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Synthesis of hexaploid (AABBCC) somatic hybrids: a bridging material for transfer of 'tour' cytoplasmic male sterility to different *Brassica* species

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Abstract Most of the alloplasmic cytoplasmic male sterility (CMS) systems are known to be associated with a number of floral abnormalities that result from nuclear-cytoplasmic incompatibilities. One such system, 'tour', which is derived from *Brassica tournefortii*, induces additional floral abnormalities and causes chlorosis in *Brassica* spp. While the restorer for this CMS has been reported to be present in *B. napus*, in *B. juncea*, where the abnormalities are more pronounced, no restorer has yet been identified. Rectification of these floral abnormalities through mitochondrial recombinations and chloroplast replacement might result in the improvement of this CMS system. As organelle recombinations can possibly be achieved only by somatic cell hybridization, fusion experiments were carried out between hygromycin-resistant *B. juncea* AABB carrying 'tour' cytoplasm and phosphinotricin-resistant, normal *B. oleracea* CC to generate AABBCC hexaploid somatic hybrids. The presence of selectable marker genes facilitated the selection of hybrids in large numbers. The resulting hybrids showed wide variation in floral morphology and organelle composition. Regenerants with normal, male-sterile flowers having recombinant 'tour'- or 'oleracea'-type mitochondria and 'oleracea'-type chloroplasts were obtained. Hybrids with male-fertile flowers were also obtained that had recombined 'tour' mitochondria. The AABBCC hexaploid hybrids synthesized in the present study were successfully utilized as a bridging material for transferring variability in the organelle genome simultaneously to all the digenomic *Brassica* species, and all of these hybrids are now being stabilized through repeated backcrosses to the allopolyploid crop brassicas.

Key words CMS *Brassica juncea* · *B. oleracea* · Somatic hybrids · Mitochondrial recombination · Chloroplast segregation

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Introduction

Cytoplasmic male sterility (CMS), a maternally inherited inability in plants to produce functional pollen, provides an extremely useful and economic way to produce heterotic F_1 hybrid seeds in crop plants. A phenotypic manifestation of nuclear-cytoplasmic incompatibility, CMS can be either spontaneous or alloplasmic in origin. Alloplasmic CMS systems are commonly found in crop plants (Kaul 1988). They develop through interspecific or intergeneric crosses and harbour alien chloroplast and mitochondria in the nuclear background of the recurrent parent. Such CMS lines, in addition to displaying male sterility, may display other phenotypic aberrations such as chlorosis, petal-less flowers, petaloid anthers, modified phenotypes of corolla and stamens, pistilloidy, abnormal nectary formation etc. (Edwardson 1970; Sand and Christoff 1973; Prakash and Chopra 1988; Berbec 1994). Recent evidence based on molecular analyses implicates mitochondria (mt) as the CMS determinant (Hanson et al. 1989). A sequence or sequences present in the mitochondrial genomes have been ascribed to be associated with CMS expression in all of the types of CMS analysed so far (Vedel et al. 1994). However, it has not yet been clearly demonstrated whether the sequences responsible for CMS expression are also responsible for the additional CMS-associated floral abnormalities in an alloplasmic CMS. In the case of *Nicotiana*, mitochondrial involvement in petal and stamen development and modifications of mtDNA causing changes in floral development have been reported (Bonnett et al. 1991; Kofer et al. 1991).

In *Brassica* species many sources of alloplasmic CMS are known (Prakash and Chopra 1990; Pradhan et al. 1991; Mekiyanon et al. 1994; Rao et al. 1994). One of the stable CMS systems in *B. juncea* and *B. napus* is 'tour', which has the cytoplasm of *B. tournefortii* (Pradhan et al. 1991). In addition to causing male sterility this CMS system, induces chlorosis in both species and also results in the manifestation of the aforementioned additional floral abnormalities in *B. juncea* and some less conspicuous irregularities in *B. napus* flower morphology. Whereas some restorers have been identified for this CMS in *B. napus* (Sodhi et al. 1994), none has been recorded so far in existing *B. juncea* germplasm. The availability of restorers is a prime necessity for the production of hybrid seeds. Rectification or minimization of floral abnormalities through the modification of the mitochondrial genome may facilitate identification of restorers for 'tour' CMS in *B. juncea*.

In this paper we report the synthesis of hexaploid (AABBCC) somatic hybrids between CMS ('tour') *B. juncea*, AABB, and normal *B. oleracea*, CC, in order to generate variability for organelle genomes. Our objectives were: (1) to improve the morphology of CMS lines by mitochondrial recombination; (2) to rectify the chlorosis problem by replacement of the '*tournefortii*' chloroplast; and (3) to use the AABBCC (hexaploid) as a bridging material for simultaneous transfer of the desirable CMS cytoplasms to all of the allopolyploid *Brassica* species.

Materials and methods

Plant material

Brassica juncea (AABB) var 'Varuna', which carries the 'tour' cytoplasm, was crossed sexually to hygromycin-resistant (Hm^+) *B. juncea* var 'RLM 198' (Pental et al. 1993). The seeds were germinated and seedlings screened for resistance to the antibiotic by re-rooting on RI medium [MS + IBA (2.0 mg/l)] supplemented with 20 mg/l hygromycin. The Hm^+ shoots of CMS AABB thus obtained were multiplied and maintained on RI medium. Seeds of phosphinotricin-resistant (Ppt^+) *B. oleracea* (CC) var 'Early kunwari' (Mukhopadhyay et al. 1991) were similarly screened for resistance to the herbicide on RII medium [MS + IBA (1.0 mg/l)] supplemented with 10 mg/l phosphinotricin and subsequently multiplied and maintained on SM medium [MS + NAA (0.005 mg/l) + Kn (0.05 mg/l) + CH (50 mg/l)].

Protoplast isolation, fusion and regeneration of somatic hybrids

The shoots of Hm^+ CMS AABB and Ppt^+ normal CC were grown on RI and RII medium, respectively, for 15 days prior to the isolation of protoplasts. The protoplasts were isolated following the protocol described in Mukhopadhyay et al. (1991), and the subsequent fusion of isolated protoplasts was done following Mukhopadhyay et al. (1994) with one modification, that the suspension solution used in the present study contained BAP (1.0 mg/l) and 2,4-D (0.05 mg/l). Protoplasts were plated at a density of 4×10^4/ml in liquid PC1 medium (Mukhopadhyay et al. 1991) supplemented with either BAP (1.0 mg/l) and 2,4-D (0.05 mg/l), BAP (1.0 mg/l) and 2,4-D (1.0 mg/l) or BAP (1.0 mg/l) and NAA (1.0 mg/l). After 10 days of culture the media were diluted with PC2 medium (PC1 modified by replacing 0.5 *M* glucose with 0.1 *M* sucrose) containing the respective hormones three times at 3-day intervals. After 4 weeks the microcolonies were plated on SL 1 medium [K_3 medium (Nagy and Maliga 1976) supplemented with hormones as in PC1 medium and 0.1 *M* sucrose, 0.25% agarose, 20 mg/l hygromycin and 10 mg/l phosphinotricin] for the selection of hybrids. For each fusion experiment the following were kept as controls: (1) AABB Hm^+ protoplasts on 10 mg/l phosphinotricin, (2) CC Ppt^+ protoplasts on 20 mg/l hygromycin, (3) AABB Hm^+ on hygromycin, (4) CC Ppt^+ on phosphinotricin and (5) a physical mixture of AABB Hm^+ and CC Ppt^+ on medium containing both antibiotics. For further growth and regeneration of hybrid shoots the colonies were transferred to SL2 medium (MS with 20 μM silver nitrate along with hormones and selection agents as in the SL1 medium). Shoots regenerated on MS supplemented with BAP (1.0 mg/l) and 2,4-D (0.05 mg/l) and MS with BAP (1.0 mg/l) and NAA (1.0 mg/l) in the presence of 20 μM $AgNO_3$. Colonies obtained on MS with BAP (1.0 mg/l) and 2,4-D (1.0 mg/l) were induced to regenerate by transfer to MS with BAP (1.0 mg/l) and NAA (1.0 mg/l).

The regenerated shoots were transferred to SM medium for further growth and rooted on RI medium containing 20 mg/l hygromycin and 10 mg/l phosphinotricin prior to transfer to the field. The plants were numbered in a sequential manner to denote the fusion, plate, colony and shoot number; e.g. the plant designated 1.2.27.3 denotes (1) the fusion number, (2) the plate number, (27) the colony number and (3) the third shoot regenerated from this colony. The hybrids were maintained *in vitro* on RI medium.

Characterization of somatic hybrids

Total DNA was isolated from the parents and 78 regenerated hybrids following the procedure of Dellaporta et al. (1983) and purified on CsCl density gradients. The hybrid nature of the regenerants was established through random amplified polymorphic DNA (RAPD) analysis following Mukhopadhyay et al. (1994) using four 10-mer primers (OPB8, OPB10, OPD13 and OPE1) supplied by Operon Technologies, Alameda, Calif., USA.

Restriction fragment length polymorphism (RFLP) analysis of the chloroplast (cp) and mitochondrial genomes of the 78 hybrids was done according to Pradhan et al. (1992). For the chloroplast genome, total DNAs were digested with *Eco*RI and hybridized to two heterologous probes of chloroplast origin, namely *rbc*L and *psb*D.

For mitochondrial genome analysis, total DNAs were digested with *Eco*RI and *Hin*dIII and hybridized to 11 mitochondrial gene probes, *atpA*, *atp6*, *atp9*, *coxI*, *coxII*, *coxIII*, *cob*, *rrn 5–18*, *rrn 26*, *nad3* and *nad4*, and eight overlapping cosmid clones of the *B. oxyrrhina* mtDNA library covering about 190 kb of the mitochondrial genome. The mitochondrial gene probes were kindly provided by Drs. C. J. Leaver, Oxford University, C. S. Levings III, North Carolina State University, G. G. Brown, McGill University and C. A. Makaroff, Miami University. The *B. oxyrrhina* mtDNA cosmid clones were generated in our laboratory.

Results

Protoplast fusion, recovery of hybrid colonies and regeneration of hybrids

Protoplasts were observed to divide within 48 h of culture, and a large number of microcolonies developed within 10–15 days. The parental protoplasts grew only on the permissive media, and no colony growth was observed in the physical mixture of the two parental protoplasts that was plated on media containing both selection agents. The hybrid colonies obtained on selection plates in the different fusion experiments are given in Table 1. All three media tested proved effective in

Table 1 Fusion of CMS *B. juncea* AABB Hm^+ and normal *B. oleracea* CC Ppt^+ protoplasts and recovery of hybrids

Fusion number	Number of protoplasts used for fusion AABB Hm^+	CC Ppt^+	Number of hybrid colonies	Number of colonies regenerating shoots
1	8×10^5	8×10^5	350	18
2	6×10^5	6×10^5	256	20
3	6×10^5	6×10^5	32	8
4	6×10^5	6×10^5	245	3
5	8×10^5	8×10^5	93	10

stimulating growth of the hybrid colonies. In all, 59 out of a total of 889 colonies obtained from different fusion experiments regenerated shoots (Table 1). The regenerants were further selected by rooting on RI medium in the presence of 20 mg/l hygromycin and 10 mg/l phosphinotricin. All of the regenerated shoots rooted on the double selection medium.

Variation in the floral morphology of the somatic hybrids

A total of 93 independent regenerants from 20 independent colonies, with 3–5 regenerants from each colony, were transferred to the field. Of these, 82 regenerants, representing all the 20 colonies, were established in the field. Seventy-eight of these hybrids grew vigorously, were morphologically intermediate between the two parents and flowered within 3 months of transplantation; the remaining 4 were stunted and had buds that either failed to open or aborted. The hybrids originating from different colonies or even independently from a single colony exhibited marked differences in floral morphologies.

The major deviations from normal flower morphology were: (1) variation in the number of petals (either absence or presence of fewer than four petals), (2) variation in the shape of the petals (narrow, crinkled, scaly or needle like), (3) variation in the length of the stamens (stamen shorter than style), (4) presence of epipetalous or petaloid anthers, (5) variation in the shape and size of the style and stigma (style 1–4, fused or winged, falcate instead of straight style, thick, tri-or quadrilobed stigma) and (6) non-opening of flower.

Flowers exhibiting any of the above mentioned deviations were termed as having abnormal flower morphology, and on the basis of these variations, the hybrids were broadly grouped into six classes:

Class I: Male-sterile plants with normal floral morphology.
Class II: Male-sterile plants with abnormal floral morphology.
Class III: Male fertile plants with normal floral morphology.
Class IV: Male fertile plants with abnormal floral morphology.
Class V: Semisterile plants with normal floral morphology.
Class VI: Semisterile plants with abnormal floral morphology.

All of the plants having morphologically normal gynoecium were backcrossed to the three allopolyploid brassicas, *B. juncea* (AABB), *B. napus* (AACC) and *B. carinata* (BBCC). Good seed set was observed in all three cases. BC_1 seeds with *B. juncea* were obtained from 72 hybrids (out of 78 pollinated), with *B. napus* from 60 hybrids (out of 68 pollinated) and with *B. carinata* from 10 hybrids (out of 10 pollinated). BC_1 progenies were grown along with the respective recurrent parents as control checks, and BC_2 seeds have been obtained. A summary of the variation in floral characteristics of AABBCC somatic hybrids has been presented in Table 2.

Table 2 Summary of variation in the floral characteristics in relation to the organelle compositions of AABBCC somatic hybrids (*N* normal flower morphology·*Ab* abnormal flower morphology·*MS* male sterile·*MF* male fertile·*SS* semi-sterile)

Organelle composition	Number of hybrids belonging to different classes based on floral morphology						
	I N, MS	II Ab, MS	III N, MF	IV Ab, MF	V N, SS	VI Ab, SS	Total number of hybrids
$T_{cp}\,T_{mt}$	0	13(12)	0	1(1)	0	0	14(13)
$T_{cp}\,T^R_{mt}$	0	3(2)	0	0	0	0	3(2)
$T_{cp}\,C^R_{mt}$	2(2)[a]	2(2)	1(1)	0	1(1)	0	6(6)
$C_{cp}\,T^R_{mt}$	1(1)	22(21)	1(1)	1	0	0	25(23)
$C_{cp}\,C_{mt}$	8(8)	0	5(5)	0	0	1(1)	14(14)
$C_{cp}\,C^R_{mt}$	3(3)	3(1)	2(2)	0	5(5)	1(1)	14(12)
$C_{cp}\,TC^R_{mt}$	0	1(1)	0	0	0	1(1)	2(2)

[a] Figure in parentheses indicates the number of hybrids backcrossed to *B. juncea* and/or *B. napus*

Molecular analysis of the somatic hybrids

RAPD analysis of 12 randomly selected hybrids of independent origin using primers OPB8, OPB10, OPD13 and OPE1 showed the presence of bands specific to both parents thereby confirming that these are true AABBCC hybrids (Fig. 1). RFLP analysis of 78 hybrids using total DNA with heterologous cpDNA probes showed that 23 hybrids had the CMS *B. juncea* ('tour')-type chloroplast (T_{cp}), while the other 55 had the *B. oleracea*-type chloroplast (C_{cp}) (Table 2). No recombination in cpDNA was observed. The Southern hybridization pattern of 12 hybrids of independent origin and their parents is presented in Fig. 2.

The mitochondrial genome composition of the 78 hybrids was determined using the eight overlapping cosmid clones of the *B. oxyrrhina* mtDNA library as probes for Southern hybridization. These clones cover most of the area on the mtDNA and detect any rearrangement/recombination that might have occurred in the intergenic sequences. On the basis of RFLP patterns, the hybrids were designated as T_{mt} [having a pattern similar to that of CMS *B. juncea* ('tour'), Fig. 3, hybrid no. 2], C_{mt} (having a pattern similar to that of *B. oleracea*, Fig. 3, hybrid no. 5), T^R_{mt} [having a rearranged mitochondrial genome with a predominance of the CMS *B. juncea* ('tour')-type pattern, Fig. 3, hybrid nos. 4 and 6] and C^R_{mt} (having a rearranged mitochondrial genome with a predominance of the *B. oleracea*-type pattern, Fig. 3, hybrid nos. 1 and 3). Two hybrids exhibited a T_{mt}-like pattern when hybridized to some cosmid clones and a C_{mt}-like pattern when hybridized to other cosmid clones; these have been designated as TC^R_{mt}. Out of 78 hybrids, 28 hybrids had true parental-type patterns, i.e. either that of CMS *B. juncea* ('tour') or of *B. oleracea*, 28 hybrids had the T^R_{mt}-and 20 hybrids the C^R_{mt}-type pattern, and the remaining 2 had the TC^R_{mt}-type pattern (Table 2).

To identify specific gene loci involved in the rearrangement and/or recombination events, we used 11 mitochondrial genes as probes for Southern hybridization in order to analyse the 12 representative hybrids. The gene probes showed that novel or recombinant bands appeared most frequently in the region of *atpA* and *atp9* (in 4 hybrids, Fig. 4) followed by the *coxI* and *rrn 5–18* region (2 hybrids).

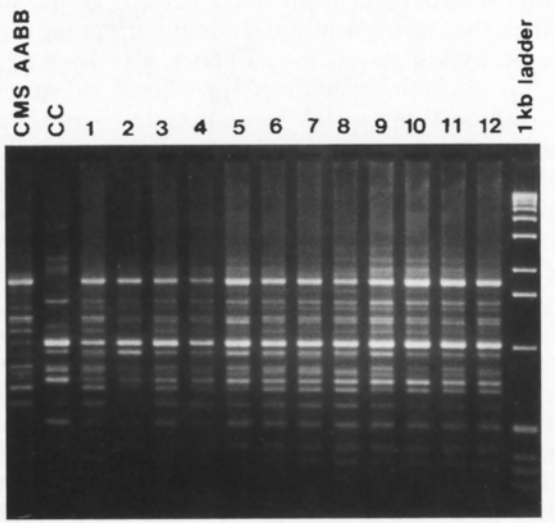
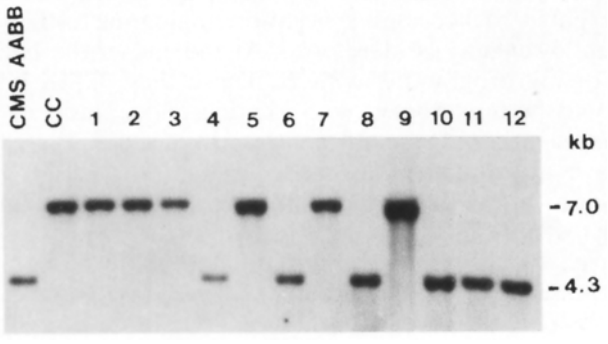


Fig. 1 RAPD analysis of parents (*CMS* AABB *B. juncea*, *CC B. oleracea*) and 12 somatic hybrids using the 10-mer oligonucleotide primer OPB10

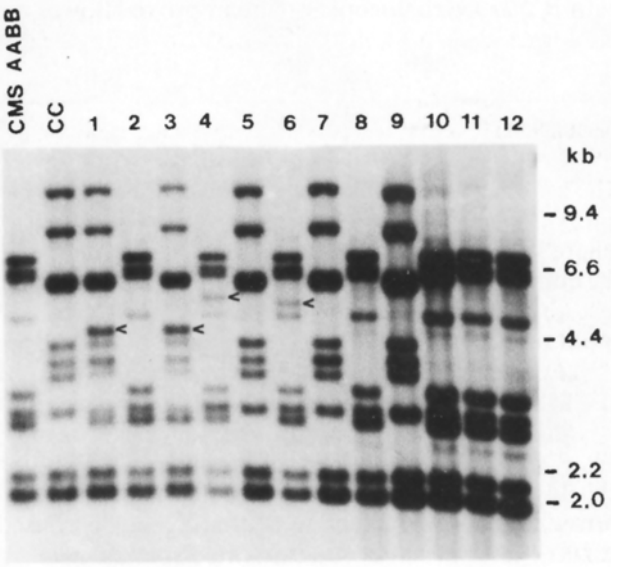
Fig. 2 Southern hybridization pattern of *Eco*RI-digested total DNAs of parents (*CMS* AABB *B. juncea*, *CC B. oleracea*) and 12 somatic hybrids probed with radioactively labelled cpDNA probe *psbD*



Correlation between organelle composition and floral characteristics

The replacement of 'tour' chloroplasts (T_{cp}) with cultivated *B. oleracea*-type chloroplasts (C_{cp}) was observed

Fig. 3 Southern hybridization pattern of *Hin*dIII-digested total DNAs of parents (*CMS* AABB *B. juncea*, *CC B. oleracea*) and 12 somatic hybrids probed with radioactive labelled cosmid clone pCos42. Hybrids showing the presence of novel bands are indicated (<).



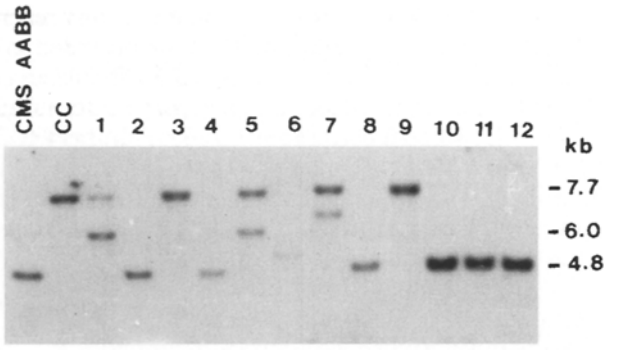


Fig. 4 Southern hybridization pattern of *Hin*dIII-digested total DNAs of parents (*CMS* AABB *B. juncea*, *CC B. oleracea*) and 12 somatic hybrids probed with radioactive labelled mitochondrial gene probe *atpA*

in hybrids belonging to all of the morphological classes (Table 2).

When different classes of floral morphologies of the AABBCC somatic hybrids were correlated with mtDNA composition (Table 2) it was observed that the largest number of hybrids belonged to class II, i.e. those having abnormal, male-sterile flowers, with the majority of these having either the T_{mt}- or T^R_{mt}-type mitochondrial genome composition. Conversely, the majority of hybrids belonging to class I, the most desirable group and the one having normal, male-sterile flowers, showed either the C_{mt}- or C^R_{mt}-type mtDNA pattern. It was observed that while all of the hybrids with the C_{mt}-type mitochondria turned fertile in the BC_1 generation, hybrids having C^R_{mt}-mtDNA maintained their male sterility (data not shown).

On the basis of organelle composition the hybrids were divided into seven groups (Table 2). The majority of the male-sterile hybrids showed either $C_{cp}T^R_{mt}$-or $C_{cp}C^R_{mt}$-type configurations and 4 of these had normal, male-sterile flowers. Ideally these are the most desirable types as they show the replacement of 'tour' chloroplasts with *B. oleracea* chloroplasts and improved flower morphology.

Discussion

The use of CMS for hybrid seed production is dependent upon the availability of restorer gene(s) in situations where seed is the harvest product. In crop plants, where the commercial production of hybrid seeds using CMS-restorer systems has been successful (mostly in CMS spontaneous in origin), two factors have played important key roles: (1) the absence of additional abnormalities in CMS lines; (2) the availability of restorer genes in the existing germ plasm of the cultivated species harbouring the CMS (Kaul 1988). In alloplasmic CMS systems hybrid seed production has not been feasible due to the non-availability of effective restorer gene(s) from the existing germ plasm of the cultivated species.

The only exception in alloplasmic CMS with respect to exploitation in commercial hybrid seed production is CMS in sunflower where the cultivated species *Helianthus annuus* contains the cytoplasm of *H. petiolaris* (Leclercq 1969). There is no apparent associated alloplasmic effect, and a large number of varieties of sunflower are known to restore this CMS (Leclercq 1984; Crouzillat et al. 1991). One of the reasons for observing so many restorer lines may be the absence of multi-loci incompatibility between the mitochondrial and nuclear genomes, as the mitochondrial genomes of *H. annuus* and *H. petiolaris* are almost similar except for a 17-kb region (Siculella and Palmer 1988) containing those gene sequences that have been shown to be associated with the CMS phenotype (Moneger et al. 1994). Both species also have the same chloroplast genome (Brown et al. 1986).

If floral abnormalities in alloplasmic systems are due to multi-loci incompatibilities between the nuclear and mitochondrial genome and if the loci controlling male sterility are different from those causing floral abnormalities, then mitochondrial recombinations induced by somatic hybridization may offer a way by which to develop CMS lines with proper floral morphology. Through mitochondrial recombinations, a situation may be created where most of the alien mitochondrial genome could be replaced by the cultivated mitochondrial genome with the exception of the region conferring CMS. The chloroplasts of the alien species can be simultaneously replaced through this approach, leading to elimination of chlorosis. Thus, the development of a CMS line with improved flower and plant morphology can be visualized. It may also be easier to find restorer functions for such recombinant lines. Mitochondrial recombinants obtained through somatic fusions have been shown to improve the quality of CMS lines in *B. napus* (Pelletier et al. 1983, 1988). In rice, Kyozuka et al. (1989) were able to transfer a small portion of the mitochondrial genome conferring the male sterility character to fertile mitochondria by protoplast fusion.

In the study presented here, we were able to generate male-sterile plants with a normal flower morphology that had an organelle composition of either $C_{cp}C^R_{mt}$ or $C_{cp}T^R_{mt}$. These plant types are the most desirable ones as they fulfill the criteria for achieving our objectives of rectification of the problems of chlorosis and abnormal flower morphology. If the cytotype $C_{cp}C^R_{mt}$, with a male-sterility character could be stably transmitted through repeated backcrosses, a situation analogous to that in sunflower may be developed. At the end of the backcrossing programme with *B. juncea* these CMS lines could be test crossed with existing germ plasm of *B. juncea* to identify the restorer lines. In *B. napus*, where we have already identified restorer lines for this CMS (Sodhi et al. 1994), such improved male-sterile lines (cytotypes $C_{cp}C^R_{mt}$, $C_{cp}T^R_{mt}$) with chloroplasts of *B. oleracea* would provide an improved CMS system in rapeseed breeding. Recently, Stiewe and Robbelen (1994) have reported synthesis of a male-sterile cybrid

using *B. napus* and *B. tournefortii* as fusion partners. Although this hybrid had *B. napus* chloroplasts it was not free from other floral abnormalities like narrow petals or petal-less flowers.

Besides the normal, male-sterile hybrids described above, hybrids with male-fertile flowers having the T^R_{mt} mitochondrial-genome were also generated in the present study. These hybrids, when stabilized in different *Brassica* nuclear backgrounds, would provide eminently suitable material for analysing CMS at genetic and molecular levels. Since 'tour' mitochondria (T_{mt}) confer male sterility, the appearance of fertile flowers in plants with the T^R_{mt} mitochondrial genome may give some clue to the sequences involved with 'tour' CMS in alloplasmic situations.

One of the most important points to be considered in generating novel organelle combinations through somatic hybridization is the number of hybrids that have to be screened for identification of the desirable mitochondrial recombinants. To identify the desirable mitochondrial recombinants that could rectify the floral abnormalities, a large population of hybrids is required for screening. Conventionally, the manual isolation of hybrid cells or the identification of hybrids by examining the population of regenerated plants have been followed. These methods suffer from the problem of limited number of hybrids that can be produced in each case. In contrast, the selectable marker genes used in this study enabled an efficient selection of a large number of hybrid colonies and plants. Since the AABBCC hexaploid hybrid is a bridging material, these marker genes could be eliminated from the progeny population during backcrossing.

In summary, our study has demonstrated that floral abnormalities associated with alloplasmic CMS can be rectified through mitochondrial recombinations obtained by somatic hybridization. The advantage of synthesizing AABBCC hybrids is that they can be maintained sexually and can be backcrossed to all of the diploid and allopolyploid *Brassica* species, thus making it possible to transfer the variability in the organelle genome that is created by protoplast fusion simultaneously to all the species. Consequently, it may allow the production of hybrid seeds in important cole crops such as cauliflower, cabbage, etc. and also provide a new set of genetic pools to be used in crop improvement programmes through conventional breeding.

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