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A. Pathania · S. R. Bhat · V. Dinesh Kumar · Ashutosh · P. B. Kirti · S. Prakash · V. L. Chopra

Cytoplasmic male sterility in alloplasmic *Brassica juncea* carrying *Diplotaxis catholica* cytoplasm: molecular characterization and genetics of fertility restoration

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Abstract The present study was aimed at characterizing cytoplasmic male sterility (CMS) and identifying the fertility restorer gene for CMS (*Diplotaxis catholica*) *Brassica juncea* derived through sexual hybridization. The fertility restorer gene was identified by crossing the CMS line with progeny plants derived from somatic hybrids of *B. juncea* and *D. cathoilca*. The CMS line is comparable to the nuclear donor *B. juncea* in all respects except for flower and silique characteristics. In CMS plants, the flowers have smaller nectaries, and anthers are converted into petals or tubular structures. Gynoecium exhibits a crooked style and trilocular ovary. Seed fertility was reduced in the CMS line. Genetic segregation data indicated that a single, dominant, nuclear gene governs fertility restoration. Restored plants showed a high female fertility and lacked gynoecium abnormalities. In fertility-restored plants, petal development was found to be variable; some flowers had the normal number of four petals, while others had zero to three petals. Interestingly, the trilocular character of the ovary was found to co-segregate with CMS and became bilocular upon male-fertility restoration. Thus, this trait appears to be affected by the interaction of nuclear and mitochondrial (mt) genomes. Restriction fragment length polymorphism analysis indicated that mt-genome of *D. catholica* is highly divergent from that of *B. juncea*. However, in Northern analysis, out of eight mt genes studied, an altered transcript pattern was recorded for only *atpA*. In fertility-restored plants, the *atpA* transcript became shorter, thereby showing its association with CMS.

Keywords *Brassica juncea* · Cytoplasmic male sterility · Fertility restoration · Silique · Transcript variation

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A. Pathania · S. R. Bhat (✉) · V. Dinesh Kumar · Ashutosh · P. B. Kirti · S. Prakash · V. L. Chopra
National Research Centre on Plant Biotechnology,
Indian Agricultural Research Institute, New Delhi 110012, India
e-mail: srbhat22@rediffmail.com
Fax: +91-11-25823984

Introduction

Cytopalsmic male sterility (CMS) is a maternally inherited trait whose determinants are located in the mitochondrial (mt) genome. In nature, CMS occurs infrequently through spontaneous mutations in the mt genome. More often, however, CMS is encountered in alloplasmics derived from interspecific or higher order hybrids (Hanson 1991). Nuclear genes capable of correcting this defect are called restorer genes. Such CMS and restorer stocks form the basis of hybrid cultivars in many crops. Molecular studies have revealed a variety of mechanisms for the expression of CMS. These include mt rearrangements, altered transcription patterns and changes in transcript processing (Brown 1999; Dill et al. 1997; Iwabuchi et al. 1993; Kempken and Pring 1999; Schnable and Wise 1998). Such changes have been proposed to produce a defective form of one or more essential mitochondrial proteins, thus causing male sterility. Fertility restorer genes act in an as yet unknown manner to bring about the production of functional protein products by altering the maturity or stability of the relevant RNA (Kennel and Pring 1989; Pruitt and Hanson 1991; Singh and Brown 1991) or protein (Abad et al. 1995; Bellaoui et al. 1999). Recently, Bentolila et al. (2002) cloned, for the first time, a nuclear gene that is directly involved in the control of the mt gene, *pcf*, associated with *Petunia* CMS. However, its exact mode of action remains to be elucidated.

In *Brassica*, a number of CMS lines of various origins have been reported. For example, *nap* (Shiga and Baba 1971) and *pol* (Fu 1981) CMS in *Brassica napus* are of spontaneous origin. Alien cytoplasms from male-sterile radish *ogura* (Ogura 1968), *B. oxyrrhina* (Prakash and Chopra 1990) *B. tournefortii* (Arumugam et al. 1996), *Erucastrum canariense* (Prakash et al. 2001), *Diplotaxis muralis* and *D. erucoides* (Malik et al. 1999) and *D. siifolia* (Rao et al. 1994) have been reported to induce male sterility in *B. napus* or *B. juncea*. Recombination between native and alien mt genomes in somatic hybrids also yields CMS (Arumugam et al. 1996; Kirti et al.

1995a; Liu et al. 1996). Whereas CMS lines can be relatively easily produced, finding restorer gene(s) has proved difficult. Restorer genes for alloplasmic CMS are expected to be available in the cytoplasm donor species. If the alien species is genetically highly divergent, the introgression of restorer gene(s) may prove difficult. Therefore, *Brassica* restorers are available for only a few alien cytoplasms such as *ogu* (Delourme et al. 1991) and *B. tournefortii* (Sodhi et al. 1994) in *B. napus*, and *Moricandia arvensis* (Prakash et al. 1998) *Trachystoma ballii* (Kirti et al. 1997) and *Erucastrum canariense* (Prakash et al. 2001) in *B. juncea*.

Even though several CMS systems are known in *Brassica*, molecular details underlying CMS expression are available for only a few. The *ogu* CMS has been shown to result from the expression of a novel *orf138* transcript in CMS *Brassica* (Grelon et al. 1994; Krishnasamy and Makaroff 1993). Upon restoration of fertility, ORF138 protein fails to accumulate in the anthers even though *orf138* transcript levels remain high (Bellaoui et al. 1999). Thus, restorer gene product appears to act post-transcriptionally. The *nap* and *pol* CMS systems have also been well-characterized, and two closely related *orfs, orf222* and *orf224*, are associated with CMS (Handa and Nakajima 1991; L'Homme et al. 1997; Singh and Brown 1991). The restoration of fertility in *pol* CMS is accompanied with the appearance of the monocistronic *atp6* transcript (Menassa et al. 1999). Similarly, in *B. tournefortii*-based CMS, a bi-cistronic transcript, *orf263*, involving the *atp6* gene has been implicated as a causal agent of male sterility (Landgren et al. 1996).

A CMS *B. juncea* line was developed by placing its nucleus in the cytoplasm of an alien wild species, *D. catholica*, through sexual hybridization followed by backcrossing (Mohanty 1996). We identified the restorer gene for this CMS in the progeny derived from the somatic hybrids of *D. catholica* + *B. juncea*. Here we report the morphology, genetics of fertility restoration and the molecular changes associated with the CMS. Further, we observed that the number of locules in the silique is also governed by nuclear cytoplasmic interaction and is linked to CMS.

Materials and methods

Plant material

The CMS line, derived by repeated backcrossing of the sexual alloploid *Diplotaxis catholica* × *Brassica juncea* with *B. juncea*, was kindly provided by Prof. K. Shivanna, University of Delhi, India. The CMS line was backcrossed to *B. juncea* cv. Pusa Bold for four generations. For identifying the restorer genes, the CMS line was allowed to open-pollinate with progenies derived from somatic hybrids of *D. catholica* + *B. juncea* (Mohapatra et al. 1998). The male-fertile plants identified in the progeny generation were selfed and crossed to the CMS line to study the genetics of fertility restoration. Pollen fertility was assessed based on staining with 1% acetocarmine and seed set upon selfing.

Molecular analysis

DNA isolation and Southern analysis

Mitochondrial DNA was isolated from flower buds, whereas total DNA was isolated from leaf tissue. Details of the protocols employed for DNA isolation, restriction, electrophoresis and blotting have been described earlier (Kirti et al. 1995b). Restriction fragment length polymorphism (RFLP) analysis was carried out to determine the differences in the organization of the mt genome of the CMS line and those of the normal *B. juncea* and *D. catholica*. Mitochondrial gene probes *atpA* (Braun and Levings 1985), *atp6* (Dewey et al. 1985a), *atp9* (Dewey et al.1985b), *coxI* (Issac et al. 1985), *coxII* (Fox and Leaver 1981), *coxIII* (Brennicke, personal communication), *nad/rps12* (C.A. Makaroff, personal communication), *cob* (Dawson et al. 1984) and *18SrRNA* (Gwynn et al. 1987) were used for Southern analysis. Genetic similarity between the parental mt genomes was calculated from the RFLP data using the Dice similarity index (Nei and Li 1979). For determining the chloroplast (cp) constitution, total DNA was digested with various restriction enzymes, and the blots were hybridized with *psbA* (Zurawaski et al. 1982) and *psbD* (Alt et al. 1984) gene probes.

RNA isolation and northern analysis

Mitochondria were isolated from young flower buds of the CMS line, fertility-restored plants, *B. juncea* and *D. catholica*, lysed in Trizol reagent and RNA isolated as per the manufacturer's protocol (Invitrogen, Carlsbad, Calif.). For Northern blot analyses, approximately 5 μg RNA was mixed with formamide, formaldehyde and MOPS buffer and heated to 65 °C for 15 min. The samples were then electophoresed on a 1.2% agarose-formaldehyde gel at a constant voltage (4 V/cm). Following electrophoresis, the RNA fragments were transferred to nylon membrane (Hybond N^+, Amersham, Piscataway, N.J.) and immobilized by UV crosslinking.

Probe preparation and hybridization

For preparing the probes, the plasmid containing the mt or cp gene was linearized with the appropriate restriction enzyme and radiolabeled using [^{32}P]-dCTP in a nick translation reaction following the manufacturer's specifications (Promega, Madison, Wis.). After the blots had been incubated for 2–4 h at 65 °C in prehybridization buffer (5 × SSPE, 5 × Denhardt's solution, 0.5% SDS and 0.25 mg sonicated salmon sperm DNA), the probe was added and hybridization allowed to continue for 14–16 h. Following hybridization, the filters were subjected to high stringency washes (2 × SSPE + 0.1% SDS for 15 min at room temperature; 1 × SSPE + 0.1% SDS, 55 °C, 10 min; 0.5 × SSPE + 0.1% SDS, 55 °C, 10 min; 0.1 × SSPE + 0.1% SDS, 55 °C, 10 min) and exposed to X-ray film in a suitable cassette with intensifying screen. The film was developed after an appropriate time to visualize the autoradiograms.

Results

Morphology

CMS plants are green and comparable to nuclear donor fertile plants of *B. juncea* with respect to growth and development. However, compared to wild-type *B. juncea*, the CMS and fertility-restored plants were about 10 days early in flowering. In addition, CMS plants continued to grow and produce flowers for a longer time and consequently, were taller than nuclear donor or fertility-restored plants (Table 1). The CMS line showed altered

Table 1 Comparative morphology of nuclear donor line *Brassica juncea* cv. Pusa Bold, CMS and fertility-restored plants

Trait	*B. juncea*	CMS	Fertility-restored plants
Plant height (cm)	184±8.3	223±10.6	196±7.6
Days to 50% flowering	60	48	48
Flower	Normal	Petaloid anthers	Normal to apetalous
Pollen fertility (%)	94–96	0	90–92
Locules/silique	Bilocular	Trilocular	Bilocular
Silique length (cm)	5.08±0.30	3.05±0.38	4.43±0.32
Seeds/silique	14.40±2.60	8.40±3.10	12.60±2.00
Seed fertility (%)	95.60±2.10	43.00±8.50	92.00±4.60

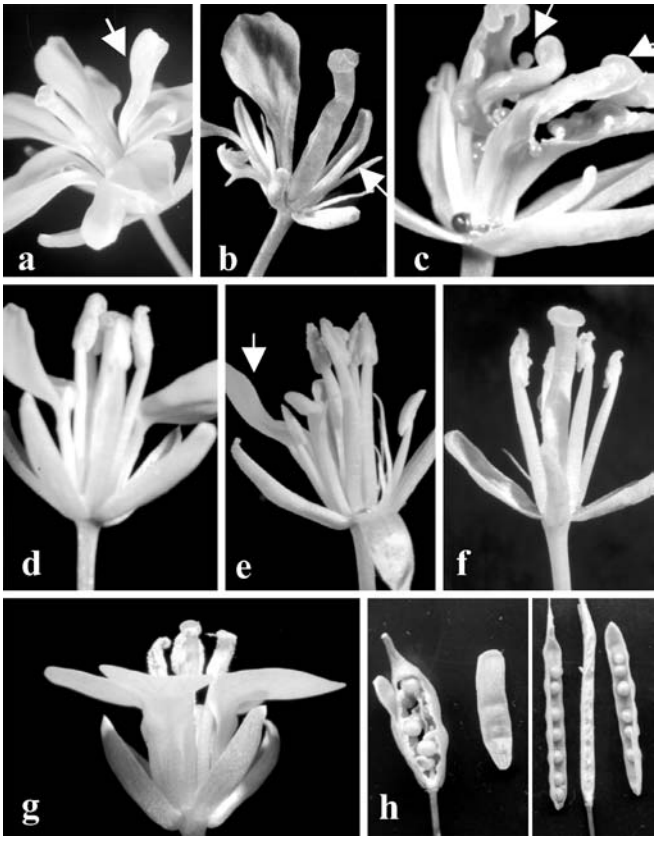

Fig. 1a–h Flower and silique morphology of CMS and fertility-restored plants of *Brassica juncea* with *Diplotaxis catholica* cytoplasm. **a–c** Flowers of the CMS line: **a** petaloid anthers, **b** anthers converted into tubular structures, **c** split ovary exposing ovules (changes indicated with *arrows*). **d–f** Flowers of fertility-restored plants: **d** normal petals, **e** single petal, **f** apetalous. **g** Flower of *B. juncea* with fertile cytoplasm. **h** Tri- and bi-locular silique of CMS (*left*) and fertility-restored (*right*) plants

floral morphology, especially with respect to the stamen and carpel (Fig. 1). In the majority of plants, stamens are converted into petals (Fig. 1a). In some, the stamens form tubular structures (Fig. 1b). Spherical ovule-like developments are also occasionally seen on modified stamens. Similarly, carpel deformities are common in CMS. The style is curved and the stigma is bi- or multi-lobed. In extreme phenotypes the ovary splits open and exposes the ovules (Fig. 1c). Nectary development is also adversely affected. The seed set upon open- or hand-pollination is low, indicating reduced female fertility (Table 1). The silique of the CMS line is trilocular and splits open along three distinct valves (Fig. 1h). In plants restored to male fertility, stamen development is normal, but petal development is variable (Fig. 1d–f). Some flowers are comparable to those of normal *B. juncea* (Fig. 1g), whereas in others zero to three petals are seen. Various floral phenotypes are found within a plant. Pollen fertility in restored plants is high (approx. 90%). No ovary abnormalities are observed in fertility-restored plants and seed set on selfing is comparable to that of normal *B. juncea* (Table 1). Further, fertile plants always produce a bilocular silique (Fig. 1h).

Identification of fertility restorer gene and genetics of restoration

The CMS trait was found to be stable under various nuclear backgrounds and no restorers could be found among the available accessions of *B. juncea*. Therefore, CMS plants were allowed to open pollinate in an isolated block with the BC_4–BC_5 progenies of the somatic hybrids *D. catholica* + *B. juncea*. Seeds collected from CMS plants were sown and examined at flowering for fertility restoration. Of the approximately 1,500 plants examined, three plants were fertile. These were selfed and crossed with the CMS line. The details of segregation in the progeny generations are presented in Table 2.

As expected, the CMS line gave only male-sterile progenies when crossed with the maintainer line *B. juncea* cv. Pusa Bold. When the three restored plants were used to pollinate CMS, a 1:1 segregation of male-sterile:male-fertile plants was observed in the progeny. In selfed progeny of restored plants, a 3:1 segregation of male-fertile:male-sterile was recorded (Table 2). Based on these results it was concluded that a single dominant gene controlled male-fertility restoration. Since the restored plants showed more than 90% pollen fertility, restoration was inferred to be sporophytic.

Molecular analyses

Southern analysis

RFLP analysis was carried out with mtDNA of CMS, *B. juncea* and *D. catholica* plants using nine mt-gene-specific probes. With the *atp6* probe, only a monomorphic

Table 2 Segregation for male sterility and fertility in progenies of different crosses of CMS (*Diplotaxis catholica*) *B. juncea*

Cross	Number of sterile plants	Number of fertile plants	$\chi 2^{a}$
CMS × *B. juncea* cv Pusa Bold	126	0	–
CMS × Restored 1	44	39	0.30 (1:1)[b]
CMS × Restored 2	58	63	0.21 (1:1)
CMS × Restored 3	32	28	0.26 (1:1)
Restored 1 self	97	31	0.04 (3:1)
Restored 2 self	39	10	0.55 (3:1)
Restored 3 self	73	19	0.93 (3:1)

[a] χ^2 values were non-significant indicating good fit to the indicated ratio
[b] Figures in parentheses indicate the segregation ratio for which χ^2 was calculated

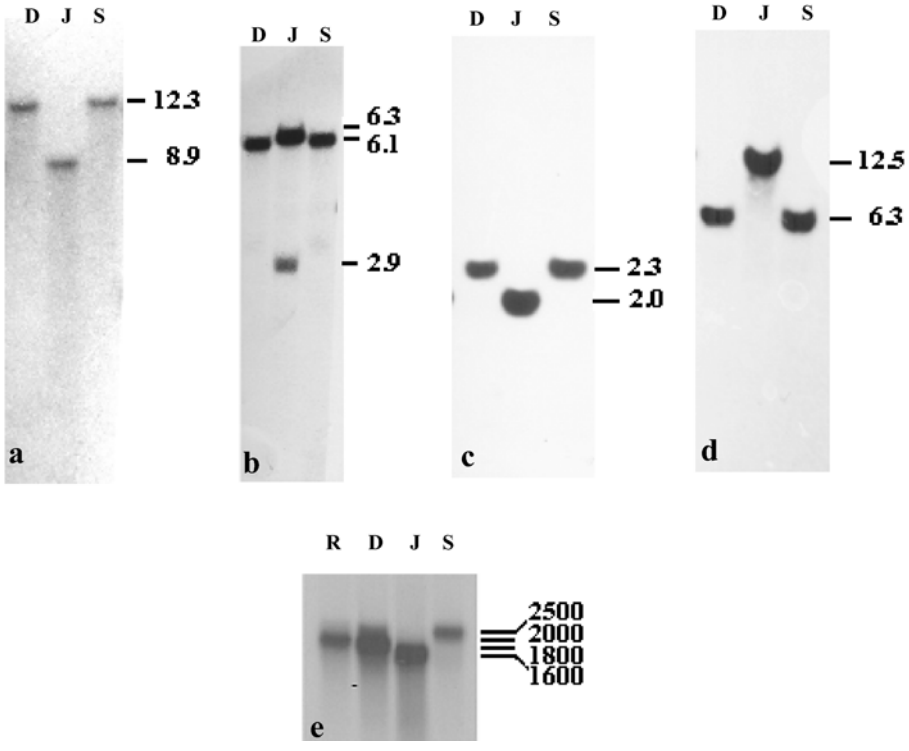

Fig. 2a–e RFLP pattern of mtDNA restricted with: **a** *Eco*RV and probed with *atpA*; **b** *Eco*RI and probed with *coxIII*. **c, d** Southern blot of genomic DNA restricted with: **c** *Eco*RI and probed with *psbA*; **d** *Bam*HI and probed with *psbD*. **e** Northern blot of mt-RNA showing variation in transcript size of *atpA* in different lines. *D Diplotaxis catholica*, *J Brassica juncea*, *S* CMS line, *R* fertility-restored plant. Size of fragments are indicated in kilobases for Southern blots and in nucleotides for the Northern blot

banding pattern was observed. Of 73 probe-restriction enzyme combinations tested, 56 showed polymorphic banding patterns between *D. catholica* and *B. juncea*. The CMS line displayed a banding pattern identical to that of *D. catholica*. For example, in mtDNA blots prepared from *Eco*RV-digested DNA, the probe *atpA* hybridized to a 12.3-kb fragment in *D. catholica* and the CMS line, whereas a 8.9-kb fragment was visualized in *B. juncea* (Fig. 2a). Similarly, with the *Eco*RI and *coxIII* restriction enzyme/probe combination, a single 6.3-kb band was observed in *D. catholica* and the CMS line, while two bands of 6.3 kb and 2.9 kb were recorded in *B. juncea* (Fig. 2b). When RFLP patterns of the parents obtained with the 73 probe-restriction enzyme combinations were compared, only 53 out of the total 246 bands were found to be common between *D. catholica* and *B. juncea*. Thus, the mt genomes of the two species seem to share about 43% similarity based on the Nei and Li (1979) formula. Chloroplast DNA organization was also found to differ between *D. catholica* and *B. juncea*. When total DNA was digested with *Eco*RI and probed with the cp-specific *psbA* gene, a single fragment of 2.3-kb hybridized to the probe in *D. catholica* and the CMS line. In *B. juncea*, a shorter, 2.0-kb fragment was found to hybridize with the probe (Fig. 2c). A polymorphic hybridization pattern was also recorded with the *psbD* and *Bam*HI probe/restriction enzyme combination (Fig. 2d). A single band of 6.3 kb was visualized in *D. catholica* and the CMS line, while *B. juncea* displayed a 12.5-kb band. Thus the CMS line was found to carry both mt and cp genomes derived from the maternal parent *D. catholica*.

Northern analysis

Since Southern analysis revealed a high degree of diversity between mt genomes of *B. juncea* and *D. catholica*, Northern analysis was carried out to identify the gene(s) associated with the CMS trait. Northern blots of mtRNA of *B. juncea*, *D. catholica* and the CMS line

were probed with eight mt-gene probes as stated earlier (except *cob*). CMS and parents displayed identical transcript patterns for seven of the eight mt genes tested. Only the *atpA* transcription pattern was found to differ among the three lines. The probe, *atpA*, hybridized to a single transcript of 1,600 nt in *B. juncea*, while 1,800-and 2,500-nt-long transcripts were visualized in the *D. catholica* and CMS lines, respectively (Fig. 2e). To verify the correlation of *atpA* with CMS, we prepared Northern blots with RNA of *B. juncea, D. catholica*, CMS and fertility-restored plants and hybridized these with the *atpA* probe. In fertility-restored plants, a change in *atpA* transcription pattern was observed: instead of the 2,500-nt-long transcript seen in the CMS line, a 2,000-nt-long transcript was recorded (Fig. 2e). This suggested that altered transcription or processing of *atpA* might be associated with the cause of CMS in this system.

Discussion

CMS is a popular pollination control system for the production of hybrid seeds and hence CMS is a much-sought trait by plant breeders. Furthermore, diversity among CMS sources is desirable to avoid disasters of the kind experienced with the extensive use of Texas CMS of maize which led to Southern corn blight epidemic (Ullstrup 1972). We have been engaged in developing CMS *B. juncea* systems and have produced CMS and restorer stocks involving cytoplasms of *Trachystoma ballii* (Kirti et al. 1997), *Moricandia arvensis* (Prakash et al. 1998) and *Erucastrum canariense* (Prakash et al. 2001). A study on species relationships among *Brassica* and its wild allies based on mtDNA RFLP patterns revealed that *D. catholica* is very distantly related to oilseed *Brassica* species (Pradhan et al. 1992). Therefore, we employed this cytoplasm for developing a CMS system.

The results of the present study showed that stable CMS is conferred by *D. catholica* cytoplasm. However, the CMS is of an extreme type and results in alteration of both the third and fourth whorls of flowers, silique characteristics and reduced female fertility. Similar phenotypes are also seen with *ogu* CMS in *B. napus* (Pellan-Delourme and Renard 1988). The CMS line was green despite the presence of the *D. catholica* cp genome. This is in contrast to other CMS systems based on *Brassica oxyrrhina* and *M. arvensis* cytoplasms where the presence of wild chloroplasts caused leaf chlorosis (Prakash and Chopra 1990; Prakash et al. 1998). The flower phenotype was variable within a plant, which may be related to differences in the expression of the concerned gene(s). It was interesting to note that in CMS, anthers were converted into petals, whereas in fertility-restored plants petal development was adversely affected.

Southern analysis confirmed the high degree of divergence between mt genomes of *B. juncea* and *D. catholica*. Polymorphic RFLP patterns were observed with eight of the nine probes tested. Organelle genomes of the CMS line were identical to those of the maternal parent, *D. catholica*. Since *D. catholica* and *B. juncea* mt genomes are highly divergent, CMS could arise due to altered transcription or post-transcriptional processing of otherwise functional mt genes under the influence of *B. juncea* nuclear genes. In radish, normal and *ogu* mt genomes were highly divergent, but transcript differences were detected for only three genes (Makaroff and Palmer 1988). In the present study, Northern analysis revealed the presence of a longer *atpA* transcript in the CMS line, This transcript was modified in fertility-restored plants, thereby opening the possibility that this gene could be involved in the CMS mechanism. In alloplasmic CMS *B. napus* with *B. tournefortii* cytoplasm, Landgren et al. (1996) found a longer *atp6* transcript, which contained a new *orf* downstream of the *atp6* gene that coded for a 29-kDa protein. Similar transcript variations in alloplasmic CMS lines have been recorded in rice (Iwabuchi et al. 1993), wheat (Ogihara et al. 1999) and tobacco (Bergman et al. 2000).

Because of wide differences between the mt genomes of *B. juncea* and *D. catholica*, we expected multiple incompatability between the nuclear and mt genomes in the CMS line leading to an altered transcription pattern for many mt genes. By the same logic, introgression of restorer genes was also thought to pose difficulties. Fortunately, we found that a single nuclear gene was able to restore full male fertility. The restorer got introgressed into the *B. juncea* genome during segregation of the somatic hybrids. In the sexual hybrid between *D. catholica* and *B. juncea*, 7 II and 1 III chromosome association were observed at meiosis (Mohanty 1996). Thus, introgression of the restorer gene may have been facilitated by the homoeology between *D. catholica* and *B. juncea* chromosomes. Although three independent male-fertile plants were identified, they showed an identical restoration pattern, suggesting a common origin. However, this must be confirmed by an allelism test. The restorer gene was found to have a pleiotropic effect; it not only conferred full male fertility but also improved female fertility and altered the locule number of the siliques. Pleiotropic effect of the restorer gene has been recorded in *Brassica* (Singh and Brown 1991) and maize (Wen and Chase 1999).

An important finding of this study concerns the control of locule number in *Brassica* siliques. Most *Brassica* species have bilocular siliques. In *B. rapa* where genotypes with tetralocular siliques are also found, the bilocular phenotype is reported to be dominant and controlled by a single nuclear gene. Mutants of *B. juncea* have indicated that the tri- or tetra-locular silique characteristic is recessive (Bhat et al. 2001) and is governed by two genes (Bhat unpublished). The CMS lines of *B. juncea* with *D. catholica* cytoplasm displayed trilocular siliques even when pollinated with *B. juncea* having a bilocular silique. All plants restored to fertility had bilocular siliques. Further, the trilocular trait was found to co-segregate with CMS. These results clearly

show that locule number is also governed by nucleo-cytoplasmic interactions. Besides CMS, only a few other phenotypes, notably, the non-chlorotic stripe mutation in maize (Newton and Coe 1986) and maternal distorted leaf mutation in *Arabidopsis* (Sakamoto et al. 1996) have been reported to be influenced by mt genes. Now we may add one more trait to this list.

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