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Original Paper

Production and characterization of intersectional hybrids between *Tricyrtis* sect. *Brachycyrtis* and sect. *Hirtae* via ovule culture

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Abstract The liliaceous perennial plants, *Tricyrtis* spp., have recently become popular as ornamental plants for pot and garden uses. In order to broaden the variability in plant form, flower form and flower color of *Tricyrtis* spp., intersectional hybridization was examined between four *T. formosana* cultivars or *T. hirta* var. *albescens* (sect. *Hirtae*) and *T. macranthopsis* (sect. *Brachycyrtis*). After cross-pollination, ovary enlargement was observed only when *T. macranthopsis* was used as a pollen parent. Ovules with placental tissues were excised from enlarged ovaries and cultured on half-strength MS medium without plant growth regulators. From five cross-combinations, 31 ovule culture-derived plantlets were obtained and 20 of them were confirmed to be intersectional hybrids by flow cytometry and inter-simple sequence repeat analyses. Almost all hybrids grew well and produced flowers 1–2 years after transplantation to the greenhouse. Hybrids had semi-cascade-type shoots, which was intermediate between *T. formosana* cultivars and *T. hirta* var. *albescens* (erect-type shoots) and *T. macranthopsis* (cascade-type shoots). They produced flowers with novel forms and colors compared with the corresponding parents, and some were horticulturally attractive. The results obtained in the present study indicate the validity of intersectional hybridization via ovule culture for breeding of *Tricyrtis* spp.

Key words: embryo rescue, FCM analysis, ISSR analysis, liliaceous ornamental plants, wide hybridization.

Introduction

Wide hybridization is one of the most effective approaches for broadening the variability in horticultural traits of ornamental plants (Küligowska et al. 2016b). Although production of wide hybrids is often hindered by two types of hybridization barriers, i.e., pre- and post-fertilization barriers, the latter can partly be overcome by embryo rescue such as embryo or ovule culture. Interspecific and intergeneric hybrids with novel and attractive traits have already been produced via embryo rescue in various ornamental plants such as *Primula* spp. (Amano et al. 2006; Hayashi et al. 2007), colchicaceous plants including *Gloriosa* spp. and *Sandersonia aurantiaca* (Amano et al. 2009), *Hydrangea* spp. (Kudo et al. 2008), *Kalanchoe* spp. (Izumikawa et al. 2008), *Lychnis* spp. (Godo et al. 2009; Nakano et al. 2013), *Rhododendron* spp. (Okamoto and Ureshino 2015), *Hibiscus* spp. (Küligowska et al. 2016a), and *Cyclamen* spp. (Ishizaka 2018).

The genus *Tricyrtis*, a member of the family Liliaceae, consists of over 20 species, which are distributed in East Asia (Kono et al. 2015). Some *Tricyrtis* spp. are cultivated as ornamental plants for pot and garden uses because of their beautiful foliage, attractive flowers, and ability to grow in the shade (Nakano et al. 2006). The most popular species as ornamental plants is *T. formosana*, which belongs to the sect. *Hirtae*, and a number of *T. formosana* cultivars have so far been produced. *T. formosana* cultivars have erect-type shoots and cup-shaped, upward-facing flowers, reddish-purple, purple, pink, pale blue or white in color. We previously produced intersectional hybrids via ovule culture between *T. formosana* cultivars and *T. flava*, which belongs to the sect. *Flavae*, for increasing the variability in flower color (Tasaki et al. 2014). Some hybrids showed novel and attractive flower colors, indicating the validity of intersectional hybridization for broadening the variability in horticultural traits of *Tricyrtis* spp.

T. macranthopsis, which belongs to the sect. *Brachycyrtis*, shows markedly different characteristics from *T. formosana*, such as cascade-type shoots and

Abbreviations: FCM, Flow cytometry; MS, Murashige and Skoog (1962); ISSR, Inter-simple sequence repeat.
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bell-shaped, pendulous flowers, yellow in color. Thus, this species is promising as a novel parent for wide hybridization. In the present study, we examined intersectional hybridization between *T. macranthopsis* and two species in the sect. *Hirtae*, *T. formosana* and *T. hirta* var. *albescens*, via ovule culture.

Materials and methods

Plant materials

Four cultivars of *T. formosana*, 'Fujimusume' (TfFu), 'Seiryu' (TfSei), 'Soten' (TfSo) and 'Tosui' (TfTo), *T. hirta* var. *albescens* (Tha), and *T. macranthopsis* (Tm) were used in the present study. All species and cultivars are diploid with $2n=26$ chromosomes. Potted plants were cultivated in the greenhouse without heating according to Tasaki et al. (2014).

Pollination and ovule culture

Cross-pollination and ovule culture were carried out according to Tasaki et al. (2014). Briefly, flowers of the seed parent were emasculated 2 days before anthesis, and pollination was made at anthesis using fresh pollen. Enlarged ovaries were collected 5–14 days after pollination. Ovules with placental tissues were isolated from ovaries and cultured on half-strength MS medium without plant growth regulators. Ovule culture-derived plantlets were acclimatized, transplanted to pots and cultivated as the parental plants.

FCM and ISSR analyses

Relative DNA content of nuclei isolated from leaf tissues was measured using a flow cytometer PA (Partec GmbH, Münster, Germany) as previously described (Amano et al. 2007; Saito et al. 2003). Leaf tissues of *Petroselinum crispum* were used as an internal standard. ISSR analysis using the primer ISSR-15 (5′-(AC)8GA-3) was performed according to Farsani et al. (2012).

Morphological characterization

Three years after cultivation of hybrid plants, morphological characterization was performed according to Nakano et al. (2006). For flower color, the center of the adaxial side of outer tepals was investigated visually with an aid of the JHS Color Chart (Japan Horticultural Plant Standard Color Chart 1984). Flower color was expressed using Inter-Society Color Council, National Bureau of Standard (ISCC-NBS) color name as well as JHS Color Chart number according to Kuwayama et al. (2005).

Results

Production of intersectional hybrid plants via ovule culture

Results of intersectional hybridization are summarized in Table 1. Enlarged ovaries could be obtained from all cross-combinations using Tm as a pollen parent, whereas no ovary enlargement was observed when Tm was used as a seed parent. For each of five cross-combinations using Tm as a pollen parent, 2–10 independent plantlets were obtained six months after the initiation of ovule culture. Some ovules produced calli and subsequently died without plantlet formation. All of the ovule culture-derived plantlets were successfully acclimatized and transplanted to pots.

In order to verify the hybridity of ovule culture-derived plants, FCM and ISSR analyses were carried out. Figure 1 shows typical FCM histograms of ovule culture-derived plants and corresponding parents. Histograms of all analyzed plants showed a single peak corresponding to nuclei in the G0/G1 phase of the cell cycle. Positions of the G0/G1 peak of *T. formosana* cultivars and Tha were apparently different from that of Tm. Therefore, the position of the G0/G1 peak was used as an index for identifying intersectional hybrids in the present study. For TfFu×Tm, TfTo×Tm and Tha×Tm, the G0/G1 peak of all ovule culture-derived plants appeared at an intermediate position between the corresponding parents, indicating that they are diploid intersectional hybrids. For TfSei×Tm, five out of nine ovule culture-derived plants were also identified as diploid

Table 1. Results of intersectional cross-pollination and ovule culture in *Tricyrtis*.

Seed parent[1]	Pollen parent[1]	No. of flowers pollinated	No. of enlarged ovaries[2]	No. of cultured ovules[2]	No. of germinated ovules[3]	No. of ovule culture-derived plants[4]	No. of hybrid plants[5]
TfFu	Tm	3	1	98	6	3	3
TfSei	Tm	79	27	2895	19	9	5
TfSo	Tm	49	30	3053	15	10	3
TfTo	Tm	17	3	400	3	2	2
Tha	Tm	3	2	226	7	7	7
Tm	TfSei	10	0	0	0	0	0
Tm	TfSo	8	0	0	0	0	0
Tm	TfTo	8	0	0	0	0	0
Tm	Tha	4	0	0	0	0	0

[1]TfFu, *T. formosana* 'Fujimusume'; TfSei, *T. formosana* 'Seiryu'; TfSo, *T. formosana* 'Soten'; TfTo, *T. formosana* 'Tosui'; Tha, *T. hirta* var. *albescens*; Tm, *T. macranthopsis*. [2]Data were recorded 5–14 days after cross pollination. [3]Data were recorded five months after the initiation of ovules culture. [4]Data were recorded six months after the initiation of ovules culture. [5]The hybridity was confirmed by FCM and ISSR analyses.

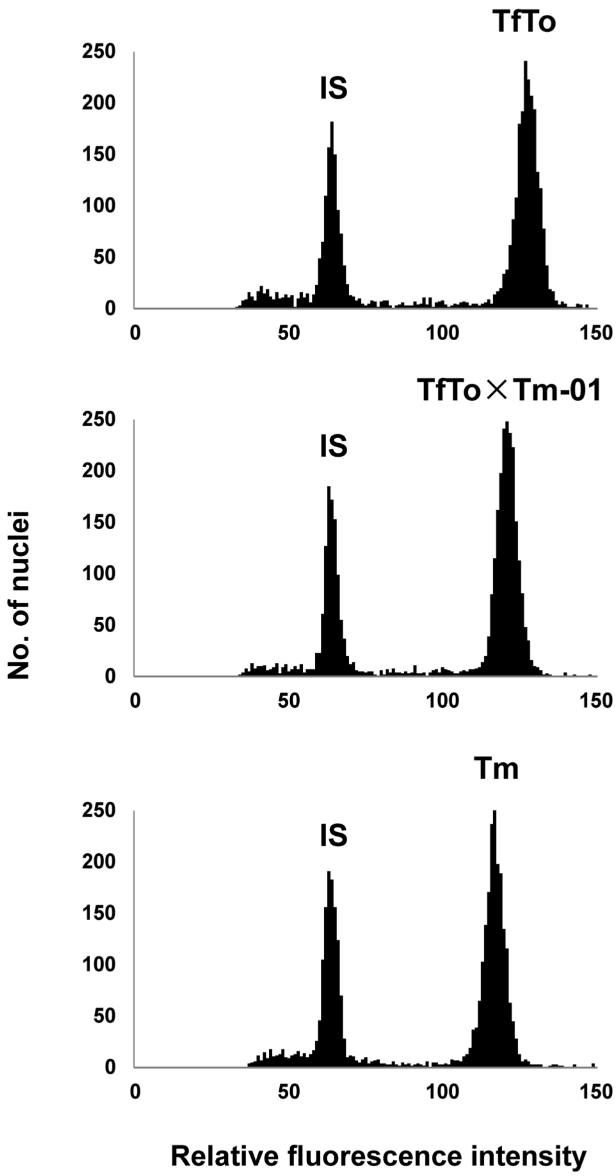

Figure 1. Histograms from FCM analysis of nuclear DNA content of *Tricyrtis formosana* 'Tosui' (TfTo), putative hybrid of *T. formosana* 'Tosui' × *T. macranthopsis* (TfTo×Tm-01), and *T. macranthopsis* (Tm). Leaf tissues of *Petroselinum crispum* were used as an internal standard (IS).

intersectional hybrids by FCM analysis. However, the G0/G1 peak of the other four plants appeared at almost the same position as the seed parent TfSei, indicating that they may be derived from self-pollination or apomixis of TfSei. For TfSo×Tm, two out of ten ovule culture-derived plants were identified as diploid intersectional hybrids and seven plants may be derived from self-pollination or apomixis of TfSo. The G0/G1 peak of the other one plant (TfSo×Tm-3) appeared at a position of nearly two-times higher than the intermediate position between the parents, indicating that this plant is a tetraploid intersectional hybrid.

The results of FCM analysis were confirmed by ISSR analysis. Figure 2 shows a typical electropherogram

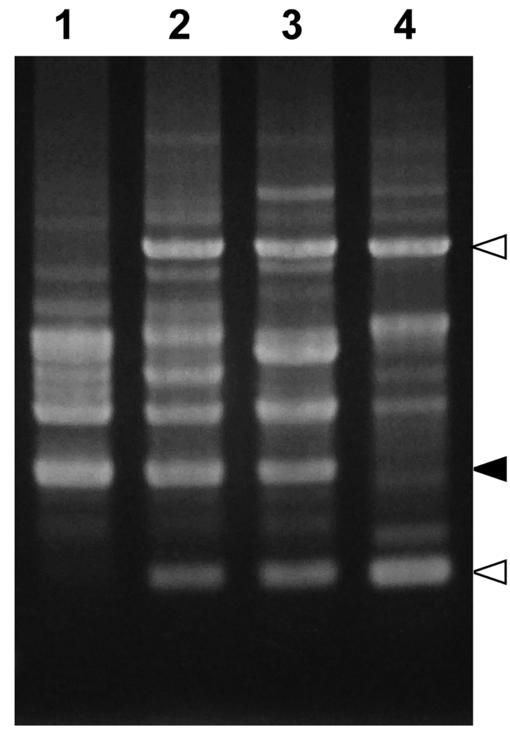

Figure 2. ISSR profiles of *Tricyrtis formosana* 'Tosui' (lane 1), independent putative hybrids of *Tricyrtis formosana* 'Tosui' × *T. macranthopsis* (lanes 2–3), and *T. macranthopsis* (lane 4). Black and white arrowheads indicate bands specific to the seed parent (*Tricyrtis formosana* 'Tosui') and the pollen parent (*T. macranthopsis*), respectively.

of ISSR analysis. All plants identified as intersectional hybrids by FCM analysis including TfSo×Tm-3 contained both seed and pollen parent-specific amplified fragments. On the other hand, plants considered to be derived from self-pollination or apomixis of the seed parent *T. formosana* cultivars showed nearly the same electropherogram as *T. formosana* cultivars.

Morphological characterization of intersectional hybrid plants

All intersectional hybrids except for TfSo×Tm-3 grew well, and 16 of them produced flowers 1–2 years after cultivation in the greenhouse. Morphological characteristics of hybrid and parental plants investigated at the flowering season are summarized in Table 2. TfSo×Tm-3 was not investigated since plants showed only poor growth even after three years of cultivation in the greenhouse.

All of the investigated hybrids had semi-cascade-type shoots, which were intermediate between *T. formosana* cultivars or *T. hirta* var. *albescens* (erect-type shoots) and *T. macranthopsis* (cascade-type shoots) (Table 2; Figure 3). Flowers of 16 hybrids were upward-facing as the seed parent *T. formosana* cultivars or Tha. Flower form and color were apparently distinguishable from the corresponding parents (Table 2; Figure 4). For

Table 2. Morphological characterization of *Tricyrtis formosana* cultivars, *T. hirta* var. *albescens*, *T. macranthopsis*, and intersectional hybrids at the flowering stage.[1]

Species, cultivars and hybrids[2]	Ploidy level	Shoot type[3]	No. of shoots per plant	Shoot length (cm)[4]	Stem diameter (mm)[4]	Leaf length (cm)[5]	Leaf width (cm)[5]	No. of flowers per shoot[4]	Flower length (cm)[6]	Flower diameter (cm)[6]	Flower color[7]
TfFu	2*x*	E	11.0±0	8.8±1.9	3.0±0	7.4±0.6	2.2±0.2	1.0±0	2.2±0.3	3.6±0	9201 (pinkish white)
TfSei	2*x*	E	12.3±0.9	40.6±2.4	2.9±0	8.3±0.6	2.6±0	8.0±1.2	2.3±0.1	3.0±0	8602 (pale purple)
TfSo	2*x*	E	26.0±1.0	26.8±1.3	2.0±0	6.9±0.4	2.6±0	3.3±0.3	2.2±0	3.2±0.1	8601 (purplish white)
TfTo	2*x*	E	22.0±2.1	21.4±0.7	2.4±0	7.2±0.2	2.3±0	2.3±0.3	2.1±0.1	3.3±0.1	2901 (yellowish white)
Tha	2*x*	E	11.0±4.5	24.0±2.1	2.4±0.3	10.7±0.5	2.8±0.2	8.0±0.6	2.1±0.2	2.3±0.2	2903 (pale greenish yellow)
Tm	2*x*	C	4.3±0.9	32.1±6.6	1.7±0.3	9.3±0.3	2.6±0.4	3.7±0.9	4.5±0.1	2.6±0.2	2506 (bright yellow)
TfFu×Tm-1	2*x*	S	17.7±4.7	16.5±6.8	1.0±0	7.8±1.9	2.7±0.6	1.0±0	3.0±0.2	3.7±0.2	8910 (soft reddish purple)
TfFu×Tm-2	2*x*	S	18.3±4.3	23.5±2.1	2.0±0	9.1±0.5	2.9±0.2	1.0±0	2.3±0	3.5±0.1	8302 (pale purple)
TfFu×Tm-3	2*x*	S	16.0±6.4	40.4±3.9	2.0±0	9.1±1.4	2.3±0.1	3.0±0.6	3.2±0.1	3.7±0	8910 (soft reddish purple)
TfSei×Tm-3	2*x*	S	20.7±2.3	28.3±0.9	2.1±0.1	10.3±0.6	2.4±0	2.0±0.6	3.0±0.1	2.6±0.2	2203 (lt. reddish yellow)
TfSei×Tm-4	2*x*	S	12.0±3.5	23.6±2.6	1.9±0.1	16.2±9.0	2.2±0.1	1.3±0.3	2.6±0.2	2.6±0.2	2205 (bright yellow)
TfSei×Tm-5	2*x*	S	17.0±5.6	26.6±3.3	2.2±0	7.5±0.7	2.2±0.1	2.3±0.9	2.9±0.1	2.3±0.2	2204 (bright reddish yellow)
TfSei×Tm-6	2*x*	S	7.5±0.3	29.1±2.3	2.2±0	9.3±0.4	2.2±0	6.3±2.8	2.8±0.1	2.8±0.1	2204 (bright reddish yellow)
TfSei×Tm-8	2*x*	S	9.7±1.3	30.8±17.4	2.5±0.3	9.4±1.9	3.1±1.3	4.0±1.0	2.8±0.1	2.5±0.1	1905 (bright yellowish orange)
TfSo×Tm-3	4*x*	—[8]	—[8]	—[8]	—[8]	—[8]	—[8]	—[8]	—[8]	—[8]	—[8]
TfSo×Tm-6	2*x*	S	8.7±3.7	7.8±0.4	1.7±0.2	5.1±0.1	1.6±0	0.3±0.3	2.3[9]	2.6[9]	2703 (lt. greenish yellow)
TfSo×Tm-7	2*x*	S	5.0±1.5	16.9±6.8	2.1±1.1	7.5±1.5	2.2±0.3	1.3±1.3	1.7[10]	3.0[10]	2703 (lt. greenish yellow)
TfTo×Tm-1	2*x*	S	9.0±3.2	39.8±7.9	2.4±0.2	9.0±1.2	2.7±0	3.3±0.9	3.2±0.1	3.1±0.1	2205 (bright yellow)
TfTo×Tm-2	2*x*	S	6.3±0.9	20.0±5.0	2.2±0.3	6.0±0.6	2.0±0.2	2.7±2.7	—[11]	—[11]	—[11]
Tha×Tm-1	2*x*	S	8.0±1.2	68.7±6.2	3.0±0	9.4±3.3	6.2±3.3	3.7±0.3	2.9±0.2	3.5±0.1	2701 (yellowish white)
Tha×Tm-2	2*x*	S	7.3±0.3	67.7±2.4	4.0±0	7.9±2.7	5.3±2.4	3.0±0.6	2.9±0	4.0±0.2	2702 (pale greenish yellow)
Tha×Tm-3	2*x*	S	6.7±0.3	54.7±8.2	4.0±0	11.0±0.1	2.8±0.2	4.3±1.9	3.4±0.1	3.8±0.2	2502 (yellowish white)
Tha×Tm-4	2*x*	S	12.0±1.0	53.8±1.9	3.0±0	10.4±0.3	2.6±0.1	4.3±0.3	3.2±0.2	3.8±0.1	2701 (yellowish white)
Tha×Tm-5	2*x*	S	8.0±0.6	67.3±5.9	3.0±0	9.9±0.6	3.2±0.7	4.7±2.0	2.5±0.3	3.3±0.1	2701 (yellowish white)
Tha×Tm-6	2*x*	S	12.7±2.2	33.7±0.9	3.0±0	9.8±0.2	2.1±0.1	2.0±0.6	2.7±0.1	3.7±0.2	2701 (yellowish white)
Tha×Tm-7	2*x*	S	11.7±1.5	46.1±0.8	2.8±0.1	9.0±0.9	2.5±0.1	7.3±0.3	2.8±0	4.5±0.1	2701 (yellowish white)

[1]Values represent the mean±standard error of three plants for each genotype. [2]TfFu, *T. formosana* 'Fujimusume'; TfSei, *T. formosana* 'Seiryu'; TfSo, *T. formosana* 'Soten'; TfTo, *T. formosana* 'Tosui'; Tha, *T. hirta* var. *albescens*; Tm, *T. macranthopsis*. [3]C, cascade; E, erect; S, semi-cascade. [4]The longest shoot was investigated for each plant. [5]Randomly selected three leaves were investigated for each plant. [6]Randomly selected three flowers were investigated for each plant. [7]Color of the center of the adaxial side of outer tepals was investigated visually with an aid of the JHS Color Chart (Japan Color Research Institute 1984). ISCC-NBS color names are shown in parentheses. [8]TfSo×Tm-3 was not investigated since plants showed only poor growth. [9]Two out of three plants produced one flower each. [10]All three plants produced flowers, but only two flowers opened. [11]All flowers were blasted before anthesis.

TfFu×Tm-2, TfSei×Tm-6 and TfTo×Tm-1, flower length and flower diameter were intermediate between the corresponding parents. Shoot length and flower diameter of most hybrids of Tha×Tm were increased compared with both parents. For all hybrids producing flowers, no anther dehiscence was observed and pollen fertility was below 1% as assessed with acetocarmine staining (data not shown).

For plants considered to be derived from self-pollination or apomixis of the seed parent by FCM and ISSR analyses showed almost the same morphology as the seed parent (data not shown).

Discussion

In the present study, intersectional hybridization between *Tricyrtis* sect. *Hirtae* (*T. formosana* cultivars or Tha) and sect. *Brachycyrtis* (Tm) was achieved by using Tm as a pollen parent. However, no enlarged ovaries were obtained when Tm was used as a seed parent, although *T. formosana* cultivars and Tha showed over 80% of pollen fertility as assessed with acetocarmine staining (data not shown). Thus, Tm plants used in the present study may possibly be female-sterile. It is also possible that interspecific unilateral incompatibility might occur between sect. *Hirtae* and sect. *Brachycyrtis*. Interspecific unilateral incompatibility has so far been reported for various genera such as *Erythronium* (Harder et al. 1993), *Nicotiana* (Murfett et al. 1996), *Dianthus* (Nimura et al. 2003) and *Capsicum* (Onus and Pickersgill 2004). In order to clarify the cause of non-enlargement of Tm ovaries after intersectional cross-pollination, it is necessary to examine self-pollination and intraspecific cross-pollination of Tm. Pollen germination and pollen tube growth in the pistil also should be observed in the reciprocal cross between sect. *Hirtae* and sect. *Brachycyrtis*.

For cross-combinations using Tm as a pollen parent, hybrid plants were successfully obtained by a simple ovule culture technique, which has initially been developed for intersectional hybridization between *T. formosana* cultivars and *T. flava* (sect. *Flavae*) (Tasaki et al. 2014). Recently, we also produced wide hybrid plants in *Tricyrtis* using transgenic plants carrying the gibberellin 2-oxidase gene from *Torenia fournieri* by the same ovule culture technique (Otani et al. 2019). Therefore, this technique may be universally usable for producing interspecific hybrids in the genus *Tricyrtis*.

Figure 3. Flowering plants of *Tricyrtis formosana* 'Seiryu' (TfSei), hybrid of *T. formosana* 'Seiryu' × *T. macranthopsis* (TfSei×Tm-8), and *T. macranthopsis* (Tm). Bar=10 cm.

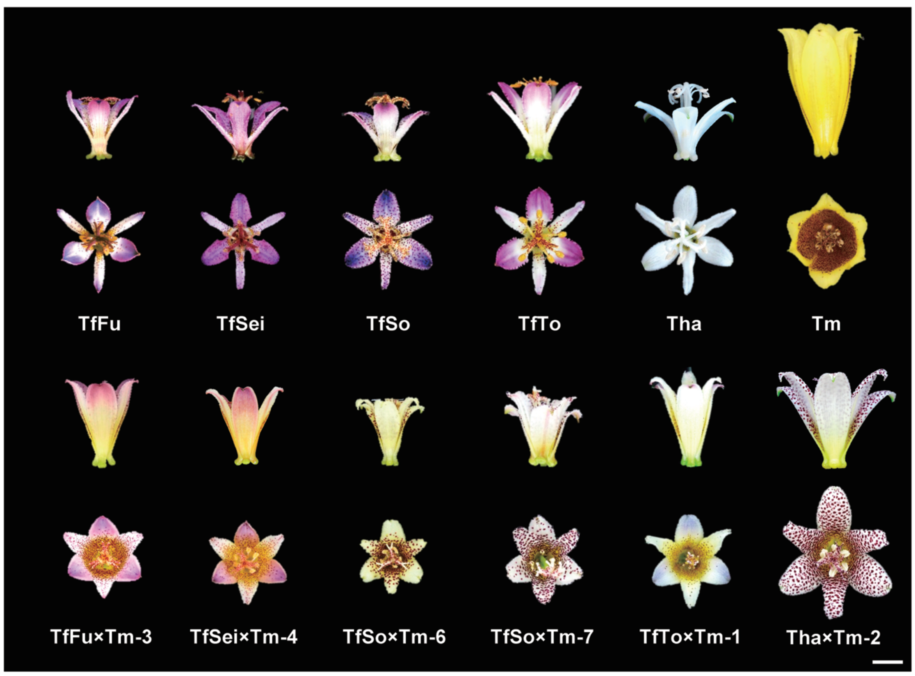

Figure 4. Flowers of *Tricyrtis formosana*, 'Fujimusume' (TfFu), 'Seiryu' (TfSei), 'Soten' (TfSo) and 'Tosui' (TfTo), *T. hirta* var. *albescens* (Tha), *T. macranthopsis* (Tm), and their hybrids. Bar=1 cm.

For TfSo×Tm, one tetraploid hybrid (TfSo×Tm-3) was obtained. TfSo×Tm-3 may be derived from fertilization of an unreduced diploid female and male gametes or chromosome doubling of a hybrid embryo (Amano et al. 2006; Izumikawa et al. 2008; Nakano et al. 2006). Although TfSo×Tm-3 shows only poor growth at present, this tetraploid hybrid may possibly have resorted pollen fertility and be usable as a further cross-breeding material.

In *Tricyrtis*, only sect. *Brachycyrtis* spp. including Tm have cascade-type shoots, and the other spp. including *T. formosana* and *T. hirta* var. *albescens* have erect-type shoots. Since all of the intersectional hybrids investigated in the present study had semi-cascade-type shoots, the shoot pattern in *Tricyrtis* may be a semi-dominant trait. In *Prunus spachiana* (Nakamura et al. 1994) and *Salix matsudana* (Liu et al. 2017), weeping shoot traits are regulated by the endogenous gibberellin level. It is necessary to analyze the endogenous gibberellin level in *Tricyrtis* plants with erect-, semi-cascade- and cascade-type shoots. Effect of exogenous gibberellin treatments on shoot type in *Tricyrtis* plants should also be examined.

In the present study, intersectional hybrid plants were successfully produced between *Tricyrtis* sect. *Hirtae* and sect. *Brachycyrtis* via ovule culture. Hybrids produced flowers with novel forms and colors, some of which were horticulturally attractive. These results together with those obtained from our previous study (Tasaki et al. 2014) strongly indicate the validity of intersectional hybridization for breeding of *Tricyrtis* spp. We are now examining propagation and cultivation characteristics of the hybrids obtained in the present study for their commercialization.

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