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Alloplasmic male-sterile *Brassica juncea* with *Enarthrocarpus lyratus* cytoplasm and the introgression of gene(s) for fertility restoration from cytoplasm donor species

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Abstract A new cytoplasmic male sterility (CMS) source in *Brassica juncea* (2n = 36; AABB) was developed by substituting its nucleus into the cytoplasm of *Enarthrocarpus lyratus* (2n = 20; EE). Male sterility was complete, stable and manifested in either petaloid- or rudimentary-anthers which were devoid of fertile pollen grains. Male sterile plants resembled the euplasmic *B. juncea* except for slight leaf yellowing and delayed maturity. Leaf yellowing was due mainly to higher level of carotenoids rather than a reduction in chlorophyll pigments. Female fertility in male-sterile plants varied; it was normal in lines having rudimentary anthers but poor in those with petaloid anthers. Each of the 62 evaluated germplasm lines of *B. juncea* was a functional maintainer of male sterility. The gene(s) for male-fertility restoration (*Rf*) were introgressed from the cytoplasm donor species through homoeologous pairing between A and E chromosomes in monosomic addition plants (2n = 18II+1E). The percent pollen fertility of restored F_1 (*lyr* CMS × putative restorer) plants ranged from 60 to 80%. This, however, was sufficient to ensure complete seed set upon by bag selfing. The CMS (*lyr*) *B. juncea* compared favourably with the existing CMS systems for various productivity related characteristics. However, the reduced transmission frequency of the *Rf* gene(s) through pollen grains, which was evident from the sporadic occurrence of male-sterile plants in restored F_1 hybrids, remains a limitation.

Keywords *Brassica juncea* · *Enarthrocarpus lyratus* · Cytoplasmic male sterility · Fertility restoration · Alien introgression

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Introduction

Mustard (*Brassica juncea* L.) is a major oilseed crop in India. It is largely self-pollinated and pure-line cultivars are mostly bred. Yield plateauing and the need to produce more has prompted interest in alternate strategies like heterosis breeding for shifting the yield frontiers beyond the level of current commercial cultivars. Cytoplasmic male sterility (CMS) coupled with fertility restoring systems has been used widely to facilitate pollination control and mass production of hybrid seeds in hermaphrodite crops. CMS is encoded in mitochondrial DNA and it may result from spontaneous mutations or through substitution of the crop nucleus into the alien cytoplasm, the alloplasmic lines. Cytoplasm from eight wild species, namely *Brassica oxyrrhina* (*oxy*), *Trachystoma balli* (*trachy*), *Moricandia arvensis* (*mori*), *Diplotaxis catholica* (*cath*), *D. siifolia* (*sii*), *D. erucoides* (*eru*), *Erucastrum canariense* (*can*) and *Raphanus sativus* (*ogu*), has been introgressed into *B. juncea* using sexual or somatic hybridization (Prakash 2001). Most of these CMS lines have been registered in India. Male sterility in such alloplasms is manifested in rudimentary or petaloid anthers and expression is environment neutral. Limitations, however, exist in the form of chlorophyll deficiency (*ogu*, *oxy*, *mori*), poor female fertility (*trachy*) and the productivity loss (biological penalty) associated with the male-sterilizing cytoplasm (Fu and Yang 1998; Banga 2001). Chloroplast substitutions or mitochondrial recombinations have proved useful to overcome many of these problems (Kirti et al. 1995; Prakash et al. 1998a, b). In spite of significant technological advances towards the development of a workable system of pollination control, the limitations with respect to efficient and stable male-fertility restoration remain due to the absence of fertility restoring genes for the target CMS systems within mustard germplasm. Attempts to obtain *Rf* genes from cytoplasm donor species have met with a varied degree of success, due mainly to their unstable introgression and inconsistent expression. In view of the complexities outlined, efforts are continuing to develop new, and

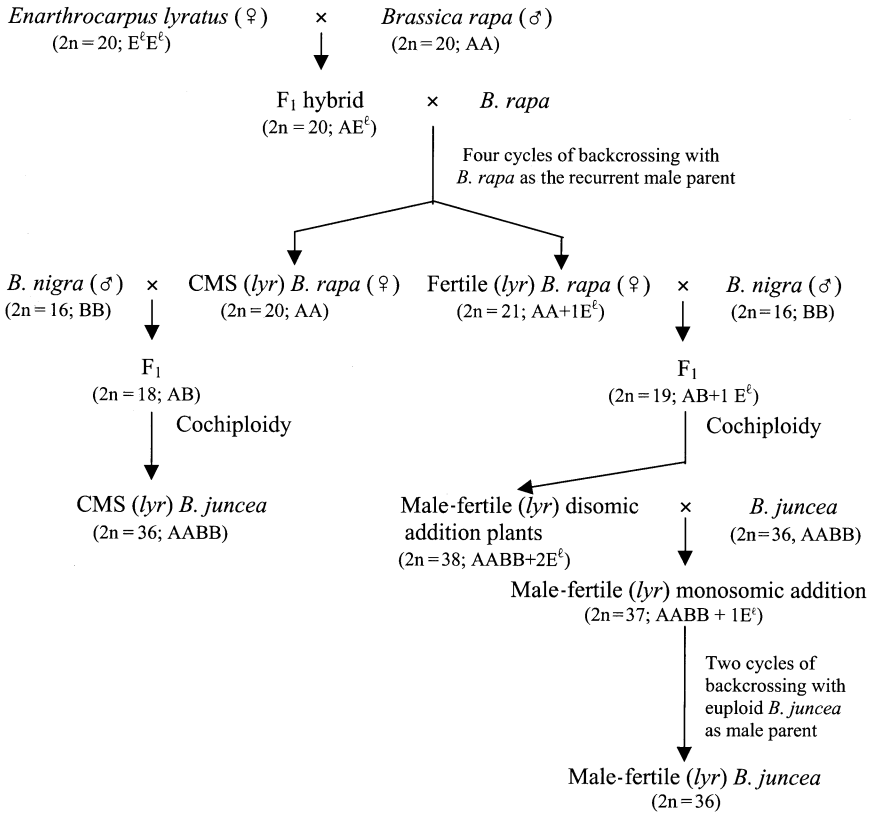

Fig. 1 Synthesis of *lyr* alloplasmic male-sterile and fertility restorer lines of *B. juncea*

perhaps better, CMS sources. In this communication, we report the synthesis of yet another CMS–fertility restorer system for *B. juncea*. This CMS was caused by the male-sterilizing cytoplasm from *Enarthrocarpus lyratus* which is a weedy crucifer endemic to the Mediterranean region and has been demonstrated earlier to possess partial homoeology with the *Brassica* genome (Gundimeda et al. 1992).

Materials and methods

The CMS line of *B. juncea* (2n = 36; AABB) with *E. lyratus* (2n = 20; EE) cytoplasm was synthesized by utilizing *Brassica rapa* (2n = 20; AA) as a bridging species (Fig. 1). The CMS (*lyr*) *B. rapa* was hybridized as female with *Brassica nigra*, the B-genome donor species for *B. juncea*. It was followed by chromosome doubling to synthesize CMS (*lyr*) *B. juncea*. For introgression of the fertility restorer (*Rf*) gene(s), male-fertile alloplasmic (*lyr*) *B. rapa* plants carrying a monosomic addition from *E. lyratus* were hybridized as a female with *B. nigra*, followed by chromosome doubling to generate (*lyr*) *B. juncea* plus a disomic addition from *E. lyratus*. Hybridization followed by two cycles of backcrossing with *B. juncea*, selection for increased pollen fertility and the euploid chromosome number (2n = 36) helped to obtain male-fertile alloplasmic (*lyr*) *B. juncea*.

For cytological analysis anthers were fixed in Carnoy's–II solution for 24 h before squashing in 2% acetocarmine. An aqueous solution of colchicine (0.2%) was applied, using small cotton plugs, to the axillary meristems of the desired plants to achieve chromosome doubling. Pollen fertility was studied by staining with 2% acetocarmine. Female fertility of the CMS lines was estimated from seed set following cross-pollination as a percent of the cross seed set in the corresponding euplasmic maintainer line.

For the purpose of making a comparison, the newly developed (*lyr*) *B. juncea* CMS line was evaluated along with a few other CMS sources (*tour*, *trachy*, *ogu* and *oxy*), all in the nuclear background (BC_5) of *B. juncea* cv HD-1. Leaf pigments associated with photosynthesis were also estimated (Vernon 1960) in *lyr* alloplasmic and corresponding euplasmic lines in a set of four combinations. These *lyr* alloplasmic lines were developed by recurrent backcrossing of four Indian *B. juncea* germplasm lines (HD-1, IJWHJ-001, USJ-15 and USJ-64) as male with the CMS (*lyr*) of *B. juncea.*

Results

Male sterility in CMS (*lyr*) *B. juncea* was manifested in flowers with petaloid or rudimentary anthers and narrow petals (Fig. 2a). While petaloid anthers were devoid of pollen grains, the rudimentary anthers harboured few small and unstained pollen grains in contrast to the deeply stained pollen grains of the euploid parent (Fig. 2b,c). Male sterility was complete and stable throughout the crop season. There was no seed set on selfing by bags in these lines. Floral nectaries were almost normal in size. Alloplasmic plants were agronomically identical to the euplasmic *B. juncea*, except for 7–10-days delay in the onset of flowering. Female fertility in the original CMS stock was low, largely due to impaired bud opening and a crooked stigma. It improved significantly, subsequent to nuclear genome diversification and advancing cycles of backcrossing. It ranged from 36 to 85% in different nuclear genotypic backgrounds. Cytological investigations on CMS plants having rudimentary anthers indicated

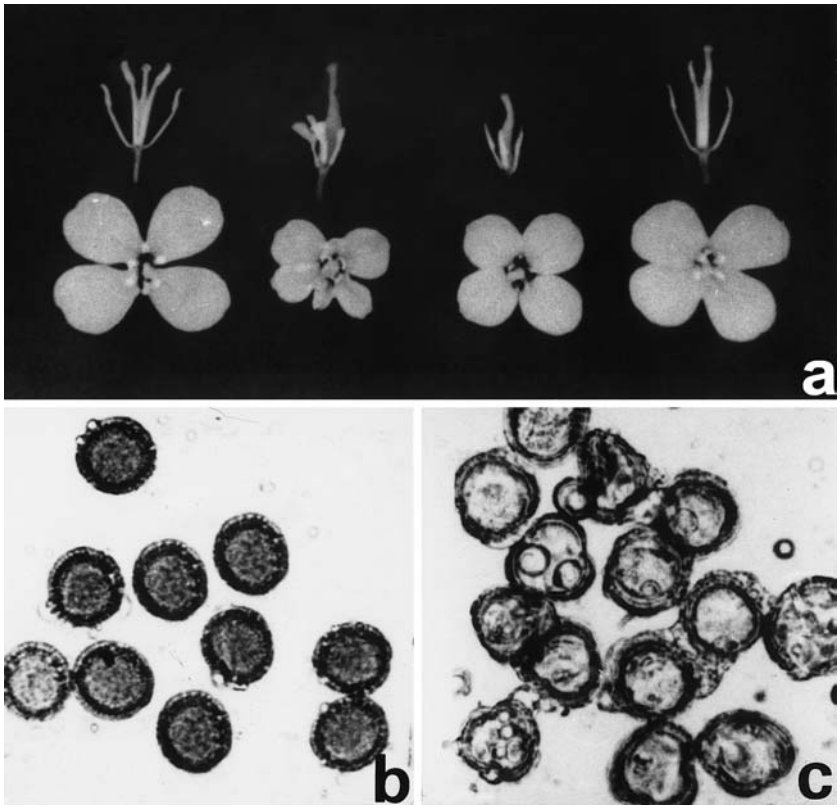

Fig. 2a–c Flower morphology and pollen grains of the euplasmic fertile and *lyr* alloplasmic CMS line. **a** (left to right): flowers of euplasmic fertile, petaloid male-sterile, vestigeal male-sterile and fertility restored F_1 plants. **b, c**: fertile and sterile pollen grains

Table 1 Meiotic analysis in a monosomic alien (*E. lyratus*) addition line of *B. juncea* and a normal diploid plant

Genotype	PMCs studied	Chromosome number	18II	18II+1I	17II+1III
SNCR-1[a]	22	37	–	17(73.3)	5(22.7)
SNCR-2T	28	36	28	–	–

[a]Monosomic addition line

a normal euploid *B. juncea* chromosome number, with regular 18IIs during metaphase–I. To identify maintainers of male sterility, the *lyr* CMS was crossed with 62 genotypes of *B. juncea*, representing a spectrum of available variability in the native and exotic germplasm. All of the resultant 62 F_1s were male-sterile. This indicated the absence of *Rf* genes in euplasmic *B. juncea*.

Synthesis of the male-fertility restorer

One male-fertile alloplasmic disomic addition plant (2n = 36 + 2E), obtained from hybridizing monosomic addition line of *B. rapa* with *B. nigra* followed by chromosome doubling, was used to initiate backcrossing with euploid *B. juncea*. The cytological studies (Table 1) with selected F_1 plant revealed a meiotic configuration of 18II+1I (Fig. 3a) in 77% of the pollen mother cells (PMC's). In about 22% of the PMC's, the extra chromosome occurred as a trivalent (Fig. 3b). Two cycles of backcrossing with euploid *B. juncea* helped in the isolation of male fertile plants having 18II chromosome configuration (Fig. 3c). Selfed generation of the selected putative fertility restorer plants revealed a gradation for pollen grain fertility. It ranged from 58 to 87%. Pollen grains from the plant having the highest male fertility were used to develop F_1 hybrids in crosses with *lyr* CMS lines. Resulting F_1s segregated for male fertility restoration. Pollen grain fertility in F_1 plants having phenotypically normal anthers was 60–80%. Such plants were classified as completely male fertile. The proportion of completely male-fertile plants was 71 to 90% in different F_1 cross combinations.

Appraisal of the fecundity and agronomic potential of the *lyr* CMS source

The male-sterility expression for *lyr* CMS was monitored during the entire crop season for over 4 years, at two locations (approximately 247 ft and approximately 11,500 ft above mean sea level) under short- and long-day length conditions. No influence of the prevailing environmental conditions and of advancing backcross generations was observed. The female fertility, low initially, improved consistently with the advancing generations of backcrossing in a majority of nuclear geno-

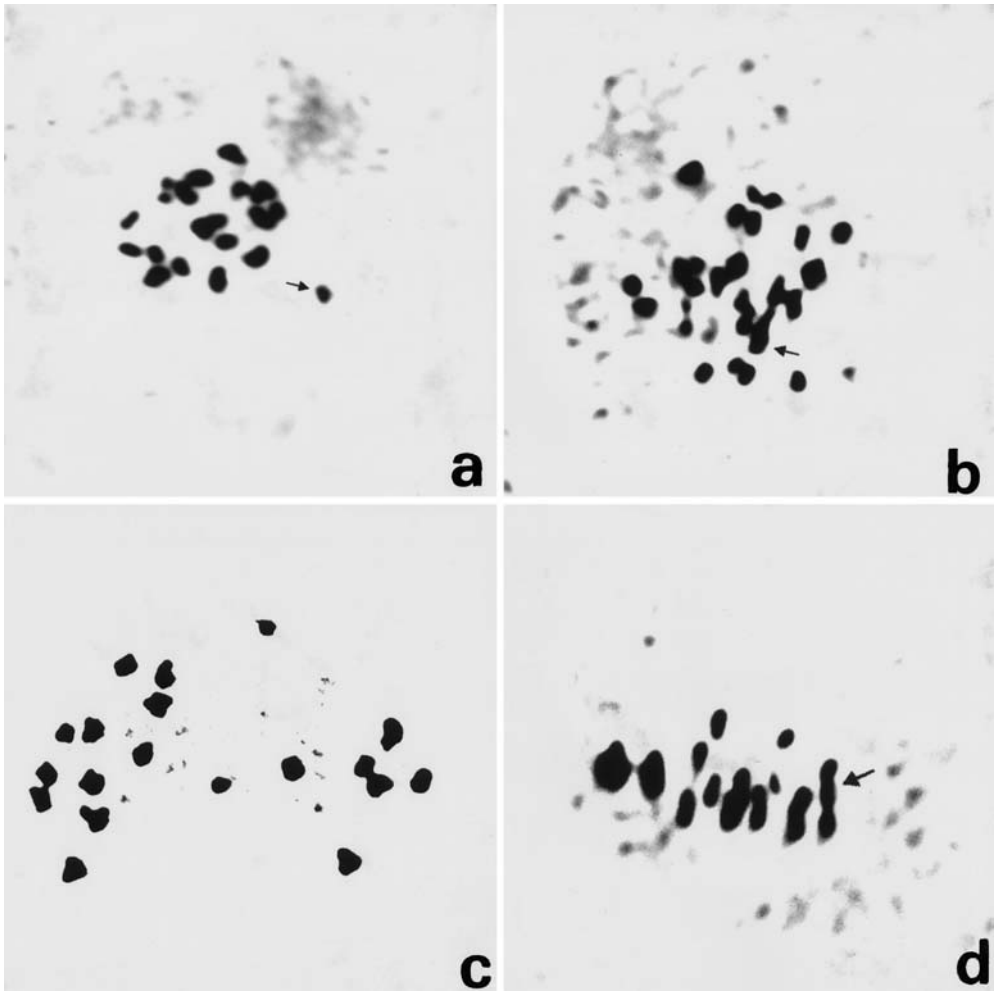

Fig. 3 Meiotic analysis in monosomic addition/euploid plants of *B. juncea* **(a–c)** and *B. rapa* **(d). a**: 18II+1I. **b**: 17II+1III. **c**: 18II. **d**: 9II+1III

Table 2 Comparative performance of various alloplasmic lines of *B. juncea* in the genotypic background of a common euplasmic nuclear donor parent HD-1

CMS source	Origin	Organelle type	Male-sterility expression	Female fertility (%)	Yield (g/plant)	Oil (%)
tournefortii	Unknown	*B. tournefortii*	Rudimentary	73.6	2.9	35.4
trachystoma	Somatic	cp : *T. balli* mt : recombinant	Rudimentary	64.5	2.5	37.0
ogura (UR)[a]	Sexual	*R. sativus*	Rudimentary	49.2	1.7	27.5
ogura (R)[a]	Somatic	cp : *B. juncea* mt : recombinant	Rudimentary	89.0	5.8	37.8
oxyrrhina	Sexual	*B. oxyrrhina*	Sterile pollen	80.5	3.9	34.2
lyratus	Sexual	*E. lyratus*	Rudimentary	78.7	4.3	35.5
B. juncea cv HD-1	–	*B. juncea*	Fertile	100.0	8.5	38.6

[a]UR = unrefined; R = refined

typic backgrounds. Response to selection for improving female fertility in the *lyr* CMS lines having petaloid anthers was significantly lower than that observed for genotypes having rudimentary anthers. A comparison of agronomic attributes for *lyr* CMS with five other alloplasmic CMS sources, all in the nuclear background of *B. juncea* cv HD-1, is presented in Table 2. The euplasmic nuclear donor parent, HD-1, had normal male and female fertility, plant vigour and seed yield. In contrast, the female fertility in CMS (*lyr*) HD-1 was only 78.7% which was otherwise better than that of the *ogu* (UR), *tour* and *trachy* alloplasmic HD-1 lines. CMS (*lyr*) HD-1 was also higher yielding (4.3 g/plant) than *ogu* (UR), *tour*, *trachy* and *oxy* CMS lines, but it was much-lower yielding than the common euplasmic parent HD-1 (8.5 g/plant). For the percent oil content, the depression

Table 3 Comparison of plant leaf pigments in some *lyr* CMS *B. juncea* lines and corresponding euplasmic parents

Genotype	Chlorophyll a (mg/g)		Chlorophyll b (mg/g)		Carotenoids (mg/g)	
	Allo.	Eup.	Allo.	Eup.	Allo.	Eup.
HD-1	0.47	0.46	0.62	0.60	0.99	0.77
IJWHJ-001	0.88	0.96	0.50	0.77	0.79	0.53
USJ-15	0.29	0.27	0.39	0.39	0.79	0.49
USJ-64	0.16	0.43	0.19	0.44	0.53	0.99

caused by *lyr* cytoplasm was again lower than that observed for *tour*, *ogu* (UR) and *oxy* alloplasmics.

Chlorophyll analysis

Various leaf pigments associated with photosynthesis were compared in four *lyr* alloplasmics (BC_5) with corresponding euplasmic maintainer (B) lines (Table 3). The data revealed in general a slight and non-significant reduction in chlorophyll level following cytoplasmic substitution. The extent of decrease was, however, nuclear genotype dependent. For example, there was hardly any difference between alloplasmic and euplasmic lines for any of the chlorophyll pigments assayed in *B. juncea* strain HD-1, whereas the alloplasmic line of *B. juncea* cv USJ 64 had drastically reduced levels of the chl-a and chl-b pigments. Alloplasmic IJWHJ-001 possessed a near normal chl-a but a low chl-b content as compared to the euplasmic IJWHJ-001. For carotenoid pigments, the alloplasmic lines had higher values than the corresponding euplasmic parents with the sole exception of USJ 64. This, rather than any significant reduction in chlorophyll content, was considered to be the primary cause of partial leaf yellowing in the *lyr* alloplasmics. The leaf yellowing was persistent at both low and high temperatures, at pre- and post- anthesis.

Discussion

Brassica coenospecies constitute a rich source of variability for both nuclear and plasma genes. Many members of this group, having close homoeology with crop *Brassica*, offer excellent opportunities for developing alloplasmics (Prakash et al. 1998a). Disturbed nucleo-cytoplasmic harmony, as a consequence of substituting the *B. juncea* nucleus in *E. lyratus* cytoplasm, manifested a petaloid or rudimentary anther type of male sterility. Male-sterility expression was stable and appeared not to be influenced by photoperiod or temperature. Petaloid as well as the rudimentary anther type of male sterility has been previously documented in several alloplasmic lines such as (*Diplotaxis muralis*) – *B. rapa* (Hinata and Konno 1979); (*B. nigra*) – *B. oleracea* (Pearson 1972); (*R. sativus*) – *B. juncea* (Kirti et al. 1995) and (*E. canariense*) – *B. juncea* (Prakash et al. 2001). That the variations for anther morphotypes in alloplasmic CMS lines result mainly from nucleo–cytoplasmic interactions was supported by the observation that different *lyr* CMS lines, all carrying the same *E. lyratus* cytoplasm in varied nuclear backgrounds, differed for the prevalence of rudimentary/petaloid anthers and/or other floral deformities. Impaired female fertility associated with male-sterilizing cytoplasms has been reported in many alloplasmics (Prakash et al. 2001). It is mostly caused by abnormal bud development and split pistils with exposed or aborted ovules. Unlike some other alloplasmic *Brassica* CMS systems (e.g. *ogu*), partial leaf yellowing in (*lyr*) *B. juncea* was not due to low temperature chlorosis, as plants maintained their yellow hue throughout the crop season. The leaf yellowing was primarily due to higher carotenoid levels rather than a reduction in chlorophyll content and was also influenced by the nuclear genotype. This alloplasmic line may have value in human nutrition as carotenoids are vitamin-A precursors. Such morphological manifestations of nucleo-cytoplasmic incongruity are of common occurrence in several crops, including wheat (Mukai and Tsunewaki 1976) and *Brassica* (Bannerot et al. 1977; Prakash et al. 1998a).

As expected for a CMS of alloplasmic origin, the euplasmic *B. juncea* germplasm acted as a male-sterility maintainer. This is due to the fact that the fertility restoring gene(s) generally occurs at higher frequency in populations carrying the male sterilizing plasmotype, whereas recessive plasmon sensitive alleles are expected to occur with a high frequency in populations with a normal plasmotype. Efforts were, therefore, made to introgress the gene(s) for fertility restoration from sterilizing cytoplasm donor species, as has been done previously for several other alloplasmic male-sterile lines like *ogu* (Heyn 1976) and *can* (Prakash et al. 2001). Formation of a trivalent in (*lyr*) *B. juncea*, having a monosomic chromosome addition from *E. lyratus*, allowed limited exchange of genetic material across the genomes. This resulted in recovery, albeit at a low frequency, of male-fertile plants ($2n = 36$) in the BC_2 generation of backcrossing *B. juncea* with the *lyratus* monosomic addition line. It is likely that the introgression of the *Rf* gene(s) in *B. juncea* (AABB) from *E. lyratus* occurred following homoeologous pairing between the A and E genome chromosomes. This was supported by the meiotic analysis involving *lyr* monosomic addition in *B. rapa*, where as many as 80% of the PMCs revealed a trivalent (Deol et al. 2003). Observation regarding the homoeologous relationship between *Brassica* and *E. lyratus* genomes is consistent with the report of Gundimeda et al. (1992) who observed a mean bivalent frequency of 2.20 in their intergeneric hybrid between *E. lyratus* × *B. rapa*. Apparently not all bivalents in that cross could be

ascribed to allosyndetic pairing, as up to two auto pairs are expected in the *B. rapa* genome alone (Röbbelen 1960). Homoeologous recombinations or translocations are rare and random events which result in the variable size of alien introgression. That the size of introgressed segment, in the present context, is large was indicated by less than normal transmission frequency of the introgressed *lyr Rf* gene(s). Repeated selfing, selection and testcrossing putative fertility restorer plants as male with *lyr* CMS lines, is expected to improve the transmission frequency of the *Rf* gene(s) by reducing the size of alien chromosome introgression in the *B. juncea* genome. In the absence of any molecular data to show the introgression of chromosomal DNA from *E. lyratus*, male-fertility restoration observed in the crosses of CMS (*lyr*) *B. juncea* with fertile (*lyr*) *B. juncea* types was construed as evidence for the alien introgression of *Rf* genes in these derivatives, especially when viewed in light of the absence of *Rf* genes in native *B. juncea* germplasm. We are initiating experiments to develop molecular tags for the introgressed *Rf* gene(s) using RAPD and microsatellite markers. Towards this goal, we hope to be benefitted by our success (Janeja et al., unpublished) in identifying RAPD markers (UPZ 06_{1300} and OPK 15_{700}) that flank the introgressed *Rfl* gene for the *lyr* CMS system in *B. napus* (AACC). The availability of linked markers, besides demonstrating genetic introgression, will also permit the estimation of *E. lyratus* DNA that accompanied the *Rf* gene during the introgression process. Such markers can be used to limit the size of donor DNA by improving the efficiency of selection programmes aimed at enhancing the transmission frequency of the introgressed *Rf* gene(s).

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