Theor Appl Genet (2000) 100:1043–1049

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Somatic cell hybridization of '*oxy*' CMS *Brassica juncea* (AABB) with *B. oleracea* (CC) for correction of chlorosis and transfer of novel organelle combinations to allotetraploid brassicas

Received: 9 August 1999 / Accepted: 14 September 1999

Abstract Alloplasmic lines of cultivated *Brassica* species with *B. oxyrrhina* cytoplasm are male-sterile and suffer from severe chlorosis. We developed male-sterile lines corrected for chlorosis by fusing protoplasts of CMS *B. juncea* (AABB) with '*oxy*' cytoplasm and normal *B. oleracea* (CC). A large number of male-sterile AABBCC somatic hybrids with desirable organelle combinations, i.e. chloroplasts of *B. oleracea* and mitochondria with recombinant genomes, were recovered. While no recombination was observed in the chloroplast genome, the mitochondrial genome showed extensive recombination that resulted in the appearance of totally novel banding patterns in some of the hybrids. Hybrids with a parental-type mitochondrial genome as well as recombinant patterns close to either of the parental types were also obtained. Using AABBCC somatic hybrids as bridging material, we transferred the desirable organelle combinations to *B. juncea* (AABB), *B. napus* (AACC), and *B. carinata* (BBCC). Many of these lines are now at advanced stages of backcrossing and show stable inheritance of the CMS character and do not suffer from chlorosis.

Key words *Brassica* · CMS · Somatic hybrids · Correction of chlorosis · Mitochondrial recombination

Introduction

Alloplasmic cytoplasmic male sterility (CMS) systems are of common occurrence in *Brassica* species. Several alloplasmic CMS systems, such as '*ogu*' (Ogura 1968), '*nap*' (Thompson 1972), *B. nigra* (Pearson 1972), *Diplotaxis muralis* (Hinata and Konno 1979), '*oxy*' (Prakash and Chopra 1990), '*tour*' (Pradhan et al. 1991; Stiewe and Robbelen 1994), *D. siifolia* (Rao et al. 1994) and *D. catholica* (Kirti et al. 1995), are known in crop brassicas. These alloplasmic CMS systems were developed by intraspecific, interspecific or intergeneric crosses in which alien material was used as the female parent followed by repeated backcrossing to the cultivated species.

The presence of *Brassica oxyrrhina* cytoplasm ('*oxy*') in *B. campestris*, *B. napus* and *B. juncea* nuclear backgrounds leads to male sterility (Prakash and Chopra 1990). These CMS lines exhibit severe chlorosis (a manifestation of incompatibility between the alien chloroplast and the nuclear genome), a reduction in plant height and delayed flowering but no obvious floral defects. Chlorosis, which leads to loss of vigour and productivity, coupled with the non-availability of restorer function renders this CMS system unsuitable for hybrid seed production. It is therefore essential to rectify these problems before '*oxy*' CMS can be effectively used for heterosis breeding.

The problem of chlorosis can be rectified through somatic cell hybridization, a technique that facilitates organelle interaction by bringing the alien and the cultivar cytoplasms together in hybrid cells. Correction of chlorosis through the exchange of chloroplasts by protoplast fusion has been reported for '*ogu*' CMS lines of both spring (Pelletier et al. 1983; Menczel et al. 1987) and winter varieties of rape (Jarl and Bornman 1988) and for '*tour*' CMS in *B. napus* (Stiewe and Robellen 1994; Arumugam et al. 1996). Correction of chlorophyll deficiency for '*oxy*' CMS *in B. juncea* has been reported by Kirti et al. (1993). This report described protoplast fusion between '*oxy*' CMS and fertile *B. juncea* and the development of a single plant corrected for chlorophyll deficiency through the recombination of chloroplast genomes.

Besides the rectification of chlorosis, somatic hybridization can induce extensive mitochondrial recombination. Somatic hybrids with recombinant mitochondrial genomes can be used for discerning the sequence(s) in

Communicated by D.R. Pring

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Table 1 Culture media used for the synthesis of '*oxy*' CMS *B. juncea* AABB + normal *B. oleracea* CC somatic hybrids

Culture medium	Basal medium[a]	Growth regulators and other supplements[b]
R1	MS	2.0 mg/l IBA
R2	MS	1.0 mg/l IBA
SM	MS	0.005 mg/l NAA, 0.05 mg/l kinetin, 50 mg/l casein hydrolysate
MSP	Kao	0.5 *M* glucose, 1 mg/l BAP, 1 mg/l NAA
SL1	K3	0.5 *M* glucose, 1 mg/l BAP, 1 mg/l NAA, 20 μ*M* $AgNO_3$, 20 mg/l hygromycin, 10 mg/l phosphinothricin, 0.25% agarose
SL2	MS	1 mg/l BAP, 1 mg/l NAA, 20 μ*M* $AgNO_3$, 20 mg/l hygromycin, 10 mg/l phosphinothricin
SL3	MS	2.0 mg/l IBA, 20 mg/l hygromycin, 10 mg/l phosphinothricin

[a] MS, Murashige and Skoog (1962); Kao, Glimelius (1984); K3, Nagy and Maliga (1976)
[b] IBA, Indolebutyric acid; NAA, 1-naphthaleneacetic acid; BAP, 6-benzylaminopurine

the mitochondrial genome that confers male sterility. A large population of recombinants is therefore necessary for the identification of sequences conferring male sterility. We have earlier developed marker gene-containing stocks of all the diploid species of U's triangle (U 1935) and *B. juncea* (Mukhopadhyay et al. 1991, 1992; Gupta et al. 1993; Pental et al. 1993) and these stocks have been used for the *in vitro* selection of hybrids (Mukhopadhyay et al. 1994; Arumugam et al. 1996). Here we report the generation of a large number of somatic hybrids by fusion of protoplasts isolated from '*oxy*' CMS *B. juncea* AABB containing a hygromycin resistance gene as a selectable marker and *B. oleracea* CC containing a marker gene for resistance to phosphinothricin. Regenerated hybrids were screened for rectification of chlorosis and extent of mitochondrial recombination. The hexaploid (AABBCC) somatic hybrids synthesized by protoplast fusion were used as bridging material for the simultaneous transfer of desirable chloroplast and mitochondrial combinations to cultivated allopolyploid brassicas.

Materials and methods

Plant material

CMS *Brassica juncea* (AABB) var. Pusa Bold carrying '*oxy*' cytoplasm was sexually crossed with hygromycin resistant (hm^+) *B. juncea* var. RLM 198 (Pental et al. 1993). F1 seeds were germinated and the seedlings screened for resistance to the antibiotic by rerooting on R1 medium with 20 mg/l hygromycin (Table 1). The hm^+ shoots of CMS *B. juncea* thus obtained were multiplied and maintained on R1 medium. Seeds of phosphinothricin-resistant (ppt^+) *B. oleracea* (CC) var. Early kunwari (Mukhopadhyay et al. 1991) were similarly screened for resistance to 10 mg/l phosphinothricin on R2 medium. Resistant shoots were multiplied and maintained on SM medium (Table 1).

Protoplast isolation, fusion and regeneration of somatic hybrids

Details of the media used in the present investigation are presented in Table 1. Shoots of '*oxy*' CMS *B. juncea* AABB hm^+ and normal *B. oleracea* CC ppt^+ were grown on R1 and R2 medium, respectively, for 15 days prior to the isolation of protoplasts. Protoplast isolation, fusion and culture were done following the protocol of Arumugam et al. (1996). The parental mix (controls) and the fusogen treated protoplasts were cultured in MSP medium at 24° ± 1°C in the dark. Four-week-old protoplast-derived microcolonies were over-layered on selection medium (SL1) containing 20 μg/ml hygromycin and 10 μg/ml phosphinothricin and incubated at 24° ± 1°C and under a 16-h (light) photoperiod for the selection of hybrid colonies. Colonies that continued to grow on the selection medium were transferred to SL2 medium for shoot regeneration. Hybrid shoots were rooted on SL3 medium. Individual shoots were given numbers, e.g. 1.2.3.4, where '1' depicted the number of the fusion experiment, '2', the dish number, '3', the colony number from which the shoot regenerated and '4', the number of the independent shoot. Shoots that could root on the double selection medium were transferred to soil in a net house.

Molecular characterization of somatic hybrids

Total DNA was isolated from the leaves of field-grown parental lines and 52 somatic hybrids following the protocol of Dellaporta et al. (1983). Chloroplast (cp) DNA was isolated according to Kemble (1987). For establishing the hybrid nature of the regenerants, random amplified polymorphic DNA (RAPD) analysis was carried out following Mukhopadhyay et al. (1994) using two 10-mer primers, OPB8 and OPB10 (Operon Technologies). Organelle genomes were characterized by hybridization of total DNA of hybrids with two chloroplast-specific heterologous probes, 11 heterologous mitochondrial (mt) gene probes and eight overlapping cosmid clones of *B. oxyrrhina* mtDNA.

Results

Regeneration of somatic hybrids

Protoplasts started dividing within 24 h of culture. By the 4th week, dividing protoplasts had developed into microcolonies 1—2 mm in diameter. Microcolonies derived from fusogen-treated protoplasts continued further growth when plated on selection medium (SL1) containing both hygromycin and phosphinothricin, whereas those derived from a physical mixture of parental protoplasts failed to do so. A total of 591 rapidly growing colonies were transferred to SL2 medium for shoot regeneration. Thirty-two of these colonies regenerated shoots (Table 2) that were subsequently rooted on SL3 medium.

Morphology of somatic hybrids

A total of 121 independent regenerants from 21 different colonies were established in soil. The plants, in general,

Table 2 Development of somatic hybrids (AABBCC, hm^+, ppt^+) through the fusion of protoplasts of '*oxy*' CMS *B. juncea* (AABB hm^+) and normal *B. oleracea* (CC ppt^+)

Fusion no.	Number of protoplasts used		Number of AABBCC hm^+, ppt^+ colonies obtained	Number of colonies regenerating plants
	CMS *B. juncea* (AABB hm^+)	*B. oleracea* (CC ppt^+)		
1	8×10^5	8×10^5	232	10
2	8×10^5	8×10^5	63	5
3	8×10^5	8×10^5	56	2
4	6×10^5	6×10^5	102	8
5	6×10^5	6×10^5	138	7

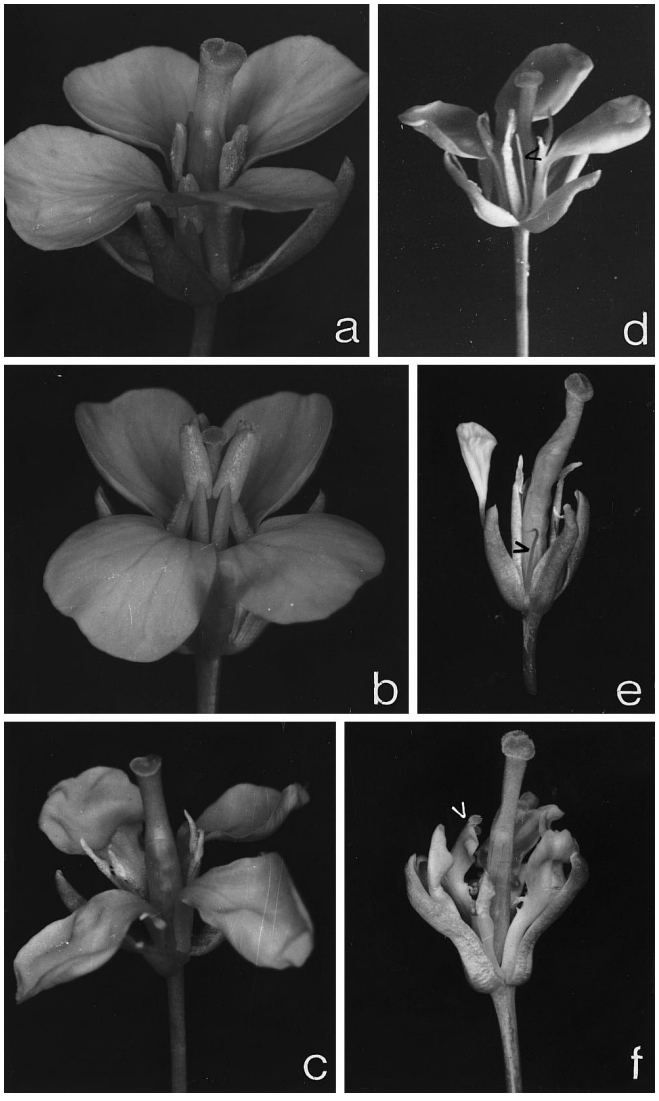

Fig 1a–f Variation in floral morphology of AABBCC somatic hybrids: **a,b** Flowers with normal petals and rudimentary (**a**) or normal-sized (**b**) anthers; **c** flower with four crinkled petals and feathery anthers; **d,e** flowers with petals modified to scale-like structures (*arrowhead*); **f** flower with sepaloid anthers (*arrowhead*)

grew vigorously and came to flowering within 3 months of transplantation. The vegetative features of the regenerated plants were, by and large, intermediate between *B. juncea* and *B. oleracea*, but their floral features exhibited variation in the number (0–3) and shape of the petals (crinkled or trumpet shaped) and shape (petaloid, sepaloid, feathery) and size (rudimentary or as large as in the fertile flower) of the anthers. In the case of flowers having fewer than four petals, scale-like structure(s) represented the missing petal(s). Some anthers produced pollen, which were subsequently analyzed for viability by staining with fluorescein diacetate (FDA). A pollen viability of up to 25% was observed in some of the petalless plants. Based on floral morphology and pollen viability the plants were divided into five phenotypic classes:

1) Normal male-sterile (NMS). Plants bearing flowers with four normal petals and rudimentary (Fig. 1a) or normal-sized (Fig. 1b) anthers without any pollen. A few of these plants produced some shriveled pollen.
2) Normal partially fertile (NPF). Plants bearing flowers having features described for NMS flowers but producing viable pollen that showed staining with FDA.
3) Abnormal male-sterile (AMS). Plants bearing male-sterile flowers with crinkled petals (Fig. 1c) or with one or more petal(s) replaced by scale-like structures (Fig. 1d,e) and/or anthers modified into some other floral structure (Fig. 1f)
4) Abnormal partially fertile (APF). Plants bearing flowers like those described for AMS but producing viable pollen.
5) Highly abnormal (HA). Plants which showed the abscission of flower buds or premature opening of flowers with gynoecium protruding out at the bud stage. Prematurely opened flowers lacked a proper stigmatic surface and therefore could not be backcrossed.

Molecular analysis of the somatic hybrids

RAPD analysis of 12 somatic hybrids of independent origin using OPB8 and OPB10 primers showed the presence of bands specific to both '*oxy*' CMS *B. juncea* and normal *B. oleracea*, thereby confirming that these were true AABBCC somatic hybrids (Fig. 2). The organelle genomes of 52 somatic hybrids, representing all the five phenotypic classes described earlier, were subjected to restriction fragment length polymorphism (RFLP) analysis using heterologous probes of chloroplast and mitochondrial origin. For cpDNA, total DNA digested with *Eco*RI was hybridized to *psb*D and *rbc*L sequences. Out of the 52 hybrids, 30 showed '*oleracea*'- and 22 showed

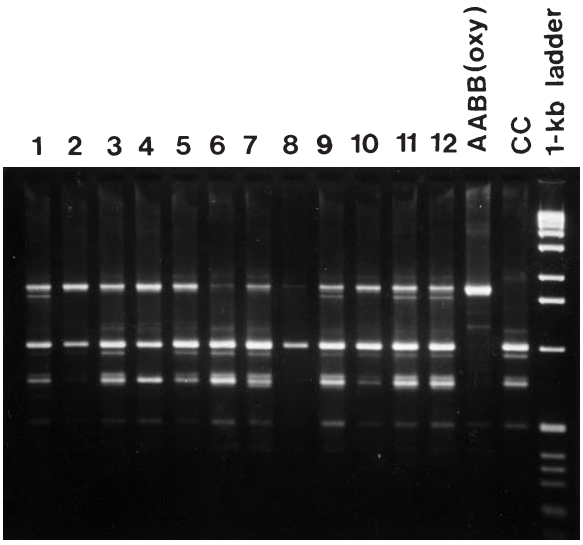

Fig 2 RAPD profile of 12 AABBCC somatic hybrids and their parents '*oxy*' CMS *B. juncea* (AABB) and normal *B. oleracea* (CC) using primer OPB 10

Table 3 Summary of variation in the floral characteristics in relation to the organelle composition of '*oxy*' CMS *B. juncea* (AABB hm^+) + normal *B. oleracea* (CC ppt^+) somatic hybrids

Organelle composition[a]	Phenotypic Classes					Total no. of hybrids
	NMS	NPF	AMS	APF	HA	
CcpCmt	0	1	0	0	0	1
$CcpCmt^R$	6	0	0	0	2	8
CcpOmt	5	0	0	0	0	5
$CcpOmt^R$	9	0	1	0	0	10
$CcpMt^R$	0	0	5	1	0	6
$OcpMt^R$	0	0	13	9	0	22

[a] C, *B. oleracea* type DNA profile; O, *B. oxyrrhina* type DNA profile, cp, chloroplast; mt, mitochondria; mt^R, recombinant mitochondria; Mt^R, highly recombinant mitochondria
[b] NMS, Normal male-sterile; NPF, normal partially fertile; AMS, abnormal male-sterile; APF, abnormal partially fertile; HA, highly abnormal. See Results for more detailed descrption

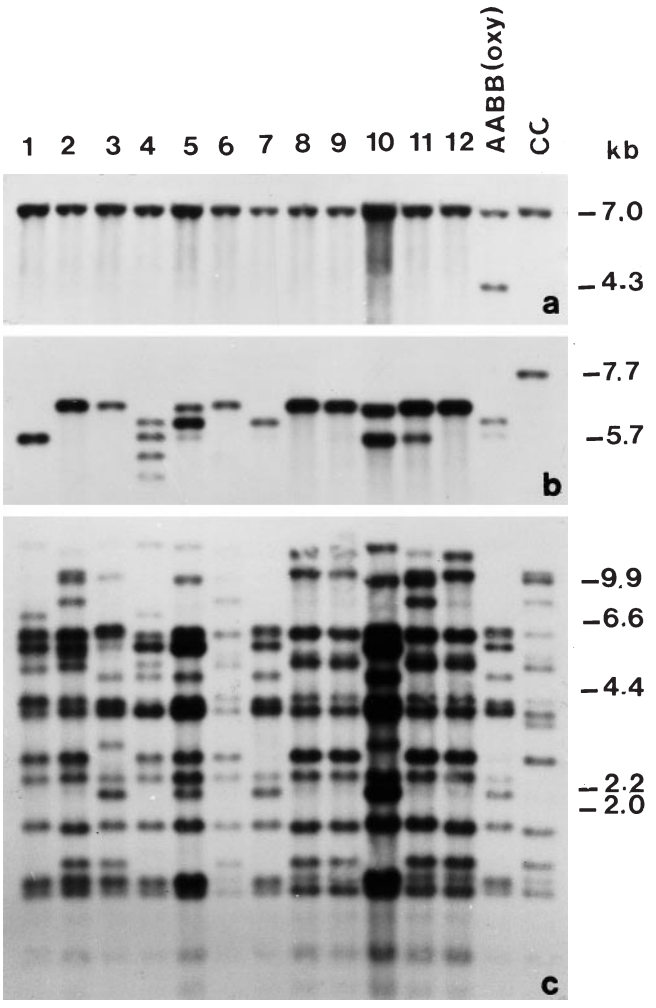

Fig 3 Southern hybridization patterns of 12 AABBCC somatic hybrids and their parents '*oxy*' CMS *B. juncea* (AABB) and normal *B. oleracea* (CC) **a** *Eco*RI-digested total DNA probed with cpDNA probe *psbD*, **b, c** *Hind*III-digested total DNA probed with mtDNA probes *atp*A (**b**) and *pCos*42 (**c**)

'*oxy*'-type chloroplasts (Table 3). The Southern hybridization pattern of 12 hybrids of independent origin and their parents is shown in Fig. 3a. Occasionally shoots regenerating independently from the same colony were found to have chloroplasts of either of the parents. None of the hybrids, however, exhibited the presence of a mixture of parental chloroplasts or chloroplast recombination. This was further confirmed by *Eco*RI and *Hind*III restriction analysis of the cpDNA of the 12 selected hybrids (data not shown).

For mtDNA analysis, total DNA from 12 hybrids of independent origin and their parents was digested independently with *Eco*RI or *Hind*III and hybridized to 11 mitochondrial gene probes, namely *atp*A, *atp*6, *atp*9, *cox*1, *cox*2, *cox*3, *cob*, *rrn*5–18, *rrn*26, *nad*3 and *nad*4. Rearrangements in the mtDNA were observed in the regions of *cox*1, *cox*2, *atp*6, *atp*9, *atp*A and *nad*3. The rearrangements were either in the form of the appearance of novel bands, of a combination of novel and one of the parental bands (Fig. 3b) or of a combination of parental bands. Mitochondrial genomic regions representing *cox*1, *cox2*, *atp*6, and *atp*A were found to show the most frequent variation in RFLP patterns. Consequently, these 4 probes were used to study variation in the mtDNA patterns of the remaining 40 hybrids. Out of the 52 hybrids studied, 46 showed rearrangements in the mitochrondrial genome. While 28 of these hybrids showed novel banding patterns for all of the four mitochondrial gene probes selected, 13 hybrids showed bands of one of the parent for one mitochrondrial region and of the other parent for the other region. Representative Southern hybridization data for 10 hybrids with four mitochrondrial gene probes is presented in Table 4.

The 52 somatic hybrids were analyzed for their overall mitochondrial genome composition using eight overlapping cosmid clones from *B. oxyrrhina* mtDNA cover-

Table 4 Correlation of floral features with mitochondrial and chloroplast composition of ten selected '*oxy*' CMS *B. juncea* (AABB hm$^+$) + normal *B. oleracea* (CC ppt$^+$) somatic hybrids

Hybrid	Chloroplast type[a]	Mitochondria type[a]	Southern hybridization pattern with mt gene probes[b]				Floral phenotype[a]	Pollen viability (%)
			atpA	*atp6*	*cox1*	*cox2*		
4.5.7.3	C	Cmt	C	C	C	C	NPF	2
4.5.8.1	C	Omt	O	O	O	O	NMS	0
2.2.2.9	C	Omt^R	N	O	O	O	NMS	0
1.8.3.6	C	Cmt^R	N	O	C	C	NMS	0
3.8.2.1	C	Omt^R	N	O	O	N	NMS	0
4.5.1.1	C	Omt^R	C	O	O	N	NMS	0
1.8.3.10	C	Cmt^R	C	O	C	C	NMS	0
2.2.2.2	C	Omt^R	C	O	N	N	NMS	0
5.6.11.2	C	Mt^R	N	N	N	N	AMS	0
5.5.9.8	O	Mt^R	N	N	N	N	APF	25

[a] See Table 3 for explanation of abbreviations
[b] C, *B. oleracea*; O, *B. oxyrrhina*; N, novel

ing about 190 kb of the mtDNA (Arumugam et al. 1996). Hybridization patterns of 12 hybrids to *pCos*42 is shown in Fig. 3c. On the basis of the overall hybridization patterns, the mitochondrial composition of the hybrids was designated as Cmt (having a pattern similar to that of *B. oleracea*), Omt (having a pattern similar to that of '*oxy*' CMS *B. juncea*), Cmt^R (having rearranged mtDNA with a predominant *B. oleracea* pattern) or Omt^R (having rearranged mtDNA with a predominant '*oxy*' CMS *B. juncea* pattern). Some of the hybrids exhibited completely novel patterns when hybridized to all 4 mitochondrial gene probes namely, *cox*1, *cox*2, *atp*6 and *atp*A and also showed many novel bands when hybridized to cosmid clones. These hybrids could not be assigned to any of the above-mentioned four classes and were designated as Mt^R.

Correlation of hybrid morphology vis-a-vis organelle composition

The organelle composition of different phenotypic classes is summarized in Table 3. Comparison of phenotypic and molecular data indicated that while '*oleracea*'-type chloroplasts were found in all of the morphological classes, '*oxy*'-type chloroplasts were present only in the AMS and APF categories. Correlation of floral morphology with mtDNA profiles indicated that the normal floral morphology (NMS class) is associated with minimum changes in the mtDNA, represented by Omt-, Omt^R- or Cmt^R-type mitochondria. Conversely, the hybrids belonging to AMS and APF class showed totally recombinant (Mt^R)-type mitochondria (Table 3). The solitary hybrid belonging to the NPF class harboured '*oleracea*'-type mitochondria and produced pollen grains with 2% viability.

A survey of the hybridization patterns of the 52 hybrids with mitochondrial gene probes revealed that all the NMS hybrids invariably had the '*oxy*'-type *atp*6 sequence, while all other gene sequences showed one or the other of the parental patterns or the novel-type pattern (data not shown). From the phenotypic analysis and the molecular characterization it can be concluded that hybrids 1.8.3.6 and 1.8.3.10 are the most desirable as these belong to the NMS category, have a *B. oleracea* chloroplast genome and recombinant mitochrondrial genomes with a predominant input from the mitochrondrial genome of *B. oleracea* (Table 4).

Absence of chlorosis and retention of male sterility in the backcross progenies

With the exception of the highly abnormal types, all the remaining hybrids were backcrossed to the allotetraploid *Brassica* species, *B. juncea* (AABB), *B. napus* (AACC) and *B. carinata* (BBCC), and good seed set was obtained. None of the hybrids, including those showing the presence of FDA-positive pollen grains, set seed on selfing. Some of the hybrids belonging to the abnormal classes (AMS and APF) maintained their floral morphology over three backcross generations, while others showed an improvement in terms of petal development. This feature was more pronounced in *B. napus* than in *B. juncea* and *B. carinata*. At the hexaploid level, chlorosis was not apparent and the hybrids with '*oxy*' or '*oleracea*' chloroplasts could not be distinguished from each other. However, when 27 out of the 29 somatic hybrids having '*oleracea*'-type chloroplasts and mitochrondrial genomes of either Omt, Cmt^R, Omt^R or Mt^R composition were backcrossed with the allotetraploids, all the progenies looked greener than the plants with '*oxy*'-type chloroplasts with the advance in backcross generations. All of these lines, as also the 22 somatic hybrids belonging to the AMS and APF classes (Table 3), tested up to the BC_3 generation, were found to stably maintain their male sterility. None of the male-sterile hybrids turned fertile upon backcrossing.

Discussion

Cytoplasmic male sterility is very useful in commercial hybrid seed production. However, the majority of alloplasmic CMS systems in *Brassica* species cannot be effectively used for hybrid seed production due to chlorosis and a corresponding lack of vigour and yield in the CMS lines and hybrids. CMS lines of *B. juncea* and *B.*

napus containing *B. oxyrrhina* cytoplasm exhibit severe chlorosis (Prakash and Chopra 1990). In the present study, attempts were made to rectify this defect by replacing '*oxy*' chloroplasts with those from cultivated species through somatic cell hybridization of '*oxy*' CMS *B. juncea* with normal *B. oleracea*. Among 52 somatic hybrids analysed 29 were corrected for chlorophyll deficiencies. The availability of such a large number of hybrids corrected for chlorosis is advantageous as it provides a scope for selecting the best CMS lines. Earlier reports on the correction of chlorosis by chloroplast replacement for many CMS systems in *Brassica* species (Pelletier et al. 1983; Menczel et al. 1987; Stiewe and Robellen 1994; Kirti et al. 1998) described the generation of only one or a few desirable hybrids.

In an earlier study on the correction of chlorosis in '*oxy*' alloplasmic lines, Kirti et al. (1993) reported the production of 123 allo-octaploid somatic hybrids, 1 out of which was corrected for chlorophyll deficiency. It was reported that this correction resulted from the recombination of chloroplast genomes of *B. oxyrrhina* and *B. juncea* though no corroborative evidence in the form of restriction profiles of cpDNA was furnished. In the present study we obtained 29 hybrids which were corrected for chlorosis, but none showed any detectable recombination in cpDNA as revealed by Southern hybridization. This was further verified by analysis of the *Eco*RI and *Hind*III restriction profiles of cpDNA of 12 selected hybrids. Chloroplast genome recombination is very rare and has been reported in interspecific and intergeneric hybrids in Solanaceae (Medgyesy et al. 1985; Thanh and Medgyesy 1989) where the rare recombinants were recovered at a frequency of 1.9×10^{-5} and 2.5×10^{-4} respectively, after marker-based selection.

In contrast to cpDNA, extensive rearrangement and/or recombination were observed in mtDNA. Out of the 52 hybrids analysed, 46 showed rearrangements in mtDNA (Table 3). Rearrangement and/or recombination in mtDNA is a common phenomenon in somatic hybrids and has been reported in several plants species (Earle 1995). The use of overlapping cosmid clones enabled us to determine the overall composition of mtDNA in the hybrids (i.e. Cmt, Cmt^R, Omt, Omt^R and Mt^R types). By using mitochrondrial gene probes, it was possible to identify intergenomic recombination between the two types of mtDNA (e.g. hybrid 1.8.3.10 has a mitochondrial genomic sequence of *atp*A, *cox*1, and *cox*2 from *B. oleracea* and *atp*6 from '*oxy*' CMS *B. juncea*; Table 4). It is evident from the data given in Table 4 that all male-sterile hybrids with normal floral morphology containing recombined mitochondria have the *atp*-6 region from '*oxy*' CMS *B. juncea*. This suggests that the *atp*-6 region might be involved with the CMS phenotype in '*oxy*' CMS. Detailed analysis of these mitochondrial recombinants in the nuclear background of allopolyploid *Brassica* species could lead to the identification of mitochondrial region(s) associated with the CMS phenotype.

The normal male-sterile hybrids with a cytoplasmic composition of CcpOmt, $CcpOmt^R$, and $CcpCmt^R$ (20, Table 3) obtained in the present study are desirable, since these fulfill our objective of rectifying of the chlorosis problem. However, male-sterile somatic hybrids 1.8.3.6 and 1.8.3.10 (Table 4) are the most desirable by virtue of possessing the maximum number of mitochondrial genomic sequences from *B. oleracea*. These types of male-sterile mitochondrial recombinants, if stabilized during subsequent sexual generations, would be ideal genetic stocks to search for potential restorer(s) for '*oxy*' CMS. The inheritance of the male-sterility trait in these lines has been found to be stable through three backcross generations. However, the stability of the mtDNA pattern needs to be confirmed after the complete reconstitution of the nuclear genome. The chlorosis-free CMS lines are being crossed with genetically divergent lines available within each crop species for diversification and identification of restorer function. CMS *B. napus* (AACC) can also be backcrossed to *B. oleracea* (CC) to develop '*oxy*' CMS lines corrected for chlorosis in different cole crops.

Acknowledgements This work was supported by the National Dairy Development Board (NDDB), India.

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