Plant Biotechnology 26, 301–305 (2009)

Original Paper

Interspecific hybridization between triploid Senno (*Lychnis senno* Siebold et Zucc., Caryophyllaceae) and allied taxa of the genus *Lychnis*

Toshinari Godo[1,*], Junko Miyazaki[2], Sachiko Kuwayama[3], Shinjiro Ogita[2], Yasuo Kato[2], Masaru Nakano[3], Masashi Nakata[1]

[1] Botanic Gardens of Toyama, Fuchu-machi, Toyama 939-2713, Japan; [2] Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, Imizu, Toyama 939-0398, Japan; [3] Faculty of Agriculture, Niigata University, Niigata 950-2181, Japan

* E-mail: godo@bgtym.org Tel: +81-76-466-4187 Fax: +81-76-465-5923

Received January 26, 2009; accepted March 27, 2009 (Edited by T. Anai)

Abstract Senno (*Lychnis senno*, Caryophyllaceae), a traditional ornamental plant in Japan, was introduced from China and all the strains of Senno found in Japan are triploid with $2n=3x=36$. Hand-pollination was conducted between triploid Senno and allied *Lychnis* taxa, *L. kiusiana* ($2n=24$), *L. miqueliana* ($2n=24$), *L. miqueliana* f. *albescens* ($2n=24$), *L. chalcedonica* ($2n=24$), *L. wilfordii* ($2n=24$), *L. sieboldii* ($2n=24$), and *L. coronata* ($2n=24$). Immature seeds 17–42 days after pollination were cultured on half-strength Murashige and Skoog media with or without 6-benzyladenine. Although immature seeds were obtained in most cross combinations, seed germination was observed only in three cross combinations, *L. senno*×*L. kiusiana*, *L. kiusiana*×*L. senno*, and *L. senno*×*L. sieboldii*. Seedlings derived from reciprocal crosses between *L. senno* and *L. kiusiana* showed an abnormal morphology or a chlorophyll-deficiency, xantha. Seedlings derived from *L. senno*×*L. sieboldii* also showed an abnormal morphology or chlorophyll-deficiency, but one seedling grew into a green plantlet. The hybridity of these seedlings was confirmed by random amplified polymorphic DNA analysis. The chromosome number of the interspecific hybrid plantlet between *L. senno* and *L. sieboldii* was determined to be $2n=32$. The present study shows the possibility of using triploid Senno for breeding by interspecific hybridization, and the effectiveness of immature seed culture for producing interspecific hybrids in the genus *Lychnis*.

Key words: Caryophyllaceae, interspecific hybrid, *Lychnis senno*, Senno, triploid.

The genus *Lychnis* belongs to the family Caryophyllaceae and consists of about 30 species distributed in the temperate regions of the Northern Hemisphere, from East Asia to Europe (Kitagawa 1982). Many species, such as *L. chalcedonica* L., *L. sieboldii* Van Houtte, and *L. coronaria* Desr., have high ornamental value and have been used as pot or garden plants. Horticultural cultivars of *Lychnis* species have been bred mainly by intraspecific hybridization.

Senno (*L. senno* Siebold et Zucc.), a traditional ornamental plant in Japan, was introduced from China about 600 years ago (Ohwi 1975, Kitamura 1983). We previously reported that all strains of Senno found in Japan are triploid with $2n=36$ (Godo et al. 2000). Since Japanese Senno strains usually set no normal seeds, plants have thus far been propagated vegetatively by conventional methods such as division and cutting. Recently, a micropropagation method has also been established for Senno (Godo et al. 2004a). Although Japanese Senno strains have a high ornamental value like other *Lychnis* species, they have not yet been utilized as cross breeding material, probably due to their rarity and sterility.

Embryo, ovule, and immature seed cultures have been successfully applied to produce interspecific or intergeneric hybrids in various ornamental plants such as *Lilium* species (Asano 1978, 1980), *Cyclamen* species (Ishizaka 1997), *Primula* species (Kato and Mii 2000; Amano et al. 2006; Hayashi et al. 2007a, b), *Cosmos* species (Oku et al. 2008), and Colchicaceous plants (Amano et al. 2007). Although all the Japanese Senno strains previously found are triploid, they had relatively high pollen fertility (about 70%) as assessed with acetocarmine staining and sometimes produced a few seeds by artificial self-pollination (Godo et al. 2004b). In the present study, we examined the production of interspecific hybrids between triploid Senno and allied *Lychnis* taxa utilizing an aseptic immature seed culture

Abbreviations: BA, 6-benzyladenine; MS, Murashige and Skoog; PGR, plant growth regulator; RAPD, random amplified polymorphic DNA.
This article can be found at http://www.jspcmb.jp/

technique.

Materials and methods

Plant materials

The strain MS (Godo et al. 2000) of Senno ($2n=36$) and other *Lychnis* taxa, *L. kiusiana* Makino ($2n=24$), *L. miqueliana* Rohrb. ($2n=24$), *L. miqueliana* f. *albescens* Honda ($2n=24$), *L. chalcedonica* L. ($2n=24$), *L. wilfordii* (Regel) Maxim. ($2n=24$), *L. sieboldii* Van Houtte (white-flowered cultivar; $2n=24$), and *L. coronata* Thunb. (white-flowered cultivar; $2n=24$) were cultivated in a glasshouse of the Botanic Gardens of Toyama and used in the present study (Table 1).

Pollination and immature seed culture

Hand-pollination with fresh pollen was conducted between the strain MS and other *Lychnis* taxa during the summers of 2002–2006. Immature fruits were harvested before dehiscence 17–42 days after pollination. The fruits were surface-sterilized with 70% ethanol for 1 min and then washed with distilled sterilized water. Immature seeds were aseptically isolated from the fruits and placed on half-strength MS media (Murashige and Skoog 1962), which consists of half-strength MS mineral salts and full-strength MS organic constituents, with or without 0.2 mg l^{-1} BA. All media used in the present study were supplemented with 20 g l^{-1} sucrose, solidified with 2 g l^{-1} gellan gum (Phytagel; Sigma Chemical Co., St. Louis, USA), and adjusted to pH 5.8 before autoclaving at 121°C for 15 min. Culture tubes (20×140 mm), each containing 10 ml of the medium and covered with a polypropylene cap, were used, and one immature seed was cultured per tube. Cultures were incubated at 25°C under a 16 h photoperiod with fluorescent lighting (40 μmol m^{-2} s^{-1}). Immature seed-derived plantlets were maintained and propagated by subculturing nodal segments every 3–4 months on half-strength MS medium without any PGRs under the same conditions as the immature seed culture.

RAPD analysis and chromosome observation

Isolation of total genomic DNA from leaves and RAPD analysis were performed according to Amano et al. (2007). For RAPD analysis, three sets of DNA Oligomer (12) Set, C43, G64, and G81 (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 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. Amplified products were analyzed by electrophoresis in a 1.7% (w/v) agarose gel.

For chromosome observation, fresh root tips (5 mm in length) of *in vitro* plantlets were pretreated with 0.5 g l^{-1} colchicine for 6 h at 20°C and fixed with Farmer's fluid (1 : 3 mixture of glacial acetic acid and ethanol, v/v) for over 24 h at 5°C. The fixed root tips were macerated in a mixture of 1N HCl and 45% acetic acid (1 : 1, v/v) for 15 s at 60°C, and the meristematic region of the root was stained with 1% aceto-orcein. Chromosome preparation was made by the squashing method.

Table 1. *Lychnis* taxa used as materials in this study and their chromosome numbers

Taxon	Chromosome number (2n)
L. senno Siebold et Zucc.	36
L. kiusiana Makino	24
L. miqueliana Rohrb.	24
L. miqueliana Rohrb. f. *albescens* Honda	24
L. chalcedonica L.	24
L. wilfordii (Regel) Maxim.	24
L. sieboldii Van Houtte (WF[a])	24
L. coronata Thunb. (WF)	24

[a] White-flowered cultivar

Results and discussion

Immature seeds were obtained from all cross combinations expect for *L. coronata*×*L. senno* (Table 2). The number of immature seeds per fruit varied from 2.3 in *L. miqueliana* f. *albescens*×*L. senno* in 2004 to 23.8 in *L. senno*×*L. sieboldii* in 2003. Environmental conditions such as temperature after pollination, physiological conditions of parental plants and/or genetic distance between the parental species may affect seed set.

When the immature seeds obtained were subjected to *in vitro* culture, seed germination was observed 3 days to 2 months after culture initiation. There was no effect of the period from pollination to fruit harvest on seed germination. Although a promotive effect of BA has been reported on the growth and development of cultured embryos of *Lilium japonicum* (Furuya and Hosoki 2005), no apparent effect of BA on seed germination was observed in the present study. In *L. senno*×*L. miqueliana*, no seed germination was observed, although, relatively, many immature seeds were obtained in both 2002 and 2003. Thus, there may be no correlation between the number of immature seeds per fruit and the seed germination frequency. Finally, seed germination was observed only in three cross combinations, *L. senno*×*L. kiusiana*, *L. kiusiana*×*L. senno*, and *L. senno*×*L. sieboldii* (Table 2).

In the crosses of *L. senno*×*L. kiusiana* and *L. kiusiana*×*L. senno*, a total of 26 and 10 immature seeds germinated, respectively. However, most seedlings showed a chlorophyll-deficient, xantha phenotype (Figure 1A) or an abnormal morphology and subsequently died. Ultimately, 14 and 10 xantha plantlets derived from independent immature seeds were obtained in *L. senno*×*L. kiusiana* and *L. kiusiana*×*L. senno*, respectively. RAPD analysis was performed to confirm the hybridity of these plantlets (Figure 2). A clear polymorphism in the RAPD profile was obtained

Table 2. Seed sets and germination frequency in interspecific hybridization between triploid *Lychnis senno* and allied *Lychnis* taxa

Year	Female	Male	No. of cross-pollinated flowers	No. of fruits containing immature seeds	Mean no. of immature seeds per fruit	Total no. of sowing seed[b]	Total no. of seed germination[c]	Seed germination frequency (%)	Total no. of seedlings growing to plantlet[d]
2002	*L. senno*	*L. kiusiana*	9	6	17.7	106	23	21.7	13
	L. senno	*L. miqueliana*	11	7	17.6	123	0	0	0
	L. senno	*L. chalcedonica*	2	2	5.0	10	0	0	0
	L. senno	*L. wilfordii*	1	1	3.0	3	0	0	0
2003	*L. senno*	*L. kiusiana*	8	3	9.7	29	3	10.3	1
	L. senno	*L. miqueliana*	2	2	3.5	7	0	0	0
	L. senno	*L. sieboldii* (WF)	12	6	23.8	133	11	8.3	2
	L. senno	*L. coronata* (WF)	1	1	9.0	9	0	0	0
2004	*L. senno*	*L. miqueliana* f. *albescens*	14	14	13.2	185	0	0	0
	L. miqueliana f. *albescens*	*L. senno*	5	3	2.3	7	0	0	0
	L. kiusiana	*L. senno*	9	3	3.7	11	0	0	0
	L. coronata (WF[a])	*L. senno*	2	0	—	—	—	—	—
2006	*L. kiusiana*	*L. senno*	5	5	19.4	95	10	9.5	10

[a] White-flowered cultivar
[b] Immature seeds were sowed on 1/2MS media with or without 0.2 mg l^{-1} BA and were incubated at 25°C under a 16 h photoperiod with fluorescent lighting (40 μmol m^{-2} s^{-1}).
[c] Measured after 3 months of sowing.
[d] Measured after 6 months of sowing.

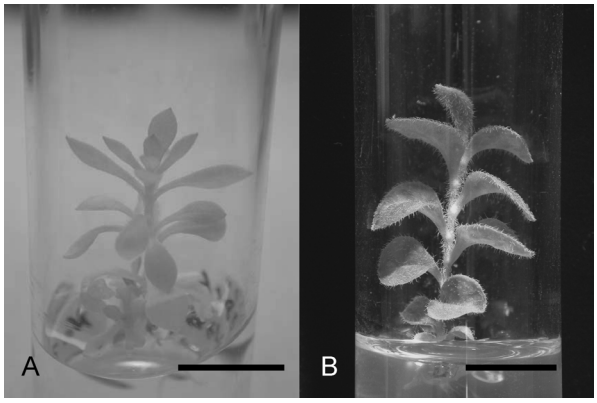

Figure 1. Interspecific hybrids in *Lychnis*. (A) Hybrid plantlet of *L. senno*×*L. kiusiana* showing xantha leaves. (B) Hybrid plantlet of *L. senno*×*L. sieboldii* showing normal green leaves. Bars = 1 cm.

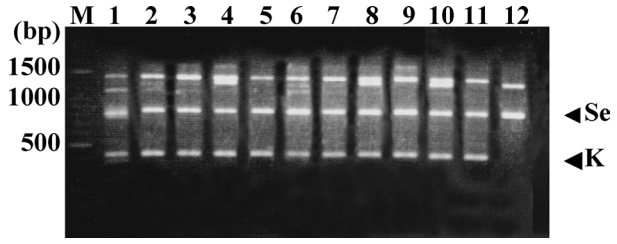

Figure 2. RAPD profiles of *Lychnis kiusiana*, *L. senno*, and their putative hybrids. Primer G64 was used. Lane M, size marker; lane 1, *L. kiusiana*; lanes 2–11, independent putative hybrid plantlets of *L. kiusiana*×*L. senno*; lane 12, *L. senno*. K, band specific to *L. kiusiana*; Se, band specific to *L. senno*.

between *L. senno* and *L. kiusiana* using the primers C43 and G64. All the plantlets analyzed showed RAPD profiles containing only pollen parent-specific amplified fragments for the primer C43 (data not shown) and those containing both seed parent-specific and pollen parent-specific amplified fragments for the primer G64 (Figure 2). Thus, these plantlets were verified as hybrids between *L. senno* and *L. kiusiana*.

Leaves of these hybrid plantlets often turned yellowish green during subculture using a medium without PGRs, but the plantlets did not develop into normal green plantlets even after 2.5 years of subculture. Chlorophyll-deficiency has also been reported in interspecific hybrids of *Trifolium* (Sawai et al. 1990), *Zantedeschia* (Yao et al. 1994), and *Rhododendron* (Ureshino et al. 1999; Ureshino and Miyajima 2002, Michishita et al. 2002). This phenomenon may be caused by plastome-genome incompatibility between the nuclear genome of the pollen parent and the plastome of the seed parent (Kirk and Tilney-Bassett 1978; Yao et al. 1994; Ureshino et al. 1999). In some cases, hybrid chlorophyll-deficiency that resulted from the plastome-genome incompatibility could be overcome by reciprocal crossing. Such a phenomenon, unidirectional plastome-genome incompatibility, was observed in interspecific hybrids between *Zantedeschia odorata* and *Z. aethiopica* (Yao et al. 1995) and between *Nierembergia scoparia* and *N. caerulea* (unpublished data). In the present study, regrettably, chlorophyll-deficiency was observed in seedlings derived from both *L. senno*×*L. kiusiana* and *L. kiusiana*×*L. senno*. It is difficult to breed new cultivars by cross hybridization between these two species.

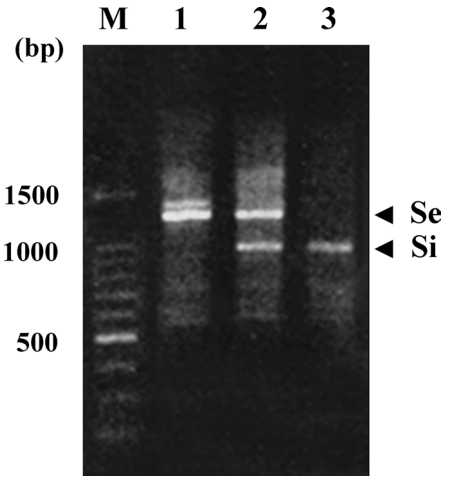

Figure 3. RAPD profiles of *Lychnis senno*, *L. sieboldii*, and their putative hybrid. Primer G81 was used. Lane M, size marker; lane 1, *L. senno*; lane 2, putative hybrid plantlet of *L. senno*×*L. sieboldii*; lane 3, *L. sieboldii*. Se, band specific to *L. senno*; Si, band specific to *L. sieboldii*.

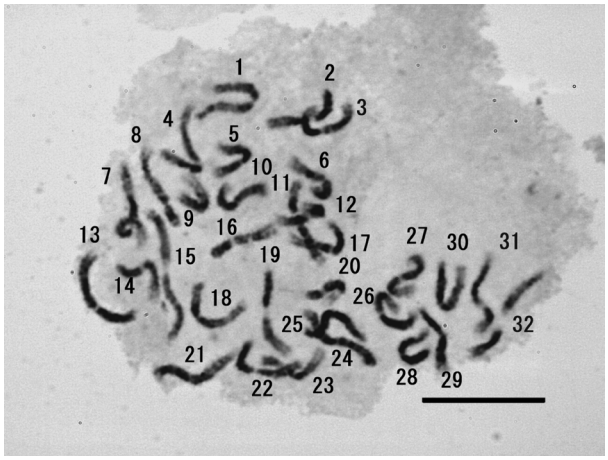

Figure 4. Somatic prometaphase chromosomes of an interspecific hybrid of *Lychnis senno*×*L. sieboldii* ($2n=32$). Bar=10 μm.

In the cross of *L. senno*×*L. sieboldii*, a total of 11 immature seeds germinated and, ultimately, 2 plantlets were obtained from independent immature seeds. Although leaves of one plantlet were green (Figure 1B), the other plantlet showed chlorophyll-deficiency. RAPD analysis was performed to confirm the hybridity of the green plantlet. A clear polymorphism was obtained in the RAPD profile between *L. senno* and *L. sieboldii* using the primer G81. The green plantlet showed RAPD profiles containing both seed parent-specific and pollen parent-specific amplified fragments (Figure 3), and thus this plantlet was verified as a hybrid between *L. senno* and *L. sieboldii*. The chromosome number of the hybrid plantlet between *L. senno* and *L. sieboldii* was determined to be $2n=32$ (Figure 4). The chromosomes of the hybrid were assumed to consist of 12 normal haploid chromosomes of *L. sieboldii* ($2n=24$) and 20 chromosomes of triploid *L. senno* ($2n=36$). Japanese triploid Senno strains sometimes produced a few seeds after artificial self-pollination (Godo et al. 2004b), and progenies obtained from these seeds were aneuploid with $2n=24$–44 (unpublished data) suggesting that they produced male and/or female gametes containing chromosomes of irregular numbers such as 20. This result shows the possibility of using triploid Senno for breeding by interspecific hybridization. *In vitro* propagation and acclimatization of the hybrid between *L. senno* and *L. sieboldii* obtained in the present study are now in progress.

The genus *Lychnis*, containing many species with beautiful flowers, is a valuable group for ornamental use, and various cultivars have been bred mainly by intraspecific hybridization in this genus. Although Senno strains cultivated in Japan also have a high ornamental value, they have not yet been used as a cross breeding material due to their triploid property. The present study showed that interspecific hybrids between triploid Senno and allied species could be obtained by *in vitro* immature seed culture. For practical breeding of *Lychnis* species, it is necessary to establish more efficient methods for rescuing interspecific hybrids in *Lychnis*. Genetic distances among taxa in *Lychnis* should also be studied by cytological and molecular genetic approaches.

Acknowledgements

We thank Ms. and Mr. Miyazako for kindly providing the plant materials.

References

Amano J, Kato J, Nakano M, Mii M (2006) Production of intersection hybrids between *Primula filchnerae* and *P. sinensis* through ovule culture. *Sci Hort* 110: 223–227

Amano J, Kuwayama S, Mizuta Y, Oomiya T, Nakamura T, Nakano M (2007) 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 analysis. *J Japan Soc Hort Sci* 76: 73–78

Asano Y (1978) Studies on crosses between distantly related species of lilies III. New hybrids obtained through embryo culture. *J Japan Soc Hort Sci* 47: 401–414

Asano Y (1980) Studies on crosses between distantly related species of lilies V. Characteristics of newly obtained hybrids through embryo culture. *J Japan Soc Hort Sci* 49: 241–250

Furuya H, Hosoki T (2005) Study on the in vitro culture conditions for raising seedlings from embryos excised from immature seeds of *Lilium japonicum* Thunb. *Hort Res* 4: 281–286 (in Japanese with English summary)

Godo T, Oku T, Mii M, Nakata M (2000) In vitro culture for preservation of triploid Senno (*Lychnis senno* Siebold et Zucc., $2n=36$), a valuable and rare ornamental plant. *Bull Bot Gard Toyama* 5: 35–43

Godo T, Oku T, Mii M, Nakata M (2004a) Micropropagation and

characterization of the regenerated plants of triploid *Lychnis senno* Siebold et Zucc., a valuable and rare ornamental plant. *Bull Bot Gard Toyama* 9: 9–17

Godo T, Oku T, Mii M, Nakata M (2004b) Triploid property of Senno (*Lychnis senno* Siebold et Zucc., Caryophyllaceae), a traditional ornamental plant conserved in Japan. *Breed Sci* 54: 105–109

Hayashi M, Kato J, Ichihashi Y, Matsubara N, Ohashi H, Mii M (2007a) Inter-sectional hybrids with various ploidy levels between *Primula denticulata* and three varieties of *P. modesta*. *Breed Sci* 57: 165–173

Hayashi M, Kato J, Ichihashi Y, Ohashi H, Mii M (2007b) Variation of ploidy level in inter-section hybrids obtained by reciprocal crosses between tetraploid *Primula denticulata* ($2n=4x=44$) and diploid *P. rosea* ($2n=2x=22$). *J Hort Sci Biotechnol* 82: 165–173

Ishizaka H (1997) Interspecific hybridization using ovule culture and haploid production by anther culture in *Cyclamen*. *Spe Bull Saitama Hort Exp Sta* 5: 1–95

Kato J, Mii M (2000) Difference in ploidy levels of inter-specific hybrids obtained by reciprocal crosses between *Primula sieboldii* and *P. kisoana*. *Theor Appl Genet* 101: 690–696

Kirk JTO, Tilney-Bassett RAE (1978) *The plastids, their chemistry, structure, growth and inheritance, 2nd ed*. Elsevier, Amsterdam

Kitagawa M (1982) Caryophyllaceae. In: Satake Y, Ohwi J, Kitamura S, Watari S, Tominari T (eds) *Wild Flowers of Japan II, Herbaceous Plants, Choripetalae*. Heibonsha, Tokyo, pp 32–45 (in Japanese)

Kitamura S (1983) Senno. *New Knowledge in Horticulture* 8: 38 (in Japanese)

Michishita A, Ureshino K, Miyajima I (2002) Plastome-genome incompatibility of *Rhododendron serpyllifolium* (A. Gray) Miq. to evergreen azalea belonging to series Kaempferia. *J Japan Soc Hort Sci* 71: 214–219

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–497

Ohwi J (1975) *Flora of Japan, New edition, revised and enlarged*. Shibundo, Tokyo (in Japanese)

Oku T, Takahashi H, Yagi F, Nakamura I, Mii M (2008) Hybridization between chocolate cosmos and yellow cosmos confirmed by phylogenetic analysis using plastid subtype identity (PSID) sequences. *J Hort Sci Biotechnol* 83: 323–327

Sawai A, Ueda S, Gau M, Uchiyama K (1990) Interspecific hybrids of *Trifolium medium* L.×4*x T. pratense* L. obtained through embryo culture. *J Japan Soc Grassland Sci* 35: 267–273

Ureshino K, Miyajima I, Ozaki Y, Kobayashi N, Michishita A, Akabane M (1999) Appearance of albino seedlings and ptDNA inheritance in interspecific hybrids of azalea. *Euphytica* 110: 61–66

Ureshino K, Miyajima I (2002) The study on the relationship between leaf colors and ptDNA inheritance in intersectional cross of *Rhododendron kiusianum*×*R. japonicum* f. *flavum*, resulting in an unexpected triploid progeny. *J Japan Soc Hort Sci* 71: 214–219

Yao JL, Cohen D, Rowland RE (1994) Plastid DNA inheritance and plastome-genome incompatibility in interspecific hybrids of *Zantedeschia* (Aradeae). *Theor Appl Genet* 88: 255–260

Yao JL, Cohen D, Rowland RE (1995) Interspecific albino and variegated hybrids in the genus *Zantedeschia*. *Plant Sci* 109: 199–206