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New tri-parental interspecific *Torenia* hybrids via ovule culture

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Abstract

Tri-parental cross is an effective way to increase variations in flower color and morphological characteristics. Recombination of three genomes would produce new phenotypes having high ornamental value. The fertile pollen of（*T. bicolor* × *T. baillonii*）was mounted on the stigma of four species, *T. concolor, T. fournieri, T. hirsutissima* and *T. siamensis*. Only the cross combination of *T. fournieri* ×（*T. bicolor* × *T. baillonii*）produced five progenies. Then three hybrids（*T. fournieri* × *T. baillonii*），（*T. fournieri* × *T. bicolor*）and（*T. bicolor* × *T. baillonii*）were used as female parents then cross with other *Torenia* species. Three progenies were obtained from（*T. fournieri* × *T. baillonii*）× *T. siamensis* and two progenies were obtained from（*T. fournieri* × *T. baillonii*）× *T. concolor*. The hybridity of progenies was confirmed by PCR-RFLP, and all tested plants were true hybrids. Tri-parental hybrids showed intermediate chromosome number between parents and all tri-parental hybrids were male sterile. Petal color of the hybrids was mostly intermediate of parents with narrow variation.

Keywords：chromosome, PCR-RFLP

Introduction

Interspecific hybridization is a powerful tool to produce novel characteristic of ornamental plants. Breeders have been attempting to combine useful traits though interspecific hybridization for many years, and have acquired great success in many ornamental plants including lily [(1)], *Dianthus* [(2)], *Cameilia* [(3)] and *Globba* [(4)] etc.Currently, *Torenia* become a popular summer bedding plant. Most of vegetative propagated commercial *Torenia* cultivars are derived from interspecific hybridization [(5)]. However, only a few of *Torenia* species, *T. fournieri*, *T. concolor* and *T. baillonii,* have been used in breeding programs of *Torenia*. Successful interspecific hybridization by use of ovule culture has been achieved in *Torenia* [(6)]. Expansion of the genetic background is essential for expanding the range of variations in hybrids. Tri-parental crossing can expect to increase the variation in interspecific hybrids. Recombination of three different genomes would produce further variations in flower color, plant form, leaf size, etc.

In this study, six *Torenia* species including newly introduced three species, *T. hirsutissima*, *T. siamensis* and *T. bicolor*, were used to produce new hybrids by tri-parental interspecific hybridization though ovule culture.

Materials and methods

Plant materials and growth conditions

T. fournieri 'Crown Violet', *T. baillonii*, *T. concolor*, *T. siamensis*, *T. bicolor*, and *T. hirsutissima* and three interspecific hybrids（*T. fournieri* × *T. baillonii*），（*T. fournieri* × *T. bicolor*）and（*T. bicolor* × *T. baillonii*）were used. Three new species, *T. siamensis*, *T. bicolor*, and *T. hirsutissima*, were introduced from Thailand according to the materials transfer agreement between Kagawa University and Queen Srikit Botanical Garden, Thailand.

Plants were planted in 15 cm pots containing commercial soil mixture（Tsuchitarou, Sumitomo Forestry Landscaping Co. Ltd）in March 2016. All pots were placed in a greenhouse under 50% shade. Complete soluble fertilizer（$N:P_2O_2:K_2O$, 6-10-5）was provided once a week.

Pollen germination test

Pollen germination of the *Torenia* species and hybrids was tested on a germination medium（0.3 mg L^{-1} Ca $(NO_3)_2$, 0.14 mg L^{-1} $MgSO_4$, 0.05 mg L^{-1} H_3BO_3, and 10% sucrose）. The cultured pollen was incubated at 100% RH and 25-30°C for 2 h. To evaluate the pollen germination ability, germination of about 100 pollen grains from randomly chosen three micro-

scopic fields were counted.

Ovule culture

Pods were collected ten days after pollination, surface sterilized with 1% NaOCl for 5 min. followed by three times rinse with distilled water. The pods were dried for 15 min. and dissected under aseptic conditions. 250 swollen ovules per cross combination were placed on a culture medium in each cross combination. The medium used for the ovule culture was Murashige & Skoog (MS) medium containing 30 $g \cdot L^{-1}$ sucrose and solidified with 2.5 $g \cdot L^{-1}$ gellan gum, adjusted to pH 5.7–5.8. All cultures were kept at 24°C under 38 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of inflorescence lamps (FL40SBRD, TOSHIBA Lighting & Technology Co. Ltd) for 16 h per day.

Chromosome number determination

Root tips of each hybrid were collected in the morning and the chromosome numbers were determined by the squash method according to Laojunta et al. [6].

Morphological observation

The obtained plants were grown in a growth chamber kept at 25 ± 2°C with natural radiation. Morphological characteristics, flower and leaf sizes, number of branches, plant height and spread, were recorded fifty days after planting.

PCR-RFLP analysis

The hybridity of the obtained plants was confirmed using PCR-RFLP analysis of the ribosomal RNA gene according to Laojunta et al. [6].

Statistical analysis

Data were analyzed using ANOVA followed by Duncan's multiple range test at $p \leq 0.05$. All statistical analyses were performed using SAS (SAS® Studio).

Experimental design

Exp. I Tri-parental crossing by use of interspecific hybrids as pollen parent.

Pollen germination of three interspecific hybrids (*T. fournieri* × *T. baillonii*), (*T. fournieri* × *T. bicolor*) and (*T. bicolor* × *T. baillonii*) were tested. Then, the fertile pollen was mounted on the stigma of four species *T. concolor, T. fournieri, T. hirsutissima* and *T. siamensis*.

Exp. II Tri-parental crossing by use of interspecific hybrids as ovule parents.

Three hybrids (*T. fournieri* × *T. baillonii*), (*T. fournieri* × *T. bicolor*) and (*T. bicolor* × *T. baillonii*) were used as female parents. Tri-parental cross combinations; (*T. fournieri* × *T. baillonii*) were crossed with *T. concolor, T. bicolor, T. hirsutissima* and *T. siamensis*, (*T. fournieri* × *T. bicolor*) were crossed with *T. concolor, T. baillonii, T. hirsutissima* and *T. siamensis*, (*T. bicolor* × *T. baillonii*) were crossed with *T. concolor, T. fournieri, T. hirsutissima* and *T. siamensis*.

In both Exp. I and II, 250 ovules per cross combination were cultured on the MS medium according to Laojunta et al. [6]. All plants obtained from ovule culture were acclimatized, and the chromosomes number and morphological characteristics were investigated as described above.

Results and Discussion

Tri-parental hybrids production

Exp. I Pollen fertility of three hybrids, (*T. fournieri* × *T. baillonii*), (*T. fournieri* × *T. bicolor*) and (*T. bicolor* × *T. baillonii*) were tested. Only (*T. bicolor* × *T. baillonii*) produced fertile pollen and the other two hybrids were male sterile. Then the pollen was mounted on the stigma of four *Torenia* species. Only the cross combination of *T. fournieri* × (*T. bicolor* × *T. baillonii*) produced progenies (Fig 1) and cultured ovules derived from the other cross combinations did not germinate (Table 1). In our previous study [6], (*T. fournieri* ×*T. bicolor*) and (*T. fournieri* ×*T. baillonii*) produced mature seeds. This suggests that these three species have a certain level of cross compatibility. However, *T. hirsutissima* × (*T. bicolor* × *T. baillonii*) did not produce progenies whereas (*T. hirsutissima* × *T. bicolor*) and (*T. hirsutissima* × *T. baillonii*) produced progenies though ovule culture [6]. The results showed that being able to get a progeny in two way crossing does not always necessarily mean of tri-parental crossing was successful.

Exp. II Three hybrids mentioned above were used as female parents and crossed with five *Torenia* species. Only two cross combinations produced hybrids. Three progenies were obtained from cultured 250 ovules of (*T. fournieri* × *T. baillonii*) × *T. siamensis* and two progenies were obtained from cultured 250 ovules of (*T. fournieri* × *T. baillonii*) × *T. concolor* (Fig 1, Table 1). No ovule germination was observed when (*T. fournieri*) × *T. bicolor* and (*T. bicolor* × *T. baillonii*) were used as mother plants (Table 1). Again, the results of two way crossings did not always support the possibility of progeny production in tri-parental crossings.

Table 1 Result of tri-parental crosses with ovule culture.

Cross combination Female × Male	No cultured ovules	Number of ovules germinated (%)
Species × hybrids		
T. concolor × (*T. bicolor* × *T. baillonii*)	250	0
T. fournieri × (*T. bicolor* × *T. baillonii*)	250	5 (2.0)
T. hirsutissima × (*T. bicolor* × *T. baillonii*)	250	0
T. siamensis × (*T. bicolor* × *T. baillonii*)	250	0
Hybrids × species		
(*T. fournieri* × *T. baillonii*) × *T. concolor*	250	2 (0.8)
(*T. fournieri* × *T. baillonii*) × *T. hirsutissima*	250	0
(*T. fournieri* × *T. baillonii*) × *T. siamensis*	250	3 (1.2)
(*T. fournieri* × *T. baillonii*) × *T. bicolor*	250	0
(*T. fournieri* × *T. bicolor*) × *T. concolor*	250	0
(*T. fournieri* × *T. bicolor*) × *T. hirsutissima*	250	0
(*T. fournieri* × *T. bicolor*) × *T. siamensis*	250	0
(*T. fournieri* × *T. bicolor*) × *T. baillonii*	250	0
(*T. bicolor* × *T. baillonii*) × *T. concolor*	250	0
(*T. bicolor* × *T. baillonii*) × *T. fournieri*	250	0
(*T. bicolor* × *T. baillonii*) × *T. hirsutissima*	250	0
(*T. bicolor* × *T. baillonii*) × *T. siamensis*	250	0

Table 2 Pollen germination percentage and chromosome number of hybrids.

Parents and hybrids	Pollen germination (%)	Chromosome number
Parents		
T. bicolor × *T. baillonii*	22.40 ± 7.29	25
T. fournieri × *T. baillonii*	0	17
T. fournieri × *T. bicolor*	0	26
Tri-parental hybrids		
T. fournieri × (*T. bicolor* × *T. baillonii*) no.1	0	21
T. fournieri × (*T. bicolor* × *T. baillonii*) no.2	0	21
T. fournieri × (*T. bicolor* × *T. baillonii*) no.3	0	21
T. fournieri × (*T. bicolor* × *T. baillonii*) no.4	0	21
T. fournieri × (*T. bicolor* × *T. baillonii*) no.5	0	21
(*T. fournieri* × *T. baillonii*) × *T. siamensis* no.1	0	18
(*T. fournieri* × *T. baillonii*) × *T. siamensis* no.2	0	18
(*T. fournieri* × *T. baillonii*) × *T. siamensis* no.3	0	18
(*T. fournieri* × *T. baillonii*) × *T. concolor* no.1	0	26
(*T. fournieri* × *T. baillonii*) × *T. concolor* no.2	0	26

Confirmation of hybridity by PCR-RFLP analysis

Interspecific crossing with ovule culture often produced escapes derived from apomixes [7]. To clarify the hybridity of the obtained progenies in this study, PCR-RFLP of rRNA analysis was applied according to Haruki et al. [8]. The expected size fragment, 520 bp, was amplified in all parents and progenies. Three restriction enzymes (*Rsa* I, *Taq* I and *Msp* I) were applied successfully to distinguish parents. The hybridity of all ten progenies from the three cross combinations was confirmed with the results of PCR-RFLP (Fig. 2). The results showed that obtained plants from three cross combinations, *T. fournieri* × (*T. bicolor* × *T. baillonii*), (*T. fournieri* × *T. baillonii*) × *T. siamensis* and (*T. fournieri* × *T. baillonii*) × *T. concolor*, were true hybrids. No apomict production was observed in tri-parental crossings.

Chromosome number and pollen fertility of hybrids

The chromosome number of hybrids, (*T. fournieri* × *T.*

Fig. 1. Flower, chromosomr number and plant form of tri-parental hybrids
A: Flower shape of *T. fournieri* and *T. bicolor* × *T. baillonii* (right).
B: Chromosome number.
C: Flower, plant form and leaf shape of *T. fournieri* × (*T. bicolor* × *T. baillonii*).
D: Flower shape of *T. fournieri* × *T. baillonii* (left), hybrids (middle) and *T. siamensis* (right).
E: Chromosome number.
F: Plant form of (*T. fournieri* × *T. baillonii*) × *T. siamensis*
G: Flower shape of *T. fournieri* × *T. baillonii* (left), hybrids (middle) and *T. concolor* (right).
H: Chromosome number.
I: Plant form of (*T. fournieri* × *T. baillonii*) × *T. concolor*.

baillonii), (*T. bicolor* × *T. baillonii*) and (*T. fournieri* × *T. baillonii*) were determined in our previous study [6] (Laojunta et al. 2019). The chromosome number of (*T. fournieri* and *T. baillonii*) was consistent with several other reports [9, 10, 11, 12]. This indicates that interspecific hybridization of (*T. fournieri* and *T. baillonii*) always produce the progenies having same chromosome number.

Chromosome number of tri-parential hybrids was determined. Newly produced interspecific hybrids showed intermediate chromosome number between parents i.e., *T. fournieri* × (*T. bicolor* × *T. baillonii*) was 2n=21, (*T. fournieri* × *T. baillonii*) × *T. siamensis* was 2n=18, and (*T. fournieri* × *T. baillonii*) × *T. concolor* was 2n=26 (Fig. 1, B, E and H, Table 2). In case of hybrids (*T. fournieri* × *T. baillonii*) × *T. siamensis*, the parent hybrid (*T. fournieri* × *T. baillonii*) can produce n=8 or 9 gametes and *T. siamensis* produces n=9 gamete. The chromosome number 18 in the hybrids (*T. fournieri* × *T. baillonii*) × *T. siamensis* is thought to be derived from matching n=9 gamete of (*T. fournieri* × *T. baillonii*) and n=9 gamete of *T. siamensis*. The chromosome numbers 26 in hybrids (*T. fournieri* × *T. baillonii*) × *T. concolor* suggested matching n=9 gamete of (*T. fournieri* × *T. baillonii*) and n=17 gamete of *T. concolor*. The chromosome numbers 21 in hybrids *T. fournieri* × (*T. bicolor* × *T. baillonii*) suggested matching n=9 gamete of *T. fournieri* and n=12 gamete of (*T. bicolor* × *T. baillonii*). The results indicate that only n=9 female gamete of (*T. fournieri* × *T. baillonii*) produced the progenies. In the back-crosses by use of (*T. fournieri* × *T. baillonii*), female gametes having n=9 gamete in (*T. fournieri* × *T. baillonii*) have higher potential for progeny production [13]. It is a very interesting fact that gametes with a certain number of chromosomes have higher potential for progeny production in interspecific hybridization.

All tri-parental hybrids were male sterile regardless their chromosome number (Table 2). It is unconfirmed whether these tri-parental hybrids produce ovules having the function.

Morphological characteristic of tri-parental hybrids

Growth types of progenies was different depending on cross combination: *T. fournieri* × (*T. bicolor* × *T. baillonii*) showed erect type growth, (*T. fournieri* × *T. baillonii*) × *T. siamensis* showed semi-erect type growth and (*T. fournieri* × *T. baillonii*) × *T. concolor* showed creeping type growth (Table 3, Fig. 1, C, F and I) In the case of the hybrids, an erect type appeared when *T. fournieri* was used as mother plant. Leaf size varied from 15.4 to 28.9 mm in width and 21.8 to 46.3 in length; leaf shape also varied depending cross combinations. The basic structure of the flower consisting four petals was same in all the hybrids. Flower size varied from 19.1 to 26.5 mm in width and 21.3 to 30.1 in length depending on cross combinations. Petal color of the hybrids was mostly intermediate of parents. Variation in petal color was small as shown in two way crossing of *Torenia* [6]. Further efforts, self-and back-crossing following chromosome doubling are required to obtain sufficient variations in floral traits in the progenies.

Acknowledgements

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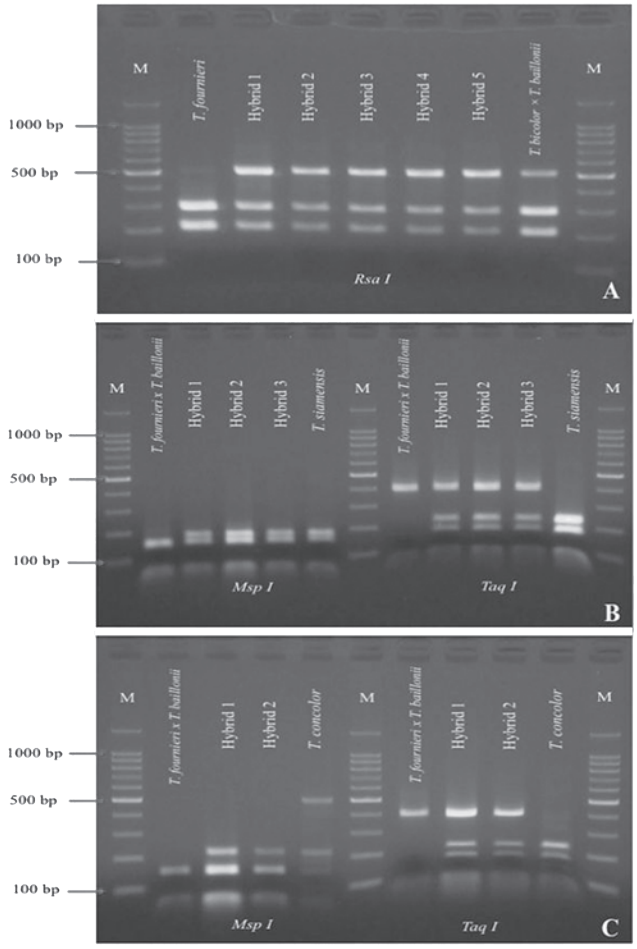

Fig. 2. Confirmation of hybridity of obtained progenies in tri-parental hybrids.
A : Tri-parental crossing of *T. fournieri* × (*T. baillonii* × *T. bicolor*).
G : Tri-parental crossing of (*T. fournieri* × *T. baillonii*) × *T. siamensis*).
C : Tri-parental crossing of (*T. fournieri* × *T. baillonii*) × *T. concolor*).
M: Ladder.

Table 3 Morphological characteristics of tri-parental hybrids.

Cross combination Female × Male		Growth type	Plant height (cm)	Plant spread (cm)	No. of branch	Leaf width (mm)	Leaf length (mm)	Flower width (mm)	Flower length (mm)
T. fournieri × (*T. bicolor* × *T. baillonii*)	no.1	Erect	18.4 ± 0.28 a	19.0 ± 0.70 e	7 ± 0.22 d	19.3 ± 0.44 e	31.2 ± 1.84 c	22.3 ± 0.34 d	29.8 ± 0.18 ab
T. fournieri × (*T. bicolor* × *T. baillonii*)	no.2	Erect	10.6 ± 0.17 c	5.6 ± 0.17 fg	1.6 ± 0.17 h	19.4 ± 0.53 e	28.8 ± 0.52 d	20.1 ± 0.04 e	28.3 ± 1.72 b
T. fournieri × (*T. bicolor* × *T. baillonii*)	no.3	Erect	10.2 ± 0.14 cd	4.2 ± 0.14 g	4.6 ± 0.17 g	15.4 ± 0.70 h	21.8 ± 0.15 f	23.2 ± 0.01 c	28.1 ± 0.04 b
T. fournieri × (*T. bicolor* × *T. baillonii*)	no.4	Erect	10.4 ± 0.17 cd	6.6 ± 0.17 f	5.4 ± 0.17 f	16.5 ± 0.11 g	25.0 ± 0.07 e	19.1 ± 0.14 f	21.3 ± 0.06 d
T. fournieri × (*T. bicolor* × *T. baillonii*)	no.5	Erect	10.6 ± 0.17 c	21.0 ± 0.17 d	12 ± 0.22 b	21.1 ± 0.18 d	35.6 ± 0.11 b	22.6 ± 0.01 d	25.1 ± 0.04 c
(*T. fournieri* × *T. baillonii*) × *T. siamensis*	no.1	Semi-erect	9.8 ± 0.23 d	33.9 ± 0.90 c	5.9 ± 0.29 ef	28.9 ± 0.16 a	36.0 ± 0.17 b	24.2 ± 0.22 b	25.3 ± 0.01 c
(*T. fournieri* × *T. baillonii*) × *T. siamensis*	no.2	Semi-erect	9.8 ± 0.25 d	33.3 ± 1.27 c	6.5 ± 0.17 de	27.1 ± 0.32 b	46.3 ± 1.43 a	23.8 ± 0.19 b	28.2 ± 0.18 b
(*T. fournieri* × *T. baillonii*) × *T. siamensis*	no.3	Semi-erect	11.9 ± 0.52 b	34.9 ± 0.50 c	6.0 ± 0.27 ef	24.5 ± 0.22 c	46.3 ± 0.44 a	26.5 ± 0.22 a	30.1 ± 0.28 a
(*T. fournieri* × *T. baillonii*) × *T. concolor*	no.1	Creeping	7.1 ± 0.16 f	44.8 ± 0.42 a	8.2 ± 0.34 c	18.1 ± 0.29 f	25.0 ± 0.22 e	20.5 ± 0.30 e	25.0 ± 0.33 c
(*T. fournieri* × *T. baillonii*) × *T. concolor*	no.2	Creeping	7.8 ± 0.13 e	41.9 ± 1.00 b	13.6 ± 0.35 a	17.1 ± 0.18 g	26.6 ± 0.26 e	23.9 ± 0.13 b	29.2 ± 0.26 ab

Means ± standard errors within a column followed by the same letter are not significant different according Duncan's multiple range test at $p \leq 0.05$.

References

(1) Van Tuyl, J.M., and De Jeu, M.J.: Methods for overcoming interspecific crossing barriers. Chapter 13. p.273-293. In: Sawhney and Shivanna (eds.), Pollen Biotechnology for Crop Production and Improvement, Cambridge University Press (1997).

(2) Nimura, M., Kato, J. and Mii, M.: Interspecific hybrid production by reciprocal cross between *Dianthus caryophyllus* L. and *Dianthus x isensis* Hirahara et Kitamura. J. Jort. Sci. and Biotech. 81, 995-1001 (2006).

(3) Yamaguchi, S., Kunitake, T. and Hisatomi, S.: Interspecific hybrid between *Camellia japonica* cv. Chochidori and *C. crysantha* produced by embryo culture.Japn. J. Breed. 37, 203-206 (1997).

(4) Nontaswatsri, C., and Suksathan, P.: Interspecific hybridization in the genus *Globba* using in vitro embryo culture. Acta Hort. 1025, 31-36 (2014).

(5) Laojunta, T., Narumi-Kawasaki, T. and Fukai, S.: Characteristics of commercial *Torenia* cultivars. Acta Hortic. 1167, 205-211 (2017).

(6) Laojunta, T., Narumi-Kawasaki, T., Takamura, T. and Fukai, S.: A new interspecific hybrid *Toren*ia obtained through ovule culture. Hort. Environ, and Biotech. (2019).

(7) Rodrigo, J.M., Zappacosta, D.C., Selva, J.P., Garbus, I., Albertini, E., and Echenique, V.: Apomixis frequency under stress conditions in weeping lovegrass (*Eragrostis curvula*). PLOS ONE 12, e0175852 (2017).

(8) Haruki, K., Hosoki, T., Nako, Y., and Ohta, K.: Possibility of classification in some species of *Lilium* by PCR-PFLP of ribulose-1,5-biphosphate carboxylase large subunit (rbcL) gene and ribosomal RNA gene. J. Japan. Soc. Hort. Sci. 66, 189-192 (1997).

(9) Kikuchi, S., Tanaka, H., Shiba, T., Mii, M., and Tsujimoto, H.: Genome size, karyotype, meiosis and a novel extra chromosome in *Torenia fournieri, T. baillonii* and their hybrids. Chromosome Res. ?? (2006).

(10) Jiranapapan, J., Kikuchi, S., Manachai, B., Taychasinpitak, T., Tanaka, H., and Tsujimoto, H.: A simple method of chromosome doubling using colchicine in *Torenia* (Linderniaceae), and the behavior of meiotic chromosome in amphidiploids. Chromosome Sci. 14, 29-32 (2011).

(11) Sawangmee, W., Taychasinpitak, T., Jompuk, P. and Kikuchi, S.: Effect of gamma- ray Irradiation in plant morphology of interspecific hybrids between *Torenia fournieri* and *Torenia baillonii*.Kasetsart J. Nat. Sci. 45, 803-810 (2011).

(12) Nuntha, B., Kikuchi, S., Taychasinpitak, T., Sassa, H., and Koba, T.: New karyotypes of an interspecific hybrid of *Torenia fournieri* and *Torenia baillonii* and its progenies. Chromosome Sci. 19, 37-40 (2016).

(13) Laojunta, T., Narumi-Kawasaki, T., Takamura, T. and Fukai. Expansion of flower trait variations in interspecific hybrids between *Torenia fournieri* and *Torenia baillonii*. Acta Horticulturae (in press)

新規トレニア三元交配種の作出

タナポーン　ラオジュンタ・鳴海貴子・深井誠一

要　　約

三元交配は，花の色と形態的特徴のバリエーションを増やす効果的な方法である．（*T. bicolor* × *T. baillonii*）の稔性花粉を，*T. concolor*，*T. fournieri*，*T. hirsutissima*および*T. siamensis*の４種の柱頭に受粉した．*T. fournieri* ×（*T. bicolor* × *T. baillonii*）の交配組合せのみが５つの後代を生み出した．次に，３つの交配種（*T. fournieri* × *T. baillonii*），（*T. fournieri* × *T. bicolor*），および（*T. bicolor* × *T. baillonii*）を雌性親として使用し，他の*Torenia*の種と交配した．（*T. fournieri* × *T. baillonii*）× *T. siamensis*から３つの後代が，（T. fournieri × *T. baillonii*）× *T. concolor*から２つの後代が得られた．得られた植物の雑種性はPCR-RFLPによって確認され，全てが真の交配種であった。三元交配種は，両親の中間の染色体数を示し，すべての交配種は雄性不稔であった．交配種の花弁の色は，ほぼ全て親の中間色であった．