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Minireview

Interspecific hybridization by embryo rescue in the genus *Cyclamen*

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Abstract   Numerous cultivars of cyclamen have been developed by improving wild plants of *Cyclamen persicum*, and they are now among the most important pot plants. However, no cultivars have yet been produced in the other 21 *Cyclamen* species. Furthermore, these 21 wild species have not yet been used for improving *C. persicum* cultivars due to hybrid inviability and hybrid sterility. In recent studies, hybrid inviability caused by abortion of hybrid embryos was overcome by an embryo rescue technique involving culture of ovules with placenta, which resulted in the production of interspecific hybrids between *C. persicum* and each of ten wild species. Hybrid sterility caused by abnormal meiotic chromosome behavior in hybrids (allodiploids) between diploid *C. persicum* and each of two diploid wild species was overcome by chromosome doubling using *in vitro* colchicine treatment, resulting in the production of two types of fertile amphidiploids (allotetraploids). On the other hand, three types of allotetraploids were produced between autotetraploid cultivars of *C. persicum* and each of three autotetraploid wild species by the embryo rescue technique without chromosome doubling. Embryo rescue and *in vitro* chromosome doubling are simple and useful methods for overcoming hybrid inviability and hybrid sterility in interspecific hybridization in the genus *Cyclamen*. Some of the fertile amphidiploids thus produced have already been incorporated into commercial breeding of novel types of cyclamens with important traits such as flower fragrance and disease resistance.

Key words: Amphidiploid, chromosome doubling, *Cyclamen*, embryo rescue, interspecific hybridization.

In some flower crops such as orchids, lily and rose, numerous cultivars with abundant variations have been developed by wide cross. In wide cross, there is a cross-sterility barrier related to reproductive isolation, and the barrier is divided into pre- and post-fertilization barriers. The pre-fertilization barrier, called cross-incompatibility, is a phenomenon in which no pollen germination occurs on the stigma or pollen tube elongation stops in the style, resulting in the failure of fertilization. This barrier can be overcome in some cases by test tube pollination or protoplast fusion. In the post-fertilization barrier, called hybrid inviability, hybrid embryos or plantlets obtained after fertilization degenerate during their growth. This barrier can also sometimes be overcome by embryo rescue using tissue culture techniques (Khush and Brar 1992). Hybrids produced by overcoming these barriers contain different genomes, and the degree of affinity between the parental genomes strongly influences the fertility of the hybrids. In the hybrids with a weak affinity of the parental genomes, normal bivalent formation does not occur in diakinesis or metaphase-I of meiosis, which leads to male and/or female sterility. This phenomenon is generally called hybrid sterility, and in some cases, normal bivalent formation and subsequent fertility can be restored by chromosome doubling (Singh 1993).

Wild plants of *C. persicum* are consistently diploid and are distributed in the eastern Greek Islands, western and southern Turkey, western Syria, Lebanon, Israel, Cyprus and Tunisia. Wild plants reach the flowering stage about two years after sowing. In Japan, wild plants bloom in February, produce seeds in May, and are dormant thereafter until the end of September. They then produce leaves at the beginning of October, and produce flowers again in February of the next year. Wild plants are single-flowered, and their petals are about 2 cm in length. Their flower color is white or pale pink with a deep purple region at the petal base (hereafter this region is referred to as the eye), or pure white without an eye. Since wild plants were introduced into western Europe in

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about 1700, diploid and autotetraploid cultivars with various characteristics have been bred from them. Unlike wild plants, *C. persicum* cultivars reach the flowering stage about 12 months after sowing and have no spontaneous dormancy in the summer. A wide range of flower size (about 3 to 7 cm in petal length), shape (single, double, fringed or crested flowers) and color (white, pink, purple, red or pale yellow with an eye; white, pink or pale yellow without an eye) is observed in the present cultivars (Grey-Wilson 2002; Takamura 2007).

Cyclamen species other than *C. persicum* have neither been developed into major commercial plants nor been used for improving *C. persicum* cultivars due to hybrid inviability and hybrid sterility. The wild *Cyclamen* species have several valuable traits such as cold hardiness, disease resistance and flower fragrance, and are therefore recognized as potentially attractive gene sources for breeding of *C. persicum* (Table 1).

In the genus *Cyclamen*, several fertile interspecific hybrids have been produced by conventional hybridization methods in the following cross combinations: *C. balearicum* ($2n=20$)×*C. repandum* ($2n=20$), *C. creticum* ($2n=20$)×*C. repandum*, *C. balearicum*×*C. peloponnesiacum* ($2n=20$), *C. coum* ($2n=30$)×*C. alpinum* ($2n=30$), *C. libanoticum* ($2n=30$)×*C. pseudibericum* ($2n=30$), *C. cyprium* ($2n=30$)×*C. libanoticum*, *C. cilicium* ($2n=30$)×*C. intaminatum* ($2n=30$), and *C. africanum* ($2n=34$)×*C. hederifolium* ($2n=34$) (Grey-Wilson 2002). However, no interspecific hybrids between species with different chromosome numbers have yet been produced by conventional hybridization methods, especially between *C. persicum* cultivars and other wild species. From an examination of interspecific hybridization in the genus *Cyclamen*, Legro (1959) concluded that introduction of useful traits of wild species into *C. persicum* cultivars would be difficult. In the present mini-review, recent studies on the cross-sterility between *C. persicum* cultivars and wild *Cyclamen* species and on the production of fertile amphidiploids by embryo rescue and chromosome doubling are summarized.

Interspecific hybridization between *C. persicum* cultivars and *C. hederifolium*

Ovule culture

Diploid *C. hederifolium* produces small flowers, about 2 cm in petal length and pale pink in color. This species has some useful traits such as fall flowering and cold hardiness. Diploid *C. persicum* cultivar 'Sonja' produces small flowers, about 3 cm in petal length and white with a deep purple eye. Autotetraploid *C. persicum* cultivar 'Salmon Scarlet' produces large flowers, about 6 cm in petal length and red in color. Flowers of 'Sonja',

'Salmon Scarlet' and *C. hederifolium* were emasculated about two days before anthesis. Crossings of 'Sonja'×*C. hederifolium*, *C. hederifolium*×'Sonja', *C. hederifolium*×'Sonja' and 'Salmon Scarlet'×*C. hederifolium* were performed immediately after emasculation. Although ovaries became enlarged after cross-pollination, they contained no viable seeds. From histological observation, embryos were found in the ovules 21 days after pollination, although no endosperms were formed around the embryos. Thereafter, the embryos aborted 35 days after pollination. Since embryos and ovules were too small to be excised without wounding, ovules with placenta were isolated from the ovary 21 to 28 days after pollination and used as an explant.

Initially 41, 61 and 55 explants derived from *C. persicum* ($2x$)×*C. hederifolium* ($2x$) were cultured on media described by Murashige and Skoog (1962), Nitsch (1951) and White (1963), respectively. All the media were supplemented with $30–100 \, \mathrm{g \, l^{-1}}$ sucrose, adjusted to pH 5.8, and solidified with $10 \, \mathrm{g \, l^{-1}}$ agar. Cultures were maintained at 25°C in the dark. As a result, a total of 249 plantlets were obtained on MS medium (Figure 1), whereas two plantlets were obtained on White medium, but none on Nitsch medium. No effect of the sucrose concentration was apparent. These results suggest that *in vitro* development of *Cyclamen* embryos is influenced more by the composition of inorganic chemicals than by sucrose concentration, and that the embryos may require ammonium ions for their development *in vitro*.

A further 27 explants derived from *C. persicum* ($4x$)×*C. hederifolium* ($2x$) were prepared 14 or 21 days after pollination and cultured on either MS3 or MS3CM10 at 25°C in the dark. As a result, 10 plantlets were obtained on MS3CM10, but none on MS3. This result suggests that embryos of *C. persicum* ($4x$)×*C. hederifolium* ($2x$) may have a different nutrient requirement from those of *C. persicum* ($2x$)×*C. hederifolium* ($2x$) (Ishizaka and Uematsu 1992).

Colchicine treatment of ovule cultures

Since ovule culture-derived plants derived from *C. persicum* ($2x$)×*C. hederifolium* ($2x$) were completely sterile, chromosome doubling by colchicine treatment of ovule cultures was examined as a method to overcome hybrid sterility. After 21 days of cross-pollination of *C. persicum* ($2x$)×*C. hederifolium* ($2x$), ovules with placenta were prepared from the ovary and subjected to culture. Forty-four explants were placed on MS3C500 for colchicine treatment and incubated at 25°C in the dark. After 10 or 15 days, they were transferred onto MS3CM10 and cultured at 25°C in the dark. As a result, 116 flowering plants were obtained from colchicine-treated ovule cultures, of which 38 were fertile (Ishizaka and Uematsu 1994).

Table 1. Chromosome number and horticultural characteristics of *Cyclamen* species

Species	Ploidy level and chromosome number ($2n$)	Dormant period[a]	Flowering period[a]	Flowering habit[b]	Useful characteristics for improving *C. persicum* cultivars	References
C. repandum	$2x=20$	May–Aug.	Mar.–Apr.	L	Cold hardiness	Greilhuber (1989), Legro (1959), Grey-Wilson (2002)
C. balearicum	$2x=20$	May–Aug.	Mar.–Apr.	L		Greilhuber (1989), Legro (1959), Grey-Wilson (2002)
C. peloponnesiacum	$2x=20$	May–Aug.	Mar.–Apr.	L		Grey-Wilson (2002)
C. creticum	$2x=20, 22$	May–Aug.	Mar.–Apr.	L		Greilhuber (1989), Legro (1959), Grey-Wilson (2002)
C. coum	$2x=30$	May–Sept.	Feb.–Mar.	L	Cold hardiness	Grey-Wilson (2002), Legro (1959)
C. elegans	$2x=30$	May–Sept.	Feb.–Mar.	L		Grey-Wilson (2002), Legro (1959)
C. alpinum	$2x=30$	May–Sept.	Feb.–Mar.	L	Cold hardiness	Grey-Wilson (2002), Legro (1959)
C. parviflorum	$2x=30$	Absent	Feb.	L	Cold hardiness	Grey-Wilson (2002), Legro (1959)
C. intaminatum	$2x=30$	May–Sept.	Oct.	L	Cold hardiness	Grey-Wilson (2002), Legro (1959)
C. cilicium	$2x=30$	May–Sept.	Oct.	L	Cold hardiness	Grey-Wilson (2002), Legro (1959)
C. mirabile	$2x=30$	May–Sept.	Oct.	L	Cold hardiness	Grey-Wilson (2002), Legro (1959)
C. libanoticum	$2x=30$	May–Sept.	Feb.–Mar.	L		Grey-Wilson (2002), Legro (1959)
C. cyprium	$2x=30$	May–Sept.	Jan.–Feb.	L		Grey-Wilson (2002), Legro (1959)
C. pseudibericum	$2x=30$	May–Sept.	Feb.- Mar.	L	Cold hardiness	Grey-Wilson (2002), Legro (1959)
C. colchicum	$2x=34$	Absent	Jul.–Oct.	L	Cold hardiness, Flower fragrance	Grey-Wilson (2002), Hravětová et al. (1983)
C. purpurascens	$2x=34$	Absent	Aug.–Mar.	L	Cold hardiness, Flower fragrance	Hravětová et al. (1983), Legro (1959)
C. purpurascens	$4x=68$	Absent	Aug.–Mar.	L	Cold hardiness, Flower fragrance	Ishizaka and Kondo (2004)
C. hederifolium	$2x=34$	May–Sept.	Oct.	F	Cold hardiness, Attractive leaves	Bennett and Grimshaw(1991), Hravětová et al.(1983)
C. hederifolium	$3x=51$	—[c]	—	—		Bennett and Grimshaw (1991)
C. hederifolium	$4x=68$	May–Sept.	Oct.	F	Cold hardiness, Attractive leaves	Bennett and Grimshaw (1991), Grey-Wilson (2002)
C. africanum	$2x=34$	May–Sept.	Oct.	F	Attractive leaves	Bennett and Grimshaw (1991)
C. africanum	$4x=68$	May–Sept.	Oct.	F	Attractive leaves	Bennett and Grimshaw (1991), Legro (1959)
C. persicum wild plant	$2x=48$	May–Sept.	Feb.–Mar.	L		Legro (1959), Pavliček et al. (2008)
C. persicum cultivar	$2x=48$	Absent	Oct.–Feb.	L		De Haan and Doorenbos (1951), Legro (1959)
C. persicum cultivar	$3x=72$	—	—	—		Takamura and Miyajima (1996a,b,c,1999)
C. persicum cultivar	$4x=96$	Absent	Oct.–Feb.	L		De Haan and Doorenbos (1951), Legro (1959)
C. persicum cultivar	$4x=90, 92, 94, 95$	—	—	—		Legro (1959)
C. somalense	—	—	—	—		Grey-Wilson (2002), Thulin and Warfa (1989)
C. graecum	$4x=84$	May–Sept.	Sep.–Oct.	F	Cold hardiness, Disease resistance	Ishizaka (1996)
C. rohlfsianum	$4x=96$	May–Sept.	Sep.–Oct.	F	Attractive leaves	Ishizaka et al. (2002a), Shibusawa and Ogawa (1997)
C. rohlfsianum	$6x=144$	May–Sept.	Sep.–Oct.	F	Attractive leaves	Ishizaka et al. (2002a)

[a] Dormant period and flowering period were examined in Saitama Prefecture, Japan.
[b] L, flowers appear after leaf appearance leaves; F, flowers appear before leaf appearance leaves.
[c] Not examined.

Cytological observation of ovule culture-derived plants

Ovule culture-derived plants of *C. persicum* ($2n=2x=48$)×*C. hederifolium* ($2n=2x=34$) had 41 somatic chromosomes consisting of 24 small chromosomes and 17 large ones (Figure 2A, B, C; Ishizaka and Uematsu 1992). The former probably originated from *C. persicum* and the latter from *C. hederifolium*. Thus, these plants were allodiploids ($2n=2x=41$), with the complete genomes of both parents. On the other hand, ovule culture-derived plants of *C. persicum* ($2n=4x=96$)×*C. hederifolium* ($2x$) had 64 somatic chromosomes consisting of 47 small chromosomes and 17 large ones. Since sesquidiploids (allotriploids, $2n=3x=65$) were expected to be obtained from this cross-combination, deletion of one small chromosome derived from *C. persicum* may have occurred in the hybrid with 64 chromosomes (Ishizaka and Uematsu 1992). Fertile
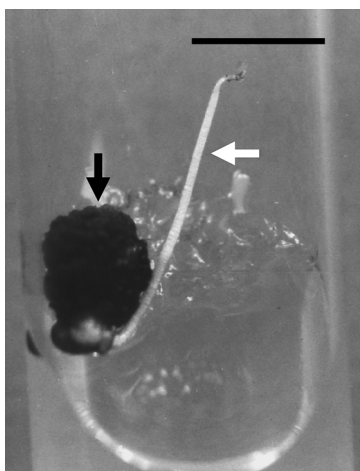
Figure 1. Culture of ovules derived from crosses between *Cyclamen persicum* (2*x*) and *C. hederifolium* (2*x*). Black and white arrows indicate ovules with placenta and a seedling germinated from the ovules, respectively. Bar＝1 cm

plants derived from colchicine-treated ovule cultures of *C. persicum* (2*x*)×*C. hederifolium* (2*x*) had 82 somatic chromosomes consisting of 48 small chromosomes and 34 large ones. These fertile plants were considered to be amphidiploids (allotetraploids) arising from chromosome doubling of the allodiploids (Figure 2D; Ishizaka and Uematsu 1994).

In *C. persicum* (2*x*) and *C. hederifolium* (2*x*), 24 small and 17 large bivalents were observed at diakinesis or metaphase-I in PMCs, respectively (Figure 2E, F). In the hybrid (allodiploid, 2*n*=2*x*=41), univalents and loosely paired bivalents were formed, resulting in production of sterile pollen (Figure 2G). In the amphidiploid (allotetraploid, 2*n*=4*x*=82), 41 bivalents consisting of 24 small bivalents and 17 large ones were observed, but no trivalents or quadrivalents were observed, suggesting that normal pairing occurred between homologous chromosomes induced by chromosome doubling of the allodiploid (Figure 2H). Bivalent formation in the amphidiploid resulted in normal microsporogenesis and production of fertile pollen. These results suggest a low degree of genomic affinity between *C. persicum* and *C. hederifolium*, and that these two species are distantly related in the genus *Cyclamen* (Ishizaka 1994).

Development of a commercial cultivar

In general, leaves appear at the flowering stage in *C. persicum* cultivars, whereas *C. hederifolium* produces flowers in late September before leaf appearance. One plant showing this unique trait of *C. hederifolium* was found in progenies of the amphidiploid of *C. hederifolium* (2*x*)×*C. persicum* 'Sonja' (2*x*). About 200 S1 progenies from this plant showed uniform characteristics with respect to flower color and size and leaf shape, and the uniformity was maintained in the S2 population. This strain has been registered as a
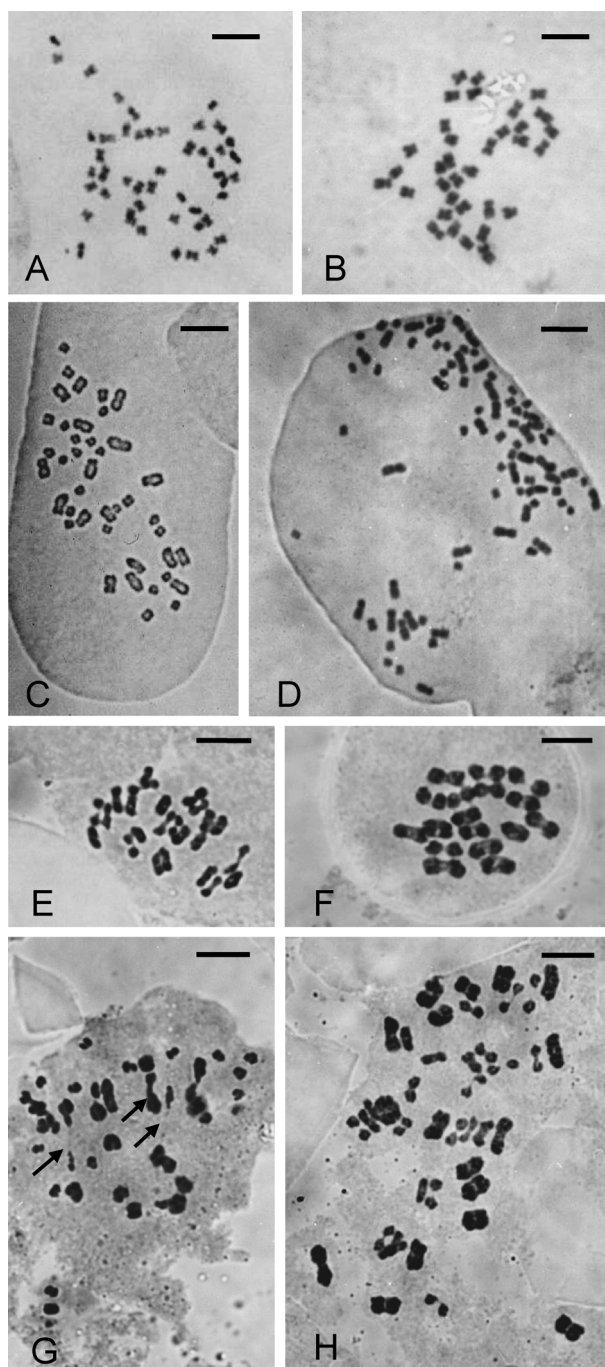

Figure 2. Somatic chromosomes in the root tip cells and chromosome pairing at metaphase-I of PMCs in *Cyclamen*. (A, B, C, D) Somatic chromosomes in, respectively, *C. persicum* (2*n*=2*x*=48), *C. hederifolium* (2*n*=2*x*=34), their hybrid (allodiploid 2*n*=2*x*=41) and an amphidiploid (allotetraploid, 2*n*=4*x*=82) derived from chromosome doubling of the allodiploid. (E, F, G, H) Chromosome pairing at metaphase-I of PMCs in, respectively, *C. persicum* (24 bivalents), *C. hederifolium* (17 bivalents), their hybrid (allodiploid, 35 univalents and 3 bivalents) and an amphidiploid (allotetraploid, 41 bivalents) derived from chromosome doubling of the hybrid. Arrows (G) indicate loosely paired bivalents. Bars＝5 *μ*m.

commercial cultivar named 'Tancho', which produces small flowers, about 3 cm in petal length and white in color with a deep purple eye like *C. persicum* 'Sonja',

Table 2. Interspecific hybridization between *Cyclamen persicum* cultivars and other wild species

Cross combination	Methods[a]	Fertility of hybrids	Chromosome number of hybrids ($2n$)	References
C. persicum ($2n=2x=48$)×*C. repandum* ($2n=2x=20$)	ER	Sterile	34	Ishizaka and Uematsu (1990)
C. persicum ($2n=2x=48$)×*C. libanoticum* ($2n=2x=30$)	ER	Sterile	39	Shibusawa and Ogawa (1997)
C. persicum ($2n=2x=48$)×*C. cyprium* ($2n=2x=30$)	ER	Sterile	39	Shibusawa (personal communication)
C. persicum ($2n=2x=48$)×*C. mirabile* ($2n=2x=30$)	ER	—[b]	—	Yamashita and Takamura (2007)
C. persicum ($2n=2x=48$)×*C. hederifolium* ($2n=2x=34$)	ER	Sterile	41	Ishizaka and Uematsu (1992)
C. persicum ($2n=2x=48$)×*C. hederifolium* ($2n=2x=34$)	ER, CD	Fertile	82	Ishizaka and Uematsu (1994)
C. hederifolium ($2n=2x=34$)×*C. persicum* ($2n=2x=48$)	ER	Sterile	41	Ishizaka and Uematsu (1994)
C. hederifolium ($2n=2x=34$)×*C. persicum* ($2n=2x=48$)	ER, CD	Fertile	82	Ishizaka and Uematsu (1994)
C. persicum ($2n=4x=96$)×*C. hederifolium* ($2n=2x=34$)	ER	Sterile	64	Ishizaka and Uematsu (1992)
C. persicum ($2n=4x=96$)×*C. hederifolium* ($2n=4x=68$)	ER	Sterile	—	Ishizaka (unpublished)
C. persicum ($2n=2x=48$)×*C. africanum* ($2n=2x=34$)	ER	Sterile	—	Takamura et al. (2002)
C. persicum ($2n=2x=48$)×*C. colchicum* ($2n=2x=34$)	ER	Sterile	—	Yamashita and Takamura 2007
C. persicum ($2n=2x=48$)×*C. purpurascens* ($2n=2x=34$)	ER	Sterile	41	Ishizaka and Uematsu (1995a), Ewald (1996)
C. persicum ($2n=2x=48$)×*C. purpurascens* ($2n=2x=34$)	ER, CD	Fertile	82	Ishizaka and Uematsu (1995b)
C. persicum ($2n=4x=96$)×*C. purpurascens* ($2n=2x=34$)	ER	Sterile	65	Ishizaka and Uematsu (1995a)
C. persicum ($2n=4x=96$)×*C. purpurascens* ($2n=4x=68$)	ER	Fertile	82	Ishizaka and Kondo (2004)
C. persicum ($2n=2x=48$)×*C. graecum* ($2n=4x=84$)	ER	Sterile	66	Ishizaka (1996, 2003)
C. persicum ($2n=4x=96$)×*C. graecum* ($2n=4x=84$)	ER	Fertile	90	Ishizaka (1996, 2003)
C. graecum ($2n=4x=84$)×*C. persicum* ($2n=4x=96$)	ER	Fertile	90	Ishizaka (unpublished)
C. persicum ($2n=2x=48$)×*C. rohlfsianum* ($2n=4x=96$)	ER	Sterile	—	Ishizaka (unpublished)
C. persicum ($2n=2x=48$)×*C. rohlfsianum* ($2n=6x=144$)	ER	Sterile	—	Ishizaka (unpublished)
C. persicum ($2n=4x=96$)×*C. rohlfsianum* ($2n=4x=96$)	ER	Fertile	—	Shibusawa and Ogawa (1997), Ishizaka (unpublished)
C. persicum ($2n=4x=96$)×*C. rohlfsianum* ($2n=6x=144$)	ER	Fertile	—	Ishizaka (unpublished)
C. rohlfsianum ($2n=6x=144$)×*C. persicum* ($2n=4x=96$)	ER	Fertile	—	Ishizaka (unpublished)

[a] ER, hybrids were produced by embryo rescue. CD, amphidiploids were produced by chromosome doubling.
[b] Not examined.

and shows a flowering habit similar to *C. hederifolium* (Table 2, Figure 3A).

Interspecific hybridization between C. persicum *cultivars and* C. purpurascens

Ovule culture

C. purpurascens ($2n=2x=34$) produces small flowers, about 2 cm in petal length and purple in color. This species has the most fragrant flowers among the 22 *Cyclamen* species. To introduce this attractive character into *C. persicum* cultivars, two diploid *C. persicum* cultivars ($2n=2x=48$), 'Schubert' and 'Pure White', and one autotetraploid *C. persicum* cultivar, 'Salmon Scarlet' ($2n=4x=96$), were crossed with *C. purpurascens*. Both 'Schubert' and 'Pure White' produce large flowers, about 5 cm in petal length. Flower color of 'Schubert' and 'Pure White' is pink with a purple eye and pure white, respectively. Flowers of three *C. persicum* cultivars were emasculated about two days before anthesis and crossed with pollen of *C. purpurascens* immediately after emasculation.

Histological observation indicated that embryos derived from interspecific crosses started to abort 35 days after pollination due to the lack of endosperms. Twenty-two ovaries were collected 28 days after pollination and ovules with placenta were cultured on

Figure 3. Interspecific hybrids in *Cyclamen*. (A) Commercial cultivar 'Tancho' developed from amphidiploids of *C. hederifolium* ($2x$)× *C. persicum* ($2x$). (B) Commercial cultivar 'Kaori-no-mai' developed from amphidiploids of *C. persicum* ($2x$)×*C. purpurascens* ($2x$). (C) Commercial cultivar 'Akifuyu-no-kisetsu' developed from amphidiploids of *C. persicum* ($4x$)×*C. graecum* ($4x$). (D) Interspecific hybrid of *C. persicum* ($4x$)×*C. rohlfsianum* ($6x$). Bars=6 cm.

MS3 at 25°C in the dark. As a result, 46 plantlets were obtained from *C. persicum* cultivars ($2x$ and $4x$)×*C. purpurascens* ($2x$), but the flowering plants developed

from these plantlets were sterile.

Colchicine treatment of ovule cultures

Chromosome doubling by colchicine treatment of ovule cultures was used to overcome the sterility of ovule culture-derived plants derived from *C. persicum* ($2x$)×*C. purpurascens* ($2x$). After 21 days of cross-pollination of *C. persicum* ($2x$) 'Schubert' and 'Pure White'×*C. purpurascens* ($2x$), 13 ovaries were collected and ovules with placenta were cultured on MS3C50 for colchicine treatment at 25°C in the dark. After 10 or 15 days, they were transferred onto MS3CM10 and cultured at 25°C in the dark. Two fertile hybrid plants were selected from seven flowering plants obtained from colchicine-treated ovule cultures (Ishizaka and Uematsu 1995a).

Cytological observation of ovule culture-derived plants

Ovule culture-derived plants of *C. persicum* ($2n=2x=48$)×*C. purpurascens* ($2n=2x=34$) had 41 somatic chromosomes, whereas those of *C. persicum* ($2n=4x=96$)×*C. purpurascens* ($2x$) had 65 somatic chromosomes. Based on the chromosome number and size, the former and the latter were considered to be allodiploids and sesquidiploids (allotriploids), respectively, both of which had the complete genomes of the parents. Fertile plants derived from colchicine-treated ovule cultures of *C. persicum* ($2x$)×*C. purpurascens* ($2x$) had 82 somatic chromosomes that corresponded to twice the 41 somatic chromosomes, indicating that they were amphidiploids (allotetraploids) (Ishizaka and Uematsu 1995a, b).

In *C. persicum* ($2x$) and *C. purpurascens* ($2x$), 24 and 17 bivalents were observed at diakinesis or metaphase-I in PMCs, respectively. Many univalents and loosely paired bivalents were observed in the hybrids (allodiploid, $2n=2x=41$) of *C. persicum* ($2x$)×*C. purpurascens* ($2x$), whereas 41 bivalents were frequently observed in the amphidiploid (allotetraploid, $2n=4x=82$) induced by chromosome doubling of the allodiploid, but trivalents and quadrivalents were not observed. In the sesquidiploid (allotriploid, $2n=3x=65$) of *C. persicum* ($4x$)×*C. purpurascens* ($2x$), 24 bivalents and 17 univalents were frequently observed, but not trivalents. These results suggest a low degree of genomic affinity between *C. persicum* and *C. purpurascens*, and these two species are considered to be distantly related in the genus *Cyclamen*.

Development of commercial cultivars

Volatile compounds in flowers of *C. persicum* 'Schubert' and 'Salmon Scarlet', *C. purpurascens* ($2x$), and their hybrids were analyzed using gas chromatography coupled with mass spectrometry. The chemical components of fragrance of *C. persicum* cultivars were sesquiterpene hydrocarbons, whereas those of *C.* *purpurascens* were alcohols, aldehydes and esters of monoterpene or aromatic classes. The chemical compositions of fragrance of the hybrid and amphidiploid were similar to that of *C. purpurascens*. By contrast, the chemical composition of fragrance of the sesquidiploid was similar to that of *C. persicum* cultivars. Flowers of *C. persicum* cultivars have a woody or powdery fragrance, whereas those of *C. purpurascens* have a rose- or hyacinth-like fragrance. Flowers of the hybrid (allodiploid) and the amphidiploid (allotetraploid) had a similar fragrance to that of *C. purpurascens*, whereas flowers of the sesquidiploid (allotriploid) had a similar fragrance to that of *C. persicum* cultivars. These results indicate that genome constitution affects the fragrance of flowers of the interspecific hybrids (Ishizaka et al. 2002b).

Two types of amphidiploids, one with pink flowers with a purple eye and the other with pale pink flowers with a purple eye, were crossed reciprocally and F1 plants were obtained by the conventional methods. Promising progenies were selected from the F2 population, and thereafter three strains were established in F5 by a pedigree breeding method. These three strains have been registered as commercial cultivars named 'Kokou-no-kaori' (light purple flowers with a purple eye), 'Uruwashi-no-kaori' (pink flowers with a deep purple eye) and 'Kaori-no-mai' (purple flowers with a deep purple eye) (Figure 3B). Flowers of these three cultivars are about 4 cm in petal length and are fragrant.

Interspecific hybridization between C. persicum *cultivars and* C. graecum

Ovule culture

C. graecum ($2n=4x=84$) is a valuable gene source for improving disease resistance and cold hardiness in cyclamen cultivars, although this species has small flowers, about 2 cm in petal length and pale pink in color. Flowers of *C. persicum* 'Pure White' ($2n=2x=48$), *C. persicum* 'Salmon Scarlet' ($2n=4x=96$) and *C. graecum* were emasculated about two days before anthesis. Crossings of 'Pure White'×*C. graecum*, 'Salmon Scarlet'×*C. graecum* and *C. graecum*×'Salmon Scarlet' were performed immediately after emasculation. According to histological observation, embryos were found in the ovules 21, 28 and 35 days after pollination, although no endosperms were formed around the embryos. Thereafter, the embryos aborted 42 days after pollination.

Seven ovaries were collected 35 days after pollination, and ovules with placenta isolated from the ovaries were cultured on MS3 or MSCM10 at 25°C in the dark. Four plantlets were obtained on both media from 'Pure White'×*C. graecum*. On the other hand, in 'Salmon Scarlet'×*C. graecum*, three plantlets were obtained on

MS3CM10 but not on MS3. Flowering plants of 'Pure White'×*C. graecum* were sterile, whereas those of 'Salmon Scarlet'×*C. graecum* were fertile (Ishizaka 1996).

Cytological observation of ovule culture-derived plants

Ovule culture-derived plants of *C. persicum* ($2n=2x=48$)×*C. graecum* ($2n=4x=84$) had 66 somatic chromosomes, and those of *C. persicum* ($2n=4x=96$)×*C. graecum* had 90 somatic chromosomes. Based on the chromosome number, both progenies were considered to be interspecific hybrids.

Diploid *C. persicum* generally formed 24 bivalents at diakinesis or metaphase-I in PMCs, while both autotetraploid *C. persicum* and *C. graecum* formed quadrivalents as well as bivalents. Hybrids of *C. persicum* ($2x$)×*C. graecum* frequently formed 21 bivalents and 24 univalents, but did not form trivalents. These hybrids showed low pollen fertility. In contrast, hybrids of *C. persicum* ($4x$)×*C. graecum* frequently formed 45 bivalents, resulting in the production of fertile pollen. Thus, *C. graecum* was considered to be an autotetraploid. In addition, the hybrids ($2n=90$) were considered to be amphidiploids (allotetraploids), and the hybrids ($2n=3x=66$) were considered to be sesquidiploids (allotriploids) (Ishizaka 2003).

As shown in the interspecific hybridization studies described above, one useful strategy for clarifying the genomic affinity between two species is to examine the frequency of chromosome pairing at diakinesis or metaphase-I in PMCs of the F1 hybrids produced between diploid plants. However, no diploid plants of *C. graecum* have yet been discovered. Therefore, we produced polyhaploids ($2n=2x=45$) containing two genomes each originating from *C. persicum* and *C. graecum* by anther culture of the amphidiploids ($2n=4x=90$) and examined their meiosis. The polyhaploids formed univalents and loosely paired bivalents, resulting in the production of sterile pollen. These results suggest a low degree of genomic affinity between *C. persicum* and *C. graecum*. Thus, these two species are distantly related in the genus *Cyclamen* (Ishizaka 2002, 2003).

Characterization of amphidiploids and development of a commercial cultivar

Amphidiploids thus produced had the nature to rest spontaneously in the summer under high temperatures, and those released from dormancy produced flowers in October after the emergence of foliage. In the winter in Saitama Prefecture, cultivars of *C. persicum* left outdoors withered due to frost and freezing injuries, whereas the amphidiploids survived. Furthermore, the amphidiploids were resistant to diseases caused by fusarium wilt (*Fusarium oxsporum* f. sp. *cyclaminis*), bacterial leaf rot (*Erwinia herbicola* pv. *cyclamenae*) and bacterial bud rot (*Pseudomonas marginalis* pv. *marginalis*). The amphidiploids also showed a high ability to cross with diploid and autotetraploid cultivars of *C. persicum*. Therefore, amphidiploids with useful traits derived from *C. graecum* are considered to be useful breeding material for introducing these traits into *C. persicum* cultivars. One strain producing mid-sized flowers, about 4 cm in petal length and pale pink in color with a purple eye, has been selected from amphidiploid progeny and registered as a commercial cultivar named 'Akifuyu-no-kisetsu' (Figure 3C). This cultivar may be usable as a bedding plant as well as breeding material.

Interspecific hybridization between diploid cultivars of C. persicum *and diploid wild species*

Besides the above-mentioned cross combinations, interspecific hybrids (allodiploids) have been produced by embryo rescue between diploid cultivars of *C. persicum* ($2n=2x=48$) and the following diploid wild species: *C. repandum* ($2n=2x=20$), *C. libanoticum* ($2n=2x=30$), *C. cyprium* ($2n=2x=30$), *C. mirabile* ($2n=2x=30$), *C. colchicum* ($2n=2x=34$) and *C. africanum* ($2n=2x=34$) (Table 2). These hybrids may contain one genome originating from diploid cultivars of *C. persicum* and one genome from diploid wild species. Although these hybrids were sterile, sterility may be overcome by amphidiploidization of these allodiploids as described above, and the genomic affinity between *C. persicum* and these species may be clarified by observing chromosome pairing at diakinesis or metaphase-I in PMCs of these allodiploids.

Interspecific hybridization between autotetraploid cultivars of C. persicum *and polyploid wild species*

Autotetraploid cultivars of *C. persicum* ($2n=4x=96$) have unique characteristics, such as large, striped, fringed, doubled and/or 'Victoria'-formed flowers, and have played an important role in the development of cyclamen production. Recently, demand for diploid F1 hybrid cultivars with high uniformity and for diploid cultivars with small flowers, the so-called 'mini cyclamen', has increased. Diploid cultivars with characteristics similar to autotetraploid cultivars have been produced by conventional cross breeding or by anther culture of autotetraploids, as reported by Ishizaka and Uematsu (1993). These trends have led to a decline in the production of autotetraploid cultivars.

On the other hand, autotetraploid cultivars of *C. persicum* are useful as a parent for interspecific

hybridization with autotetraploid wild species, from which fertile amphidiploids (allotetraploids) can be obtained without chromosome doubling as described above. Fertile amphidiploids have also been produced by interspecific hybridization between an autotetraploid cultivar of *C. persicum* and an autotetraploid wild species, *C. rohlfsianum* (Shibusawa and Ogawa 1997). Therefore, autotetraploid cultivars of *C. persicum* are indispensable for further production of fertile amphidiploids with autotetraploid wild species as well as chromosome-doubled diploid wild species.

In *C. rohlfsianum*, autohexaploid wild plants ($2n=6x=144$) have also been found and successfully used as a male parent to produce interspecific hybrids with autotetraploid cultivars of *C. persicum* (Figure 3D). Although these hybrids were considered to be allopentaploids, they produced viable seeds by self-pollination. The mechanism of fertility remains to be clarified.

Since no autotetraploid plant is found in *C. purpurascens*, ovules 14 days after self-pollination of *C. purpurascens* ($2n=2x=34$) were treated with colchicine as described above for producing amphidiploids of *C. persicum* ($2x$)×*C. hederifolium* ($2x$). Autotetraploids of *C. purpurascens* ($2n=4x=68$) thus obtained were successfully used to produce fertile amphidiploids (allotetraploids, $2n=4x=82$) with autotetraploid culti-vars of *C. persicum* ($2n=4x=96$) by embryo rescue. The amphidiploids produced striped or 'Victoria'-formed flowers, which had fragrance with volatile compounds similar to those of *C. purpurascens* ($4x$) (Ishizaka and Kondo 2004).

In *C. hederifolium*, both diploid and autotetraploid wild plants ($2n=2x=34$, $4x=68$) are found, and the latter are classified as *C. hederifolium* var. *confusum* (Grey-Wilson 2002). Although interspecific crossing between autotetraploid cultivars of *C. persicum* and autotetraploid *C. hederifolium* was performed for producing fertile amphidiploids (allotetraploids), hybrids obtained by embryo rescue were sterile. Cytological studies should clarify the cause of sterility.

Conclusions

As described in this mini-review, embryo rescue and *in vitro* chromosome doubling are simple and useful methods for overcoming hybrid inviability and hybrid sterility in interspecific hybridization between *C. persicum* cultivars and wild *Cyclamen* species. Interspecific hybrids with various genome constitutions have been obtained by applying these methods and introduced into the gene pool as novel available germplasms of *Cyclamen*. Thus, *Cyclamen* breeding, which had previously been targeted only *C. persicum*, will enter a new phase by exploiting interspecific hybridization.

References

Bennett ST, Grimshaw JM (1991) Cytological studies in *Cyclamen* subg. *Cyclamen* (Primulaceae). *Plant Syst Evol* 176: 135–143

De Haan I, Doorenbos J (1951) The cytology of the genus *Cyclamen*. *Meded Landbouwhogeschool Wageningen* 51: 151–166

Ewald A (1996) Interspecific hybridization between *Cyclamen persicum* Mill. and *C. purpurascens* Mill. *Plant Breed* 115: 162–166

Greilhuber J (1989) Karyotype structure and evolution in *Cyclamen* L. subgen. *Psilanthum* Schwz. (Primulaceae). *Flora* 183: 103–113

Grey-Wilson C (2002) *Cyclamen* (*A guide for gardeners, horticulturists and botanists*). B.T. Bastsford, England.

Hravětově A, Dvořák F, Dadáková B (1983) Chromosome morphology of three species of the genus *Cyclamen* L. *Biológia* (*Bratislava*) 38: 401–409

Ishizaka H (1994) Chromosome association and fertility in the hybrid of *Cyclamen persicum* Mill. and *C. hederifolium* Aiton and its amphidiploid. *Breed Sci* 44: 367–371

Ishizaka H (1996) Interspecific hybrids of *Cyclamen persicum* and *C. graecum*. *Euphytica* 91: 109–117

Ishizaka H (2002) Characteristics of polyhaploid derived by anther culture of an amphidiploid of *Cyclamen persicum* ($4x$)×*C. graecum* ($4x$). *J Japan Soc Hort Sci* 71 (suppl 1): 356 (in Japanese)

Ishizaka H (2003) Cytogenetic studies in *Cyclamen persicum*, *C. graecum* (*Primulaceae*) and their hybrids. *Plant Syst Evol* 239: 1–14

Ishizaka H, Kondo E (2004) Production of amphidiploid of tetraploid *Cyclamen persicum* and tetraploid *C. purpurascens* by ovule culture. *J Japan Soc Hort Sci* 73 (suppl 2): 207 (in Japanese)

Ishizaka H, Uematsu J (1990) Production of interspecific hybrids between *Cyclamen persicum* Mill. and *C. repandum* Sibth. Sm. through ovule culture. *Japan J Breed* 40 (suppl 1): 60–61 (in Japanese)

Ishizaka H, Uematsu J (1992) Production of interspecific hybrids of *Cyclamen persicum* Mill. and *C. hederifolium* Aiton by ovule culture. *Japan J Breed* 42: 353–366

Ishizaka H, Uematsu J (1993) Production of plants from pollen in *Cyclamen persicum* Mill. through anther culture. *Japan J Breed* 43: 207–218

Ishizaka H, Uematsu J (1994) Amphidiploids between *Cyclamen persicum* Mill. and *C. hederifolium* Aiton induced through colchicine treatment of ovules *in vitro* and plants. *Breed Sci* 44: 161–166

Ishizaka H, Uematsu J (1995a) Interspecific hybrids of *Cyclamen persicum* Mill. and *C. purpurascens* Mill. produced by ovule culture. *Euphytica* 82: 31–37

Ishizaka H, Uematsu J (1995b) Amphidiploids between *Cyclamen*

persicum Mill. and *C. purpurascens* Mill. induced by treating ovules with colchicine *in vitro* and sesquidiploids between the amphidiploid and the parental species induced by conventional crosses. *Euphytica* 86: 211–218

Ishizaka H, Oku T, Mishiba K, Mii M (2002a) Ploidy levels of *Cyclamen rohlfsianum*. *Breed Res* 4 (suppl 1): 150 (in Japanese)

Ishizaka H, Yamada H, Sasaki K (2002b) Volatile compounds in the flowers of *Cyclamen persicum*, *C. purpurascens* and their hybrids. *Sci Hort* 94: 125–135

Khush GS, Brar DS (1992) Overcoming the barriers in hybridization. In: Kallo G, Chowdhury JB (eds) *Distant Hybridization of Crop Plants*. Springer-Verlag, Berlin, pp 47–61.

Legro RAH (1959) The cytological background of cyclamen breeding. *Meded Landbouwhogeschool Wageningen* 59: 1–51

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

Nitsch JP (1951) Growth and development *in vitro* of excised ovaries. *Amer J Bot* 38: 566–577

Pavlíček T, Bureš P, Horová L, Raskina O, Nevo E (2008) Genome size microscale divergence of *Cyclamen persicum* in evolution canyon, Israel. *Cent Eur J Biol* 3: 83–90

Shibusawa N, Ogawa K (1997) Production of interspecific hybrids between *Cyclamen persicum* Mill. and *C. rohlfsianum* Aschers. or *C. persicum* and *C. libanoticum* Hildebr. by ovule culture. *Bull Tokyo Met Agri Exp Sta* 27: 9–15 (in Japanese with English summary)

Singh RJ (1993) *Plant Cytogenetics*. CRC Press, Boca Raton, pp 255–283

Takamura T (2007) Cyclamen. Cyclamen persicum Mill. In: Anderson NO (ed) *Flower Breeding and Genetics*. Springer, Netherlands, pp 459–478

Takamura T, Miyajima I (1996a) Cross-compatibility and ploidy of progenies in crosses between diploid and tetraploid cyclamen (*Cyclamen persicum* Mill.). *J Japan Soc Hort Sci* 64: 883–889

Takamura T, Miyajima I (1996b) Embryo development in crosses between diploid and tetraploid cyclamen (*Cyclamen persicum* Mill.). *J Japan Soc Hort Sci* 65: 113–120

Takamura T, Miyajima I (1996c) Production of the inter-ploidy hybrids of diploid and tetraploid cyclamen by ovule culture. *Japan J Breed* 46 (suppl 1): 231 (in Japanese)

Takamura T, Miyajima I (1999) Varietal and individual differences in crosses-compatibility in the $2x \times 4x$ crosses of cyclamen (*Cyclamen persicum* Mill.). *J Japan Soc Hort Sci* 65: 113–120

Takamura T, Matsumoto Y, Yoshimura N, Tanaka M (2002) Effects of nitrogen source in medium on the production of interspecific hybrid between cyclamen and *C. africanum*. *J Japan Soc Hort Sci* 71 (suppl 1): 284 (in Japanese)

Thulin M, Warfa AM (1989) *Cyclamen* (Primulaceae) in tropical Africa. *Plant Syst Evol* 166: 249–252

White PR (1963) The Cultivation of Animal and Plant Cells. Roland Press, New York, p 228

Yamashita H, Takamura T (2007) Production of interspecific hybrids between *Cyclamen persicum* and *C. colchicum*, or *C. persicum* and *C. mirabile*. *Hort Res* (*Japan*) 6 (suppl 2): 587 (in Japanese)