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Early Identification of Intra- and Intergeneric Hybrids among Colchicaceous Ornamentals, *Gloriosa* spp., *Littonia modesta* Hook. and *Sandersonia aurantiaca* Hook., by Flow Cytometry and Random Amplified Polymorphic DNA Analyses

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Plantlets derived from ovule culture following intra- or intergeneric crosses among Colchicaceous ornamentals, *Gloriosa* spp., *Littonia modesta* Hook. and *Sandersonia aurantiaca* Hook., were subjected to flow cytometry (FCM) and random amplified polymorphic DNA (RAPD) analyses in order to verify their hybridity. For crosses between plants with apparently distinct relative fluorescence intensity (RFI) of nuclei, i.e., intrageneric crosses between *Gloriosa* genotypes with different ploidy levels, and most intergeneric crosses, detection of hybrid plantlets could be readily accomplished by FCM analysis. The results of hybrid identification by RAPD analysis supported those of FCM analysis. In addition, RAPD analysis allowed the verification of the hybridity of intra- or intergeneric cross-derived plantlets, which could not be identified as hybrids by FCM analysis due to the similarity of RFI of the parents or appearance of the RFI peak in an unexpected position. Totally, 110 independent hybrid plantlets (60 intrageneric and 50 intergeneric hybrids) have so far been identified by FCM and/or RAPD analyses. Thus, FCM in combination with RAPD analyses offer simple and rapid means for the early detection of intrageneric and intergeneric hybrids in Colchicaceous ornamentals.

Key Words: Colchicaceous ornamentals, flow cytometry, hybrid identification, RAPD, wide hybridization.

Introduction

Gloriosa ssp., *Littonia modesta* Hook. and *Sandersonia aurantiaca* Hook., are tuberous plants belonging to Colchicaceae. Among them, *Gloriosa* spp. and *S. aurantiaca* Hook. have recently become popular worldwide as cut flowers. In order to widen their variability in horticultural traits such as plant form, flower color, and shape, broad hybridization has been carried out in Colchicaceous ornamentals, and intergeneric hybrid plants between *S. aurantiaca* Hook. and *L. modesta* Hook. (Eason et al., 2001; Morgan et al., 2001) and between *S. aurantiaca* Hook. and *G. rothschildiana* O'Brien (Nakamura et al., 2005) have successfully been produced via ovule culture. We also previously examined the production of intra- and intergeneric hybrid plants using six *Gloriosa*, one *L. modesta* Hook., and two *S. aurantiaca* Hook. genotypes, and obtained a number of putative hybrid plantlets via ovule culture (Kuwayama et al., 2005).

For efficient cross breeding in Colchicaceous ornamentals, early identification of hybrids is necessary since these plants require 2–3 years from seedling to flowering stages (Azuma, 1995a, 1995b). Several methods, such as a comparison of morphological characteristics, chromosome observation, and isozyme and molecular analyses, have so far been employed for verifying the hybrid nature. In addition, FCM analysis of the relative nuclear DNA content has recently been performed for hybrid identification in some ornamentals (Kato et al., 2001; Nimura et al., 2003). For FCM analysis, samples should not be large, and thus this method enables the early detection of hybrids. FCM analysis also has the advantages of accuracy, convenience, simplicity, and quickness as compared with conventional chromosome observation

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(Dolezel, 1997; Mishiba et al., 2000; Ozaki et al., 2004; Saito et al., 2003; Roux et al., 2003; Shiba and Mii, 2005), and thus a large number of samples can be analyzed in a short period of time. Although FCM analysis was also performed for hybrid identification in intergeneric hybridization between *S. aurantiaca* Hook. and *L. modesta* Hook. (Morgan et al., 2001) and between *S. aurantiaca* Hook. and *G. rothschildiana* O'Brien (Nakamura et al., 2005), the applicability of this method for detecting hybrids of a wide range of cross combinations in Colchicaceous ornamentals has not yet been examined. On the other hand, among various molecular analyses, RAPD analysis requires only small samples for DNA extraction and only simple PCR techniques, and has been utilized for hybrid identification in various ornamentals (Kato et al., 2001; Nimura et al., 2003; Obata et al., 2000; Takatsu et al., 2001). In the present study, we examined early confirmation of the hybridity by FCM and RAPD analyses of a number of plantlets derived from various intra- and intergeneric crosses among Colchicaceous ornamentals (Kuwayama et al., 2005).

Materials and Methods

Plant materials

Potted plants of six *Gloriosa* genotypes, one genotype of *L. modesta* Hook., and two genotypes of *S. aurantiaca* Hook. (Table 1), which had been used as the parents for intra- and intergeneric crosses (Kuwayama et al., 2005), were cultivated in a greenhouse without heating. All the plantlets derived from ovule culture following intra- or intergeneric crosses (Kuwayama et al., 2005) were cultured *in vitro* on half-strength MS (Murashige and Skoog, 1962) medium lacking plant growth regulators but with 30 $g \cdot L^{-1}$ sucrose and 0.2% gellan gum, and adjusted to pH 5.7, under a 16-h photoperiod with fluorescent light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

FCM analysis

The relative DNA contents of nuclei isolated from leaf tissues were measured using a flow cytometer PA (Partec GmbH, Münster, Germany) according to Saito et al. (2003). Briefly, young leaf segments (ca. 5 mm × 5 mm) were harvested from potted plants or *in vitro*-grown plantlets and chopped with a razor blade in 0.2 mL of Solution A (Plant High Resolution DNA-staining Kit, Type P, Partec GmbH). Following filtration through a 30-μm nylon mesh, 1 mL of a staining solution, 50 mM sodium citrate, 2 mM $MgCl_2$, 1% (w/v) polyvinylpyrolidone K-30, 0.1% (v/v) Triton X-100, and 2 $mg \cdot L^{-1}$ 4,6-diamidino-2-phenylindole in 10 mM Tris-HCl, pH 7.5, was added to the nuclear suspensions and incubated for 5 min prior to FCM analysis. Parsley (*Petroselinum crispum*) was used as an internal standard for FCM analysis. Instrument gain was adjusted so that the RFI of nuclei isolated from parsley leaves was located at around channel 50. The ratio of RFI of nuclei from the Colchicaceous genotype and parsley (Colchicaceous genotype/parsley RFI ratio) was calculated for each genotype.

RAPD analysis

Total genomic DNA was isolated from leaves of potted plants or *in vitro*-growing plantlets and tubers of *in vitro*-growing plantlets according to Rogers and Bendich (1985) and Hasebe and Iwatsuki (1990), respectively. RAPD analysis was performed as previously described (Obata et al., 2000) with several modifications. Three sets of DNA Oligomer (12) Set, C-3, G-4, and G-5 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were tested. PCR amplification reactions contained 100 ng of template DNA, 0.5 μM of each primer, 200 μM of a dNTP mixture, 1 × *Taq* DNA polymerase reaction buffer, and 0.5 U of *Taq* DNA polymerase (Takara Bio, Inc., Shiga, Japan) in a final volume of 20 μL. DNA fragments were amplified

Table 1. Colchicaceous ornamentals used as the parents for intra- and intergeneric crosses.

Species and cultivar[z]	Abbreviation	Colchicaceous genotype/ parsley RFI ratio[y]	Chromosome number ($2n$)
Sandersonia aurantiaca Hook.	Sau	1.08±0.01 (group 1)	$2x=24$
S. aurantiaca Hook. 'Phoenix'	Sph	1.08±0.01 (group 1)	$2x=24$
Littonia modesta Hook.	Lit	1.54±0.01 (group 2)	$2x=22$
Gloriosa superba L. 'Lutea'	Gsu	1.69±0.01 (group 2)	$2x=22$
G. 'African Gold'	Gaf	1.68±0.01 (group 2)	$2x=22^x$
G. 'Marron Gold'	Gma	3.22±0.02 (group 3)	$4x=44^x$
G. rothschildiana O'Brien	Gro	5.36±0.02 (group 4)	$6x=66$
G. 'Rothschildiana Pink'	Grp	5.44±0.05 (group 4)	$6x=66^x$
G. 'Verschild'	Gve	5.95±0.05 (group 5)	$7x=77^x$

[z] Several recent studies have regarded *Gloriosa* as a monotypic genus with large morphological variation and several ploidy levels under the name *G. superba* L. (Vinnersten and Reeves, 2003).

[y] The ratio of RFI of nuclei from each Colchicaceous genotype and parsley (Colchicaceous genotype/parsley RFI ratio) was calculated. Values represent the mean±standard error of 5 replicates. RFI ratio values were classified into 5 groups.

[x] Ploidy level and chromosome number were estimated by FCM analysis.

using the GeneAmp PCR System 2400 (PerkinElmer, Inc., MA, USA) or DNA Engine OPTICON System (Bio-Rad Laboratories, Inc., CA, USA) by repeating 45 cycles of the following thermal treatments: 94°C for 1 min, 40°C for 1 min, and 72°C for 1 min. Amplification of each primer-DNA sample combination was replicated at least three times to confirm the stability of the RAPD profile. Amplified products were analyzed by electrophoresis in a 1.7% (w/v) agarose gel.

Results and Discussion

Nine Colchicaceous genotypes used as the parents for intra- and intergeneric crosses were initially subjected to FCM analysis. Histograms of all nine genotypes obtained from the young leaves of potted plants showed a single peak corresponding to nuclei in the G0/G1 phase of the cell cycle. Little background noise was detected. In our preliminary experiments, tubers, stems, and tepals of potted plants and leaves of *in vitro*-grown plantlets also showed a single peak of the same RFI as young leaves of potted plants for each genotype (data not shown). Neither cytochimeras (Väinölä, 2000) nor polysomaty (Mishiba and Mii, 2000) were found in Colchicaceous ornamentals. Thus, the position of the single peak obtained by FCM

Table 2. Number of independent plantlets identified as intra- or intergeneric hybrids by FCM and/or RAPD analyses in crosses using Colchicaceous ornamentals.

Cross combination and parents[z]		No. of plantlets examined	No. of plantlets identified as hybrids by FCM or RAPD analyses (%)	
Seed parent	Pollen parent		FCM	RAPD
Cross between the same RFI groups				
Sau	Sph	1	0 (0)	0 (0)
Lit	Gsu	3	0 (0)	3 (100)
Lit	Gaf	2	0 (0)	0 (0)
Gsu	Gaf	6	0 (0)	6 (100)
Gro	Grp	2	0 (0)	0 (0)
Total between the same RFI groups		14	0 (0)	9 (64.3)
Cross between different RFI groups				
Sau	Gsu	8	5 (62.5)	6 (75.0)
Sau	Gaf	3	3 (100)	3 (100)
Sau	Gma	13	13 (100)	13 (100)
Sau	Gro	3	1 (33.3)	1 (33.3)
Sau	Gve	4	2 (50.0)	2 (50.0)
Sph	Gma	1	1 (100)	1 (100)
Lit	Sau	3	3 (100)	3 (100)
Lit	Sph	5	4 (80.0)	4 (80.0)
Lit	Gma	5	2 (40.0)	2 (40.0)
Lit	Grp	1	1 (100)	1 (100)
Gsu	Sau	5	3 (60.0)	5 (100)
Gsu	Sph	4	3 (75.0)	3 (75.0)
Gsu	Gma	9	9 (100)	9 (100)
Gsu	Gro	2	2 (100)	2 (100)
Gsu	Grp	4	4 (100)	4 (100)
Gsu	Gve	1	1 (100)	1 (100)
Gaf	Gma	4	4 (100)	4 (100)
Gaf	Grp	1	1 (100)	1 (100)
Gma	Sau	2	0 (0)	2 (100)
Gma	Gsu	1	1 (100)	1 (100)
Gma	Gaf	11	11 (100)	11 (100)
Gma	Gro	4	4 (100)	4 (100)
Gma	Grp	2	2 (100)	2 (100)
Gro	Gma	7	7 (100)	7 (100)
Gro	Gve	2	2 (100)	2 (100)
Grp	Gaf	6	6 (100)	6 (100)
Gve	Sau	1	1 (100)	1 (100)
Total between different RFI groups		112	96 (85.7)	101 (90.2)

[z] Refer to Table 1 for abbreviations.

analysis may be usable as an index for hybrid identification in Colchicaceous ornamentals. The Colchicaceous genotype/parsley RFI ratio of each genotype is shown in Table 1. The values could be clearly classified into five groups: 1.08 (group 1) for *S. aurantiaca* Hook. (Sau) and *S. aurantiaca* Hook. 'Phoenix' (Sph); 1.54–1.69 (group 2) for *G. superba* L. 'Lutea' (Gsu), *G.* 'African Gold' (Gaf), and *L. modesta* Hook. (Lit); 3.22 (group 3) for *G.* 'Marron Gold' (Gma); 5.36–5.44 (group 4) for *G. rothschildiana* O'Brien (Gro) and *G.* 'Rothschildiana Pink' (Grp); and 5.95 (group 5) for *G.* 'Verschild' (Gve). Gaf, Gma, Grp, and Gve, for which the ploidy level and chromosome number has not yet been reported, were estimated by FCM analysis to be diploid ($2n=2x=22$), tetraploid ($2n=4x=44$), hexaploid ($2n=6x=66$), and heptaploid ($2n=7x=77$), respectively, as the basic chromosome number of 11 has been demonstrated for *Gloriosa* spp. and Gsu and Gro are known as diploid and hexaploid, respectively (Lemattre, 1980). The RFI peak of Colchicaceous genotypes belonging to different RFI ratio groups appeared at apparently different positions, whereas those of genotypes belonging to the same group appeared at almost the same positions.

Totally, 126 independent plantlets derived from intra- or intergeneric crosses (Table 2) were then subjected to FCM analysis. Out of 104 plantlets from crosses between genotypes belonging to 'different' RFI ratio groups except for crosses using Gve as one parent, the RFI peak of 90 plantlets appeared at an intermediate position between the corresponding parents, indicating that these plantlets were hybrids (Table 2 and Fig. 1a, b, c, e). For example, the positions of the RFI peak of Gsu and Gma were apparently different, and the peak of ten independent hybrid plantlets derived from reciprocal crosses between Gsu and Gma appeared at an intermediate position between them (Table 2 and Fig. 1a, b). On the other hand, when Gve was used as one parent, the RFI peak of six out of eight cross-derived plantlets appeared at near intermediate positions (Table 2 and Fig. 1h). Since Gve was estimated to be heptaploid, these six out of eight plantlets may be hybrids with 3–4 genomes of Gve. However, the RFI peak of 11 plantlets derived from crosses between genotypes belonging to different RFI ratio groups; i.e., two plantlets from Sau × Gsu, two from Sau × Gro, two from Sau × Gve, one from Lit × Sph, three from Lit × Gma, and one from Gsu × Sph, appeared at the same position as the seed parent. Thus, these 11 plantlets were not hybrids and may have been derived from self-pollination of the seed parent. When Sau was used as one parent, the RFI peak of some intergeneric cross-derived plantlets appeared at an unexpected position, and not an intermediate position between the parents (Table 2). The RFI of two independent plantlets derived from Gma × Sau were lower than an expected intermediate RFI between the parents (Fig.1d). In contrast, the RFI of one plantlet derived from Sau × Gsu and two plantlets from the reciprocal cross were higher than the expected RFI for each cross combination

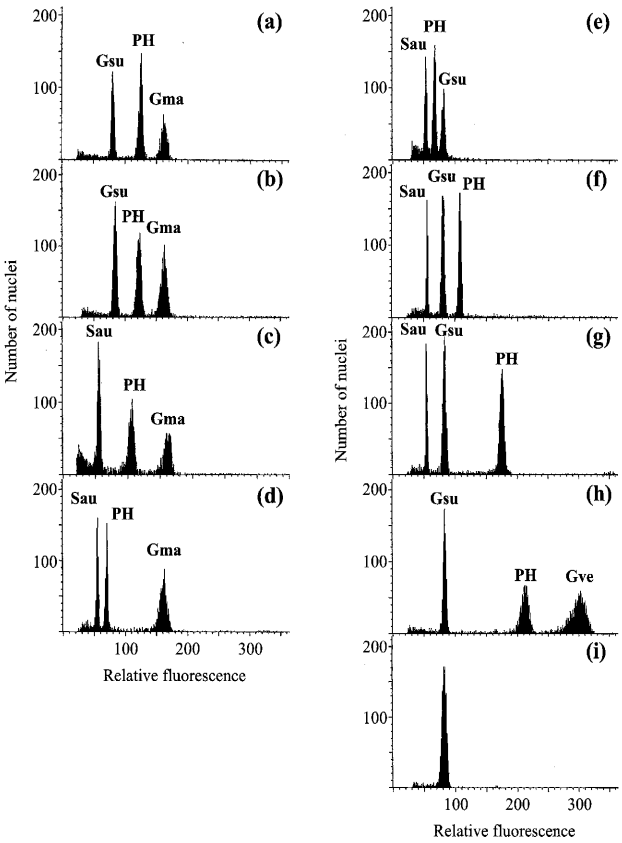

Fig. 1. Histograms from FCM analyses of the nuclear DNA content of the parents and their putative hybrid plantlets in Colchicaceous ornamentals. (a) *Gloriosa superba* L. 'Lutea' (Gsu), *G.* 'Marron Gold' (Gma), and a putative hybrid from Gsu × Gma (PH). (b) Gsu, Gma, and a putative hybrid from Gma × Gsu (PH). (c) *Sandersonia aurantiaca* Hook. (Sau), Gma, and a putative hybrid from Sau × Gma (PH). (d) Sau, Gma, and a putative hybrid from Gma × Sau (PH). (e) Sau, Gsu, and a putative hybrid from Sau × Gsu (PH). (f) Sau, Gsu, and a putative hybrid from Sau × Gsu (PH). (g) Sau, Gsu, and a putative hybrid from Gsu × Sau (PH). (h) Gsu, *G.* 'Verschild' (Gve), and a putative hybrid from Gsu × Gve (PH). (i) Cross between the same RFI group: *Littonia modesta* Hook. (Lit), Gsu, and a putative hybrid from Lit × Gsu.

(Fig. 1f, g). Thus, the hybridity of these five plantlets could not be verified by FCM analysis. For crosses between Colchicaceous genotypes belonging to the 'same' RFI ratio group, such as Sau × Sph, Lit × Gsu, Lit × Gaf, Gsu × Gaf, and Gro × Grp, the positions of the RFI peak of cross-derived plantlets and both parents were nearly the same (Fig. 1i). Thus, the hybridity of 14 independent plantlets derived from such crosses could not be verified by FCM analysis (Table 2). In consequence, a total of 96 independent hybrid plantlets (54 intrageneric and 42 intergeneric hybrids) were identified only by FCM analysis (Table 2).

For verifying the hybridity of cross-derived plantlets, which could not be identified as hybrids by FCM analysis due to the similarity in RFI of the parents or appearance of the RFI peak in an unexpected position, as well as for confirming the results of FCM analysis, RAPD analysis was carried out. Nine Colchicaceous genotypes used as parents for intra- and intergeneric crosses were initially

subjected to RAPD analysis using various primers in order to obtain stable and genotype-specific profiles. Polymorphisms in the RAPD profile between *Gloriosa* spp. and Lit, between *Gloriosa* spp. and two *S. aurantiaca* Hook. genotypes (Sau and Sph), between Lit and two *S. aurantiaca* Hook. genotypes, and among *Gloriosa* spp., were obtained using the primers C49, G67, and G87, the primers C49, C51, G67, and G86, the primers C49, G67, and G87, and the primers C45, C49, G63, G67, and G87, respectively. Unfortunately, no differences in the RAPD profiles between two *S. aurantiaca* Hook. genotypes could be detected using any primers, and therefore, the hybridity of one plantlet derived from Sau × Sph could also not be verified by RAPD analysis. Further experiments are necessary for detecting DNA polymorphisms between Sau and Sph.

In the present study, cross-derived plantlets with RAPD profiles containing only pollen parent-specific amplified fragment(s) as well as those containing both seed parent-specific and pollen parent-specific amplified fragments were considered to be hybrids. The hybridity of all the 96 independent plantlets, which were identified as hybrids by FCM analysis, was also confirmed by RAPD analysis (Table 2, Fig. 2a lanes 2–7, Fig. 2b lane 2, Fig. 2c lanes 2–11, Fig. 2d lanes 2 and 3, Fig. 2e lane 2, Fig. 2f lane 3, and Fig. 2g lane 2). In addition, RAPD analysis facilitated verification of the hybridity of 14 independent plantlets, which could not be identified as hybrids by FCM analysis (Table 2, Fig. 2c lanes 12 and 13, Fig. 2e lane 3, Fig. 2f lanes 2 and 4, and Fig. 2h lanes 2 and 3). No pollen parent-specific amplified fragments were detected in RAPD profiles of two plantlets derived from Gro × Grp and two plantlets derived from Lit × Gaf (Fig. 2i lanes 2 and 3). These four plantlets were not hybrids and may have resulted from self-pollination of the seed parent. Totally, 110 independent hybrid plantlets (60 intrageneric and 50 intergeneric hybrids) have so far been identified by FCM and/or RAPD analyses (Table 2).

In the present study, we succeeded in the early confirmation of the hybridity of a large number of plantlets derived from various crosses among Colchicaceous ornamentals by FCM in combination with RAPD analyses. The effectiveness of these analyses for the simple and rapid detection of hybrids has also been reported for some ornamentals such as *Primula* spp. (Kato and Mii, 2000; Kato et al., 2001) and *Dianthus* spp. (Nimura et al., 2003). In the present study, the RFI peak of some intergeneric hybrid plantlets derived from crosses using *S. aurantiaca* Hook. as a seed or pollen parent appeared at lower or higher positions than an intermediate

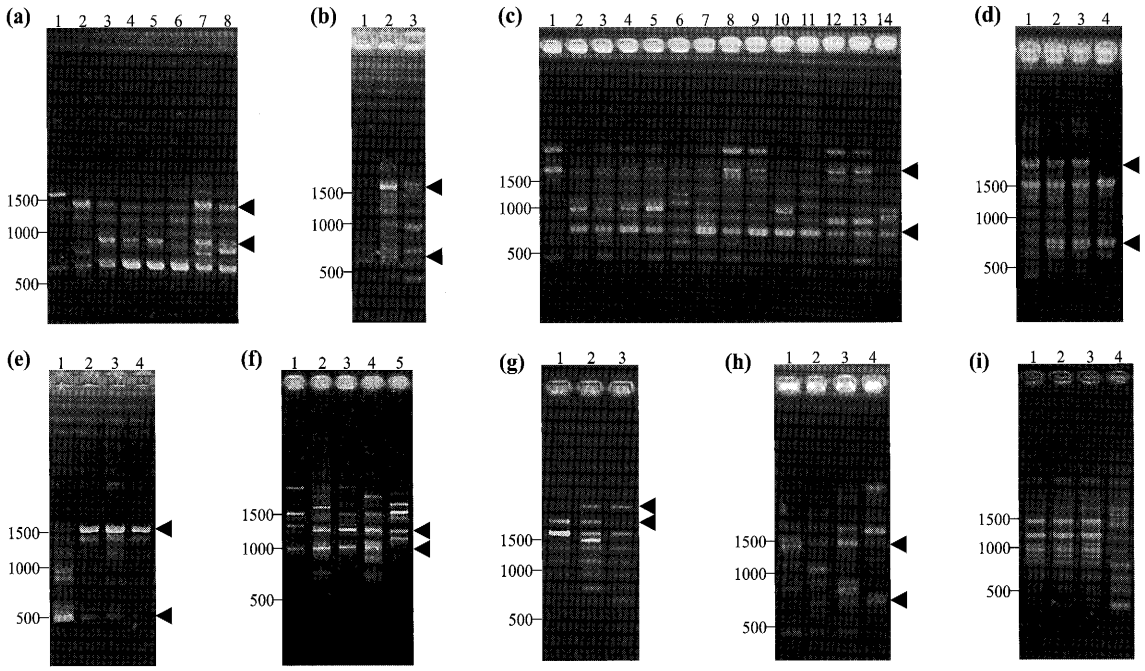

Fig. 2. RAPD profiles of the parents and their putative hybrid plantlets in Colchicaceous ornamentals. (a) *Gloriosa superba* L. 'Lutea' (Gsu) × *G.* 'Marron Gold' (Gma). Primer C49 was used. Lane 1, Gsu; lanes 2–7, independent putative hybrid plantlets from Gsu × Gma; lane 8, Gma. (b) Gma × Gsu. Primer G87 was used. Lane 1, Gma; lane 2, putative hybrid plantlet from Gma × Gsu; lane 3, Gsu. (c) Reciprocal crosses between *Sandersonia aurantiaca* Hook. (Sau) and Gma. Primer G86 was used. Lane 1, Sau; lanes 2–11, independent putative hybrid plantlets from Sau × Gma; lanes 12 and 13, independent putative hybrid plantlets from Gma × Sau; lane 14, Gma. (d) Sau × Gsu. Primer C49 was used. Lane 1, Sau; lanes 2 and 3, independent putative hybrid plantlets from Sau × Gsu; lane 4, Gsu. (e) Sau × Gsu. Primer G67 was used. Lane 1, Sau; lanes 2 and 3, independent putative hybrid plantlets from Sau × Gsu; lane 4, Gsu. (f) Gsu × Sau. Primer C51 was used. Lane 1, Gsu; lanes 2–4, independent putative hybrid plantlets from Gsu × Sau; lane 5, Sau. (g) Gsu × *G.* 'Verschild' (Gve). Primer G63 was used. Lane 1, Gsu; lane 2, putative hybrid plantlet from Gsu × Gve; lane 3, Gve. (h) *Littonia modesta* Hook. (Lit) × Gsu. Primer C49 was used. Lane 1, Lit; lanes 2 and 3, independent putative hybrid plantlets from Lit × Gsu; lane 4, Gsu. (i) Lit × *G.* 'African Gold' (Gaf). Primer G87 was used. Lane 1, Lit; lanes 2 and 3, independent plantlets derived from Lit × Gaf; lane 4, Gaf. Numbers on the left indicate bp. Arrowheads indicate bands specific to the pollen or seed parent.

position between the parents. Nakamura et al. (2005) also reported that the RFI peak position of one out of two intergeneric hybrid plants from *S. aurantiaca* Hook. × *G. rothschildiana* O'Brien was lower than an intermediate position between the parents, and they concluded through chromosome observation that this hybrid was aneuploid and some chromosomes may be lost during ovule culture. On the other hand, hybrids with a DNA content higher than an intermediate value of the parents were frequently obtained from interspecific crosses in *Primula*, probably due to unreduced gamete formation in a seed or pollen parent (Kato and Mii, 2000; Kato et al., 2001). Therefore, the five intergeneric hybrid plantlets with unexpected RFI values obtained in the present study might have resulted from unreduced gamete formation in the parent(s), chromosome elimination, and/or chromosome doubling during ovule culture. Further investigations such as genomic in situ hybridization (GISH) and observation of meiotic chromosome behavior are necessary to determine the genomic constitution of these hybrids. The horticultural characterization of all hybrids obtained in the present study is also necessary for the development of novel cultivars.

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