Title: Unreduced 3x gamete formation of allotriploid hybrid derived from the cross of *Primula denticulata* (4x) × *P. rosea* (2x) as a causal factor for producing pentaploid hybrids in the backcross with pollen of tetraploid *P. denticulata*

Subtitle: Unreduced 3x gamete formation of triploid hybrid in *Primula*

Authors

Mai Hayashi · Juntaro Kato · Hiroaki Ohashi · Masahiro Mii

M. Hayashi · M. Mii (✉)

Laboratory of Plant Cell Technology, Graduate school of Horticulture, Chiba University, 648 Matsudo, Matsudo-City, Chiba 271-8510, Japan

Correponding author. Tel. : +81-47-308-8852; Fax: +81-47-308-8852

e-mail: miim@faculty.chiba-u.jp

J. Kato

Department of Biology, Aichi University of Education, 1 Hirosawa, Igaya-cho, Kariya-City, Aichi 448-8542, Japan

H. Ohashi

Faculty of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama-City, Ehime 790-8566, Japan

Abstract

A triploid hybrid, which was obtained from interspecific crosses between tetraploid *Primula denticulata* (2n = 4x = 44) and *P. rosea* (2n = 2x = 22), successfully produced 11 plants by backcrossing with pollen of tetraploid *P. denticulata*. Analysis of ploidy level using flow cytometry and chromosome counting in the 11 BC_1 plants revealed that all progeny had much larger DNA contents and chromosome number than both parents. In this triploid-tetraploid (3x-4x) crossing, progeny was predominantly true or near pentaploid presumably produced by the fertilization between true or near triploid female gamete produced from triploid hybrid and diploid pollen of tetraploid *P. denticulata*. These results suggest that unreduced (3x) or near triploid female gametes were partially produced by single step meiosis, either FDR (first-division restitution) or SDR (second-division restitution) process. The zygotes formed by the fertilization between true or near triploid egg produced by single step meiosis in triploid hybrid and diploid pollen produced by normal meiosis of tetraploid *P. denticulata* might be the only survivors in embryogenesis.

Keywords: Backcrossing; Ploidy level; Ploidy chimera; Primrose; Unreduced gamete

Introduction

In current breeding, important horticultural hybrids have been produced mainly by two strategies; one is interspecific hybridization and the other is polyploidization (Van Tuyl and Lim 2003). Interspecific or inter-generic hybridization is one of the most important source for variation in plants breeding. Interspecific hybrids have the potential to capture new type of characteristics as well as combine diverse gene pools. Polyploidization has been playing an important role for crop evolution as well as development of new plants. There are two types of polyploidization; one is mitotic polyploidization such as chromosome doubling and the other is meiotic polyploidization such as unreduced (2n) gamete formation (Van Tuyl and Lim 2003). In the case of mitotic polyploidization, no homologous recombination can be seen in subsequent progenies, although sterile intersspecific hybrids can restore their fertility. On the other hand, meiotic polyploidization such as unreduced gamete formation has occasionally been observed in plant species or interspecific hybrids, and 2n gametes were produced instead of normal haploid (n) gametes (Nimura et al 2006, Lim et al. 2001). Hermsen (1984) described that diploid gamete formation could occur in diploid plant as a result of first-division restitution (FDR) or second-division restitution (SDR) in meiosis. Recently, third mechanism was reported as indeterminate meiotic restitution (IMR), which produces microspores with numerically disproportionate numbers of chromosomes due to a restitution mechanism (Lim et al. 2001). Particularly, FDR gamete is expected to be utilized for breeding of polyploid crops because FDR gametes contain an equal number of parental chromosomes and maintain maximum heterozygosity.

Triploid plants are generally difficult to utilize for breeding because of their high sterility. However, autotriploids have been successfully used as parents to produce aneuploids such as trisomics because some autotriploids sometimes have fertility to some extent, which explained relatively high degree of chromosome pairing during meiosis (Brandham 1982). Autotriploid might produce trivalent or bivalent + univalent between homologous chromosomes and have a chance to produce functional balanced haploid (x), diploid (2x), or

triploid (3x) gamete, and ploidy levels of progeny plants obtained by the cross using triploid depend on the ploidy levels of gamete. On the other hand, allotriploids including one genome of alien species tend to show more restricted chromosome paring during meiosis and consequently a higher degree of sterility because of the higher frequency of miss-pairing (Kato et al. 2001). However, allotriploids have successfully been used in crossing mostly as female parent and the resulting progenies have been used for backcrossing in several plants. Pentaploid progenies were yielded successfully in *Lilium* from allotriploids in 3x-4x crosses (Lim et al. 2003).

In breeding for evolutionary studies, crosses were conducted to monitor the introgression of alien genes conferring new characters and to trace the genomic relationships between closely related species (Barthes and Ricroch 2001). Moreover, chromosome addition lines of various plant species, such as wheat, have been produced from interspecific crosses in order to introduce valuable genes from wild or cultivated relatives into host plant species (Islam and Shepherd 1991; Taketa and Takeda 2001).

In our previous study, triploid interspecific hybrids were successfully produced by the cross between tetraploid *Primula denticulata* and diploid *P. rosea* (Hayashi et al. 2007a). In the present study, we report the successful production of BC_1 progeny by backcrossing the triploid hybrid with pollen of tetraploid *P. denticulata*. The BC_1 progeny had larger DNA contents in F_1 hybrids. The possible mechanisms involved in this phenomenon are also discussed.

Materials and methods

Plant materials

Three triploid hybrids (DDR genome) with two genomes of *P. denticulata* (2n = 4x = 44, DDDD genome) and one genome of *P. rosea* (2n = 2x = 22, RR genome), obtained by interspecific cross between *P. denticulata* and *P. rosea* (Hayashi et al. 2007a), and two plants

were used for each *P. denticulata* and *P. rosea* in the present study. Triploid hybrids, *P. denticulata* and *P. rosea* had both pin and thrum-type flowers.

Crossing and embryo rescue

Back crossings were carried out between triploid hybrids used as female parent and both parental species of triploid hybrid, i.e., *P. denticulata* and *P. rosea*, used as pollen parent (Table 1). Crossing was only made with legitimate combinations between pin (long style) and thrum (short style) type. Although pollen of triploid hybrids was mostly sterile, they were emasculated carefully just after blooming to avoid accidental occurrence of pollination with rarely produced fertile pollen, and pollinated with pollen of *P. denticulata* and *P. rosea*. The tips of corolla of a pollinated flower were then covered with a thread to prevent from pollination with the pollen of other plants. Four weeks after pollination, immature placenta with ovules were cultured on 0.25% (w/v) gellan gum-solidified half-strength MS medium (Murashige and Skoog 1962) containing 5% (w/v) sucrose, 50 mg/l gibberellic acid (GA_3), 0.1 mg/l 1-naphthaleneacetic acid (NAA) and 0.1 mg/l 6-benzylaminopurine (BAP) as described by Hayashi et al. (2007a).

Ploidy level analysis

The ploidy level of the parental plants and the progeny were determined by two methods, measurement of nuclear DNA content by flow cytometry (FCM) using PA (Partec, GmbH, Germany) on isolated nuclei stained with 4,6-diamidino-2-phenylindole (DAPI) solution and counting chromosome number according to the methods of Hayashi et al. (2007a). For FCM analysis, leaf tissues were mainly used. In case if different two major peaks were detected, petiole and root tissues were also subjected for the analysis. DNA contents of the parents and progeny were estimated by using *P. obconica* as an internal standard. DNA values in the present study were showed as ‘units’ rather than ‘picograms’ because the DNA values were

estimated by only DAPI.

PCR-RFLP and RAPD analysis

Total genomic DNAs of the parental plants and progeny were extracted from 100 mg of leaf tissue by CTAB method (Doyle and Doyle 1987), and used for confirming the hybrid nature by both PCR-RFLP analysis of ITS region and RAPD analysis.

The ITS region of nuclear rDNA was amplified *via* polymerase chain reaction (PCR), according to the methods of Hayashi et al. (2007b). Sequences of the primers used were ITSAB101 (5'-ACG AAT TCA TGG TCC GGT GAA GTG TTC G-3') and ITSAB102 (5'-TAG AA T TCC CCG GTT CGC TCG CCG TTA C-3'), respectively as reported by Douzery et al. (1999). Approximately 0.5 μg of PCR products were digested with 2 units of restriction endonuclease, *Hha*I at 37°C for 24h.

PCR of RAPD analysis was carried out using two common primers (BEX Co. Ltd., Tokyo, Japan), A02 (5'-GCC AGC TGT ACG-3') and A04 (5'-GCC CCG TTA GCA-3') (Nakamura 1990). Ex Taq polymerase (Takara Co., Japan) was used for RAPD analysis and each 25 ml reaction mixture contained 1 ng total plant DNA. DNA fragments were amplified with 42 cycles of 30 sec at 94ºC, 30 sec at 45ºC, 1 min at 72ºC in a thermal cycler PC-700 (ASTEC Co. Ltd., Japan).

Electrophoresis of the restricted fragments and PCR products was conducted on a 1.5 % agarose gel in a TAE buffer system. After electrophoresis, agarose gel was soaked in 0.5 μg/l ethidium bromide solution for 30 min and the bands were photographed under ultraviolet illumination.

Results

In the back crosses of triploid interspecific hybrid with parental species, either tetraploid *P. denticulata* or diploid *P. rosea*, progeny were successfully yielded in only the cross

combination between ‘25-5’ used as female parent and *P. denticulata* used as pollen parent (Table 1). In this successful cross combination, viable seeds were obtained in four out of six pollinated flowers and totally 11 progeny plants were obtained. In contrast, no viable seeds were observed in the cross combination using *P. rosea* as pollen parent, despite all pollinated flowers produced seeds. All 11 progeny plants grew vigorously and were acclimatized after four months of germination.

Both *P. denticulata* and *P. rosea*, parents of interspecific hybrid ‘25-5’, could be identified by the difference in nuclear DNA content using flow cytometry. When relative nuclear DNA content (2C value) of *P. obconica* used as an internal standard was provided to be 1.0 unit, those of *P. denticulata* and *P. rosea* were approximately 2.3 and 1.5 unit, respectively (Table 2 and Fig. 1A). Furthermore, the ploidy level revealed by chromosome counting of these two species was tetraploid (2n = 4x = 44; Fig. 2A) and diploid (2n = 2x = 22; Fig. 2B), respectively. Triploid hybrid ‘25-5’ showed an intermediate DNA content, approximately 1.9 unit, and chromosome number of 2n = 3x = 33, values lie just middle of the both parental species (Table 2, Fig. 1B and Fig. 2C, Hayashi et al. 2007a). By postulating the genome types of tetraploid *P. denticulata* and diploid *P. rosea* were DDDD and RR, respectively, the genome combination of ‘25-5’ was expressed as DDR. The C value of each one genome of D and R was calculated to be approximately 0.6 unit and 0.8 unit, respectively. Flow cytometric analysis revealed that all the progeny obtained by the cross between ‘25-5’ and *P. denticulata* showed much higher DNA contents than that of both parents. Based on the flow cytometric analysis of DNA contents, four types of BC_1 progeny plant were obtained. Eight out of 11 progeny showed the relative DNA content of approximately 3.1 unit (Table 2 and Fig. 1C). Among the remaining three plants, one plant (BC-11) showed larger DNA content of approximately 3.4 unit (Table 2 and Fig. 1D), and two plants showed two different peaks of DNA contents in a plant. Since two different peaks of DNA contents were detected in all the 5 different leaves, they are considered to be periclinal chimera consisting of two kinds of cells with different DNA contents. In these two chimeric plants, ‘BC-1’ showed two peaks of 2.9 and 3.6 unit, and ‘BC-6’ had two different peaks for larger DNA contents, 3.9 and 4.8 unit

(Table 2 and Fig. 1E).

Observation of somatic chromosomes of root tips revealed that six types of chromosome numbers were found in these 11 progeny plants. Four out of eight BC_1 plants with 3.1 unit of relative DNA contents had 55 chromosomes (Table 2, and Fig. 2D), which coincided with chromosome number of DDDDR genome (2n = 5x). This could be expected by the fertilization between unreduced 3x female gamete of '25-5' and reduced 2x pollen of tetraploid *P. denticulata*. Among four remaining plants with the same relative DNA contents (3.1 unit), however, three plants had 54 chromosomes, and one progeny 'BC-2' with smallest DNA content as approximately 3.0 unit had 52 chromosomes (Table 2). One progeny 'BC-11' with approximately 3.4 unit had 56 chromosomes (Table 2 and Fig. 2E). The two progeny plants with chimerical nature of DNA contents exhibited only one type of chromosome numbers; i.e., 61 (BC-1) and 88 (BC-6) chromosomes, respectively, through the repeated sampling of at least 10 root tips at different growth stages (Table 2 and Fig. 2G and 2H).

PCR-RFLP analysis of ITS region was performed to detect specific DNA fragments for parents of triploid F_1 hybrid in the BC_1 progeny. PCR products in ITS region of DNA from *P. denticulata* and *P. rosea* were the same in length of approximately 900 bp (Fig. 3). After digestion of PCR products by *Hha*I, polymorphisms in length of restricted fragments were observed between the two species, approximately 270 bp for *P. denticulata* and approximately 550 bp for *P. rosea*, respectively (Fig. 3). F_1 triploid hybrid '25-5'with a DDR genome showed both parental bands. Furthermore, BC_1 progeny including two progeny with chimeric DNA content had also specific bands of both *P. denticulata* and *P. rosea*, except for 'BC-11' with 2n = 56 chromosomes which had only *P. denticulata* specific band (Fig. 3).

RAPD analysis was performed to detect specific maker for *P. rosea* using two common primers, A02 and A04. DNA fragments of approximately 1400 bp and 1200 bp were shown in BC_1 progeny when A02 was used as primer, except for three plants, 'BC-2' with 2n = 52, 'BC-5' with 2n = 54, and 'BC-11' with 2n = 56 chromosomes, respectively (Fig. 4A). Furthermore, fragment of approximately 1500 bp was shown in BC_1 progeny when A04 was used as primer, except for 'BC-7' with 2n = 54 (Fig. 4B).

Discussion

In the present study, few BC_1 progeny were successfully obtained only by the cross between allotriploid hybrid '25-5' with DDR genome and tetraploid *P. denticulata*, whereas no viable seeds were obtained by the cross using diploid *P. rosea* as pollen parent. However, all BC_1 progeny plants had much larger DNA contents than triploid hybrid and both parental species, i.e., *P. denticulata* and *P. rosea*. These BC_1 plants were classified into six types based on their chromosome number and relative values of their DNA contents. These included expected pentaploid hybrids with a DDDDR genome, three types of aneuploid hybrids and two types of chimeric hybrids. These results suggest that triploid hybrid formed unreduced female gamete with 3x or 3x number of chromosomes or even plants with higher chromosome number. There might be preferential fertilization between 3x (unreduced) female gametes and 2x pollen of tetraploid *P. denticulata*, ultimately resulted into the formation of expected pentaploid or near pentaploid BC_1 progenies. Since backcross of 3x interspecific hybrid with pollen of diploid *P. rosea* resulted in the production of non-viable seeds, zygotes with postulated genomes of DDRR might have aborted due to some post fertilization barrier as previously shown in *Cyclamen* 2x × 4x crosses (Takamura and Miyajima 1996, 1999).

Pentaploid (5x) or near pentaploid progeny with postulated genome of DDDDR obtained by the cross of allotriploid hybrid (DDR) with tetraploid *P. denticulata* (DDDD) were considered to be produced by the parthenogenetic gametogenesis, such as first division restitution (FDR), and second division restitution (SDR), which occurred in this allotriploid hybrid. FDR gametes contain an equal number of parental chromosomes, but SDR gametes express maximum homozygosity due to absence of crossing-over (Lim et al. 2004) and produce disomic(s) gamete at high frequency in allotriploid. Therefore, based on chromosome counting, four BC_1 progeny expected to have a DDDDR genome (2n = 55) were obtained by fertilization between FDR female gamete of '25-5' (with 33 chromosomes) and

reduced male gamete of *P. denticulata* (with 22 chromosomes). On the other hand, 'BC-11' with 56 chromosomes was considered to be obtained by fertilization between SDR female gamete of '25-5' (with 34 chromosomes) and reduced male gamete of *P. denticulata*. During SDR meiosis in allotriploid '25-5', 11 univalents inherited from *P. rosea* and 11 bivalents inherited from tetraploid *P. denticulata* were theoretically formed at metaphase I and then at anaphase I were divided to 11 univalents of *P. denticulata* and additional random number of univalents of *P. rosea*, such as six univalents in the case of 'BC-11' in anaphase I, and produced the female gamete with 22 chromosomes of *P. denticulata* and 12 chromosomes of *P. rosea* by SDR caused by unknown factor. As the results of PCR-RFLP in ITS region digested with *Hha* I, occurrence of SDR was showed in 'BC-11' by the fact that the specific band of *P. rosea* (approximately 550 bp) was not detected in this plant whereas all of the other progeny showed this band (Fig. 3). Therefore, this result suggests that 'BC-11' did not have some chromosomes of *P. rosea* including the chromosome localizing ITS region. Three BC_1 plants with 54 chromosomes and 'BC-2' with 52 chromosomes, that did not have the specific bands of *P. rosea* in RAPD analysis, could be obtained by either fertilization of SDR female gamete or partial deletion of *P. rosea* chromosomes from FDR gamete as previously reported in wheat-barley crossing (Taketa et al. 1995).

In the two types of chimeric BC_1 plants, 'BC-1' and 'BC-6', only one type of DNA content was detected in root tissues whereas shoot, such as leaf and petiole, showed two types of DNA content commonly by FCM analysis. In both progenies, chromosome number of the root tip cells approximately coincided to theoretically calculated chromosome number of larger DNA content detected in the shoot of each chimeric plant. In the triploid interspecific hybrid '25-5' with DDR genome, maximum chromosome number of zygotes produced by SDR is postulated to be 44, which is the sum of normal DD genome (22 chromosomes) and normal RR genome (22 chromosomes) caused by completely uneven segregation of *P. rosea*-derived chromosomes at first meiosis. Therefore, 'BC-1' mainly consisted of the cells with 61 chromosomes was possibly obtained by fertilization between SDR female gamete of '25-5' with 40, 42 or 44 chromosomes and normally reduced male gamete of *P. denticulata*

(22 chromosomes), followed by elimination of few chromosomes at the early stage of embryogenesis as reported previously in barley (Kasha and Kao 1970). Moreover, further elimination of some more chromosomes occurred at the early stage of shoot growth after germination or in upper part of zygotic embryo after polarized differentiation of embryonic axis, which resulted in chimeric shoot formation in this plant as also reported in barley (Taketa et al. 1995). Although there might be several possible explanations for forming 'BC-6' which had 88 chromosomes, it is necessary to apply GISH to clarify the detailed mechanism involved in the formation of this strain, which is now in progress. These two chimeric progenies might have been stable after becoming partial chimera owing to chromosome elimination or increase because they were hyper polyploidy with unbalance genomic constitutions. In interspecific hybridization of wheat and barley, somatic chromosome elimination or duplication mostly occurred at an early stage of embryogenesis because chromosome number of most anueploid hybrid was stable and sometimes produced mosaic chromosome number plants (Taketa et al. 1995). Our results might suggest that some chromosomes of BC_1 progeny were partially eliminated or duplicated at early stage of embryogenesis, which resulted in chimeric plants consisting of the tissues with two different chromosome numbers.

In the present study, it was revealed that triploid hybrid '25-5' produced unreduced female gamete. We reported previously that both parental species of triploid hybrid, *P. denticulata* and *P. rosea*, had some capacity of producing unreduced female gamete in interspecific crossing (Hayashi et al. 2007a; Hayashi et al. 2007b). Therefore, it is considered that capacity of producing unreduced female gamete was inherited from both or either of these two parental species to the triploid hybrid. Moreover, in backcrossing of triploid hybrid '25-5' with tetraploid *P. denticulata*, almost all progeny were nearly pentaploid. This result may suggest that female gametes with near or less than diploid level of chromosomes were sterile, or embryogenesis might be inhibited when such female gametes were fertilized. On the other hand, unreduced triploid (3x) or near triploid female gametes were partially produced by either FDR or SDR process, and zygotes formed by the fertilization between

these triploid egg and diploid pollen of tetraploid *P. denticulata* might only have survived in embryogenesis. A similar result was reported in crosses of allotriploid *Lilium* cultivar, that the progeny obtained by triploid-diploid (3x-2x) crosses were predominantly near-diploid whereas in the case of triploid-tetraploid (3x-4x) crosses they were mostly near-pentaploid (Lim et al. 2003). In this 3x-4x crossing, they suggested that triploid female parent had predominantly contributed for the production of pentaploid progeny through the formation of near triploid (2n) gametes. Furthermore, in our previous study of interspecific crossing between diploid *P. rosea* and tetraploid *P. denticulata* (2x-4x), relatively high rate of tetraploid hybrid was produced by the fertilization of unreduced female gamete in *P. rosea* (Hayashi et al. 2007a), suggesting that the growth of zygotes with higher ratio of foreign male genome than female genome might be inhibited after fertilization in this interspecific cross combination, and that hybrids produced by fertilization of unreduced gamete could only survive. In the present study, progeny was not successfully obtained in the cross of triploid hybrid with pollen of diploid *P. rosea* (3x-2x cross). Therefore, fertilization between gametes with nearly the same ploidy level might survive and fertilization with much larger or less ploidy level might result in inhibited embryogenesis in the backcrossings of the present study.

References

Barthes L, Ricroch A (2001) Interspecific chromosomal rearrangements in monosomic addition lines of *Allium*. Genome 44: 929-935

Brandham PE (1982) Inter-embryo competition in the progeny of autotriploid Aloineae (Liliaceae). Genetica 59: 29-42

Douzery EJP, Pridgeon AM, Kores P, Linder HP, Kurzweil H, Chase MW (1999) Molecular phylogenetics of Diseae (Orchidaceae): A contribution from nuclear ribosomal ITS sequences. Amer J Bot 86: 887-899

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of flesh leaf tissue. Phytochem Bull 19: 11-15

Hayashi M, Kato J, Ohashi H, Mii M (2007a) Variation of ploidy level in inter-section hybrids obtained by reciprocal crosses between tetraploid *Primula denticulata* (2n = 4x = 44) and diploid *P. rosea* (2n = 2x = 22). J Hort Sci Biotechnol 82: 5-10

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

Hermsen JG (1984) Mechanisms and genetic implications of 2n-gamete formation. Iowa State J Res 58: 421-434

Islam AKMR, Shepherd KW (1991) Alien genetic variation in wheat improvement. In “Biotechnology in Agriculture and Forestry vol 13. Wheat” Bajaj, Y.P.S. (ed.),

Springer-Verlag, Berlin Heidelberg. pp128-151

Kato J, Ishikawa R, Mii M (2001) Different genomic combinations in inter-section hybrids obtained from the crosses between *Primula sieboldii* (Section Cortusoides) and *P. obconica* (Section Obconicolisteri) by the embryo rescue technique. Theor Appl Genet 102: 1129-1135

Kasha KJ, Kao KN (1970) High frequency haploid production in barley (*Hordeum vulgare* L.). Nature 225: 874-876

Lim KB, Ramanna MS, De Jong JH, Jacobsen E, Van Tuyl JM (2001) Indeterminate meiotic restitution (IMR): a novel type of meiotic nuclear restitution mechanism detected in interspecific lily hybrids by GISH. Theor Appl Genet 103: 696-701

Lim KB, Ramanna MS, Jacobsen E, Van Tuyl JM (2003) Evaluation of BC_2 progenies derived from 3x-2x and 3x-4x crosses of *Lilium* hybrids; a GISH analysis. Theor Appl Genet 106: 568-574

Lim KB, Shen TM, Barba-Gonzalez R, Ramanna MS, Van Tuyl JM (2004) Occurrence of SDR 2N-gametes in *Lilium* hybrids. Breed Sci 54: 13-18

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

Nakamura I (1990) New DNA fingerprinting procedure: Amplified fragment length polymorphism of hazy associations (ALPHA). Ann Rep Natl Inst Genet Mishima 41: 105–106

Nimura M, Kato J, Mii M (2006) Interspecific hybrid production by reciprocal crosses between *Dianthus caryophyllus* L. and *Dianthus* × *isensis* Hirahata *et* Kitamura. J Hort Sci Biotechnol 81: 995-1001

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

Takamura T and Miyajima I (1999) Varietal and individual differences in cross-compatibility in the 2x × 4x crosses of cyclamen (*Cyclamen persicum* Mill.). J. Japan. Soc. Hort. Sci 68: 55-60

Taketa S, Kato J, Takeda K (1995) High crossability of wild barley (*Hordeum spontaneum* C. Koch) with bread wheat and the differential elimination of barley chromosomes in the hybrids. Theor Appl Genet 91: 1203-1209

Taketa S, Takeda K (2001) Production and characterization of a complete set of wheat-wild barley (*Hordeum vulgare* ssp. *spontaneum*) chromosome addition lines. Breed Sci 51: 199-206

Van Tuyl JM, Lim KB (2003) Interspecific hybridization and polyploidisation as tools in ornamental plant breeding. Acta Hort 612: 13-22

Tables

Table 1 Production and germination of seeds obtained by back crossing triploid hybrid female parent with both tetraploid *P. denticulata* and diploid *P. rosea*

[a] Genome combination (D; genome complement of *P. denticulata*, R; genome complement of *P. rosea*)

Table 2 Relative nuclear DNA content and chromosome number of BC_1 progeny

[a] Each value was expressed as the relative value compared to *P. obconica* (1.0), which was used as an internal standard after staining with DAPI.

[b] Provided that average single chromosome DNA value is (2.34 + 1.51) / (44 + 22) by dividing the sum of relative DNA content by the sum of the chromosome number of both parents.

[c] Plants with chimeric DNA content.

[d] The values in parentheses were obtained only in leaf.

Figure legends

Fig. 1 Flow cytometric profiles of the plants obtained from backcrosses of '25-5', interspecific hybrid *P. denticulata* × *P. rosea*, with *P. denticulata*. (**a**) Parental species of '25-5', *P. denticulata* (peak D; DDDD genome) and *P. rosea* (peak R; RR genome), triploid hybrid '25-5' (peak 25-5: DDR genome). (**b**) BC_1 hybrid 'BC-9' with DDDDR genome. (**c**) BC_1 hybrid 'BC-11'. (**d**) BC_1 hybrid 'BC-1' with two chimeric DNA contents. Peak O indicates *P. obconica* used as an internal standard.

Fig. 2 Somatic chromosomes of the BC_1 hybrids obtained by the cross between '25-5', interspecific hybrid *P. denticulata* × *P. rosea*, and *P. denticulata*. (**a**) *P. denticulata* 2n = 4x = 44 (DDDD genome) and (**b**) diploid *P. rosea* 2n = 2x = 22 (RR genome) used as parents of '25-5'. (**c**) Triploid hybrid '25-5' 2n = 3x = 33 (DDR genome). (**d**) BC_1 hybrid 'BC-3' 2n = 5x = 55 (DDDDR genome). (**e**) BC_1 hybrid 'BC-4' 2n = 54. (**f**) BC_1 hybrid 'BC-2' 2n = 52. (**g**) BC_1 hybrid 'BC-11' 2n = 56. (**h**) BC_1 hybrid 'BC-1' with chimeric DNA content 2n = 61. (**i**) BC_1 hybrid 'BC-6' with chimeric DNA content 2n = 88. All bar = 10 μm.

Fig. 3 Analysis of PCR-RFLP in ITS region. Profiles of PCR products (**a**) and restriction fragments of the products digested with *Hha*I (**b**). Lane D, *P. denticulata*; Lane R, *P. rosea*; Lane H, triploid hybrids '25-5' (DDR); Lane 1-11, BC_1 progeny of 'BC-1' - 'BC-11'. Lane M, ϕX174/*Hae*III-digestion size marker, Open circle and solid circle indicate the specific band of *P. denticulata* and *P. rosea*, respectively.

Fig. 4 RAPD-PCR analysis, using primer A02 (**a**) and A04 (**b**). Lane D, *P. denticulata*; Lane R, *P. rosea*; Lane H, triploid hybrids '25-5' (DDR); Lane 1-11, BC_1 progenies 'BC-1' - 'BC-11'. Lane M, ϕX174/*Hae*III-digestion size marker. Arrows indicate the specific bands of *P. rosea*.

Table 1 Production and germination of seeds obtained by back crossing triploid hybrid female parent with both tetraploid *P. denticulata* and diploid *P. rosea*

Female	Male	No. of flowers pollinated	No. of flowers with seeds	No. of flowers with germinable seeds	No. of germinated seeds
'25-5' (DDR[a]; pin)	*P. denticulata* (DDDD; thrum)	6	6	4	11
'26-3' (DDR; thrum)	*P. denticulata* (DDDD; pin)	5	0	0	0
'26-22' (DDR; thrum)	*P. denticulata* (DDDD; pin)	3	0	0	0
'25-5' (DDR; pin)	*P. rosea* (RR; thrum)	5	5	0	0
'26-3' (DDR; thrum)	*P. rosea* (RR; pin)	4	0	0	0
'26-22' (DDR; thrum)	*P. rosea* (RR; pin)	1	0	0	0

[a] Genome combination (D; genome complement of *P. denticulata*, R; genome complement of *P. rosea*)

Table 2 Relative nuclear DNA content and chromosome number of BC1 progeny

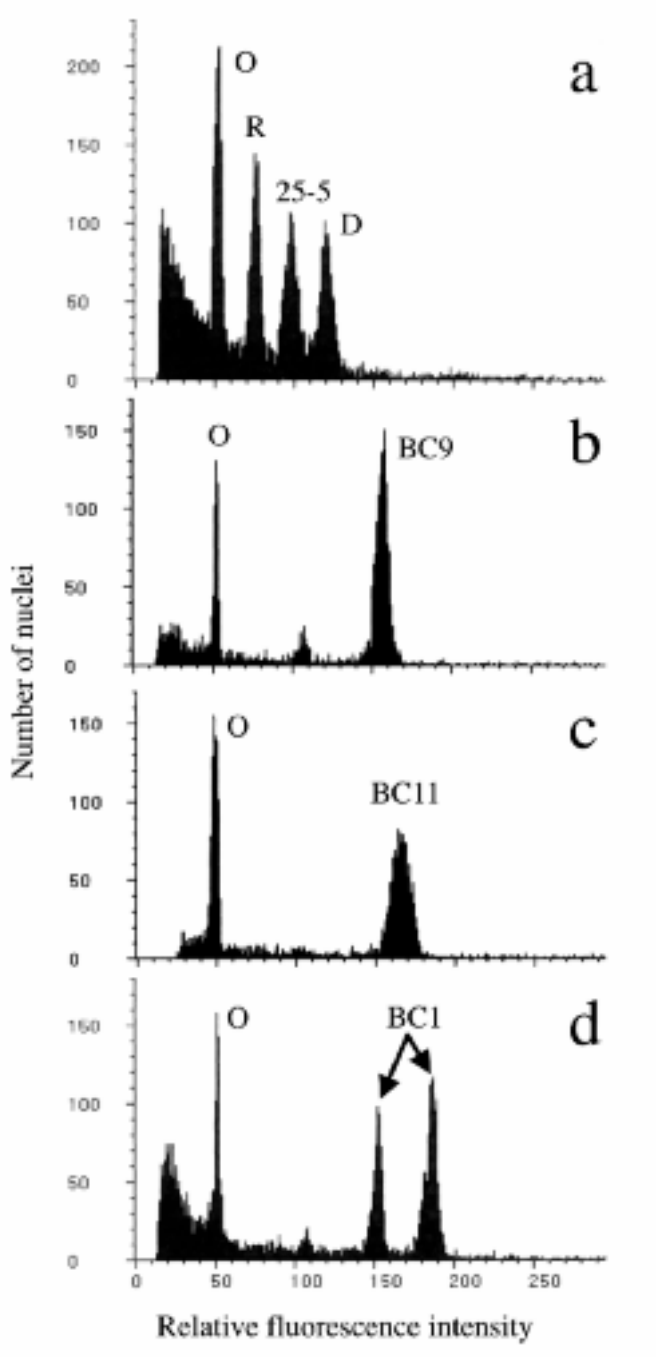
	Relative nuclear DNA content [a]	Chromosome number	
		Estimated by the results of FCM analysis [b]	Counted in root tip cells
P. denticulata	2.34	44	44
P. rosea	1.51	22	22
'25-5' (F_1 triploid hybrid)	1.91	32.8	33
BC_1 progeny			
'BC-1' [c]	3.64 (2.93) [d]	62.5 (50.3) [d]	61
'BC-2'	2.97	51.0	52
'BC-3'	3.09	53.0	55
'BC-4'	3.07	52.6	54
'BC-5'	3.07	52.7	54
'BC-6' [c]	4.78 (3.85) [d]	81.9 (66.0) [d]	88
'BC-7'	3.01	51.6	54
'BC-8'	3.10	53.2	55
'BC-9'	3.08	52.8	55
'BC-10'	3.09	53.0	55
'BC-11'	3.36	57.5	56

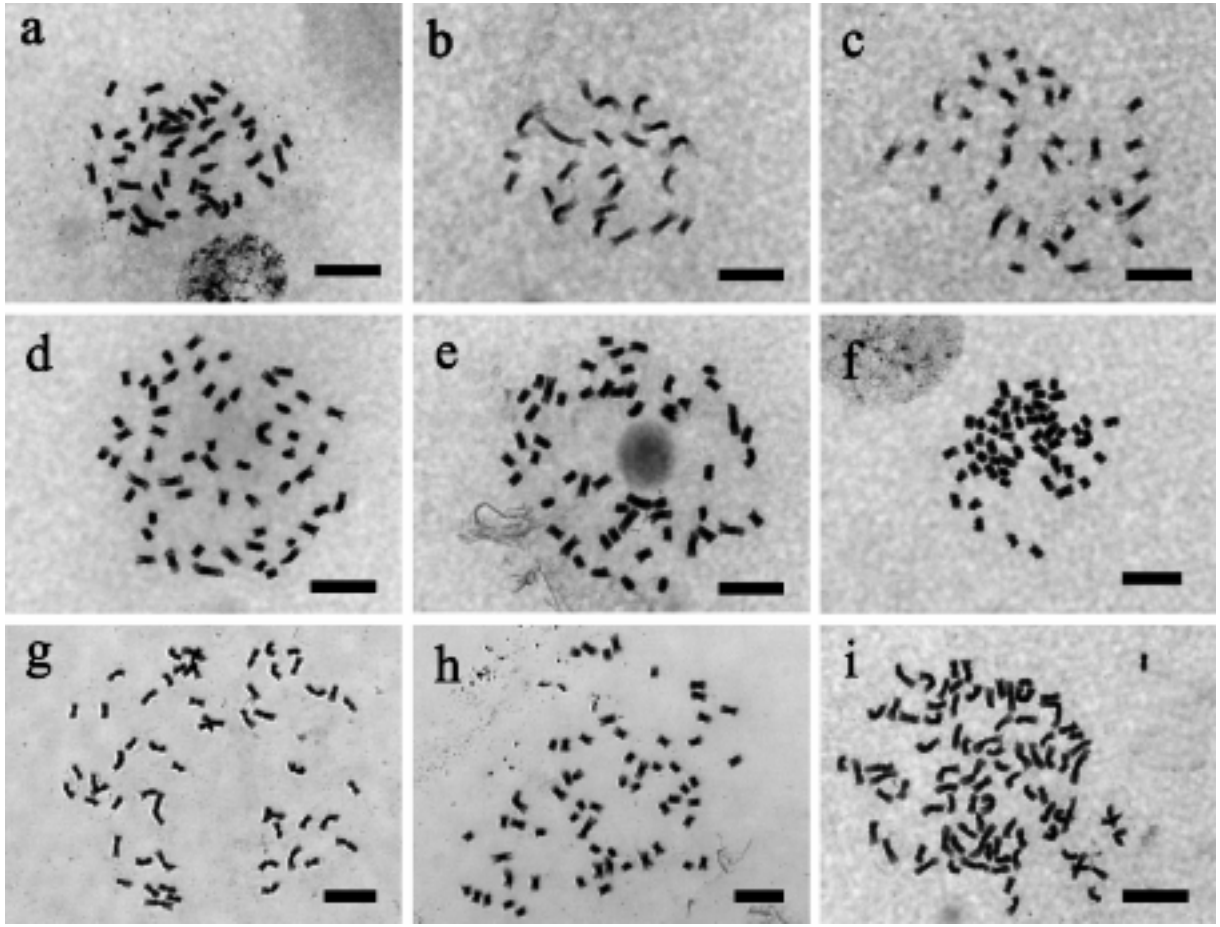
[a] Each value was expressed as the relative value compared to *P. obconica* (1.0), which was used as an internal standard after staining with DAPI.

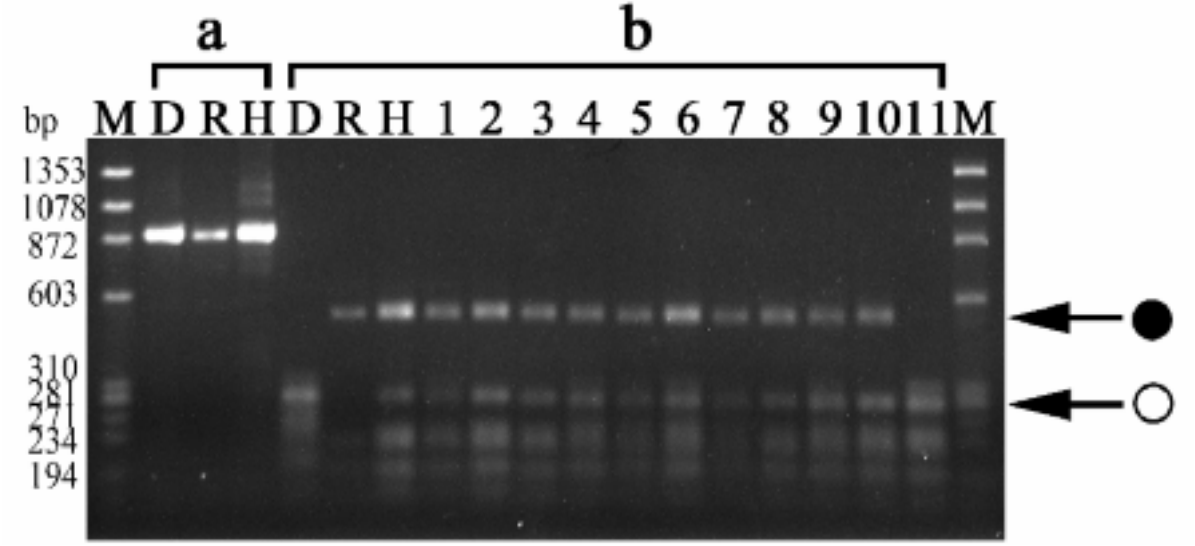
[b] Provided that average single chromosome DNA value is (2.34 + 1.51) / (44 + 22) by dividing the sum of relative DNA content by the sum of the chromosome number of both parents.

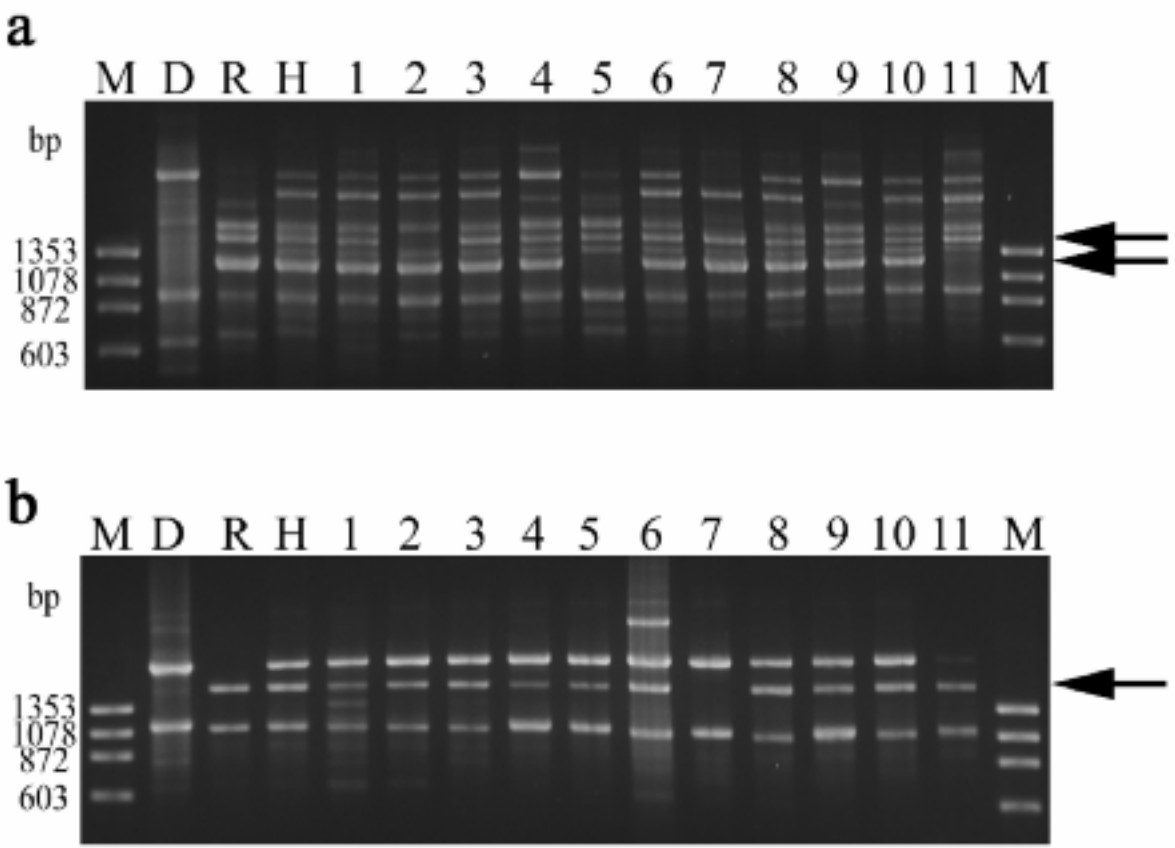
[c] Plants with chimeric DNA content.

[d] The values in parentheses were obtained only in leaf.


a
O
R
25-5
D
b
O
BC9
c
O
BC11
d
O
BC1
Number of nuclei
Relative fluorescence intensity
0
50
100
150
200
250


a
b
c
d
e
f
g
h
i


a
b
bp
M D R H D R H 1 2 3 4 5 6 7 8 9 10 11 M
1353
1078
872
603
310
234
194


a
M D R H 1 2 3 4 5 6 7 8 9 10 11 M
bp
1353
1078
872
603
b
M D R H 1 2 3 4 5 6 7 8 9 10 11 M
bp
1353
1078
872
603