12

Breeding of Interspecific Hybridization Among Carnation (*Dianthus caryophyllus* L.), *Dianthus japonicus* Thunb., and *Dianthus* × *isensis* Hirahata *et* Kitam.

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Abstract

Interspecific hybridization is one of the most important strategies used for creating variations in ornamental plants because it has the potential to combine useful traits, such as favorable morphology, disease resistance, and some environmental tolerances, of both parents that could not be achieved by crossing within a single species. Because carnation (*Dianthus caryophyllus* L.) cannot tolerate hot and humid climates (e.g., hot summers in Japan), the quality and yield of cut flowers decrease under these conditions. Therefore, for stable and quality carnation production in Japan and elsewhere, it is important to breed cultivars with heat tolerance and early flowering. Herein, two indigenous species to Japan, *Dianthus japonicus* Thunb. and *Dianthus* × *isensis* Hirahata *et* Kitam., were cross-hybridized with carnation to introduce useful traits of these two species into carnation. On reciprocal interspecific hybridization between carnation and *D. japonicus*, hybrid plants were obtained only when carnation was used as the seed parent. Although hybrid plants rarely obtained were sterile, they successfully restored fertility by producing amphidiploids through artificial chromosome-doubling treatment. When reciprocal interspecific crosses were employed between carnation and *D.* × *isensis*, fertile hybrid plants were obtained in both cross-directions. Because these two types of interspecific hybrids exhibited useful traits of the donor species, they will be efficiently used as valuable germplasm for further carnation breeding.

Abbreviations

CC	Genome constitution of *Dianthus caryophyllus* L.
JJ	Genome constitution of *Dianthus japonicus* Thunb.
II	Genome constitution of *Dianthus* × *isensis* Hirahata *et* Kitam.

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12.1 Introduction

Carnation (*Dianthus caryophyllus* L.) is one of the major floricultural crops in Japan and worldwide. It is a member of the family Caryophyllaceae and belongs to the genus *Dianthus*. The original species of *D. caryophyllus* L. was probably native to Mediterranean areas, and most of the carnation cultivars have been bred in Europe and the USA, which have a relatively cool climate.

As a result, most cultivars do not have tolerance to hot and humid climate and tend to grow unfavorably in the hot, humid summer of Japan, which results in various disease problems and production of lower-quality cut flowers. To increase the yield and quality of cut flowers, carnation should be cultivated to produce flowers before the hot summer arrives. To circumvent these problems, and to achieve more stable carnation production in Japan, it is important to breed cultivars with both heat tolerance and early flowering traits.

In plant breeding, interspecific hybrids have been produced among various species for the creation of novel genetic variability in the crops or for introgression of agriculturally useful traits from alien species into cultivated species. However, interspecific hybrid formation between distantly related species is commonly difficult because of cross incompatibility expressed as inhibition of pollen tube growth, failure of fertilization, abortion of early hybrid embryo, production of sterile seed, and lethal hybrid formation at the seedling stage. The embryo rescue technique using in vitro culture is an excellent tool for surmounting several lethal stages. With this technique, various cultivars have been produced via wide hybridization in several ornamental crops such as lilies (Asano 1980, 1982, 1984).

Interspecific hybridization is one of the most important strategies used for producing variations in ornamental crop breeding. Interspecific hybrids have the potential to show hybrid vigor and to combine the traits that in general do not occur within a single species (Volker and Orme 1988). Moreover, interspecific or intergeneric hybrids have the enormous potential to extend not only their qualitative but also their quantitative traits, such as the type of flower, plant morphology, environmental adaptation abilities, and flower vase life. Although natural hybrids may occasionally be produced between species whose flowering time overlaps, pre- and post- fertilization barriers hinder the formation of these hybrids.

There have been several reports on interspecific hybridization in the genus *Dianthus*, such as crosses of carnation with *Dianthus superbus* L. (Kanda 1992), crosses among 22 species of *Dianthus* (Ohtsuka et al. 1995), acquisition of hybrids between carnation and *Dianthus capitatus* Bald. Ex DC. (Onozaki et al. 1998), somatic hybridization through protoplast fusion between *Dianthus chinensis* L. and *Dianthus barbatus* L. (Nakano and Mii 1993a), and between carnation and *D. chinensis* (Nakano and Mii 1993b). However, only a few trials that focus on introducing the superior traits of other *Dianthus* species into carnation have so far been conducted.

Dianthus japonicus Thunb., a perennial species native to Japan, is characterized by easy seed propagation, a fasciculate cyme with many small flowers, an upright robust stem, broad and thick evergreen foliage with a developed cuticle, and high heat tolerance (Ito et al. 1994). It is also resistant to carnation bacterial wilt (Kagito and Tsuchiya 1968). Because of these valuable characteristics, *D. japonicus* is now expected to be used as breeding material for carnation, despite the fact that it has a different morphology and ecology from carnation, as well as several characteristics—such as easy seed set, season flowering (midsummer to autumn flowering), and single type of flower—that are considered to be undesirable for carnation breeding.

Dianthus × *isensis* Hirahata *et* Kitam., one of the classical, floricultural crops in Japan, is considered to be a hybrid between *D. chinensis* L. and *D. superbus* var. *longicalycinus* (Maxim.) F.N. Williams produced in the nineteenth century (Ito et al. 1994). *D.* × *isensis* is characterized by a unique flower shape, with curled, hung-down fimbriate petals, a narrow leaf lamina with a gray-green or bright-green color, a narrow stem nearly 30 cm in height, and a branching habit at the base of the stem. In addition, it has very early and perpetual flowering traits and is easy to reproduce via seed and vegetative propagation (Ito et al. 1994).

12.2 Carnation × *D. Japonicus*

Reciprocal interspecific crosses were carried out as 12 combinations between six lines of carnation and one line of *D. japonicus* (Nimura et al. 2003). When six lines of carnation were used as maternal parents, the interspecific hybrids were obtained only when one line of carnation, '98sp1651' was used as the seed parent, whereas seedlings obtained from other combinations yielded maternal-type progeny. One possible reason for this difference in hybrid productivity in reciprocal interspecific crosses might be that '98sp1651' has an effective genetic background for producing the hybrids. When the other carnation lines were used as maternal parents, most ovules died within 3 weeks after pollination. To rescue the abortive embryos, embryo culture was also carried out before the death of ovules in the crosses with some carnation lines as maternal parents, and the obtained putative interspecific hybrids were evaluated by morphological characters. Therefore, it is possible that the development of hybrid embryos was inhibited by some physiological factors possessed by most carnation lines used as maternal parents.

Another possible reason for the difference in hybrid production in the reciprocal interspecific crosses might be that the combination between the hybrid nuclear genome and '98sp1651' cytoplasmic genome allowed for the survival of the hybrid embryo or for the development of endosperm. In the present study, it was considered that nuclear–cytoplasmic interaction might result in the stunted development of the endosperm when *D. japonicus* was used as the maternal parent. In reciprocal interspecific crosses, direction of the crossing alters the genome composition of endosperm in the hybrid seeds because of the fusion between two genomes derived from the seed parent and one genome from the pollen parent at the double fertilization event. It is also possible that *D. japonicus* does not support the growth of hybrid embryo nutritionally when it is used as the maternal parent. In these two possible cases, it might be possible to produce interspecific hybrids by examining the timing of hybrid embryo rescue culture and/or culture medium constitution. However, if abortion of the hybrid embryo is caused by the influence of the cytoplasmic gene, it might be extremely difficult to obtain a hybrid.

The nuclear DNA content of the parental plants determined using flow cytometry were 1.48 pg in carnation and 2.57 pg in *D. japonicus*, and *D. japonicus* had about 1.7 times larger DNA content than carnation. Because the hybrid was expected to have an intermediate DNA content of the parents, the value of DNA content was used as an indicator for detecting the true intermediate hybrids. Consequently, 12 out of 25 progenies obtained from the cross of carnation line '98sp1651' with pollen of *D. japonicus* had an expected DNA content of 2.03 pg, which was intermediate to the parents. However, the DNA contents of 13 other progenies and a total of 167 progenies derived from other carnation lines used as the seed parent were 1.48 pg, which was the same as that of carnation. On the other hand, the DNA contents of six progenies obtained by culturing 2380 ovules, derived from the crosses using *D. japonicus* as a seed parent, showed the same DNA content as *D. japonicus* (Table 12.1).

Random amplified polymorphic DNA (RAPD) analysis was conducted on the putative hybrids to detect the specific marker of *D. japonicus* used as the pollen parent. All of the 12 progenies obtained from the carnation line '98sp1651,' which had already shown an intermediate DNA content of the two parental species, had specific bands from pollen parent *D. japonicus* 'HAMA 1.' These DNA fragments were not detected in the 13 progenies that had not shown the hybrid nature by flow cytometry.

In the present study, approximately half of the progenies obtained from '98sp1651' and all those

Table 12.1 Flow cytometric analysis of the DNA content of progenies in reciprocal interspecific crosses between *Dianthus caryophyllus* and *D. japonicus*

Female	Male	No. of progenies	DNA content		
			caryophyllus-like	Intermediate	*japonicus*-like
D. japonicus × *D. caryophyllus*					
HAMA1	98sp1441	4	0	0	4
	98sp1651	–	–	–	–
	98sp1960	–	–	–	–
	98spLPB	–	–	–	–
	99sp367-11	–	–	–	–
	99sp594-16	2	0	0	2
D. caryophyllus × *D. japonicus*					
98sp1441	HAMA1	104	104	0	0
98sp1651		25	13	12	0
98sp1960		3	3	0	0
98spLPB		4	4	0	0
99sp367-11		53	53	0	0
99sp594-16		3	3	0	0

obtained from the other lines of carnation used as the maternal parents showed very similar morphologies to maternal carnation plants that showed no evidence of hybrid nature by DNA analysis. However, some of the carnation-like progenies showed stunted growth or death, and others showed different color and morphology of flowers from those of the mother carnation plants. In barley (*Hordeum vulgare*), it is known that haploid is efficiently produced by crossing with *Hordeum bulbosum*, a wild relative species, because of the selective elimination of *H. bulbosum* chromosomes at the early stage of embryo development (Kasha and Kao 1970). Furthermore, it has been reported in carnation that doubled haploids were obtained by inducing pseudogamy via pollinating with X-ray-irradiated pollen (Sato et al. 2000). These results may suggest that the carnation-like progenies obtained in the present study are doubled haploid. Because cross-fertilization is general in carnation, it is possible that recessive lethal or unfavorable genes heterozygously possessed by carnation were expressed in these doubled haploids, which resulted in the death or growth depression of plants. Because progenies from '98sp1651' showed a segregation ratio of approximately 1 hybrid: 1 carnation-like plant, hybrid-type progenies were considered to be produced by the effect of a few genes that act to prevent the elimination of alien chromosomes, that is, those of *D. japonicus*. However, these possibilities need to be clarified in further studies.

All 12 interspecific hybrids obtained from the cross using '98sp1651' had an intermediate flower size between carnation and *D. japonicus* and a uniform flower color of reddish purple. The hybrids also had a *D. japonicus*-like fasciculate cymose inflorescence but had fewer flowers, like carnation. The shape of their leaves rather resembled that of carnation, but was broader. Most of the hybrids that were planted in late January flowered from June 10 to July 17. Among the 12 hybrids, 7 had double flowers and 5 showed single flowers. Although three out of five single-flowered hybrids had fully developed anthers, they did not dehisce and contained only a few pollen grains, which had an abnormal shape. When carnation or the hybrid plants were pollinated with these abnormal pollen grains, no

Fig. 12.1 Characteristics of the hybrids obtained from the cross between *D. caryophyllus* and *D. japonicus*. **a** Flowering plant of *D. caryophyllus* '98sp1651' (left), hybrid '43B' (center), and *D. japonicas* 'HAMA1' (right). **b** Flower variations in the interspecific hybrids

seed was produced. On the other hand, the remaining two single-flowered hybrids as well as seven double-flowered hybrids had no anthers (Fig. 12.1). In cutting propagation using lateral buds, all 12 hybrids showed high-rooting ability compared with carnation. All hybrids had stronger stems and showed more vigorous growth compared with carnation in the summer season.

The six progenies obtained by ovule culture of *D. japonicus* × *D. caryophyllus* grew normally and showed almost the same morphology as *D. japonicus* in terms of leaf shape, which is round, wide, and thick. These results supported those obtained on nuclear DNA contents using flow cytometry (Table 12.1).

However, the hybrids had several undesirable characters such as fewer flowers per stem and segregation of single-flowered individuals. To remove these undesirable characters, it is necessary to backcross the hybrids with carnation. By culturing more immature embryos, it might be possible to obtain more hybrids from various genotypes of carnation as the maternal parents.

It is difficult to use the hybrids obtained in this study as the pollen parent or as the seed parent for further breeding because of complete sterility. To restore fertility, it is necessary to produce an amphidiploid using artificial chromosome-doubling. After amphidiploids have been successfully produced, they will be efficiently used for introducing desirable characters of *D. japonicus*—such as broad leaf, strong stem, heat tolerance, and disease resistance—into carnation.

12.3 Artificial Chromosome-Doubling and Fertile Amphidiploid Induction

Both greenhouse and in vitro-grown plants of one hybrid line between carnation and *D. japonicus*, '47B,' which displayed a double flower, were used for the artificial chromosome-doubling treatment with colchicine or amiprophos-methyl (APM) (Nimura et al. 2006b). Colchicine dropping treatments onto the shoot tips of the greenhouse-grown interspecific hybrid plants yielded one amphidiploid (4.7%) at a concentration of 2000 mgL^{-1} for 1 day, and two amphidiploids (6.6%) at a concentration of 1000 mgL^{-1} for 5 days. Moreover, one octoploid hybrid (4.7%) was induced via treatment with 2000 mgL^{-1} for 1 day. The survival rate of the shoot tips after the colchicine treatment ranged from 40 to 70% depending on the concentration and the duration of the treatment (Table 12.2). In general, a higher concentration and longer duration of treatment reduced the survival of shoot tips, which resulted in failure to induce shoots at the node. Moreover, a higher concentration and longer duration of treatment increased the number of mixoploid shoots consisting of diploid shoots with tetraploid or hyperploid cells as well as tetraploid ones with octoploid cells.

In vitro shaking culture of the hybrid shoots led to the production of one tetraploid (5%) via the application of APM at 5–10 mgL^{-1}. With APM treatment, the survival rate of hybrid shoots was 100% at 5 and 10 mgL^{-1} but this figure drastically reduced to 20% at 20 mgL^{-1}, whereas those with colchicine treatment were 67% at 500 mgL^{-1} and 30% at 1000 mgL^{-1}. Many mixoploids were obtained by in vitro shaking culture compared with the dropping treatment of intact plants grown in the greenhouse (Tables 12.2 and 12.3). Three months after the chromosome-doubling treatment, the plants obtained from axillary buds were classified into six types according to the ploidy level (as shown in Fig. 12.2): diploid (**a**), tetraploid (**d**), octoploid (**f**) and mixoploids (**b**, **c**, **e**). Results of the flow cytometric analysis conducted after 1 year showed that all mixoploids showing the **b** type (73 plants) and **c** type (11 plants) reverted to diploid plants. The plants considered to be tetraploid (9 plants) or mixoploid showing the **e** type pattern (4 plants) were confirmed to be tetraploid (13 plants), whereas those considered to be diploid and octoploid in the first year did not show any change in ploidy levels.

Table 12.2 Effects of colchicine dropping treatment for apical meristem on the survival rate and induction of polyploids in F1 hybrid of *Dianthus caryophyllus* × *D. japonicus*

Treatment		No. of shoots treated	No. of shoots that survived (%)[a]	DNA C-values (min – max)					
Concentration (mg L^{-1})	Duration (days)			Diploid	Tetraploid	Octoploid	Mixoploid		
				2C-4C	4C-8C	8C-16C	2C-8C	2C-16C	4C-16C
0	1	20	20 (100)	20					
500	1	20	14 (70)	12			2		
1000	1	17	10 (59)	6			4		
2000	1	21	13 (62)	4	1	1	4	1	2
0	5	20	20 (100)	20					
500	5	28	19 (68)	7			12		
1000	5	30	14 (47)	2	2		8	2	
2000	5	32	13 (40)	2	3		5	3	

[a] Recorded at 3 months after treatment

Table 12.3 Effect of in vitro colchicine or amiprophos-methyl treatment for 24 h on the survival rate and induction of polyploids in F1 hybrid of *Dianthus caryophyllus* × *D. japonicus*

Plant materials	Treatment		No. of shoots treated	No. of shoots that survived (%)[a]	DNA C-values (min – max)					
					Diploid	Tetraploid	Octoploid	Mixoploid		
	Antimitotic agents	Concentration (mg L^{-1})			2C-4C	4C-8C	8C-16C	2C-8C	2C-16C	4C-16C
F1 hybrid '47B'	Cholchicine	0	30	30 (100)	30					
		500	30	20 (67)	12	1		7		
		1000	30	9 (39)	4			4	1	
	Amiprophos-methyl	0	20	20 (100)	20					
		5	20	20 (100)	6	1		12		1
		10	20	20 (100)	2	1		14	2	1
		20	20	4 (20)	1			1	2	
Carnation '98sp1651'	Cholchicine	0	30	30 (100)	30					
		500	30	10 (33)		2		7		1
		1000	30	8 (27)		1		5		2
	Amiprophos-methyl	0	20	20 (100)	20					
		5	20	20 (100)	11			9		
		10	20	20 (100)	3	3		10	2	2
		20	20	10 (50)	2			3	5	

[a] Recorded at 3 months after treatment

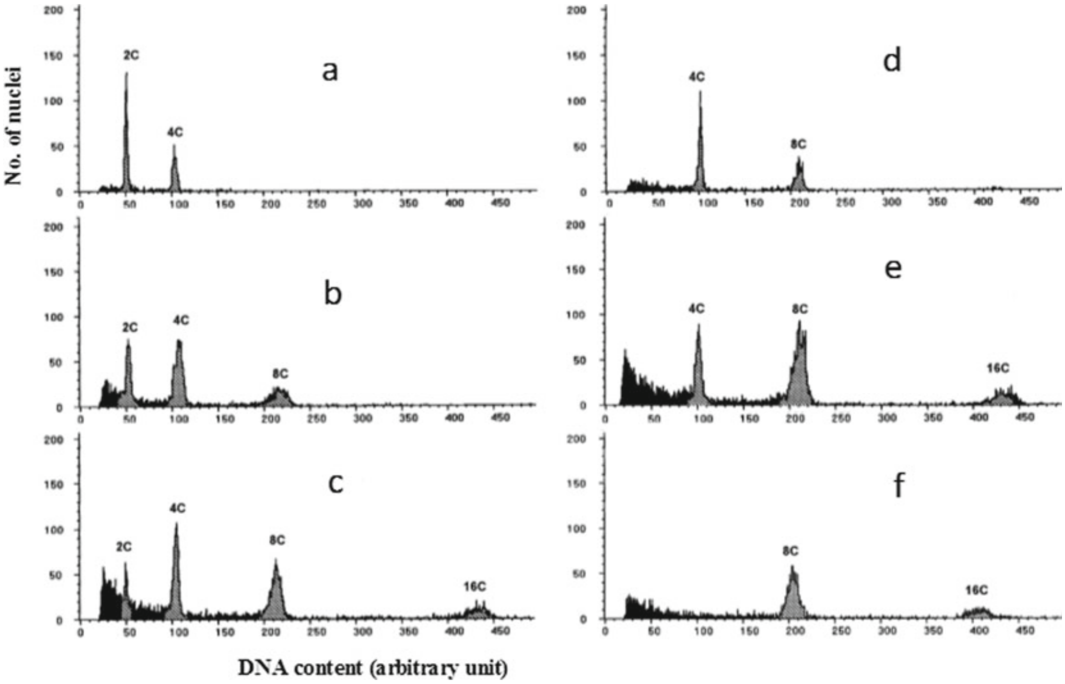

Fig. 12.2 Flow cytometric types of the plantlets obtained from artificial chromosome-doubling in F1 hybrid '47B' of *D. caryophyllus* × *D. japonicus*. **a** No treatment or 2C-4C, **b** 2C-8C, **c** 2C-16C, **d** 4C-8C, **e** 4C-16C, and **f** 8C-16C

Dropping was performed onto naked shoot tips after the removal of immature nonexpanded leaves. Although amphidiploids could be induced with this method, it was necessary to treat many plants as target material because of the low efficiency of the production of plants with chromosome-doubling, as well as to supply a large area for maintaining the treated plants. Furthermore, it takes a long time to propagate a sufficient number of amphidiploid plants necessary for testing the field performance of amphidiploids. Therefore, it was also evaluated using the in vitro treatment, which requires neither a field for cultivation nor many experimental plant materials, and the amphidiploids could be rapidly micropropagated by continuing in vitro culture.

In carnation, tetraploids have already been produced by artificial chromosome-doubling with in vitro dropping treatment of colchicine onto shoot tips of excised lateral buds (Yamaguchi and Kakei 1985). In the present study, therefore, carnation was used as control plant material in addition to the interspecific hybrid with the use of in vitro shaking culture treatment. Moreover, in addition to colchicine, APM was evaluated because it was recognized as a more efficient agent and a less toxic substance than colchicine (Sri Ramuluet al. 1991). In the present study, the use of APM was found to be effective in inducing chromosome-doubling of the hybrids at low concentrations (5 or 10 mgL^{-1}) without showing any toxic effect. The yield of the tetraploids in carnation was higher than that in the interspecific hybrid, and the maximum yield of tetraploids (25%) was obtained by in vitro shaking culture in 1/2 MS liquid medium containing APM at 10 mgL^{-1}. Therefore, it was concluded that 10 mgL^{-1} was the optimum concentration for inducing chromosome-doubling without causing damage to the explants in the APM treatment. As no apparent differences in morphological and physiological characters were observed among all 13 amphidiploid plants, it was assumed that differences in the concentration of APM did not affect the nature of the resulting amphidiploids in the present study.

A total of 13 completely nonchimeric amphidiploid plants were obtained from the interspecific hybrid ‘47B,’ 1 year after the chromosome-doubling treatments, regardless of differences in treatment conditions. These amphidiploids showed larger values in various morphological characters—including the diameter of stems, leaves, and flowers, and the number of petals per flower—than the original diploid hybrids. Moreover, the amphidiploid hybrids produced fertile pollen grains, and their guard cells were larger than those of diploid hybrids. No significant morphological differences were found among the 13 amphidiploids. However, in mixoploids (shown in histogram **b** or **c** in Fig. 12.2), the leaves and flowers were larger than those of the diploids, although the number of petals per flower was the same as that of diploids. Moreover, the size of the guard cells of mixoploids was almost the same as that of diploids.

Progeny plants were successfully obtained by selfing of the amphidiploid hybrid. When the amphidiploid hybrid was backcrossed with the pollen of carnation and *D. japonicus*, respectively, germinable seeds were produced from the former but not from the latter. The seedlings obtained from the backcross with carnation grew normally, and were confirmed to be triploid based on flow cytometric analysis. The results of the flow cytometric analysis were also confirmed on the basis of counting of the chromosome number in the root-tip cells of the hybrid, the parental plants, and the presumed amphidiploid. Both parental species and the hybrid showed the same diploid chromosome number ($2n = 2x = 30$), whereas in the presumed amphidiploid, a tetraploid chromosome number ($2n = 4x = 60$) was obtained, as expected. Moreover, the presumed octoploid plant showed an octoploid chromosome number ($2n = 8x = 120$).

Because the amphidiploids obtained in the present study displayed several desirable characters of *D. japonicus*, such as vigorous growth in summer, an upright robust stem, and broad leaves, they are expected to be used for the breeding of carnation.

12.4 *D. Japonicus* × *Dianthus* × *isensis*

Reciprocal interspecific hybridization was conducted as four combinations between one line of *D. japonicus* and two lines of *D.* × *isensis* (Nimura et al. 2006a). At attempt was made to cross the resulting interspecific hybrid with carnation. In cross combinations using two different *D.* × *isensis* lines as maternal parents, seeds were obtained only when ‘ISE 1’ was crossed with the pollen of *D. japonicus*. The number of seeds obtained from each ovary varied, and the average number was 17.3, which was less than that obtained from the intraspecific cross of *D.* × *isensis* or *D. japonicas* (approximately 30–100 seeds). A total of 53 seeds were thus obtained. Among the 48 seeds (91%) that germinated, 46 seedlings grew into plants and bloomed.

Only one line of *D.* × *isensis* showed cross-compatibility with *D. japonicus*. Similarly, in interspecific hybridization between carnation and *D. japonicas* as the male donor parent, hybrids were obtained in only one out of six lines of carnation used as female parents. These results suggest that broad intraspecific variations exist in the ability to cross with other *Dianthus* species. It is therefore important to select genotypes with a wide and high ability to cross with other species of particular target species.

Flowers on one branch of each of the two hybrids that flowered in late May were larger than the others, and their stems were thicker. The anthers of these large flowers dehisced and contained many pollen grains that were found to be fertile because of their morphology and their ability to be stained with acetocarmine (Fig. 12.3).

Usually, it is difficult or impossible to use interspecific hybrids directly as breeding material because of sterility. In most cases, disturbance of normal chromosome segregation during meiosis results in the formation of gametes with an unbalanced chromosome constitution, which eventually causes sterility (van Tuyl and Lim 2003). Therefore, it is necessary to restore

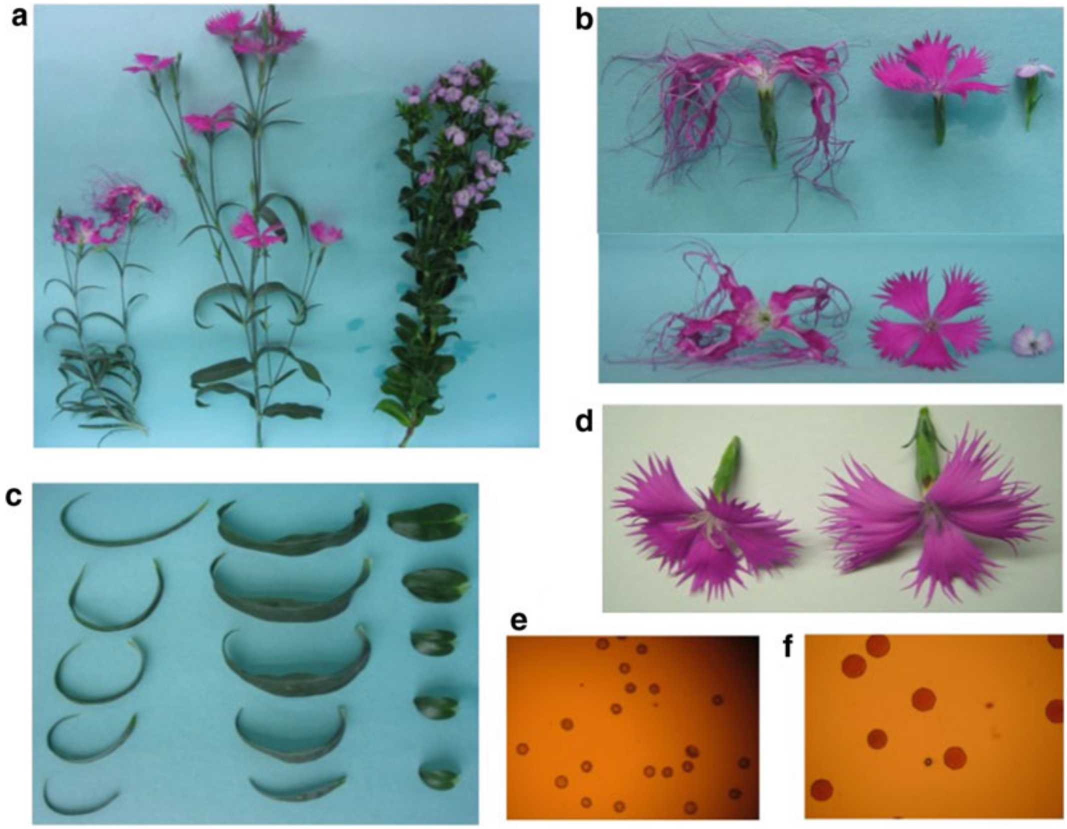

Fig. 12.3 Characteristics of hybrids obtained from a cross between *D.* × *isensis* and *D. japonicus*. **a** Flowering plants of *D.* × *isensis* (left), the hybrid (center), and *D. japonicus* (right). **b** Flowers of the parental species, *D.* × *isensis* (left), *D. japonicus* (right), and the hybrid (center). **c** Leaves of *D.* × *isensis* (left), the hybrid (center), and *D. japonicus* (right). **d** Flowers of the "normal" hybrid (left) and the amphidiploid created by natural chromosome-doubling (right). **e** Pollen of the "normal" hybrid. **f** Pollen of the amphidiploid hybrid produced by natural chromosome-doubling

fertility by inducing amphidiploidy through artificial chromosome-doubling with the use of colchicine or other chemicals with mitotic inhibitor activity once a hybrid has been successfully obtained.

In the present study, however, spontaneous chromosome-doubling was confirmed in one branch in two out of 46 interspecific hybrids of *Dianthus*, and thus it seems that this is not exactly a rare occurrence. Tetraploid branches might have arisen through the formation of a chimera in the original hybrid tissues (allodiploid), with natural chromosome-doubled tissues (amphidiploid or allotetraploid). As amphidiploids usually restore fertility because of normal chromosome pairing at meiosis, the next generation can be successfully obtained via self-pollination. As natural chromosome-doubling is considered to play an important role in plant evolution and speciation because of the acquisition of fertility, it is possible that some species with high ploidy levels in the genus *Dianthus* have evolved through natural chromosome-doubling.

D. × *isensis* is considered to be a hybrid between *D. chinensis* L. and *D. superbus* var. *longicalycinus* (Maxim.) F. N. Williams (Ito et al. 1994). Although no tetraploid has been reported in *D.* × *isensis*, there are several tetraploid varieties of *D. chinensis*, and among its interspecific hybrids is "Daimonji," a hybrid with carnation (Ito et al.

1994). In the present study, two interspecific hybrids underwent natural chromosome-doubling, and it is possible that somatic cell-doubling occurs relatively frequently in this interspecific hybrid combination. On the other hand, natural chromosome-doubling was not observed in interspecific hybrids between carnation and *D. japonicus* as the pollen parent. Moreover, tetraploids could not easily be obtained by applying artificial chromosome-doubling treatments to the interspecific hybrids obtained in this study. Therefore, it is possible that *D. chinensis* has a "genetic factor," which might exist in the line of *D.* × *isensis* used here. Because *D.* × *isensis* could have passed this "genetic factor" to its interspecific hybrids with *D. japonicus*, natural chromosome-doubling might have occurred in some of the hybrids. The relatively low frequency (2/46) of hybrids showing spontaneous chromosome-doubling suggests limited expression of the putative "genetic factor." More recent results of reverse crosses showed that all of the progeny were diploid, and no natural chromosome-doubling was observed in these plants, suggesting the possibility of maternal inheritance of this "genetic factor.".

Hybrids obtained from a cross between *D.* × *isensis* (♀) and *D. japonicus* (♂) displayed an intermediate leaf width and flower-size morphology compared with the parents. Favorable *D. japonicus* characters such as its strong stem, multiflorous character, and larger leaf width were also transmitted successfully to all 46 hybrids. Moreover, all hybrids showed more vigorous growth compared with *D.* × *isensis* during summer and were easy to propagate vegetatively by cuttings. As the hybrids had the single-flower character inherited from both parents, the tetraploid hybrid (single-flowered plant) was crossed with a double-flowered diploid carnation, which resulted in 18 progenies consisting of 14 double-flowered plants and four single-flowered ones. A similar segregation pattern was also obtained in my preliminary study on carnation, in which a cross between double- and single- flowered plants yielded both double- and single- flowered progeny.

The amphidiploids (tetraploids) obtained in the present study could produce triploid progeny via reciprocal crossing with a diploid carnation, and the triploid progeny then produced few seeds when crossed with pollen from a diploid carnation. As fewer than 20 of the seeds from such a triploid × diploid cross germinated successfully, the seedlings with aneuploidic DNA contents might be used for further breeding via recurrent backcrossing with diploid carnation. It might also be possible to obtain fertile tetraploid progeny by crossing the amphidiploids with a tetraploid carnation, which could be produced via artificial chromosome-doubling to introduce the desirable characters of *D. japonicus* (broad leaf, strong stem, and heat tolerance) and *D.* × *isensis* (early flowering and unique petal shape) into carnation.

12.5 Natural and Artificial Chromosome-Doubling in Interspecific Hybrids

Interspecific hybrids produced by the cross between species with different genomes are usually sterile because of the abnormal segregation of chromosomes at meiosis I. However, amphidiploids obtained by chromosome doubling of such an interspecific hybrid were able to have their fertility restored because of the acquisition of a complete diploid set of chromosomes derived from each ancestral species. Because interspecific hybrids obtained from carnation × *D. japonicus* were completely sterile, artificial chromosome-doubling was attempted to restore the fertility of the hybrids. Consequently, an efficient artificial doubling method was established for both interspecific hybrids and carnation, and amphidiploid of the interspecific hybrid and autotetraploid of the carnation were successfully obtained. Chromosome-doubling is known to be induced by inhibition of spindle fiber formation during mitosis, which results in the formation of cells with restituted nucleus. Spontaneous chromosome-doubling is also known to occasionally occur during growth; in fact, this was observed in two hybrid plants between *D.* × *isensis* and *D. japonicus*. In these plants, chimera with a tetraploid branch was induced by natural chromosome-doubling. Complete amphidiploids were obtained by selfing of the fertile flowers on the tetraploid branch of the chimeric plant (Nimura et al. 2006a).

Amphidiploids are usually obtained from the interspecific hybrid via artificial chromosome-doubling of somatic cells as described above. Amphidiploids are also produced by fertilization of unreduced gametes and by interspecific crossing between the tetraploids produced from diploid parents. The occurrence of amphidiploids in nature is induced by interspecific hybridization followed by chromosome-doubling, which is known to have played an important role in the speciation and evolution of *Triticum*, *Brassica*, *Nicotiana*, and others. Triticale was obtained via interspecific hybridization between *Triticum durum* and *Secale cereale*, which is an example of artificial production of amphidiploid (Stace 1987). In Japan, a vegetable crop of *Brassica napus* "Hakuran" ($2n = 4x = 38$, AACC) was produced from the cross between *Brassica rapa* ($2n = 2x = 20$, AA) and *Brassica oleracea* ($2n = 2x = 18$, CC) (Nishi 1980). In ornamentals, interspecific hybrids have been produced in various crops such as *Lilium* (Asano 1982; van Tuyl et al. 2000, 2002), *Cyclamen* (Ishizaka and Uematsu 1994), and *Iris* (Yabuya 1985). Because the amphidiploid exhibits traits from both parents including numerous undesirable ones, it is usually necessary to cross these hybrids with some cultivars for further improvement.

12.6 Carnation × *Dianthus* × *isensis*

Reciprocal interspecific crosses were carried out in 14 combinations between one line of *D.* × *isensis* and seven lines of carnation (Nimura et al. 2006c). When the carnation was crossed with the pollen of *D.* × *isensis*, the number of seeds obtained from each ovary varied, and the average was 6.7 seeds. Of the seven carnation lines used as the maternal parent, germinated seeds were obtained from four lines. In total, 79 seedlings grew normally after germination. When *D.* × *isensis* was used as the maternal parent, most crosses produced sterile seeds, and fertile seeds were obtained from the pollination of only two carnation lines. Thus, a total of only five seedlings were obtained. In the present study, six out of 14 cross combinations in reciprocal interspecific crosses between carnation and *D.* × *isensis* yielded progeny plants.

In contrast to the results obtained with interspecific hybridization between carnation and *D. japonicus*, hybrid plants were obtained more efficiently in the present interspecific cross combination irrespective of the direction of the cross. Moreover, because the hybrids were fertile like the diploids, they can be used as breeding material without the need for artificial chromosome-doubling.

Among the 79 progenies obtained from crosses where carnation was used as the maternal parent, 30 had the expected intermediate DNA content between the parents. However, the remaining 49 progenies had the same DNA content as carnation. Meanwhile, three out of five progenies from crosses of *D.* × *isensis* as maternal parent had the expected intermediate DNA content between the parents. In the remaining two progenies, one had the triploid hybrid DNA content (2.74 pg) corresponding to the sum of two genomes of *D.* × *isensis* and one genome of carnation, whereas the other had the same content as *D.* × *isensis*.

Based on the results of flow cytometric analysis, RAPD analysis was carried out on the putative hybrids obtained from the crosses of carnation as maternal parent and three out of five progenies from the crosses of *D.* × *isensis* as maternal parent. The hybrids had specific marker bands from both parents. Moreover, these RAPD bands were also present in the presumed triploid hybrid. The results of flow cytometric analysis were further confirmed by counting chromosome numbers in the root-tip cells of the presumed diploid hybrids, the presumed triploid hybrid, and the parental plants. Both parental species and the presumed diploid hybrids had the same diploid chromosome number ($2n = 2\text{x} = 30$), whereas the presumed triploid hybrid had the expected chromosome number of triploid ($2n = 3\text{x} = 45$).

When *D.* × *isensis* was pollinated using carnation '01sp48H18A' pollen, both diploid hybrids (IC genome) and a triploid hybrid (IIC genome) with two genomes of *D.* × *isensis* and one genome of carnation were obtained. The triploid hybrid could be formed via fertilization of an unreduced diploid egg of *D.* × *isensis* by normal carnation pollen, as observed in previous studies on *Cyclamen* (Takamura and Miyajima 1996, 1999) and *Primula* (Kato and Mii 2000).

The 30 progenies derived from crosses of carnation × *D.* × *isensis*, which showed hybrid nature by flow cytometric and RAPD analyses, had roughly the intermediate morphology of both parents, whereas the other 49 seedlings showed carnation-like morphology and looked similar to the respective line of carnation used as maternal parent. On the other hand, three out of five progenies from crosses of *D.* × *isensis* used as maternal parent showed the intermediate morphology of both parents, whereas one out of the remaining two progenies was a triploid hybrid and had single flowers with larger petals compared with diploid hybrids (Fig. 12.4). However, another one of the progenies had a *D.* × *isensis*-like morphology.

All 33 interspecific diploid hybrids thus obtained had an intermediate flower size between that of carnation and *D.* × *isensis*, and the flower colors varied from purple-pink to purple-red. In addition, the hybrids had flower petals with deep serrations, which gave a different impression from that of traditional carnations. However, the hybrids had a carnation-like inflorescence and were taller than *D.* × *isensis*. The leaf shape of the hybrids resembled that of carnation, but was more curled (Fig. 12.5a, c). The hybrids showed early flowering and bloomed from the end of May until the middle of June, whereas carnation-like progenies flowered in July or later. The hybrids were separated into two groups according to their flower types: 15 double-flowered plants and 18 single-flowered plants. The anthers of the diploid hybrids dehisced normally and contained many fertile pollen grains with a normal morphology, which were stainable with acetocarmine. Accordingly, plants of the second filial (F2) generation were obtained by selfing of the diploid hybrid flowers, which set seeds normally. Moreover, in propagation by cutting using lateral shoots, all hybrids had a high rooting ability, comparable to that of carnation, and almost all cuttings were rooted successfully.

The hybrids obtained by reciprocal crosses between carnation and *D.* × *isensis* showed an intermediate morphology between the parents in terms of leaf width and flower size. As a result, several favorable characters of *D.* × *isensis*, such as early flowering and large flowers with deep sinuses, were successfully transmitted to the hybrids. Moreover, the hybrids showed a branching habit at the base of the stem, and were easy to propagate by cutting. However, the F1 hybrids had several undesirable characters such as thinner stems and leaves compared with carnation and segregation of single-flowered individuals. By using an embryo rescue technique, it might be possible to obtain more hybrids from

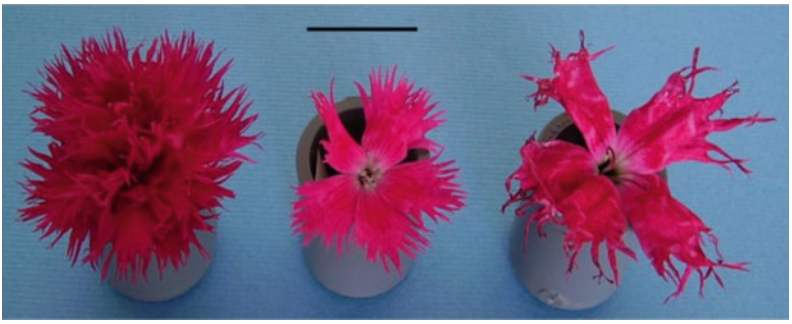

Fig. 12.4 Flowers of the hybrids obtained from the cross between *D.* × *isensis* and *D. caryophyllus*. The diploid hybrid with double flower 'IC-1' (left), the diploid hybrid with single flower 'IC-2' (center), and the triploid hybrid with single flower 'IIC-1' (right). Bar = 30 mm

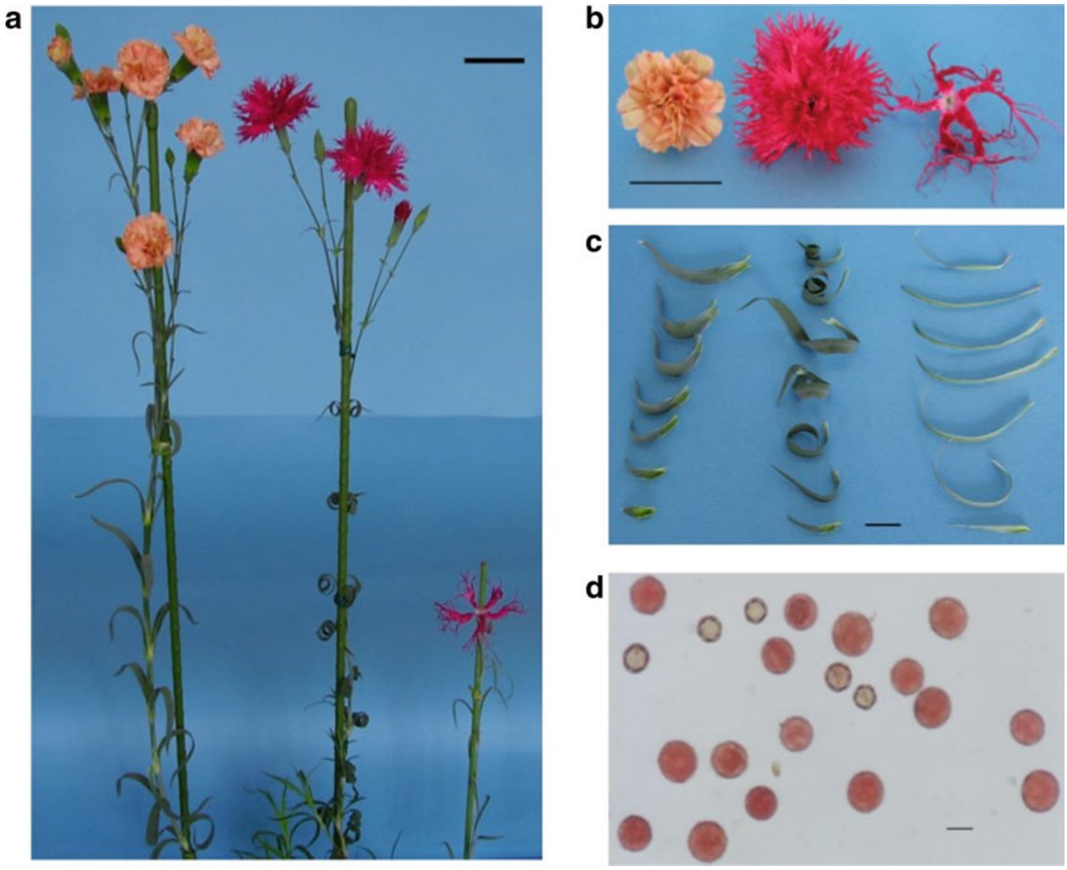

Fig. 12.5 Characteristics of the diploid hybrid obtained from the cross between *D. caryophyllus* and *D.* × *isensis*. **a** Flowering plant of *D. caryophyllus* '98sp1651' (left), the diploid hybrid 'CI-1' (center), and *D.* × *isensis* 'ISE1' (right). **b** Flowers of the parental species and the hybrid, *D. caryophyllus* '98sp1651' (left), the diploid hybrid 'CI-1' (center), and *D.* × *isensis* 'ISE1' (right). **c** Leaves of *D. caryophyllus* '98sp1651' (left), the diploid hybrid 'CI-1' (center), and *D.* × *isensis* 'ISE1' (right). **d** Fertile pollen of the interspecific diploid hybrid. Bars: **a** 50 mm; **b** 40 mm; **c** 30 mm; **d** 50 µm

various genotypes of carnation as maternal parents. In this study, only one triploid interspecific hybrid, which was an allotriploid ($2n = 3x = 45$) with two genomes of *D.* × *isensis* and one genome of carnation, was obtained. Allotriploidy has been used efficiently to produce alien monosomic addition lines through backcrossing with the diploid genome donor in various crosses such as alliums (Shigyo et al. 1996) and wheat (Taketa and Takeda 2001; Taketa 2003). Therefore, the production of alien monosomic addition lines of *D.* × *isensis* with an extra chromosome from carnation using the same procedure may also be expected.

12.7 Nondiploid Hybrid Formation by Unreduced Gametes

Triploid hybrid was produced when *D.* × *isensis* was pollinated with carnation. Assessment of relative DNA contents by flow cytometric analysis revealed that this triploid hybrid had two genomes of *D.* × *isensis* and one genome of carnation. Therefore, it is considered that *D.* × *isensis* used as maternal parent produced unreduced female gamete. Unreduced gamete formation is a consequence of failure in meiosis. Although gametes (e.g., pollen and embryo sac) should be normally haploid, failure in meiosis sometimes occurs and produces the so-called "unreduced gametes" or "2n gametes," which have somatic chromosome number. Unreduced gametes are produced by either nonreductional segregation of homologous chromosomes at first meiotic division or nonequational segregation of sister chromatid at the second division. Moreover, in either interspecific hybrids or intergeneric hybrids, there are some cases in which a restored nucleus is produced owing to insufficient or lack of chromosome pairing at first meiotic division. There are some cases in which the restored nucleus is induced owing to failure in meiosis attributed to environmental factors such as low temperature and chemicals (Ramanna and Jacobsen 2003). If these fertile gametes are fertilized, polyploids will be formed (van Tuyl and Lim 2003; Lim et al. 2004; Hayashi et al. 2007; Nimura et al. 2008). In general, triploids are sterile and of little interest in breeding. However, in vegetatively propagated ornamentals such as *Alstroemeria* and lily, the triploid is preferred over diploids and tetraploids. Triploids have been used successfully used as parents in planned crossings to produce various ploidy levels in *Lilium* (Lim et al. 2003). An interesting feature of triploids is that it is sometimes possible to generate progenies with near-diploid (circa diploid) and near-tetraploid chromosome constitutions by making the crosses as female parents with diploid and tetraploid, respectively. The progeny with a near-diploid constitution is potentially useful for breeding at the diploid level as demonstrated in the analytical breeding of potato and other polyploid crops.

12.8 Relationship Among *D. caryophyllus*, *D. japonicus*, and *D.* × *isensis*

Relationship among the different taxa has formerly been examined through similarity of morphological traits, mode of reproduction, cross-compatibility, geographical distribution, etc. In addition, information on DNA sequence similarity or DNA mutation similarity has recently been incorporated to clarify the relationships based on the progress in molecular evolutionary research.

Results on the cross-compatibility among *D. caryophyllus*, *D. japonicus*, and *D.* × *isensis* (Fig. 12.6) show that there is some reproductive isolation between carnation and *D. japonicus* because crossing of *D. japonicus* (♀) × carnation (♂) was incompatible. Moreover, although hybrids were obtained from the opposite crossing, they were sterile and their reproductive ability was almost lost. Reproductive isolation was also found between *D. japonicus* and *D.* × *isensis*. Although interspecific hybrids were obtained in both directions of reciprocal crossing, all hybrids were sterile. In contrast, interspecific hybrids between carnation and *D.* × *isensis* were easily obtained in both directions of reciprocal crossing, and they produced F2 progeny by self-pollination. Therefore, these two species might have closely related genomes to each other and might have almost no reproductive isolation. Accordingly, interspecific hybrids between these two species will be useful for diploid-level carnation breeding.

The sequence analysis of chloroplast DNA has been effectively utilized for phylogenetic study in various taxonomic groups such as several genera that belong to Rosaceae (Potter et al. 2002; Wu et al. 2002; Shaw and Small 2004; Ohta et al. 2006) and Japanese rice populations (Ishikawa et al. 2002). The reference genome sequence of carnation was reported by Yagi et al. (2014), and the chloroplast genome was also determined by Li et al. (2019). In the future, it is expected that DNA level analysis will be enforced in clarifying relationships among the three *Dianthus* species used in the present study.

12.9 Conclusions

D. japonicus Thunb., which is a perennial species native to Japan with a high tolerance for heat, and *D.* × *isensis* Hirahata *et* Kitam., which is a hybrid species indigenous to Japan with very early flowering trait, were used to introduce these useful traits into carnation (*D. caryophyllus* L.).

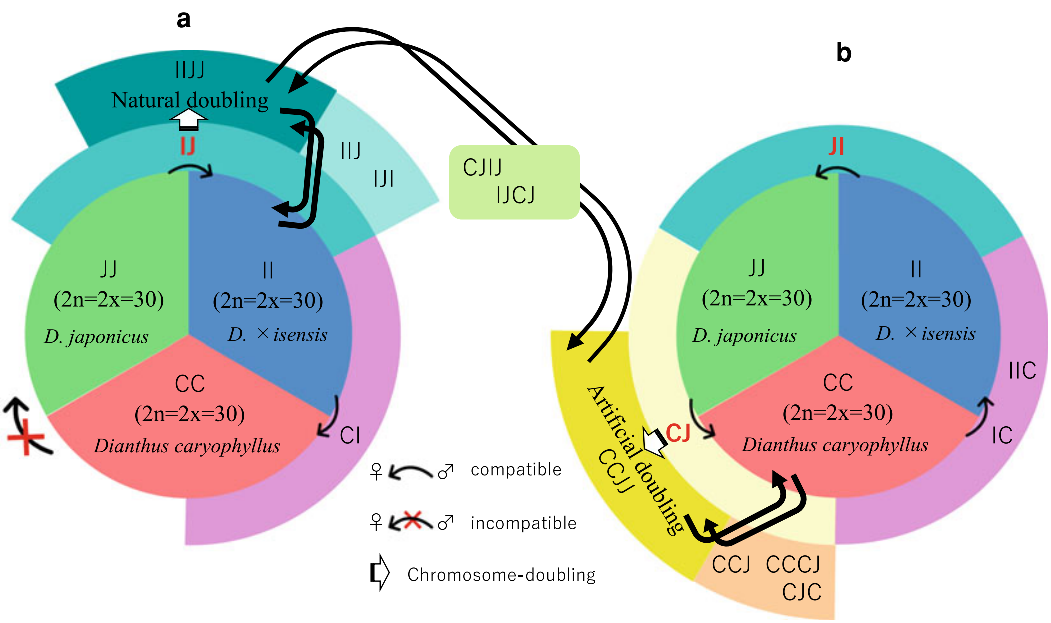

Fig. 12.6 Cross-compatibility and hybrid fertility among *D.caryophyllus*, *D. japonicus,* and *D.* × *isensis.* **a** Clockwise crosses: II was crossed with the pollen of JJ, CC was crossed with the pollen of II, and JJ was crossed with the pollen of CC. **b** Counter-clockwise crosses: JJ was crossed with the pollen of II, CC was crossed with the pollen of JJ, II was crossed with the pollen of CC

First, when reciprocal crosses were carried out between six lines of *D. caryophyllus* and *D. japonicus*, interspecific hybrids were obtained only when carnation was used as seed parent, and genotypic differences in crossability were found among carnation genotypes. Because the interspecific hybrid obtained from this cross combination was sterile, an attempt was made to establish an efficient method of artificial chromosome-doubling and thereby obtain fertile amphidiploids. Next, reciprocal interspecific crosses were carried out between *D.* × *isensis* and *D. japonicus*, which resulted in the successful production of interspecific hybrids irrespective of cross direction. Although all hybrids were sterile, fertile amphidiploids were obtained via occurrence of spontaneous chromosome-doubling in some hybrid plants produced from the cross in which *D.* × *isensis* was used as seed parent. Interspecific hybrids were also successfully obtained between *D.* × *isensis* and carnation irrespective of the cross direction. Furthermore, F2 progeny was directly obtained from the diploid hybrids without producing amphidiploids, suggesting that they might have closely related genomes to each other.

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