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A *Moricandia arvensis* – based cytoplasmic male sterility and fertility restoration system in *Brassica juncea*

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Abstract A cytoplasmic male-sterility system has been developed in mustard (*Brassica juncea*) following repeated backcrossings of the somatic hybrid *Moricandia arvensis* (2n = 28, MM) + *B. juncea* (2n = 36, AABB), carrying mitochondria and chloroplasts from *M. arvensis*, to *Brassica juncea*. Cytoplasmic male-sterile (CMS) plants are similar to normal *B. juncea*; however, the leaves exhibit severe chlorosis resulting in delayed flowering. Flowers are normal with slender, non-dehiscent anthers and excellent nectaries. CMS plants show regular meiosis with pollen degeneration occurring during microsporogenesis. Female fertility was normal. Genetic information for fertility restoration was introgressed following the development of a *M. arvensis* monosomic addition line on CMS *B. juncea*. The additional chromosome paired allosyndetically with one of the *B. juncea* bivalents and allowed introgression. The putative restorer plant also exhibited severe chlorosis similar to CMS plants but possessed 89% and 73% pollen and seed fertility, respectively, which subsequently increased to 96% and 87% in the selfed progeny. The progeny of the cross of CMS line with the restorer line MJR-15, segregated into 1 fertile : 1 sterile. The CMS (*Moricandia*) *B. juncea*, the restorer (MJR-15), and fertility restored F_1 plants possess similar cytoplasmic organellar genomes as revealed by 'Southern' analysis.

Key words Cytoplasmic male sterility · Fertility restoration · *Moricandia arvensis* · *Brassica juncea* · Protoplast fusion · Somatic hybrids

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Introduction

A major hurdle to developing commercial heterotic hybrids of crop plants is the non-availability of a reliable pollination control system, particularly for self-pollinated crops. Among the available systems, a combination of male sterility induced by cytoplasmic genes and restoration of fertility by nuclear genes is considered to be the most efficient. Although cytoplasmic male-steriles in crop species originate spontaneously, or have been synthesized following cytoplasmic substitutions, attempts at developing fertility restorers have not been very successful. Because cytoplasmic male sterility can be associated with disease susceptibility, diversification of the cytoplasmic base is desirable.

Mustard (*Brassica juncea*) is an important oil-yielding crop of the Indian subcontinent. In recent years, several male-sterile systems of alloplasmic origin have been developed in *B. juncea*. These include *B. oxyrrhina* (Prakash and Chopra 1990), *Diplotaxis siifolia* (Rao et al. 1994) and *Trachystoma ballii* (Kirti et al. 1995). However, the lack of suitable fertility restoration genes limits utilization of these lines. Investigation of chloroplast and mitochondrial diversity by RFLP analysis has established that wild and weedy germplasm related to crop Brassicas is a rich source of variable cytoplasms (Warwick and Black 1991; Pradhan et al. 1992) and can be used to generate an array of alloplasmic male-steriles of diverse origin. An advantage with this approach is that fertility restoring nuclear genes are available in the nuclear genome of the cytoplasmic donor and can be introgressed through chromosome manipulation.

We have synthesized a stable CMS system in *B. juncea* by placing its nucleus in the cytoplasm of a wild species *Moricandia arvensis*, through repeated backcrossings of the somatic hybrid *M. arvensis* + *B. juncea*. *M. arvensis* is a weed in the Mediterranean region. We

have also been successful in introgressing genetic information for fertility restoration from *M. arvensis* to *B. juncea* through homoeologous recombination, and have thus perfected a cytoplasmic male sterility-fertility restorer system. These developments are presented in this paper.

Materials and methods

Development of cytoplasmic male-steriles

The somatic hybrid *M. arvensis* (2n = 28 MM) + *B. juncea* (2n = 36, AABB) (Kirti et al. 1992), carrying the mitochondrial (mt) and chloroplast (cp) genomes of *M. arvensis*, was repeatedly crossed to *B. juncea* cv Pusa Bold (Fig. 1). Male-sterile plants, identified in the BC_3 generation, had no fertile pollen and their seed fertility ranged from 24 to 53%. BC_4 and BC_5 plants had improved seed fertility averaging 87 and 92% respectively. A study of meiosis of male-sterile plants in the BC_5 generation revealed that only the *B. juncea* genome had been retained, forming 18 bivalents regularly, while *M. arvensis* chromosomes had been completely eliminated. No seed set was obtained on selfing the male-steriles.

Cytological studies

Anthers were fixed in 3:1 ethanol and glacial acetic acid and squashed in 2% acetocarmine.

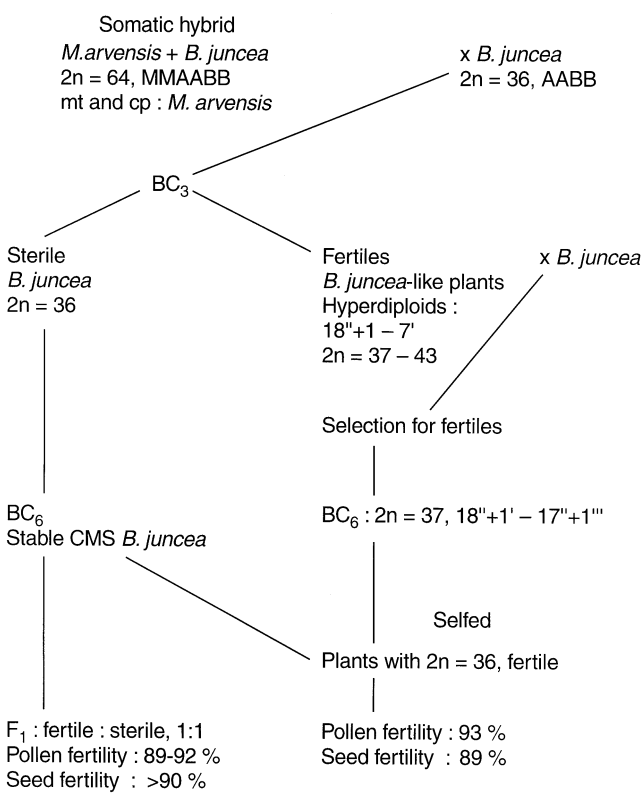

Fig. 1 Development of CMS (*Moricandia*) *Brassica juncea* and introgression of a fertility restorer gene

Molecular analysis

To confirm that the CMS plants, the fertility restored plants, and the restorer possessed similar organelles, i.e. chloroplasts and mitochondria, Southern hybridizations were performed. Total cellular DNA was isolated from leaves frozen in liquid nitrogen following the method of Saghai Maroof et al. (1984). Southern hybridizations were carried out according to Kirit et al. (1995). A mitochondrial probe, *Cox* III, and a chloroplast probe, *psb D*, were used in the Southern analysis.

Results

CMS (*Moricandia*) *B. juncea*

CMS plants show normal growth and development, similar to that of fertile *B. juncea*. However, their leaves are highly chlorotic, turning almost yellow . Flowering is delayed by 30–35 days as compared to normal *B. juncea* cv Pusa Bold, the maintainer line. Flowers have normal appearance but possess slender, small-sized anthers which fail to dehisce and contain only sterile pollen. Nectary development is excellent. Cytological studies on CMS plants in the BC_5 generation indicate a normal chromosome status (2n = 36) with regular bivalents at M_1. Subsequent stages were also normal up to tetrad formation. Pollen grains aborted during microsporogenesis. Pods are normal in shape. On pollination with the maintainer line (cv Pusa Bold), or when allowed to open pollinate, a seed set of 92% was obtained in th BC_6 generation.

Construction of the restorer

In the BC_3 segregating population a chlorotic plant was identified which had around 18% pollen fertility. This plant was selfed and a population of 11 plants was raised in the BC_4 generation. These plants had pollen fertility ranging from 19 to 53%. On chromosome analysis they were found to be hyperdiploids; in addition to a normal *B. juncea* complement, they had 1–3 extra chromosomes from *M. arvensis*. In the majority of the PMCs, *Moricandia* chromosomes remained as univalents; however, pairing with *B. juncea* chromosomes was also seen. Particular attention was given to a 2n = 36 + 1 chromosome plant which had 53% pollen fertility. The extra chromosome paired with *B. juncea* chromosomes to form a trivalent in 27% pollen mother cells (Fig. 2). This plant closely resembled *B. juncea* in morphology but also had some *M. arvensis* characters, particularly for leaf and pod shape. A population of 62 plants was obtained from selfed seeds of this plant, of which 14 were fertile and 48 were sterile. One of the fertile plants, MJR-15, had 93% fertile pollen and regular meiosis (18·II at MI). This plant, whose leaves were highly chlorotic, was marked

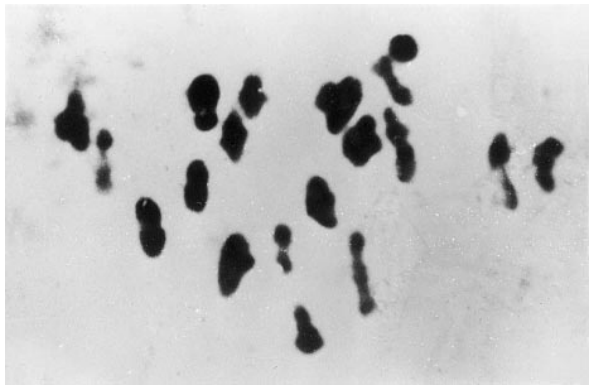

Fig. 2 Metaphase-I of meiosis in the *B. juncea-Moricandia* univalent addition line, 17·II + 1·III

as a putative restorer. All other fertile plants had one extra chromosome each and their pollen fertility centred around 50%. The pollen of the putative restorer was used to pollinate male-sterile plants. The progeny of the cross between the male-sterile line and the fertile line MJR-15 segregated into 239 fertile: 247 sterile (x^2 1 : 1 = 0.13). MJR-15 thus appears to carry a dominant fertility gene (*Rf*) in the heterozygous condition. We believe that the *Rf* gene was introgressed into the *B. juncea* genome through allosyndesis in the $2n = 36 + 1$ chromosome plant. A plant with an 18-chromosome egg bearing the restorer gene (*Rf*), when fertilized by normal *B. juncea* pollen, will result in a heterozygous *Rf rf* plant. Since only one plant of this genotype was recovered, it must be a rare event.

Molecular analysis of the fertility restored plants

To confirm the organellar genomes in the fertlilty restored plants, Southern hybridization was employed. When *Eco*RV-digested total cellular DNA was probed with the mitochondrial probe *cox* III, *B. juncea* lacked a 13.8-kb fragment hybridizing to *cox* III which is specific to *M. arvensis* (Fig. 3a). The CMS (*Moricandia*) *B. juncea*, the restorer plant, and the F_1 hybrid between CMS *B. juncea* × restorer possessed the 13.8-kb *M. arvensis* fragment. Similarly, when *Hpa*II digested DNA fragments were hybridized with a chloroplast-specific probe *psb* D (Alt et al. 1984), *B. juncea* was characterized by a 1.2-kb fragment and *M. arvensis* by 0.6- and 0.4-kb fragments (Fig. 3 b). The CMS (*Moricandia*) *B. juncea*, the restorer, and the F_1 hybrid had a hybridization pattern similar to that of *M. arvensis*. These observations clearly show that the CMS (*Moricandia*) *B. juncea*, the restorer, and the fertility restored plants all possessed the same organellar genome constitution.

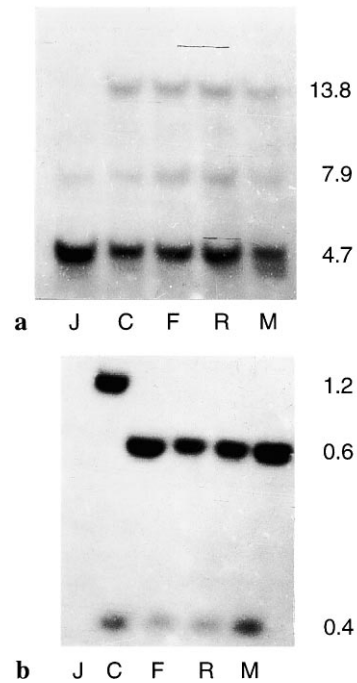

Fig. 3a *Eo*RV-digested DNA hybridized with the mitochondrial-specific probe *Cox III*. **b** The *HPa*II DNA digest probed with the chloroplast-specific *Psb D* probe. *M. arvensis* (*M*), *B. juncea* (*J*), CMS plant (*C*), restorer (*R*) and fertility restored plant (*F*)

Discussion

Several CMS systems of alloplasmic origin have been systhesized in *B. juncea* in recent years. They have been derived either from sexual alloploids or from somatic hybrids (Downey and Rimmer 1993; Prakash et al., 1995). However, non-availability of a fertility restorer has precluded their use in hybrid variety development. At the same time, many sources of cytoplasmic male sterility in *Brassica* suffer from one or other developmental or morphological abnormalities, such as leaf chlorosis, floral deformities including petaloid anthers, a crooked style, absence or poorly developed nectaries and low female fertility. Some of these abnormalities have been rectified by protoplast fusion. The CMS (*Moricandia*) *B. juncea* does not show any floral abnormalities, has excellent nectaries and normal female fertility. However, it exhibits severe leaf chlorosis which delays flowering by 30–35 days. Presently, efforts are aimed at correcting the leaf chlorosis through protoplast fusion.

In most synthetic alloplasmics, where the cytoplasm donor and the crop species are genetically very distant, a search for restorer genes among the cultivar accessions has proved futile. A strategy to overcome this difficulty is the introgression of *Rf* gene(s) from the cytoplasmic donor if homoeologies permitting chromosome pairing exist between the two species. Such a situation does exist in the system reported here which has

allowed successful introgression of the fertility restoration gene from *M. arvensis* to CMS *B. juncea*. Monosomic addition of *M. arvensis* on CMS *B. juncea* was developed where the additional chromosome carried the fertility restoring genes. It has been demonstrated that in presence of the sterility inducing cytoplasm, 37-chromosome plants were fertile while 36-chromosome plants were sterile. The additional chromosome sharing homoeology with a *B. juncea* chromosome forms a trivalent. This homoeology has allowed introgression of genetic information for fertility restoration from *M. arvensis* to *B. juncea* so that 36-chromosome fertile *B. juncea* plants carrying cytoplasm similar to the CMS plants, which had fertility restoring genes from *Moricandia*, would result.

M. arvensis was earlier considered a genetically very distant entity and was placed in the subtribe *Moricandinae*, distinct from the *Brassicinae*, by taxonomists such as Schulz (1919). However, recent investigations on chloroplast DNA restriction patterns indicate close affinities and do not support its placement in a separate subtribe (Warwick and Black 1994). Such affinities are also expressed in chromosome pairing in hybrids–up to 1·III + 5·II in *M. arvensis* × *B. nigra* (Takahata and Takeda 1990), up to 5·II in *M. arvensis* × *B. campestris* (Takahata and Takeda 1990) and up to 2·III + 6·II in *M. arvensis* × *B. oleracea* (Takahata 1990). The somatic hybrid *M. arvensis* + *B. juncea* (2n = 64) also shows up to three quadrivalents and one trivalent (Kirti et al. 1992). These multivalent associations indicate intergenomic homoeology between the *M. arvensis* and *Brassica* chromosomes. The *Moricandia* chromosome in the addition line carries the fertility restoring gene, and since it failed in 73% of PMCs to associate with any of the *B. juncea* bivalents this suggests its alien nature. A similar situation was observed in *Nicotiana* alloplasmics where normal floral morphology and fertility were restored in male-steriles carrying *N. repanda*, *N. debneyi* and *N. undulata* cytoplasms (Burns and Gerstel 1978, 1981). Fertility was also restored by a chromosome from a cytoplasmic donor in CMS *Triticum aestivum* carrying *Aegilops ovata* (Tsunewaki 1982), *Elymus ciliaris* (Jiang et al. 1993) or *Agropyron glaucum*/*A. trichophorum* cytoplasms (Suzuki et al. 1994). In all these cases the extra chromosome possessed nucleolar-organizing satellite regions which led to the suggestion that an interaction between the NOR and the cytoplasm was critical for fertility restoration. However, in the present investigation, as in wheat with rye cytoplasm (Friebe et al. 1993), the fertility restoring chromosome was not a satellited chromosome.

Homoeologous chromosome pairing has been exploited for introgressing gene/s restoring fertility to CMS (*ogu*) *B. napus* from *Raphanus sativus* through the production of the synthetic alloploids *R. sativus* × *B. napus* (Heyn 1976) and *R. sativus* × *B. oleracea* (Rouselle and Dosba 1985; Paulman and Robbelen 1988). A similar route was employed for transferring fertility restoring genes from *B. tournefortii* to *B. napus* (Stiewe et al. 1995). However, the radish fertility restoring gene was associated with reduced female fertility because of high ovule abortion. Also, meiosis was disturbed in restored plants and multivalents and univalents were frequent (Pellan-Delourme and Renard 1988).

In recent years several CMS systems of alloplasmic origin have been synthesized in Brassicas following sexual or somatic hybridisations (see Prakash et al. 1995). Fertility restoration could not be achieved in sexually derived alloplasmics, possibly because of multilocus incompatibility reactions between the unaltered alien mitochondria and the nuclear genome of crop species. It is important that in the CMS alloplasmics the alien mitochondrial contribution is restricted to a minimum, preferably no more than the sterility inducing segment, and this can be achieved only in somatic hybrids where the mitochondrial genomes of the two partners can be brought together for recombination events to occur.

In conclusion the male sterility and fertility restoration in the present case is absolute and both pollen and seed fertility in the restorer and the hybrid are normal without any meiotic disturbance. Thus, these components provide a new system for hybrid seed production in *B. juncea*.

References

Alt J, Morris J, Westhoff P, Hermann RG (1984) Nucleotide sequence of clustered genes for 44-Da chlorophyll apoprotein and 32-Da like protein of the photosystem-II reaction centre in the spinach plastid chromosome . Curr Genet 8:597–606

Burns JA, Gerstel DU (1978) Cytoplasmic male sterility in *Nicotiana*, restoration of fertility and the nucleous. II. *N. debneyi* cytoplasm. Genetics 90:151–159

Burns JA, Gerstel DU (1981) Role of nucleolar chromosomes in anther-restoration of male-sterile tobacco. J Hered 72:413–418

Downey RK, Rimmer SR (1993) Agronomic improvement in oilseed *Brassicas*. Adv Agron 50:1–66

Friebe B, Gill BS, Mukai Y, Maan SS (1993) A non compensating wheat-rye translocation maintained in perpetual monosomy in alloplasmic wheat. J Hered 84:126–129

Heyn FW (1976) Transfer of restorer gene from *Raphanus* to cytoplasmic male-sterile *B. napus*. Cruciferae Newslett 1:15–16

Jiang J, Chen P, Friebe B, Raupp WJ, Gill BS (1993) Alloplasmic wheat-*Elymus ciliaris* chromosome addition lines. Genome 36:327–333

Kirti PB, Mohapatra T, Baldev A, Prakash S, Chopra VL (1995) A stable male sterile line of *Brassica juncea* carrying restructured organelle genomes from the somatic hybrid *Trachystoma ballii* + *B. juncea*. Plant Breed 114:434–438

Kirti PB, Narasimhulu SB, Prakash S, Chopra VL (1992) Somatic hybridization between *Brassica juncea* and *Moricandia arvensis* by protoplast fusion. Plant Cell Rep 11:318–321

Paulman W, Robbelen G (1988) Effective transfer of cytoplasmic male sterility from radish (*Raphanus sativus* L.) to rape (*Brassica napus* L.). Plant Breed 100:299–309

Pellan-Delourme R, Renard M (1988) Cytoplasmic male sterility in rapeseed (*Brassica napus* L.): female fertility of restored rapeseed with *ogura* and hybrid cytoplasms. Genome 30:234–238

Pradhan AK, Prakash S, Mukhopadhyay A, Pental D (1992) Phylogeny of *Brassica* and allied genera based on variation in chloroplast and mitochondrial DNA patterns: molecular and taxonomic classifications are incongruous. Theor Appl Genet 85:331–340

Prakash S, Chopra VL (1990) Male sterility caused by cytoplasm of *Brassica oxyrrhina* in *B. campestris* and *B. juncea*. Theor Appl Genet 79:285–287

Prakash S, Kirti PB, Chopra VL (1995) Cytoplasmic male sterility (CMS) system other than *ogu* and polima in *Brassica*: Current Status. Proc. 9th Int Rapeseed Congr, Cambridge UK 1:44–48

Rao GV, Batra-Sarup V, Prakash S, Shivanna KR (1994) Development of a new cytoplasmic male-sterile system in *Brassica juncea* through wide hybridization. Plant Breed 112:171–174

Rousselle P, Dosba F (1985) Restauration de la fertility pour l' androsterilite genocytoplasmiaque chez le calza (*Brassica napus* L.). Utilization des *Raphano Brassica*. Agromonie 5: 431–43

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RS (1994) Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritence. Proc Natl Acad Sci USA 81: 8014–8018

Schulz OE (1919) *Curciferae-Brassiceae* Part I, subtribes *Brassicinae* and *Raphaninae*. In: Engler A (ed) Das Pflanzenreich IV, 68–70. Withelm Engelman, Leipzig, pp 1–290

Steiwe G, Sodhi YS, Robbelen G (1995) Establishment of a new CMS system in *Brassica napus* by mitochondrial recombination with *B. tournefortii*. Plant Breed 113:294–304

Suzuki T, Nakamura C, Mori N, Iwasa Y, Kaneda C (1994) Homoeologous group-1 chromosomes of *Agropyron* restore nucleus-cytoplasmic compatibility in alloplasmic common wheat with *Agropyron* cytoplasms. Jpn J Genet 69:41–51

Takahata Y (1990) Production of intergeneric hybrids between a C_3-C_4 intermediate species *Moricandia arvensis* and C3 species *Brassica oleracea* through ovary culture. Euphytica 46:259–264

Takahata Y, Takeda T (1990) Intergeneric (Intersubtribe) hybridisation between *Moricandia arvensis* and *Brassica* A and B genome species by ovary culture. Theor Appl Genet 80: 38–42

Tsunewaki K (1982) Monosomic analysis on the fertility restoration by *Triticum aestivum* cv Chinese spring against *Aegilops ovata* cytoplasm. Jpn J Genet 57:513–525

Warwick SI, Black LD (1991) Molecular systematics of *Brassica* and allied genera (subtribe *Brassicinae*, *Brassiceae*)–chloroplast genome and cytodeme congruence. Theor Appl Genet 82: 81–92

Warwick SI, Black LD (1994) Evaluation of the subtribes *Moricandiinae*, *Savignyinae*, *Vellinae* and *Zillinae* (*Brassicaceae*, tribe *Brassiceae*) using chloroplast DNA restriction-site variation. Can J Bot 72:1692–1701