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Characterization of sexual progenies of male-sterile somatic cybrids between *Brassica napus* and *Brassica tournefortii*

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Abstract Cytogenetic studies were performed on four male-sterile progenies derived from four different cybrids produced between *Brassica napus* and *B. tournefortii* using the donor-recipient protoplast fusion method. The objective of these studies was to characterize the nuclear constitution of the plants. Mitotic investigation revealed that three of the four male-sterile lines had 38 chromosomes, which is equal to that of *B. napus*. The fourth line, C6, had variable chromosome numbers, ranging from 39 to 42 in different plants. The meiotic behavior in each progeny varied distinctly. Of the plants having 38 chromosomes, fairly high chromosome pairing, on average 18.08 bivalents per cell, was detected at metaphase-I. However, univalents with an average of 1.39 per cell, and very low frequencies of trivalents and/or tetravalents, were also observed in the lines. These results revealed that male-sterile cybrid lines were obtained with 38 chromosomes and a relatively high level of chromosome-pairing ability, indicating their potential for establishing a stable male-sterile rapeseed line.

Key words *B. napus* · *B. tournefortii* · Cybrids · Cytogenetics · Cytoplasmic male sterility

Introduction

Somatic cybrids combining the nucleus of one species with the cytoplasm of another using the donor-recipient protoplast fusion method are especially attractive for modifying the cytoplasmic composition and for creating cytoplasmic male sterility (Pelletier et al. 1983; Pelletier 1986; Earle 1995). Usually, the donor-recipient fusion method is used for the production of somatic cybrids which theoretically contain nuclear DNA from only one parent, the nuclear donor. However, irradiation can generate fractionations of the donor chromosomes, which are putative candidates for chromosome recombination between the donor and the recipient chromosomes after cell fusion (Piastuch and Bates 1990; Parokonny et al. 1992, 1994; Forsberg et al. 1998; Skarzhynskaya et al. 1998). Therefore, the chromosome number and ploidy level in the original cybrids can vary to a large degree (Pelletier et al. 1983). Usually, it takes several generations of selfing or backcrossing with the nuclear donor to stabilize the cybrid nuclei.

In order to produce an alloplasmic, cytoplasmic male-sterile (CMS) *Brassica napus* line, somatic cybrids combining the nucleus of *B. napus* and the mitochondria of *Brassica tournefortii* were produced (Liu et al. 1996). Based on the nuclear, mitochondrial and fertility investigations performed, four groups of cybrids were identified. Among these, plants in groups one and two were male-fertile, while plants in groups three and four were male-sterile. The male-sterile cybrids of interest had a mtDNA pattern identical to or similar, to *B. tournefortii* and a nuclear DNA content either similar to *B. napus* or to a hybrid RFLP-pattern according to the Southern-blot and flow-cytometry analyses. However, after being backcrossed to rapeseed, male-fertile progenies were found in all the male-sterile cybrid lines studied except one. The male-fertile progenies continued to segregate for fertility in the subsequent backcrosses, while the male-sterile progenies were stabilized with respect to the male-sterile trait. This indicates that the nuclear genome in the original cybrids may not have been pure *B. napus* DNA.

Theoretically, the cybrids produced are expected to contain chromosomes only from the nuclear donor, *B. napus*. However, irradiation often results in incomplete

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elimination of the chromosomes from the donor and causes nuclear imbalances (Yarrow et al. 1990; Wolters et al. 1991; Trick et al. 1994; Forsberg et al. 1998). Thus, the fertile progenies obtained might be due to the presence of *B. tournefortii* nuclear DNA which could restore the fertility. Based on the nuclear analyses performed utilizing flow-cytometry analysis and RFLP, three of the four male-sterile cybrids were characterized as having only *B. napus* nuclear DNA (Liu et al. 1996). However, RFLP analysis allowed us to check only that part of the genome corresponding to the probes employed and flow cytometry is not a precise enough method to determine the exact chromosome numbers of the plants due to the small size of the *Brassica* chromosomes.

In order to establish whether stable CMS lines were obtained and whether nuclear restorer genes had been introgressed into the nuclear genome, we have started a backcross program of the male-sterile and male-fertile cybrids with *B. napus*. From the first backcross generation (BC_1), fertile progenies occurred among the male-sterile cybrid populations. In the subsequent backcrosses, the male-fertile cybrids continued to segregate from male-fertile to both male-fertile and male-sterile. In contrast, all the male-sterile cybrid plants showed stable male sterility regardless of whether they originated from a male-sterile cybrid or were segregants from a male-fertile cybrid. To be able to use these materials in our studies and to produce a stable CMS line of *B. napus*, it is necessary to reveal the nuclear constitution and stability of the cybrid progenies by performing cytogenetic analyses. Here, we present the results of meiotic and mitotic studies on the third backcross (BC_3) generation derived from the original cybrids. The nuclear stability and potential practical value of these lines are also discussed.

Materials and methods

Plant material

Somatic cybrids between *B. napus* cv Hanna and *B. tournefortii* were produced by the fusion of X-irradiated mesophyll protoplasts of *B. tournefortii* with iodoacetamide (IOA)-treated hypocotyl protoplasts of *B. napus* (Liu et al. 1996). The four cybrid lines, C6, C7, C8 and C9, were backcrossed to *B. napus* cv Hanna, as the recurrent parent, for three generations (Fig. 1). For cybrid C6, to be able to obtain pollen mother cells (PMCs) for our meiotic study, a restored line of *B. napus* cv Mangun, which was kindly provided by Dr Y. S. Sodhi (Tata Energy Research Institute, India), was used as the pollinator in the BC_3 generation instead of *B. napus* cv Hanna (Fig. 1). Seeds of *B. napus* cv Hanna and *B. tournefortii* were kindly provided by Svalöv Weibull AB.

Cytogenetic analyses

Mitotic analysis

For chromosome counting, seeds of the cybrid progenies and the parental species were sown into small pots in the greenhouse. Root-tips were excised from these plants and pre-treated with 0.05% colchicine for 2 h. The pre-treated root-tips were transferred to ice water and kept in a refrigerator for 24 h. The roots were subsequently fixed in Carnoy I solution (3:1) for 4 h at room temperature. The meristematic portion of the root-tips was squashed in 45% acetic acid after hydrolysis in 1 N HCl at 60°C for 8 min and stained with Schiff's staining. Fifty cells from each line were examined. Permanent preparations were made as described by Merker (1971).

Fig. 1 Schematic presentation of the cybrid lines produced and the backcrossed progenies used in the current study; *ms* male-sterile; *mf* male-fertile

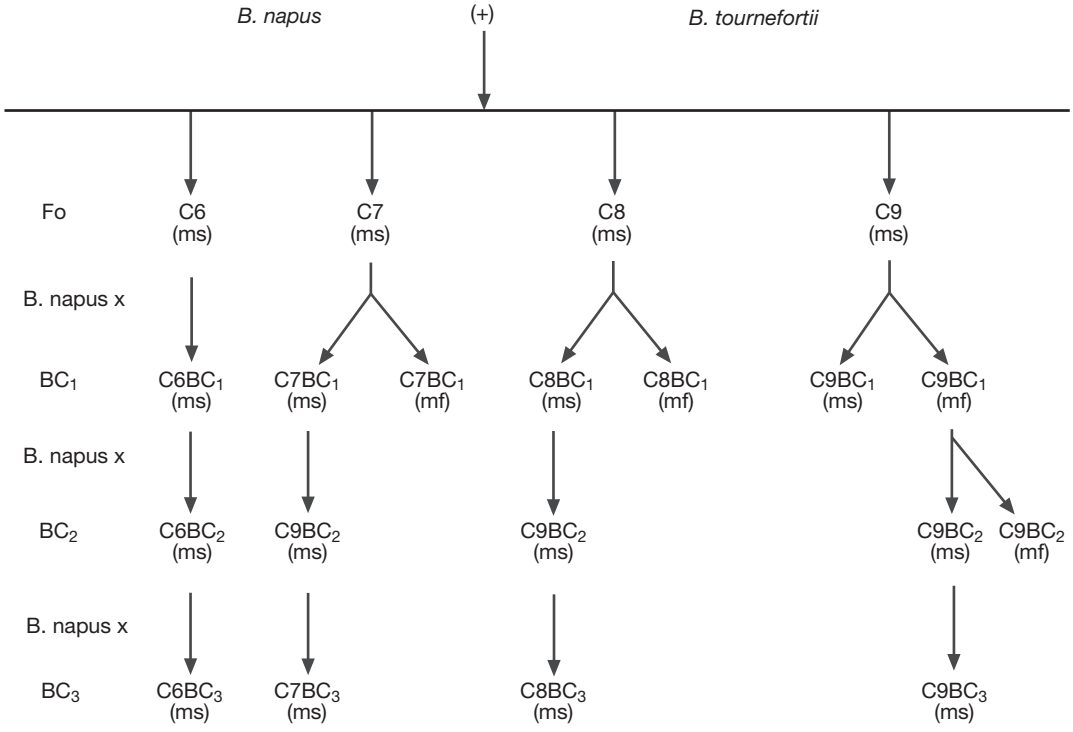

Table 1 Chromosome numbers and meiotic configurations of back-crossed progenies of male-sterile *B. napus* (+) *B. tournefortii* cybrids. Ranges of uni- bi- tri- and quadrivalents are given within parentheses; mf indicates male-fertile and ms male-sterile plants

Plant material	Fertility	No. of. chrom.	No. of PMCs	Univ.	Biv.	Triv.	Quadriv.
B. napus	mf	38	50	–	18.60 (15–19)	–	0.20
B. tour.	mf	20	50	–	10.00	–	–
$C6BC_3$[a]	ms	39	33	1.18 (1–3)	18.79 (17–19)	–	0.06 (0–1)
$C6BC_3$[b]	ms	40	19	2.10 (0–4)	18.95 (18–20)	–	–
$C7BC_3$	ms	38	36	1.17 (0–4)	18.27 (16–19)	0.06 (0–1)	0.03 (0–1)
$C8BC_3$	ms	38	20	1.80 (0–4)	17.90 (16–19)	0.10 (0–1)	–
$C9BC_3$	ms	38	30	1.20 (2–4)	18.07 (16–19)	–	0.17 (0–1)

[a] and [b] represent different plants

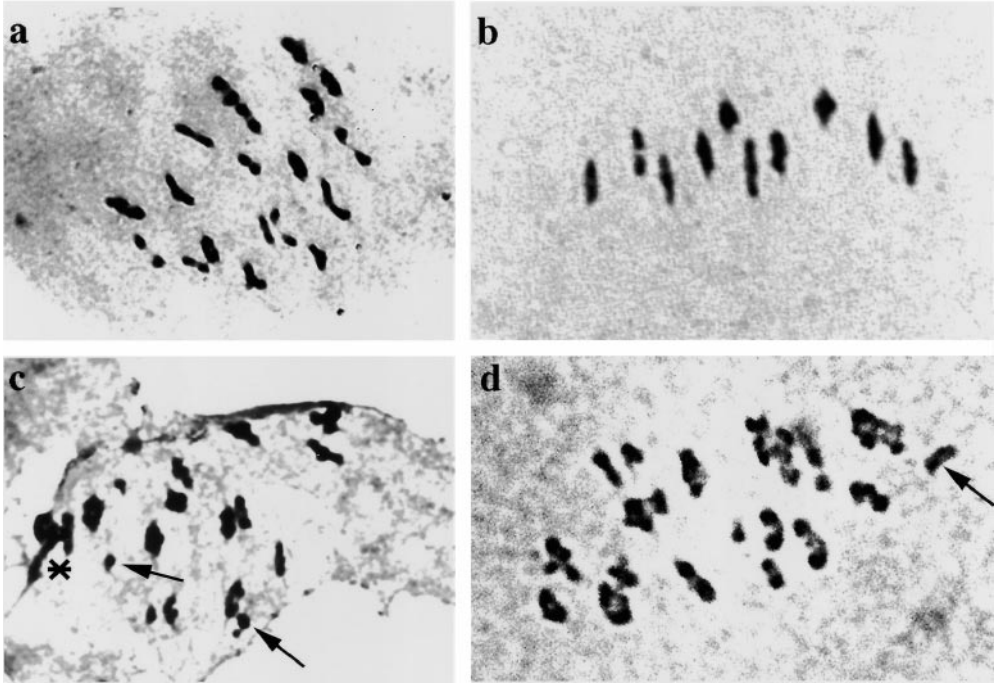

Fig. 2a–d Meiotic configurations at metaphase-I of *B. napus*, *B. tournefortii* and one male-sterile cybrid. **a** *B. napus*, 19 bivalents. **b** *B. tournefortii*, ten bivalents. **c** $C6BC_3$ with two univalents, 17 bivalents and one quadrivalent. **d** $C6BC_3$ with 19 bivalents and one univalent. The quadrivalent is indicated with * and the univalents with arrows

Meitotic analysis

For meiotic analysis, floral buds were fixed in Carnoy II solution (6:3:1 ethanol:chloroform:acetic acid) for 24 h and then transferred to 50% ethanol and stored at 4°C for at least 24 h or until use. Anthers containing PMCs at the metaphase-I were stained with acetocarmine and subsequently squashed according to Jaheir et al. (1989). Slides were made permanent as described above.

Flower morphology and fertility

Flower morphology and fertility were examined in four plants from each male-sterile line. Fertility was determined as described by Liu et al. (1995).

Results

Chromosome numbers in somatic cells

The chromosome number was examined in the four BC_3 cybrid descendants and their parents (Table 1). Of the plants studied, all the male-sterile plants except C6 had 38 chomosomes in their somatic cells, which is equivalent to the chromosome number of *B. napus*. In progenies from C6 a variation in chromosome number was found.

Meiotic configurations

The results of the meiotic behavior of the parental species and their BC_3 cybrid progenies are summarized in

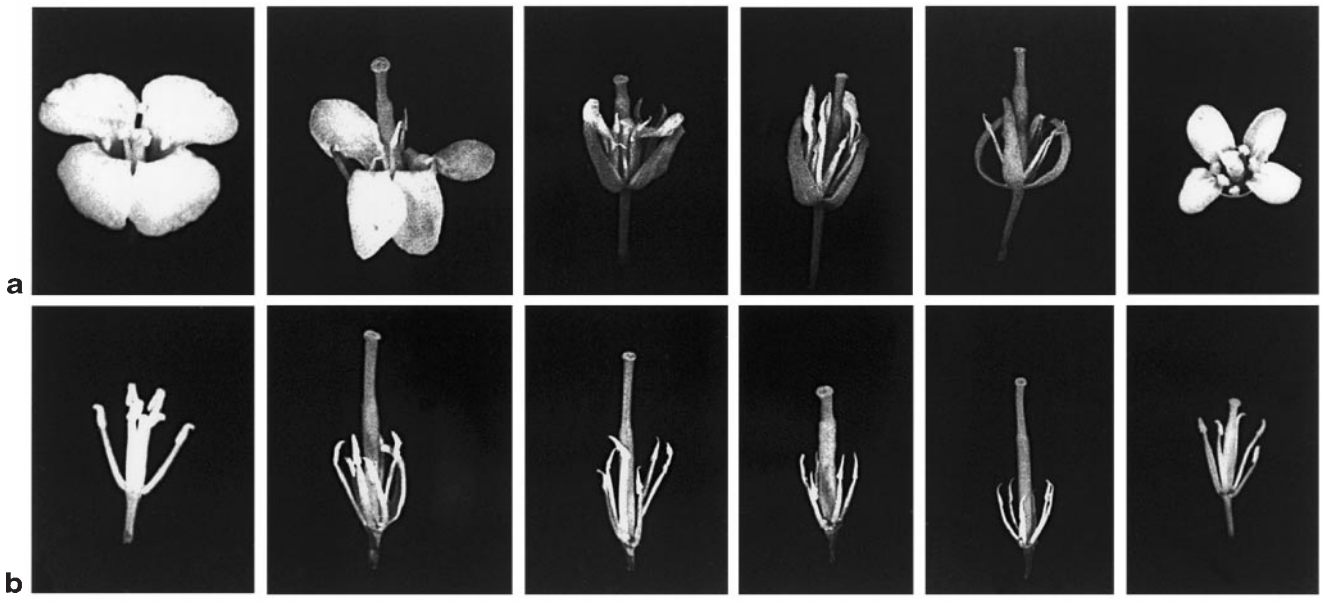

Fig. 3a, b Flower morphology. Complete flowers (**a**) and flowers with detached sepals and petals (**b**) from (left to right) *B. napus*, $C9BC_3$, $C8BC_3$, $C7BC_3$, $C6BC_3$ and *B. tournefortii*

Table 1 and Fig. 2. The two parental species had a regular meiotic behavior (Fig. 2a,b). In the diploid, *B. tournefortii*, all the chromosomes were paired as bivalents and neither univalents nor multivalents were formed, whereas in the amphidiploid, *B. napus*, the situation was different. A mean value of 18.6 bivalents per cell, with a range from 15 to 19 and 0.2 quadrivalents, with a range of 0 to 2 per cell, were recorded in *B. napus*. No univalents were found. Difficulties were encountered in obtaining meiotic configurations from the male-sterile plants. Nevertheless, the cybrid progenies from C7, C8, and C9 produced enough PMCs to conduct a meiotic analysis and to determine the meiotic configurations that were present. However, for C6, this was not possible due to the few PMCs produced. To be able to establish the meiotic configuration indirectly, the male-sterile C6 plants were crossed to a restorer line and several fertile progenies had to be analysed in order to assess the chromosome number of the mother plant.

Of the four cybrid progenies studied, large variations in meiotic configurations were detected. Of the male-sterile plants having 38 chromosomes, C7 showed the highest number of bivalents (18.27II), but the lowest number of univalents (1.17II) per cell. Univalents were found in all the plants, while trivalents occurred in C6 and C9. Quadrivalents were present in most cybrid lines (Fig. 2c). In C6 progenies a variation in chromosome number was found. This is also clearly shown in the meiotic configurations where the choromosome numbers according to our observations varied from 39 to 42 (Fig. 2c,d). The PMCs of the plants having 41 or 42 chromosomes were too few to determine their meiotic configurations.

Morphology

Among the male-sterile BC_3 progeny plants studied, a range of variations in flower morphology were observed (Fig. 3a,b, Table 2). The plants were classified into three groups. In group I the flowers contained small and needle-like anthers and the petals were narrow and abnormal. No PMCs were found. The original cybrid C6 as well as all the descendants from that line were representative of this group. In group II the two cybrids C7 and C8, as well as their stable male-sterile segregants, were characterised. They had narrow petals and degenerate

Table 2 Fertility and anther morphology of back-crossed progenies of male-sterile (ms) *B. napus* (+) *B. tournefortii* cybrids

Plant material	Fertility of original cybrid	Fertility of progenies (BC_3 generation)	Production of PMCs (%)[a]	Anther morphology
Group 1				
$C6BC_3$	ms	ms	0	Degenerated
Group 2				
$C7BC_3$	ms	ms	5	Abnormal
$C8BC_3$	ms	ms	5	Abnormal
Group 3				
$C9BC_3$	ms	ms	33	Partially normal

[a] % denotes the production of PMCs compared with that of *B. napus*

anthers exhibiting a low number of PMCs. In spite of the production of PMCs they were male-sterile. Male-sterile progenies from C9 were placed in the third group. The flowers in the original C9 cybrid, as well as in the male-sterile segregants, had fully developed petals. Anther morphology and the production of PMCs was relatively normal, but a low number of pollen was produced and thus the plants were male-sterile. The stamens among the different male-sterile cybrid lines studied were of variable length but shorter than normal *B. napus* anthers. They ranged from 2/3rds of the normal male-fertile anthers of rapeseed plants in C9 to 1/2 in the flowers of C6, C7 and C8 (Fig. 3a,b).

Discussion

In our current study, the aim was to find a stable male-sterile line with the same genomic and agronomic characteristics as oilseed rape. The male-sterile cybrids produced are of potential value for further studies of CMS traits and for future breeding applications. Based on the mitotic and meiotic studies conducted, we found that three of the four male-sterile cybrid progenies had 38 chromosomes, which is equal to that of *B. napus*. Thus, the desired chromosome number was obtained. However, the meiotic configurations of the plants differed slightly from those of *B. napus*. Univalents were present in all the PMCs of the cybrid lines C7, C8 and C9, but not in *B. napus*. This indicates nuclear instability of the original cybrids, since univalents can randomly move to either pole or lose their orientation and eventually get lost during meiotic division. The resulting daughter cells will thus contain varying chromosome numbers. The origin of the nuclear instability is most likely due to the chromosome rearrangements that occurred after combining the *B. napus* and *B. tournefortii* genomes. Mizushima (1980) reported that allosyndesis may take place between the A, C and T genomes. In addition, Nagpal et al. (1996) showed that the presence of a diploid genome in addition to the presence of a haploid genome enhanced homoeologous pairing between the divergent genomes. In our study, the segregation of male fertility in the male-sterile lines after backcrossing to *B. napus* indicated that either (1) chromosome instability could be responsible for the fertility segregation, or (2) a restorer gene(s) from *B. tournefortii* was maintained in the C7, C8 and C9 lines. In fact, Stiewe et al. (1995) showed that restorer genes are present in *B. tournefortii*. The univalents in C7, C8 and C9 may possibly represent *B. tournefortii* chromosomes. To confirm this hypothesis, genetic analysis will be performed by crossing male-sterile and fertile plants from the same origin.

In all the cybrid lines investigated, except for C6, some PMCs were found. This might be linked to the instability of these lines. Comparing the meiotic configurations of C7, C8 and C9, the highest percentage of PMCs having 19 bivalents was found in C7. This indicates that this line is the most stable line cytogenetically and may be of value for *Brassica* hybrid-seed production in the future. From the morphological point of view, C7 also showed a relatively uniform flower morphology. However, it displayed an instability in male sterility. The descendants from C9 developed the most normal flowers, including the anthers. However, the production of a relatively large number of PMCs and also pollen resulted in an instability in the male-sterile trait. In contrast, cybrid C6 showed a stable inheritance of the male-sterile trait from the original cybrid to the BC_3 progenies by never segregating to fertility. The cybrid line C6 did not produce any PMCs, which is similar to the already described morphology of the Anand CMS line, i.e. a premeiotic inhibition of microspore division. Similar results were reported for the cybrids produced by Stiewe and Röbbelen (1994). Furthermore, degeneration of PMCs prior to meiosis was described by Kerlan et al. (1993) in the *B. napus*-B. *adpressa* hybrids, which might also be the case in C6. Regardless of the stable male-sterile morphology of C6, a range of variations in chromosome number were found in the C6 plants. The current cytogenetic and morphological data suggest that the nucleus of C6 might have one of the following compositions:(1) chromosomes from the nuclear donor, *B. napus*, supplemented with extra *B. napus* chromosomes due either to partial polyploidization induced by protoplast fusion or because more than one *B. napus* protoplast was involved in fusion with the irradiated *B. tournefortii* protoplasts; or (2) a complete set of *B. napus* chromosomes and a few of *B. tournefortii* but with no restorer gene(s) present.

Since protoplast fusion and the post-fusion tissue culture could induce cytogenetic instability, such as alterations of chromosome number and structure or abnormal mitotic/meiotic divisions, the resulting somatic hybrids or cybrids may possess variable chromosome numbers (Keller et al. 1982; Joachimiak et al. 1995). A large number of cases of limited chromosome elimination of the cytoplasmic donor, as well as genetic imbalance caused by irradiation, have been reported in asymmetric hybridization or somatic cybridization (Gleba et al. 1988; Famelaer et al. 1989; Yamashita et al. 1989; Wijbrandi et al. 1990; Kovtun et al. 1993; Trick et al. 1994; Forsberg et al. 1998). This could also be the reason why the BC3 progeny derived from the C6 cybrid have variable chromosome numbers. To stabilize the nucleus of C6, further backcrosses to *B. napus* have been carried out. Ongoing restoration tests on C6 will reveal its potential value for hybrid-seed production in rapeseed.

In conclusion, the present investigation revealed that male-sterile cybrid lines with 38 chromosomes, which is equal to that of *B. napus*, were obtained. A relatively high level of chromosomal pairing ability among the 38 chromosomes in C7 with regular meiotic behavior and recombined mitochondria (Liu et al. 1996) indicates its potential value for further rapeseed breeding programmes. The stable male-sterile cybrid line C6 was proven to have variable chromosome numbers in different plants. To stabilize the C6 line, and to select stable

male-sterile lines from C6 with 38 chromosomes, subsequent backcrossing to *B. napus* and further genetic studies will be performed. The male-sterile C9 was the only line showing normal flower morphology, including normal stamens, which may permit the selection of a male-sterile line with normal flower morphology in the successive backcrossed progenies. Male-fertile lines with 38 chromosomes segregating from C7, C8 and C9 are of value as putative restorer lines and will, thus, be followed in subsequent generations to determine their restoring capacity.

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