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Chloroplast substitution overcomes leaf chlorosis in a *Moricandia arvensis*-based cytoplasmic male sterile *Brassica juncea*

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Abstract A male sterile *Brassica juncea* line based on *Moricandia arvensis* cytoplasm was developed previously by backcrossing the somatic hybrid *M. arvensis* + *B. juncea*, and the gene for restoring fertility was introgressed. The CMS line is very severely chlorotic because of the presence of alien chloroplasts and flowering is delayed by 30–40 days, making it unsuitable for the exploitation of heterosis. We have resorted to another cycle of protoplast fusion between green fertile *B. juncea* and chlorotic male sterile *B. juncea*, and developed green male-sterile plants. Molecular analysis revealed that in green male-sterile plants chloroplasts of *M. arvensis* origin were substituted by those from *B. juncea*, giving rise to intergeneric cytoplasmic hybrids with mitochondria of *M. arvensis* origin. With the development of dark-green male-sterile plants, the CMS fertility restoration system is suitable for the production of hybrid mustard.

Key words *Brassica juncea* · *Moricandia arvensis* · Cytoplasmic male sterility · Chlorosis correction · Cytoplasmic hybrids · Chloroplast substitution

Introduction

Cytoplasmic male sterility in several crop species has been generated by substituting their cytoplasms with those of allied species. However, in addition to male sterility, these resultant alloplasmics often exhibited developmental or floral abnormalities which include leaf chlorosis of varying degrees, transformation of anthers into petaloid structures and low female fertility. Mustard (*Brassica juncea*) is an important oil seed crop particularly in the Indian subcontinent and several male-sterile (CMS) systems have been developed through alloplasmic substitutions in this crop (Prakash et al. 1998 b). One such system is based on *M. arvensis* cytoplasm and the gene for fertility restoration has also been introgressed (Prakash et al. 1998 a). It was accomplished by backcrossing the somatic hybrid *M. arvensis* + *B. juncea* (Kirti et al. 1992) with *B. juncea* cv Pusa Bold. However, the leaves of the CMS plants exhibited a high degree of chlorosis, turning almost yellow due to incompatibilities between the *M. arvensis* plastids and the *B. juncea* nucleus. Because of low photosynthetic activity, plants are poorly developed and flowering is delayed by 30–40 days. Chlorosis in alloplasmic *Brassica* has previously been reported in CMS (*ogura*) *B. oleracea*/*B. napus* (Bannerot et al. 1974) and (*oxyrrhina*) *B. juncea*/*B. campestris* (Prakash and Chopra 1990), and has been rectified following protoplast fusion (Pelletier et al. 1983; Jarl and Bornman 1988; Kirti et al. 1993, 1995). We have resorted to an additional cycle of protoplast fusion, involving the chlorotic male-sterile (*Moricandia*) *B. juncea* and green fertile *B. juncea*, to achieve chlorosis rectification. As a result, green male-sterile intergeneric cytoplasmic hybrids (cybrids) carrying *M. arvensis* mitochondria and *B. juncea* chloroplasts have been obtained. The results of these fusion experiments are reported here.

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Materials and methods

The materials used in this study were a green male-fertile RLM-198, for which an efficient regeneration protocol was standardized earlier (Kirti and Chopra 1990), and the chlorotic male-sterile line of *B. juncea* cv Pusa Bold carrying *M. arvensis* cytoplasm.

Scheme of selection

Protoplasts of the green fertile line were treated with iodoacetate leading to mitochondrial inactivation. Protoplasts of CMS *B. juncea* are non-regenerable. Regenerated plants from such a fusion experiment would be chlorotic male-sterile, because of the reconstitution of parental cytoplasm, or green male-sterile cytoplasmic hybrids (cybrids).

Protoplast isolation, fusion and culture

Protoplasts of hypocotyl origin from *B. juncea* cv RLM-198 can be easily regenerated to plants (Kirti and Chopra 1990). The only modification in the present study is the addition of 20 μM of silver nitrate to the regeneration medium consisting of K3 medium +0.1 mg/l IAA + 2.0 mg/l BAP + 2.0 mg/l zeatin riboside + 0.5% Agarose (Type I, low EEO, Sigma). Hypocotyl protoplasts of both fusion partners were prepared and cultured according to the method described by Kirti and Chopra (1990). Protoplasts of RLM-198 were directly treated with iodoacetamide (3, 5, 10 mM) in the last 20 min of enzyme treatment. Protoplast fusion was carried out using PEG 8000 and dimethyl sulphoxide (Kirti et al. 1992).

Cytological analysis and pollen fertility

For cytological studies, flower buds were fixed in 1:3 acetic acid:ethanol and anthers smeared in 2% acetocarmine. Pollen fertility was assessed by smearing freshly dehiscing anthers in 2% acetocarmine.

Southern analysis for confirming the origin of organelles

To determine the origin of mitochondria and chloroplasts in the putative cytoplasmic hybrids, genomic DNA was isolated from fresh leaves, using a CTAB (cetyl trimethyl ammonium bromide) procedure described earlier, and used in Southern hybridization analysis (Kirti et al. 1992). *Hpa*II-digested DNAs were blotted onto a Hybond N^+ nylon membrane (Amersham International, UK) and probed with chloroplast-specific *psb*D (Alt et al. 1984) and *psb*A (Zurawski et al. 1982), and mitochondria-specific *atp*A (Braun and Levings 1985) genes. Standard protocols were employed in the molecular studies (Sambrook et al. 1982).

Results and discussion

A simple selection system has been employed in the cell fusion experiments for selecting the green male-sterile cytoplasmic hybrids. Mitochondria of the green self-fertile cv RLM 198 were inactivated by treating with iodoacetamide, which inhibits further protoplast development. Protoplasts of the chlorotic male-sterile *B. juncea* cv Pusa Bold could not be regenerated using the protocol described earlier (Kirti and Chopra 1990). Only fusion products developed further. All the regenerated plants were sterile, and either chlorotic or green. From three protoplast-fusion experiments, a total of 144 plants were regenerated. These plants were identified to be green or chlorotic by subjecting them to a low temperature (10°C) when chlorosis became very conspicuous in this system. Of these plants, four were dark-green and 140 chlorotic (Table 1). All plants subsequently turned out to be male-sterile at the field level.

Table 1 Plant regeneration from fusion experiments involving iodoacetate-treated protoplasts of green fertile *B. juncea* and chlorotic male-sterile (*Mori*) *B. juncea*

Exp. no.	Iodoacetate concentration (mM)	Chlorotic male-sterile	Green male-sterile	Total
1	3	87	0	87
2	5	23	1	24
3	10	30	3	33
Total		140	4	144

Cytology and pollen sterility

Chromosome numbers were determined for all four dark-green plants by smearing anthers in meiosis. Three of them showed a chromosome number of 2n = 72 whereas one plant (plant no. 3) possessed 36 chromosomes, the normal number of *B. juncea*. It is evident that the three plants having 72 chromosomes are the products of fusion between protoplasts carrying a diploid, 2n = 36, complement of chromosomes, while the one with 36 chromosomes presumably has its origin from a protoplast–cytoplast fusion. Variation in chromosome number is of frequent occurrence in plants regenerated from protoplasts and their progenies. Pelletier et al. (1983) observed a wide range of chromosome numbers in the progeny of fusion products between chlorotic male-sterile (*ogu*) *B. napus* and green fertile *B. napus*. They recorded a normal diploid *B. napus*, in addition to hyperdiploids. The anthers of the four green and ten randomly selected chlorotic plants had completely sterile pollen.

Molecular analysis of putative cytoplasmic hybrids

The chloroplast constitution of the cybrids was determined by Southern analysis of total DNA with the chloroplast probes *psb*D and *psb*A. The origin of the mitochondrial constitution of the cybrids was confirmed by the hybridization pattern of *atp*A to total DNA digests.

When *Hpa*II genomic-DNA digests were probed with the mitochondrial *atp*A probe, *B. juncea* was characterized by a 2.1-kb fragment whilst the CMS line used in fusion had a 1.0-kb fragment as in the wild parent *M. arvensis*, which was one of the parents of the somatic hybrid that gave rise to the present CMS line (Fig. 1 A). All the regenerants examined from the fusion experiments had a hybridization pattern similar to the CMS line indicating that the mitochondria were derived from the CMS line, as expected, because

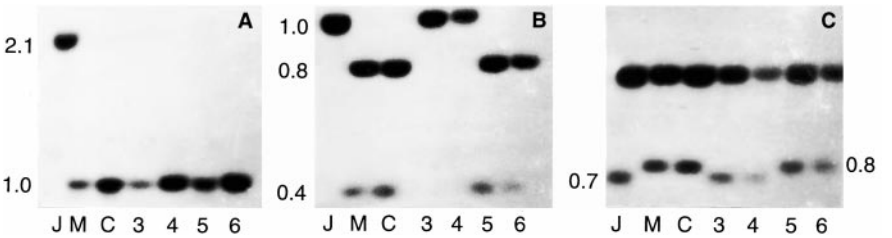

Fig. 1 Genomic DNA samples digested with *Hpa*II and hybridized with mitochondrial and chloroplast probes, *J* – *B. juncea* cv RLM-198 (green fertile), *M* – *M. arvensis*, *C* – *CMS* (*Mori*) *B. juncea* (chlorotic male-sterile) *3*, *4*, *5*, *6*-regenerated plants from fusion experiments. **A** *Hpa*II-*atp*A (mitochondria), **B** *Hpa*II-*psb*D (chloroplast), **C** *Hpa*II-*psb*A (chloroplast) enzyme-probe combinations. Fragments hybridizing to the different probes are indicated in kb

the protoplasts of the green fertile cv RLM 198 were treated with iodoacetamide for mitochondrial inactivation.

The *Hpa*II digests of genomic DNA were hybridized with the nick-translated probes for *psb*A and *psb*D genes for studying the origin of chloroplasts in the regenerants from the fusion experiment. The *Hpa*II + *psb*D combination distinguished *B. juncea* from the CMS line and *M. arvensis*. It had a 1.0-kb fragment binding to the probe whereas, in the latter, the probe bound to 0.8- and 0.4-kb fragments (Fig. 1B). Regenerants 3 and 4 exhibited a hybridization pattern similar to *B. juncea* whilst regenerants 5 and 6 were like the CMS line. When the same *Hpa*II digests were probed with the *psb*A gene, *B. juncea* was characterized by a specific 0.7-kb fragment and the CMS line by a 0.8-kb fragment (Fig. 1C). Regenerants 3 and 4 showed the *B. juncea*-specific 0.7-kb fragment whereas 5 and 6 carried the CMS-specific 0.8-kb fragment indicating that plants 3 and 4 carried mitochondria of *M. arvensis* – CMS origin and chloroplasts of *B. juncea* origin. These are the cytoplasmic hybrids generated for chlorosis correction. Plant no. 5 is also of special interest. This has been identified as a dark-green plant. However, the hybridization pattern of *Hpa*II digests of the genomic DNA with chloroplast probes was idential with that of the chlorotic CMS line – *M. arvensis*, indicating that the chloroplast genome had probably undergone some change. This plant needs further analysis to account for its green phenotype.

The predominant occurrence of chlorotic male-sterile plants in the regenerants from the fusion experiments shows that there is a strong bias for the maintenance of the wild species, i.e. *M. arvensis*, chloroplasts. This is in sharp contrast with the earlier fusion experiments for chlorosis-correction involving *B. napus* and *B. juncea*. Chloroplast segregation has often been reported to be biased in somatic hybrids involving *Brassica* species. In somatic hybrids of *Brassica* spp. with *Raphanus*, *Brassica* chloroplasts were preferentially retained (Jourdan et al. 1989; Earle et al. 1992). Cell type could not be established as a causative factor for biased segregation (Sundberg et al. 1991; Walters et al. 1992). However, *Raphanus* chloroplasts could be recovered in the regenerants from fusions involving *ogura* mesophyll protoplasts, but not when *ogura* hypocotyl protoplasts were used in fusion (Earle et al. 1992). The nuclear DNA content and the size of the cells of the fusion partners were attributed a role in influencing biased segregation as they affect the number and DNA content of plastids (Butterfass 1989). Similarly, the ploidy level of the fusion partners was attributed a role in biased segregation (Bonnett and Glimelius 1983; Earle et al. 1992). In our earlier somatic cell-fusion experiment between hypocotyl protoplasts of CMS (*ogu*) *B. juncea* and green fertile RLM 198, chloroplast assortment was fairly random (Kirti et al. 1995). However, in the present investigation, cell type, ploidy level and nuclear genotype could not have contributed to the biased segregation as hypocotyl protoplasts of two *B. juncea* lines were employed in the fusion experiments.

Chloroplast segregation was also reported to be biased in cell-fusion experiments involving the treatment of protoplasts with iodoacetamide or ionizing radiation. Sidorov et al. (1981) observed a preferential maintenance of chloroplasts from the X-irradiated fusion partner after double-inactivation experiments in *Nicotiana* species. In sharp contrast to this, in the regenerants from donor recipient fusions between CMS *B. napus* lines carrying *Raphanus sativus* cytoplasm and original *B. napus* cytoplasm, Morgan and Maliga (1987) found that the chloroplasts of iodoacetate-treated hypocotyl protoplasts were retained after double inactivation. They attributed the complete sorting out of CMS chloroplasts to better compatibility of fertile *B. napus* chloroplasts with a *B. napus* nucleus or to the lack of recovery of irradiated *R. sativus* chloroplasts. In our experiments, chloroplasts of the iodoacetate-treated partner, i.e. the green fertile line, were preferentially eliminated and the chloroplasts of *M. arvensis* origin in the chlorotic male-sterile line were retained in combination with the *B. juncea* nucleus. This indicates that there is a bias towards selective replication of the wild species chloroplasts in the given nuclear environment and that a co-transmission of chloroplasts and mitochondria of the chlorotic male-sterile line occurred. Co-transmission of chloroplasts and mitochondria was also observed in the donor-recipient method of cell fusion for the transfer of organelles in experiments involving *Nicotiana* species (Medgyesey et al. 1985; Barsby et al. 1987; Aviv and

Galun 1988). In contrast to this observation, Glimelius and Bonnett (1986) and Bonnett and Glimelius (1990) found that in *Nicotiana* (*Petunia*) cybrids, organelle segregation resulted in a combination of chloroplasts from *Petunia* and mitochondria either identical or similar to *N. tabacum*. They did not observe co-transmission of *Petunia* chloroplasts and mitochondria. Pelletier (1986) and Earle et al. (1992) noted that only certain specific cybrids were obtained and that certain organelle combinations were not realizable in fusions involving *Brassica* species for the transfer of *Raphanus* cytoplasm. In all fusion experiments involving *B. napus* or *B. oleracea*, there was a strong preference for *Brassica* chloroplasts, either atrazine-resistant or -sensitive, over *Raphanus* chloroplasts for facilitating the recovery of non-chlorotic *ogura* cytoplasm by the replacement of *Raphanus* chloroplasts (Earle et al. 1992). The present results are at variance with these findings in that most of the regenerants were chlorotic. However, the recovery of green, male-sterile cybrids with a desirable combination of organelles, mitochondria from *M. arvensis* and chloroplasts of *B. juncea* origin, facilitates the development of this male-sterile cytoplasm into a system for producing hybrid varieties.

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