Theor Appl Genet (2003) 106:1164–1170
DOI 10.1007/s00122-002-1181-0

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Unilateral compatibility and genotypic difference in crossability in interspecific hybridization between Dianthus caryophyllus L. and Dianthus japonicus Thunb

Received: 29 August 2002 / Accepted: 19 October 2002 / Published online: 25 January 2003

Abstract Reciprocal interspecific crosses were carried out between six lines of *Dianthus caryophyllus* L. and one line of *Dianthus japonicus* Thunb. Although no seed was set when *D. japonicus* was used as the seed parent, six seedlings were successfully obtained from 2,380 immature ovules by applying the embryo-rescue technique. However, they showed seed parent-like morphology and no evidence for the hybridity by flow cytometry and RAPD analyses. When six lines of *D. caryophyllus* were used as seed parents, a total of 192 seedlings were successfully obtained without using the embryo-rescue technique. Among these seedlings, 12 out of 25 progenies obtained from the carnation line '98sp1651' were confirmed to be the hybrids. The remaining 13 progenies of this line, and the total 167 progenies obtained from the other carnation lines, had carnation-like morphology without any evidence of hybridity by flow cytometry and RAPD analyses. The progenies confirmed as hybrids had intermediate characters of the parents with respect to leaf width and flower size, but they had a uniform flower color, reddish purple, which was different from that of either parent. Since the hybrids obtained in the present study have some profitable characters such as vigorous growth in summer time, upright robust stem, broad leaves and early flowering, they are expected to be used for the breeding of carnation which is suitable for growing under the Japanese climate.

Communicated by H.F. Linskens

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Keywords Carnation · *Dianthus japonicus* · Embryo rescue · Interspecific hybrid · Unilateral cross incompatibility

Introduction

Carnation (*Dianthus caryophyllus* L.) is one of the main floricultural crops and is cultivated all year round in Japan. Since the varieties of carnation are highly heterozygous due to the difficulty of selfing and/or inbreeding depression, most of the commercially important varieties are the clones of selected favorable individuals that are propagated vegetatively. Hence, neither pure-bred varieties nor F_1 hybrids have been produced. Since carnation is not so tolerant to the hot and humid climate like the Japanese summer, the quality of cut flowers tends to decrease in such a condition. Moreover, at the hot and humid conditions, carnation is easily infected with carnation bacterial wilt, a disease caused by *Burkholderia caryophylli* (Burkholder) Yabuuchi et al. [formerly *Pseudomonas caryophylli* (Burkholder) Starr and Burkholder]. Therefore, for stable carnation production in Japan, it is important to breed cultivars with both heat tolerance and resistance to carnation bacterial wilt.

There have been several reports on interspecific hybridization in the genus *Dianthus*, i.e. interspecific crosses between carnation and *Dianthus deltoids*, *Dianthus japonicus*, *Dianthus knappii* or *Dianthus superbus* (Kanda 1992), interspecific crosses among 22 allied species of *Dianthus* (Ohtsuka et al. 1995), acquisition of hybrids between carnation and *Dianthus capitatus* (Onozaki et al. 1998), somatic hybridization through protoplast fusion (Nakano and Mii 1993) and so on. However, there has been no report to produce interspecific hybrids between carnation and *D. japonicus* Thunb., which is a native perennial species to Japan. *D. japonicus* is characterized by a fasciculate cyme with many small flowers, an upright robust stem, broad and thick evergreen foliage with a developed cuticle, and strong heat tolerance (Tukamoto 1968). In addition, it does not suffer from the

Table 1 Plant materials used for the interspecific crosses between *D. caryophyllus* and *D. japonicus*

Species	Number of lines	Name of line	Flower type	Flower color	No. of petals	Flower diameter (mm)
D. caryophyllus	6	98sp1441	Double	Pale yellow green, purplish pink edging	26	48
		98sp1651	Double	Yellowish pink, deep yellowish red spots	33	45
		98sp1960	Double	Purplish pink	36	54
		98spLPB	Double	Pale purplish pink	27	49
		99sp367-11	Double	Strong purplish pink	43	45
		99sp594-16	Double	Vivid red purple	25	44
D. japonicus	1	HAMA1	Single	Rosy purplish pink	5	22

carnation bacterial wilt (Kagito and Tuchiya 1968). Since *D. japonicus* has these profitable characters, it is desired to use as it as breeding material for carnation.

In this study, we report the successful results on the production of interspecific hybrids between *D. caryophyllus* and *D. japonicus* by conducting reciprocal crosses.

Materials and methods

Plant materials

One line of *D. japonicus* Thunb., 'HAMA 1', which was collected from a sandy beach in Kyusyu, Japan, and six lines of spray type carnation, '98sp1441', '98sp1651', '98sp1960', '98spLPB', '99sp367-11' and '99sp594-16', were used for the experiments. These plant materials were grown at the Ornamental Plant Institute, Aichi-ken Agricultural Research Center. The flower color of each carnation line is described in Table 1.

Interspecific hybridization

Interspecific crosses were carried out on a total of 12 combinations, six combinations of *D. caryophyllus* L. (♀) × *D. japonicus* (♂) and six combinations of *D. japonicus* × *D. caryophyllus* (Tables 2 and 3). The flowers of seed parents were emasculated 2 or 3 days before anthesis. When the pistil ripened, the flowers were pollinated with prescribed pollen grains. Pollination was carried out from November to December 2000.

Acquisition of seedlings derived from interspecific crosses

Seeds were collected 45–50 days after pollination in the cross between carnation as the seed parent and *D. japonicus* as the pollen parent. Harvested seeds were sown in a cell-tray on late January and seedlings were planted in the greenhouse 3 months after sowing. On the other hand, seed setting was not observed in the cross between *D. japonicus* and carnation. Therefore, ovaries were collected approximately 3 weeks after pollination. They were surface-sterilized with a spray of 70% ethanol. The ovaries were then soaked in sodium hypochlorite solution (1% available chlorine) for 15 min and rinsed with sterilized water three times. Ovules with a placenta were isolated from the ovaries and placed on 1/2 MS medium (Murashige and Skoog 1962) with 3% (w/v) sucrose and 0.8% (w/v) agar. The pH of the medium was adjusted to 5.8 with 0.1 N KOH before autoclaving. The plantlets regenerated from the ovules were subcultured twice on the same composition medium. Several characters of these seedlings such as flowering time, flower shape and flower color were compared with both parents, carnation and *D. japonicus*.

Measurement of nuclear DNA content using flow cytometry

The nuclear DNA contents of the parental plants and the plants derived from interspecific crosses were measured by flow cytometry (model EPICS XL; Coulter Corporation, Hialeah, Fla.). Hybridity of the progenies was confirmed by their nuclear DNA contents. Young leaf tissues (0.5–1.0 g f.w.) were chopped with a razor blade in 2 ml of 10 mM Tris-HCl (pH 7.0) buffer solution containing 0.1% (v/v) Triton X-100, 100 mM of NaCl, 10 mM of Na_2EDTA, 20 $\mu g \cdot ml^{-1}$ of propidium iodide (PI) and 0.2 $mg \cdot ml^{-1}$ of RNase. After 5 min-staining on ice, the nuclei suspension was filtered through a 50 µm nylon mesh to remove debris and subjected to flow cytometric analysis. Nuclei from young leaves of barley (*Hordeum vulgare*) with a 2C DNA content = 11.12 pg (Michaelson et al. 1991) were used as an internal standard. Flow cytometric measurements were repeated three times using three different leaves for each progeny plant.

Extraction of DNA and random amplified polymorphic DNA (RAPD) analysis

Total genomic DNA was extracted from the parental plants and the plants derived from interspecific crosses using the plant DNA extraction kit, Nucleon Phyto Pure (Amersham Pharmacia Biotech Co.), and were used as templates for RAPD analysis. PCR was carried out using a total of 40 primers included in two kits of Operon 10-mer primers, KIT I and KIT K (Operon Co.). The rTth DNA polymerase (Toyobo Co.) was employed for PCR analysis, and a 25-µl reaction solution containing 1 ng of total plant DNA was subjected to analysis according to the manufacturer's protocol. DNA fragments were amplified by repeating 40 cycles of the following thermal treatments; 94 °C for 1 min, 41 °C for 1 min and 72 °C for 1 min, in a Takara PCR Personal Thermal Cycler (Takara Co.). Electrophoresis of the amplified DNAs was conducted on a 5% acrylamide gel in a TBE buffer system. Digested Ø × 174/*Hae*III was used as the size marker.

Result

Cross compatibility

In cross combinations of the six lines of carnation with pollen of *D. japonicus*, the number of seeds obtained from each ovary was three or less, which was much less than those obtained from intra-specific crosses of carnation (approximately 30–100 seeds). The seeds thus obtained were sown in a cell-tray in late January 2001, and 50–70% of the seeds grew into plants, which were able to bloom (Table 2).

In the cross of *D. japonicus* with the pollen of carnation, normal seeds were not obtained in our preliminary

Table 2 Seed production and germination in interspecific crosses between *D. caryophyllus* and *D. japonicus*. Pollination was carried out during the period from November to December 2000. Harvested seeds were sown in a cell-tray in the late January 2001 and the seedlings were planted in greenhouse 3 months after seeding

Carnation line	No. of flowers pollinated	No. of flowers producing seeds	Total no. of seeds	No. of seeds germinated	No. of plants obtained
98sp1441	56	49	158	106	104
98sp1651	23	21	43	26	25
98sp1960	4	2	4	3	3
98spLPB	3	3	6	4	4
99sp367-11	27	26	85	57	53
99sp594-16	4	3	6	3	3

Table 3 Ovule culture in interspecific crosses between *D. japonicus* and *D. caryophyllus*. Pollination was carried out during the period from November to December 2000. Ovaries were collected approximately 3 weeks after pollination. Ovules isolated from the ovaries were placed on 1/2 MS medium with 3% (w/v) sucrose and 0.8% (w/v) agar

Carnation line	No. of flowers pollinated	No. of ovules cultured	No. of ovules germinated	Growth of seedlings 3 months after germination			
				Died after cotyledon expansion	Growth stopping at cotyledon expansion	Dwarf	Normal
98sp1441	12	340	11	4	3	2	2
98sp1651	14	400	0	–	–	–	–
98sp1960	16	480	0	–	–	–	–
98spLPB	13	380	2	1	1	–	–
99sp367-11	18	520	0	–	–	–	–
99sp594-16	9	260	3	1	–	–	2

Table 4 Flow cytometric analysis of the DNA content of progenies in reciprocal interspecific crosses between *D. 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

experiment because of the death of ovaries about 1 month after pollination. In this cross, therefore, ovule culture was performed approximately 3 weeks after pollination. Totally 16 ovules which were derived from the crosses with pollen of three out of six lines of carnation, i.e. '98sp1441', '98spLPB' and '99sp594-16', showed germination. However, only six out of 16 ovules grew smoothly after germination and the others stopped growth or died at the stage of cotyledon expansion (Table 3).

Measurement of hybridity

The nuclear DNA content of the parental plants determined by flow cytometry was 1.48 pg in carnation and 2.57 pg in *D. japonicus*, respectively, and *D. japonicus* had about a 1.7-times larger DNA content than carnation. Since the hybrid was expected to have an intermediate DNA content of the parents, the value of the DNA content was used as an indicator for detecting the true intermediate hybrids. Consequently, 12 out of 25 progenies

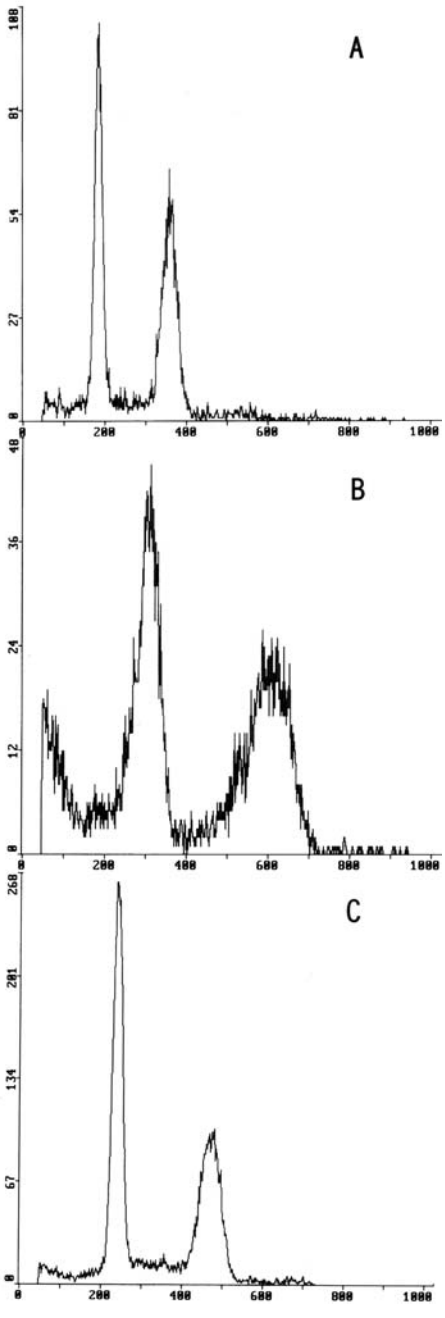

Fig. 1A–C Flow cytometric profiles of DNA contents. **A**: *D. caryophyllus* '98sp1651', **B**: *D. japonicus*, **C**: the hybrid obtained from the interspecific cross between *D. caryophyllus* '98sp1651' used as maternal parent and *D. japonicus* used as pollen parent. Left peak: 2C, right peak: 4C

obtained from the cross of carnation line '98sp1651' with the pollen of *D. japonicus* had an expected DNA content of 2.03 pg, which was intermediate to the parents (Fig. 1 and Table 4). However, the DNA content of the other 13 progenies and the total of 167 progenies derived from the other carnation lines used as the seed parent was 1.48 pg, which was the same as that of carnation. On the other hand, the DNA contents of six progenies obtained by culturing 2,380 ovules, which were obtained from the crosses using *D. japonicus* as a seed parent, showed the same DNA content as *D. japonicus* (2.57 pg) (Table 4).

RAPD analysis was carried out on the putative hybrids to detect the specific marker of *D. japonicus* used as pollen parent. Twelve out of 25 progenies obtained from the carnation line '98sp1651', which had already shown the intermediate DNA content of the two parental species, had more than two specific band markers of the pollen parent *D. japonicus*. In these putative hybrids, one out of 40 primers used, OPI-4 (5′ CCG CCT AGT C 3′), amplified two bands of approximately 300 and 275 bp, which were present in the pollen parent *D. japonicus* 'HAMA 1' but absent in carnation. However, these DNA fragments were not detected in the 13 progenies, which had not shown hybridity by flow cytometry (Fig. 2).

Morphological characters of the hybrid

Morphology of the progenies obtained from the crosses between six lines of carnation and *D. japonicus* 'HAMA 1' was mostly carnation-like and looked similar to the respective lines of carnation used as seed parents. However, 12 progenies derived from the cross using '98sp1651', which had confirmed their hybridity by flow cytometry and RAPD analyses, showed intermediate characters of the parents (Fig. 3A), whereas the other 13 progenies obtained from the same seed parent were carnation-like.

All of the 12 interspecific hybrids obtained in the present study had an intermediate flower size between carnation and *D. japonicus*, and an uniform flower color of reddish purple (Fig. 3B). The hybrids also had a *D. japonicus*-like fasciculate cymose inflorescence but had a lesser number of flowers like carnation. The leaf shape of the hybrids rather resembled carnation but was broader (Fig. 3C). Most of the hybrids flowered from 10th June to 17th July when they were sown on late January. Among the 12 hybrids, seven had double flowers and five showed single flowers (Fig. 3D). Although three out of the five single-flowered hybrids had fully developed anthers, they did not dehisce and contained few pollen grains with an abnormal shape. When carnation or the hybrid plants were pollinated with these abnormal pollen grains, no seed was obtained. On the other hand, the remaining two single-flowered hybrids as well as the seven double-flowered hybrids had no anthers (data not shown). In propagation by cutting using lateral buds, all of the 12 hybrids showed a comparably high ability of rooting to carnation. All of the hybrids had stronger stems than carnation and showed more vigorous growth than carnation in the summer season.

Six progenies obtained by ovule culture of *D. japonicus* × *D. caryophyllus* grew normally and showed almost the same morphology to *D. japonicus* in the leaf shape which was round, wide and thick. These results coincided with those obtained on the nuclear DNA content by flow cytometry.

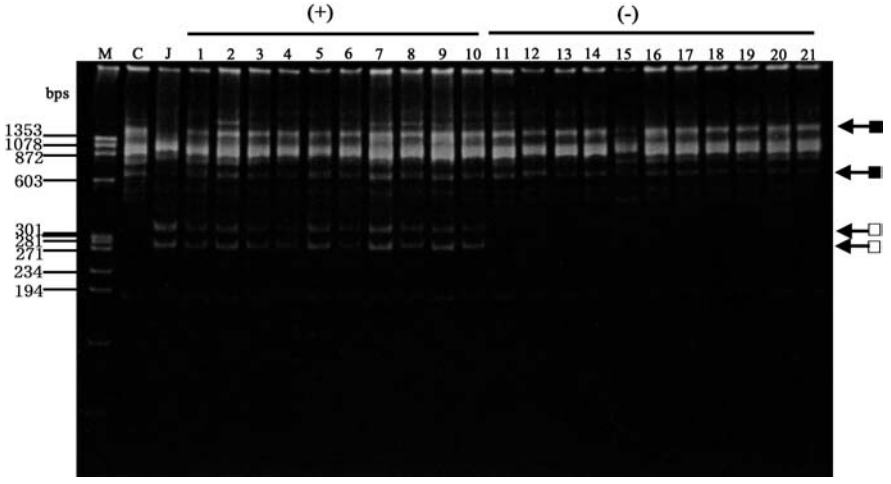

Fig. 2 PCR analysis using the OPI-4 primer on the hybridity of the progenies in the interspecific cross between *D. caryophyllus* and *D. japonicus*. **M** Ø×174/*Hae*III-digested size marker, **C** maternal parent, **J** pollen parent, **1–10** hybrids obtained from the crosses between *D. caryophyllus* and *D. japonicus*, **11–21** non-hybrids obtained from the cross between *D. caryophyllus* and *D. japonicus*, ■ the specific band of *D. caryophyllus*, □ the specific band of *D. japonicus*

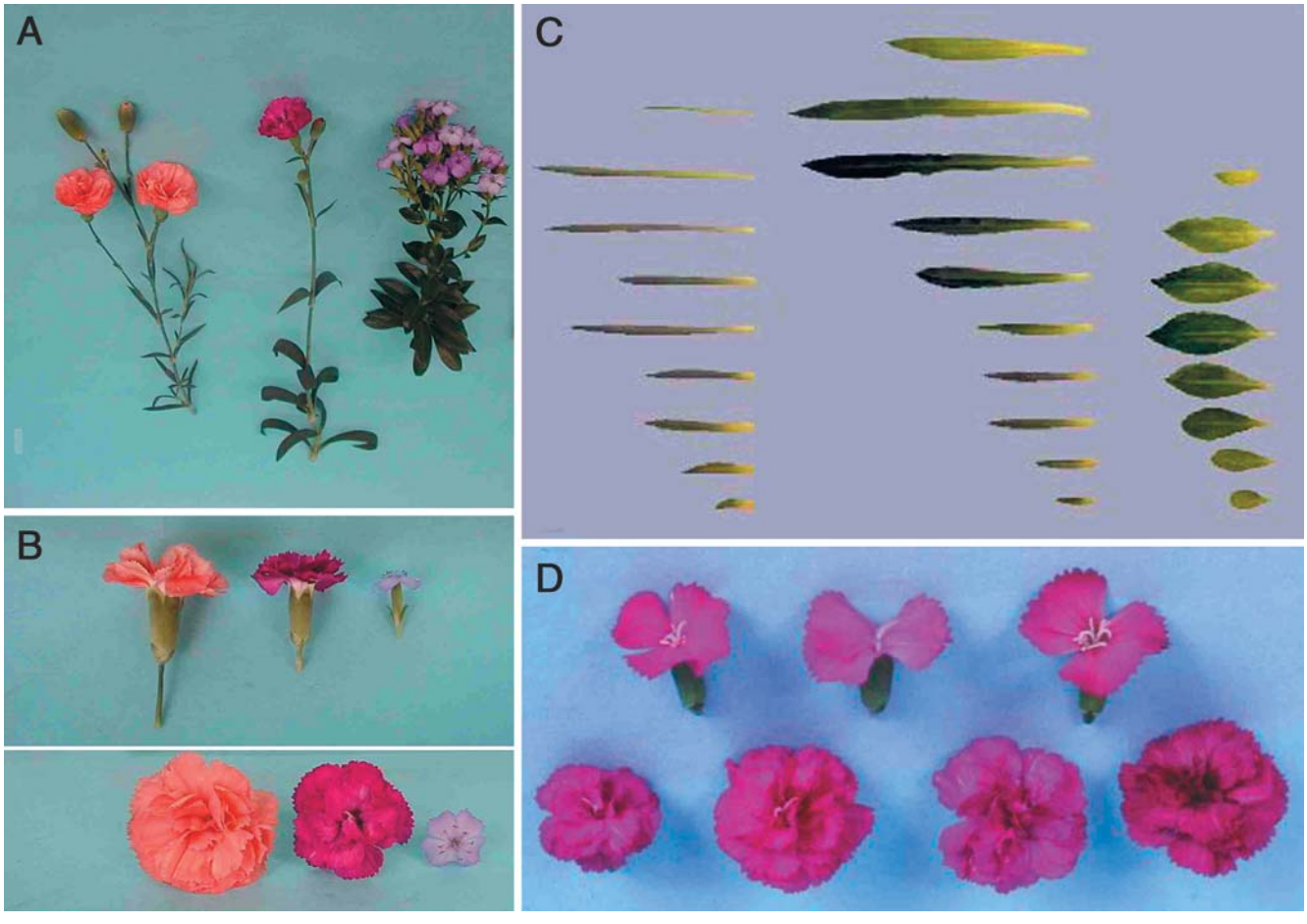

Fig. 3A–D Characteristics of the hybrids obtained from the cross between *D. caryophyllus* and *D. japonicus*. **A** Flowering plant of *D. caryophyllus* '98sp1651' (left), hybrid '43B' (center) and *D. japonicus* 'HAMA1' (right). **B** Flowers of the parental species and the hybrid, *D. caryophyllus* '98sp1651' (left), the hybrid '43B' (center) and *D. japonicus* 'HAMA1' (right). **C** Leaves of *D. caryophyllus* '98sp1651' (left), hybrid '43B' (center) and *D. japonicus* 'HAMA1' (right). **D** Flower variations in the interspecific hybrids

Discussion

Direction of cross compatibility

The results of the reciprocal crosses between six lines of carnation and one line of *D. japonicus* show that the interspecific hybrids were obtained only when one line of carnation, '98sp1651', was used as the seed parent. One of the possible reasons for this difference in hybrid productivity in the 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, we recently carried out embryo culture in the crosses with some carnation lines and obtained putative interspecific hybrids evaluated by morphological characters (unpublished result). Therefore, it is possible that the development of hybrid embryos was inhibited by some physiological factors possessed by most of the carnation lines used as maternal parents. Although abortion of the hybrid embryo has not been confirmed when *D. japonicus* 'HAMA 1' was used as the maternal parent, it might be possible to produce interspecific hybrids with carnation by using other lines of *D. japonicus*, which have an adequate genetic background.

The other possible reason for the difference in hybrid production in the reciprocal interspecific crosses might be that the combination between the hybrid nucleus and '98sp1651' cytoplasm was appropriate for survival of the hybrid embryo or for the development of the endosperm. In the interspecific cross between *Capsicum annum* and *Capsicum sinense*, normal progenies were produced when *C. annum* was used as the maternal parent, but the hybrids obtained in the reverse cross showed seriously stunted growth. The cause of this phenomenon has been considered to be the mismatch between the nuclear gene and the cytoplasmic gene (Inai et al. 1993). In the present study, nuclear-cytoplasmic interaction might cause 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 due to 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 the hybrid embryo nutritionally when it is used as the maternal parent. In these two cases, it might be possible to produce the interspecific hybrids by examining the timing of hybrid embryo-rescue culture and/or the culture medium constitution. However, if the abortion of the hybrid embryo is caused by the influence of the cytoplasmic gene, it might be extremely difficult to obtain the hybrid.

Maternal plant-like progenies having no evidence of hybridity

In the present study on interspecific hybridization, approximately half of the progenies obtained from '98sp1651', and all from the other lines of carnation used as the maternal parents, showed very similar morphologies to maternal carnation plants with no evidence of hybridity by DNA analysis. However, some of the carnation-like progenies showed stunted growth or death, and some others showed a different color and morphology of the flowers from the maternal carnation plants (data not shown). It is well known in barley (*H. vulgare*) that haploidy is efficiently produced by crossing with *Hordeum bulbosum*, a wild relative species, due to the selective elimination of *H. bulbosum* chromosomes at the early stage of embryo development (Kasha and Kao 1970). Furthermore, there is a report in carnation that doubled-haploids were yielded by inducing pseudogamy by 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-haploids. Since 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 the plants. Since the 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 which act to prevent the elimination of alien chromosomes, i.e. those of *D. japonicus*.

Double flower-gene expression in the hybrid

The 12 hybrids obtained from '98sp1651' as the maternal parent were separated into seven '98sp1651'-like double-flowered plants and *D. japonicus*-like single-flowered ones. A similar segregation pattern was also obtained in our preliminary study, in which the cross between double- and single-flowered plants yielded both double- and single-flowered progenies (unpublished result). It is conjectured from these results that the double-flowered gene(s) of carnation might be expressed by the combination of more than two genes, which are dominant to the single-flowered gene(s) of *D. japonicus*.

Among the previous studies on interspecific hybridization in *Dianthus* species, it has been reported that the difficulty in producing interspecific hybrids depends on the crossing combinations (Kanda 1992; Ohtsuka et al. 1995; Gatt et al. 1998; Onozaki et al. 1998). Although some seedlings were produced in the cross between carnation and *D. japonicus* using ovule culture (Kanda 1992), no detailed characterization of the seedlings was conducted in this study. Consequently, hybridity of the plants produced by the cross between carnation and *D. japonicus* was first confirmed in the present study.

The hybrids obtained by the cross between carnation '98sp1651' and *D. japonicus* showed an intermediate morphology of the parents in leaf width and flower size. As a result, favorable characters of *D. japonicus* such as the strong stem and wide leaf width were successfully transmitted to the hybrids. Moreover, the hybrids showed more vigorous growth than carnation and were easy to propagate vegetatively by cutting. However, the hybrids had several undesirable characters such as the less number of flowers at one stem and the segregation of single-flowered individuals. To remove these undesirable characters, it is necessary to conduct backcrossing the hybrids with carnation. Moreover, by culturing

more immature embryos, it might be possible to obtain more hybrids from the various genotypes of carnation as the maternal parents. Carnation-like progenies obtained in the present study will also be utilized for F_1 breeding of carnation if they are confirmed to be double-haploid.

The hybrids obtained in the present study are difficult to use as the pollen parent for further breeding because of pollen sterility. To restore the pollen fertility, it is necessary to produce amphidiploids by artificial chromosome doubling. After successful production of the amphidiploids, 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.

Acknowledgements The authors thank assistant Prof. Dr. T. Tanaka, Graduate School of Bioagricultural Sciences, Nagoya University, for his technical advice on flow cytometry.

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