Rhododendron serpyllifolium* (A. Gray) Miq. to Evergreen Azalea Species Belonging to Series Kaempferia

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Summary

To confirm that unilateral cross–incompatibility exists between *Rhododendron serpyllifolium*, series Serpyllifolia, and six evergreen azaleas belonging to series Kaempferia, interspecific crosses were conducted. In crosses with *R. serpyllifolium* as seed parents, few seeds of the progenies germinated; the survivors developed into plants with green leaves which PCR–RFLP analysis showed as having *R. serpyllifolium* type ptDNA. In the reciprocal crosses with *R. serpyllifolium* as pollen parents, many seeds germinated but many were non–viable albinos which had Kaempferia type ptDNA. In crosses between *R. transiens* (Accession 1) × *R. serpyllifolium* (Accession 1), however, relatively high frequency of green seedlings having *R. serpyllifolium* type ptDNA appeared. From these results, we conclude that it is better to use *R. serpyllifolium* as pollen parent for obtaining many seedlings, and that, in this case, *R. transiens* (Accession 1) is useful as bridge plants for overcoming plastome–genome incompatibility between *R. serpyllifolium* and series Kaempferia species.

Key Words: albino, PCR–RFLP, plastome–genome incompatibility, series Serpyllifolia, unilateral cross incompatibility.

Introduction

Azalea (*Rhododendron* spp.) is known as one of the horticulturally important ornamental plants (Galle, 1985). There are many azalea species in Japan, especially, the evergreen species that belong to subsection Tsutsusi which are distributed in Kyushu Island (Kunishige and Kobayashi, 1980). This subsection is divided into three series, i.e., Kaempferia, Tsutsusi, and Serpyllifolia (Yamazaki, 1996), among which the evergreen species belonging to series Kaempferia and Tsutsusi have contributed to breeding cultivated varieties. To date, they consist of several horticultural groups such as ‘Kurume tsutsusi’, ‘Edo–kirishima’ and ‘Satsuki’. Inter–series crossings between Kaempferia and Tsutsusi have produced cultivars with wide morphological variations, whereas the series Serpyllifolia has been rarely used as breeding materials.

Series Serpyllifolia in Eastern Asia consists of two species, among which *R. serpyllifolium* is distributed in Japan (Yamazaki, 1996). It is a semi–deciduous shrub with small flowers. It is distributed in rocky slopes on

mountain valleys at 300–900 m elevation on Honshu (Shizuoka and Wakayama Prefectures), Southern Shikoku and Southern Kyushu areas. Their flowers are light, pastel purple; this color is lacking in the series Kaempferia and Tsutsusi. A pale yellow–flowered form exists in *R. serpyllifolium* f. *usukiuizen*. Therefore, the species *R. serpyllifolium* and *R. serpyllifolium* f. *usukiuizen* possess the potential as breeding stocks for introducing the pastel purple and yellow colors into the evergreen azalea cultivars. However, there is little information on the cross compatibility between *R. serpyllifolium* and other species. In this study, we conducted inter–series crosses between *R. serpyllifolium* and evergreen azalea species and compared their cross compatibilities.

Materials and Methods

Plant materials

Parental stocks (Table 1) were used for inter–series crosses between *Rhododendron serpyllifolium* and six evergreen azalea species of the series Kaempferia (Table 2) in April 1996 or 1997. Flowers of pistillate parents were emasculated and covered with paper bags about seven days before anthesis and pollinated at anthesis with fresh or one–year–old pollen stored at –20 °C. All

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Table 1. *Rhododendron* species used as parents.

Species	Code	Collection site
Series Kaempferia		
<i>R. kaempferi</i> (Accession 1, 2 and 3)	KAE1, 2, 3	Mt. Kurokamiyama, Saga Pref.
<i>R. kaempferi</i> var. <i>macrogemma</i> (Accession 1 and 2)	MCR1, 2	Izuoshima Is., Tokyo.
<i>R. kiusianum</i> (Accession 1 and 2)	KIU1, 2	Mt. Aso, Kumamoto Pref.
<i>R. simsii</i> (Accession 1 and 2)	SIM1, 2	Iriomote Is., Okinawa Pref.
<i>R. tosaense</i> (Accession 1 and 2)	TOS1, 2	Hyuga City, Miyazaki Pref.
<i>R. transiens</i> (Accession 1 and 2)	TRA1, 2	Kanto area, unknown
Series Serpyllifolia		
<i>R. serpyllifolium</i> (Accession 1 and 2)	SER1, 2	Mt. Onogaradake, Kagoshima Pref.

pollinated flowers were covered again with paper bags to avoid contamination. The frequency of capsule set was observed 90 days after pollination.

In vitro seed culture

Capsules were collected 180 days after pollination and air-dried at 25 °C for two weeks. Seeds were excised from the capsules and sterilized in 5% sodium hypochlorite solution for 15 min, followed by three washes in sterile distilled water. They were then cultured on Anderson's rhododendron medium (Anderson, 1984) supplemented with 50 mg · liter⁻¹ GA₃ (pH 5.0). Germinated seedlings were transplanted into a medium that contained 10 mg · liter⁻¹ N⁶-[2-isopentenyl] adenine (2ip) (pH 5.0) to induce multiple shoots. The shoot cultures were incubated at 25 °C in a growth chamber under light (ca. 44.73 μmol · sec⁻¹ · m⁻²) and a 16-hr photoperiod. The leaf colors of the seedlings segregated into green, pale-green, and albino; some sectorial chimera were noted. Some green seedlings had elongated leaves but formed new albino shoots during multiplication, which were scored as sectorial chimeric plants. The germinated seedlings from some crosses were used for isozyme analysis.

Isozyme analysis

Isozyme analysis was conducted by 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) to confirm the hybridity of the seedlings. Detailed procedures were reported by Ureshino et al. (1998).

PCR-RFLP

Total genomic DNA was extracted from 300 mg frozen multiple shoots by following the CTAB method (Murray and Thompson, 1980) modified by Kobayashi et al (1998). *RbcL* region of ptDNA was amplified via polymerase chain reaction (PCR). Primer sequences were 5'-TGTCACCAAAAACAGAGACT for *rbcL*-1 and 5'-TTCCATACTTCACAAGCAGC for *rbcL*-2, which were designed from *Pseudotsuga menziesii* by Hipkins et al. (1990). Reaction mixtures (25 μl) for PCR contained 25 ng of template DNA, 0.5 μM of each

primer, 2.0 mM of MgCl₂, 0.1 mM of dNTPs, 2.5 μl of 10 × reaction buffer, and 0.5 unit of *Taq* DNA polymerase (Boehringer Mannheim GmbH, Mannheim, Germany). Amplification was carried out by using Program Temp Control System (ASTEC Co., Ltd., Fukuoka, Japan) with one cycle at 94 °C for 5 min, 40 cycles of 1 min at 94 °C, 1 min at 52 °C, and 2 min at 72

Table 2. Frequency of capsule set and number of seeds per capsule in reciprocal crosses between *Rhododendron serpyllifolium* and species in series Kaempferia.

Cross	Capsule set ^z	No. of seeds per capsule ^y
<i>Kaempferia</i> × <i>R. serpyllifolium</i>		
KAE1 × SER1	100 (4) ^x	225 ± 25.2
MCR1 × SER1	100 (2)	501
MCR2 × SER2	38 (8)	221 ± 19.4
KIU1 × SER1	60 (10)	145 ± 19.5
KIU2 × SER2	100 (7)	174 ± 35.7
SIM1 × SER1	33 (3)	399
SIM2 × SER2	100 (2)	169
TOS1 × SER1	100 (4)	210 ± 34.3
TOS2 × SER2	88 (8)	197 ± 78.7
TRA1 × SER1	100 (3)	99 ± 13.3
TRA2 × SER2	100 (3)	220 ± 48.3
<i>R. serpyllifolium</i> × <i>Kaempferia</i>		
SER1 × KAE2	100 (3)	257 ± 38.8
SER2 × KAE3	100 (3)	277 ± 16.1
SER1 × MCR1	100 (3)	60 ± 18.3
SER2 × MCR2	83 (6)	180 ± 35.9
SER1 × KIU1	100 (3)	145 ± 53.3
SER2 × KIU2	100 (3)	267 ± 40.0
SER1 × SIM1	83 (6)	157 ± 19.4
SER2 × SIM2	38 (8)	28 ± 18.2
SER1 × TOS1	25 (8)	43
SER2 × TOS2	100 (3)	260 ± 6.0
SER1 × TRA1	60 (5)	90 ± 13.0
SER2 × TRA2	80 (5)	29 ± 5.9

^z Capsule set was observed 90 days after pollination.

^y Mean ± SE.

^x Percentage of capsule set (No. of pollinated flowers).

℃, and one cycle at 72 °C for 5 min. PCR products were digested with 0.5 unit of *Hinf*I (TaKaRa Shuzo Co., Ltd, Shiga, Japan) at 37 °C for 1 hr. After digestion, the restriction fragments were separated by electrophoresis in 2.0% agarose (Sigma Chemical Co., St. Louis, MO, USA) gel, containing 0.00005% ethidium bromide at 100 V for 40 min. The gel plates of restriction fragments were photographed under ultraviolet illumination.

Results

The number of capsules and of seeds per capsule produced from all the crosses varied among the cross combinations, and produced more than 60 seeds per capsule in most crosses, except for SER2 × SIM2 and SER1 × TOS1 (Table 2).

When *R. serpyllifolium* was used as a pollen source, the germination percentage ranged from 33 (KIU2 × SER2) to 94% (SIM2 × SER2) (Table 3). When *R. serpyllifolium* was used as the seed parent, seeds from most crosses failed to germinate. Seedlings were obtained from the three crosses, SER2 × MCR2, SER1 ×

KIU1 and SER2 × KIU2 whose seeds had a germination percentage of less than 18%.

In the crosses with *R. serpyllifolium* as pollen parent, most seedlings were pale-green and albino; they withered and died during acclimation (Table 3). Green seedlings which were obtained from seven crosses amounted to 0.4 to 11% of the progenies, except for TRA1 × SER1 which yielded 65%. Although seedlings were obtained from only three crosses using *R. serpyllifolium* as seed parent, the frequency of green plants ranged from 50 to 82% as compared to the reciprocal crosses.

To confirm the hybridity of the seedlings, isozyme analysis was conducted on progenies from seven crosses using the three enzyme systems (Table 4). Parental species exhibited different genotypes at *Pgm*-2 for TOS2 × SER2, *Idh*-1 for all crosses, and at *Mdh*-2 for TRA2 × SER2. Seedlings that indicated expected genotypes at those loci were confirmed to be of hybrid origin regardless of the leaf colors.

PCR products in *rbcL* region indicated that DNA fragment size (1350 bp) between SER1 and KIU1 was

Table 3. Comparison of percentages of seed germination and green plants in reciprocal crosses between *Rhododendron serpyllifolium* and species in series *Kaempferia*.

Cross	No. of seeds cultured	Germination percentage ^z	Leaf color of seedlings ^y				% of green seedlings
			G	PG	AL	Sec	
<i>Kaempferia</i> × <i>R. serpyllifolium</i>							
KAE1 × SER1	391	69	5	92	172	0	1.9
MCR1 × SER1	223	55	0	41	81	0	0
MCR2 × SER2	271	90	0	90	155	0	0
KIU1 × SER1	353	71	1	21	222	5	0.4
KIU2 × SER2	277	33	1	7	78	5	1.1
SIM1 × SER1	274	82	3	204	18	0	1.3
SIM2 × SER2	52	94	0	31	18	0	0
TOS1 × SER1	280	66	0	47	139	0	0
TOS2 × SER2	185	72	9	62	61	1	6.8
TRA1 × SER1	66	79	34	10	1	7	65.4
TRA2 × SER2	73	75	6	25	24	0	10.9
<i>R. serpylliforlium</i> × <i>Kaempferia</i>							
SER1 × KAE2	100	0	–	–	–	–	–
SER2 × KAE3	71	0	–	–	–	–	–
SER1 × MCR1	40	0	–	–	–	–	–
SER2 × MCR2	88	2	1	0	1	0	50.0
SER1 × KIU1	96	18	14	1	1	1	82.4
SER2 × KIU2	63	6	2	0	2	0	50.0
SER1 × SIM1	140	0	–	–	–	–	–
SER2 × SIM2	32	0	–	–	–	–	–
SER1 × TOS1	36	0	–	–	–	–	–
SER2 × TOS2	35	0	–	–	–	–	–
SER1 × TRA1	81	0	–	–	–	–	–
SER2 × TRA2	106	0	–	–	–	–	–

^z Germination until four months after sowing was observed.

^y G; Green, PG; Pale-green, AL; Albino, Sec; Sectorial chimera.

the same; the same size of PCR products was also obtained in their F₁ hybrid seedlings. After digestion of PCR products with *Hinf* I, polymorphism of restriction fragments was observed between the parents (Fig. 1). The specific restriction fragment was 140 bp for SER1 and 170 bp for KIU1. For their progenies, irrespective of

cross direction, all green plants showed the same banding patterns as SER1, whereas the banding patterns of pale-green and albino plants were the same as KIU1. The banding patterns of a sectorial chimeric hybrid were the same as SER1 in the green leaf segment and as KIU1 in the albino leaf segment.

PtDNA inheritance of hybrids from several crosses was investigated to clarify the origin of albino plants (Table 5). Banding patterns of species (KIU2, TOS2, TRA1 and TRA2) belonging to series *Kaempferia* were same as KIU1 having the specific restriction fragment of 170 bp (*Kaempferia* type). All individuals of *R. serpyllifolium* had another specific restriction fragment of 140 bp (*R. serpyllifolium* type). In all crosses between evergreen species and *R. serpyllifolium*, albino and pale-green progenies contained the *Kaempferia* type, whereas most green progenies contained the *R. serpyllifolium* type. One green progeny from TRA1 × SER1, however, had both *Kaempferia* specific and *R. serpyllifolium*

Fig. 1. Gel plate showing the inheritance patterns of ptDNA *rbcL* gene restricted with *Hinf* I in the interspecific crosses between *Rhododendron kiusianum* (Accession 1) and *R. serpyllifolium* (Accession 1). G; Green, PG; Pale-green, AL; Albino, Sec (G); Green segment of sectorial chimeric progeny, Sec (A); Albino segment of sectorial chimeric progeny.

Table 4. Segregation analysis of PGM, IDH and MDH isozymes in offsprings from crosses between *Rhododendron serpyllifolium* and species in series *Kaempferia*.

Cross	Locus	Parentage genotype	Offspring genotype ² (No. of offsprings)			
			G	PG	AL	Sec
<i>Kaempferia</i> × <i>R. serpyllifolium</i>						
KIU1 × SER1	<i>Pgm</i> -2	bb × bb	—	bb (8)	bb (45)	bb (5)
	<i>Idh</i> -1	bb × aa	—	ab (8)	ab (45)	ab (5)
	<i>Mdh</i> -2	ad × dd	—	ad (3): dd (5)	ad (25): dd (20)	ad (2): dd (3)
KIU2 × SER2	<i>Pgm</i> -2	bb × bb	bb (1)	bb (2)	bb (76)	bb (5)
	<i>Idh</i> -1	bb × aa	ab (1)	ab (2)	ab (76)	ab (5)
	<i>Mdh</i> -2	ad × dd	dd (1)	ad (1): dd (1)	ad (43): dd (33)	ad (3): dd (2)
TOS2 × SER2	<i>Pgm</i> -2	de × bb	bd (4): be (4)	—	bd (3)	—
	<i>Idh</i> -1	bb × aa	ab (8)	—	ab (3)	—
	<i>Mdh</i> -2	dd × dd	dd (8)	—	dd (3)	—
TRA1 × SER1	<i>Pgm</i> -2	bb × bb	bb (29)	—	bb (1)	—
	<i>Idh</i> -1	ef × aa	ae (15): af (14)	—	af (1)	—
	<i>Mdh</i> -2	ad × dd	ad (12): dd (17)	—	ad (1)	—
TRA2 × SER2	<i>Pgm</i> -2	ab × bb	ab (2): bb (4)	—	ab (1): bb (1)	—
	<i>Idh</i> -1	be × aa	ab (2): ae (4)	—	ab (1): ae (1)	—
	<i>Mdh</i> -2	aa × dd	ad (6)	—	ad (2)	—
<i>R. serpyllifolium</i> × <i>Kaempferia</i>						
SER1 × KIU1	<i>Pgm</i> -2	bb × bb	bb (14)	—	bb (1)	bb (1)
	<i>Idh</i> -1	aa × bb	ab (14)	—	ab (1)	ab (1)
	<i>Mdh</i> -2	dd × ad	ad (4): dd (10)	—	dd (1)	dd (1)
SER2 × KIU2	<i>Pgm</i> -2	bb × bb	bb (1)	—	—	—
	<i>Idh</i> -1	aa × bb	ab (1)	—	—	—
	<i>Mdh</i> -2	dd × ad	ad (1)	—	—	—

² G; Green, PG; Pale-green, AL; Albino, Sec; Sectorial chimera.

Table 5. ptDNA type of hybrids from the crosses between *Rhododendron serpyllifolium* and species in series *Kaempferia*.

Cross	Leaf color ^z	No. of hybrids tested	Type of ptDNA		
			Kaempferia (170 bp)	<i>R. serpyllifolium</i> (140 bp)	Both ^y
<i>Kaempferia</i> × <i>R. serpyllifolium</i>					
KIU1 × SER1	PG	7	7	0	0
	AL	43	43	0	0
	Sec	5	5 ^x	5 ^x	0
KIU2 × SER2	G	1	0	1	0
	PG	2	2	0	0
	AL	76	76	0	0
	Sec	4	4 ^x	4 ^x	0
TOS2 × SER2	G	1	0	1	0
	AL	3	3	0	0
TRA1 × SER1	G	29	0	28	1
	AL	1	1	0	0
TRA2 × SER2	G	6	0	6	0
	AL	2	2	0	0
<i>R. serpyllifolium</i> × <i>Kaempferia</i>					
SER1 × KIU1	G	14	0	14	0
	AL	1	1	0	0
	Sec	1	1 ^x	1 ^x	0
SER2 × KIU2	G	1	0	1	0
	AL	2	2	0	0

^z G; Green, PG; Pale-green, AL; Albino, Sec; Sectorial chimera.^y Biparental ptDNA inheritance.^x Green and albino leaf segments contained *R. serpyllifolium* type and *Kaempferia* type of ptDNA, respectively.

specific bands, which indicated a biparental ptDNA inheritance. Sectorial chimeric progenies contained *Kaempferia* type in albino leaf segments and *R. serpyllifolium* type in green leaf segments.

Discussion

Interspecific hybridization is one useful method to create new cultivars with new characteristics. The difficulty in obtaining hybrids, however, increases with the increase in phylogenetic distance between the parental taxa involved (Sharma, 1995). Fundamental knowledge on the nature of cross incongruity is important for a systematic design of efficient breeding procedures.

Among the genus *Rhododendron*, an interspecific cross barrier is reported in many remote crosses (Rouse et al., 1993), and the barrier operates at many different stages both before and after fertilization by the degree of relatedness of parental species (Williams et al., 1982; Rouse et al., 1993). The occurrence of albino plants, which are not viable, is an obstacle to breeding with remote crosses (Ureshino et al., 1999). Capsules, which set, formed seeds in all crosses, but the frequency of viable seeds was apparently dependent upon whether *R. serpyllifolium* was used as the seed parent or pollen parent. This indicates that unilateral cross-incompatibility exists in an inter-series cross between series *Serpyllifolia* and *Kaempferia* and that viable seeds are obtained only when *R. serpyllifolium* is used as pollen

source.

Albino seedlings and all albino leaf segments had ptDNA from series *Kaempferia* species, whereas green progenies and green chimeras had ptDNA from *R. serpyllifolium*. Hybrid albinism is 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) and is considered to be caused by the incompatibility between plastid genome and nuclear genome, i.e., 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 was inherited from *Z. odorata* (Yao et al., 1994). In azalea, it was reported that the appearance of albino seedlings from an intersectional cross between evergreen azalea species and deciduous species of *R. japonicum* was caused by the plastome-genome incompatibility between plastid genome from evergreen azalea species and nuclear genome of *R. japonicum* (Ureshino et al., 1999). From these results, it is considered that the albino seedlings in this study were caused by the plastome-genome incompatibility between the plastid genome from series *Kaempferia* species and the nuclear genome from *R. serpyllifolium*.

PtDNA of progenies was mainly inherited from the seed parent in all crosses, whereas paternal ptDNA

inheritance was observed in some crosses. Plastid DNA is usually the inherited mother in angiosperm genera because the elimination of paternal ptDNA occurred through pollen and pollen tube development before fertilization (Corriveau and Coleman, 1988; Harris and Ingram, 1991). In some *Rhododendron* species, however, it was reported that disappearance of paternal ptDNA does not occur during pollen and pollen tube development (Kuroiwa, 1991). Such a phenomenon was observed in other angiosperm genera, e.g., *Oenothera* and *Campanula* (Harris and Ingram, 1991) in which the elimination of paternal ptDNA may have occurred after fertilization and during embryo development (Birky, 1995). In the present study, paternal ptDNA inheritance was observed in some crosses but its frequency was lower than that of maternal inheritance. Sorting out of paternal ptDNA in *Rhododendron* might occur late, and such relaxed mechanism might allow it to happen.

As mentioned above, ptDNA of most of progenies is inherited from a seed parent, so that it is preferable to use *R. serpyllifolium* as a seed parent in crosses with other evergreen azalea species. In such crosses, however, the frequency of viable seeds is predictably very low because of cross-incompatibility. To overcome incompatibility, a breeding technique such as ovule culture might be employed.

Although most seedlings were albino in the cross with *R. serpyllifolium* as pollen parent, several green seedlings, having *R. serpyllifolium* type of ptDNA (paternal inheritance), were obtained in the crosses with KIU2, TOS2, TRA1, and TRA2 as seed parents. Paternal ptDNA inheritance is reported in interspecific crosses with *R. kiusianum* as the seed parent (Kobayashi, 1996; Ureshino et al., 1998). Ureshino et al. (1998, 1999) reported the usefulness of the species as the bridge plant for increasing the frequency of green seedlings having paternal ptDNA. In this study, many green plants, having paternal ptDNA, were obtained in the cross with *R. transiens* (Accession 1) as the seed parent. Consequently, the species might be useful as the bridge plant for overcoming the cross-incongruity between the series *Serpyllifolia* and *Kaempferia* species.

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ウンゼンツツジ [*Rhododendron serpyllifolium* (A. Gray) Miq.] とヤマツツジ列に属する常緑性ツツジ種との交配における葉緑体ゲノム-核ゲノム間の不和合性道下紋乃¹・嬉野健次^{2*}・宮島郁夫³¹ 九州大学大学院生物資源環境科学研究科 812-8581 福岡市東区箱崎² 九州大学大学院農学研究科 812-8581 福岡市東区箱崎³ 九州大学大学院農学研究院 812-8581 福岡市東区箱崎

摘 要

ウンゼンツツジ列に属するウンゼンツツジとヤマツツジ列に属する常緑性ツツジ5種1変種との一側交雑不和合性の存在を明らかにするため、両者の種間交雑を行った。ウンゼンツツジを種子親とした交配では、種子はほとんど発芽しなかったが、得られた実生の多くはウンゼンツツジ由来の葉緑体ゲノムを持つ緑色実生であった。一方、ウンゼンツツジを花粉親に用いた交配では、得られた種子の多くは発芽したが、ほとんどの組合せにおいてヤマツツジ列に属する常緑性ツツジ種由来の葉緑体ゲノムを持つアルビノ実生となった。しかしながら、オオヤマツツジ1を種子親に用いた交配では、比

較的多くの緑色実生が得られた。以上の結果より、ヤマツツジ列の常緑性ツツジ種とウンゼンツツジとの交配において、ウンゼンツツジを花粉親に用いれば高い割合で実生が得られること、また、この場合オオヤマツツジ1は、ウンゼンツツジ列とヤマツツジ列との間の葉緑体ゲノム-核ゲノム間の不和合性を打破するための有効な橋渡し植物となる可能性が示唆された。

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