Theor Appl Genet (1995) 91:517–521

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Transfer of *Ogu* cytoplasmic male sterility to *Brassica juncea* and improvement of the male sterile line through somatic cell fusion

Received: 27 May 1994 / Accepted: 29 December 1994

Abstract Male sterility conferred by *ogu* cytoplasm of *Raphanus sativus* has been transferred to *Brassica juncea* cv 'RLM 198' from male-sterile *B. napus* through repeated backcrossing and selection. The male-sterile *B. juncea* is, however, highly chlorotic and late. It has low female (seed) fertility and small contorted pods. To rectify these defects, protoplasts of the male sterile were fused with normal 'RLM 198' (green, self fertile). Four dark green, completely male-sterile plants were obtained and identified as putative cybrids. All the plants were backcrossed three times with 'RLM 198'. Mitochondrial and chloroplast DNA analysis of backcross progeny confirmed hybridity of the cytoplasm. The restriction pattern of the chloroplast DNA of progeny plants of three cybrids (Og 1, Og 2, Og 3) was similar to that of the green self-fertile 'RLM 198' and indicated that the correction of chlorosis resulted from chloroplast substitution. The chloroplast DNA of the lone progeny plant of the fourth cybrid (Og 10) could not be analyzed because the plant was stunted and had only a few leaves. When total cellular DNA was probed with mitochondrial probes *coxI* and *atpA* it was found that the cybrids had recombinant mitochondria. The chlorosis-corrected plants were early flowering and had vastly improved seed fertility.

Key words *Ogu* cytoplasmic male sterility · *Brassica juncea* · Chlorosis correction · Protoplast fusion · Mitochondrial recombination

Communicated by K. Tsunewaki

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Introduction

A spontaneously occurring stable male sterility-inducing cytoplasm for *Brassica* was discovered in a wild population of radish (*Raphanus sativus*) by Ogura (1968). Subsequently, this cytoplasm (commonly referred to as *ogu*) was introgressed into *B. oleracea* and *B. napus* (Bannerot et al. 1974). The expression of sterility was associated with a high degree of leaf chlorosis at low temperatures and the poor development of nectaries. These defects have been corrected in *B. napus* through cytoplasmic hybridization, and green male-sterile plants with well-developed nectaries have been obtained (Pelletier et al. 1983). An advantage of the *ogu* system of *B. napus* for developing hybrid cultivars is the availability of a monogenic restorer that does not impair female fertility (Renard et al. 1992). So far, this cytoplasm has not been transferred to *B. juncea*, an important oilseed crop of the Indian subcontinent, China and East Europe. We have transferred the sterility-inducing *ogu* cytoplasm to this species. As with other *ogu* alloplasmics, the plants were highly chlorotic and harbored several floral deformities (petaloid anthers, poorly developed nectaries, non-opening of the flower buds and low female fertility). We fused protoplasts of chlorotic male steriles with normal green plants of *B. juncea*, and subsequently isolated green male-sterile plants having normal inflorescences. The results of this work are reported in this paper.

Materials and methods

Transfer of *ogu* cytoplasm to *B. juncea*

Cytoplasmic male-sterile (CMS) (*ogu*) *B. napus* (AACC) was crossed with *B. juncea* cv 'RLM 198' (AABB). The interspecific F_1 hybrid (2n = 37, AABC) was intermediate in morphology and completely pollen-sterile. This hybrid was repeatedly backcrossed to *B. juncea*. In the BC_1 and BC_2 generations, selection was made for plants having *B. juncea* morphology. Male-sterile *B. juncea* plants could be isolated only in the BC_3 generation (Fig. 1).

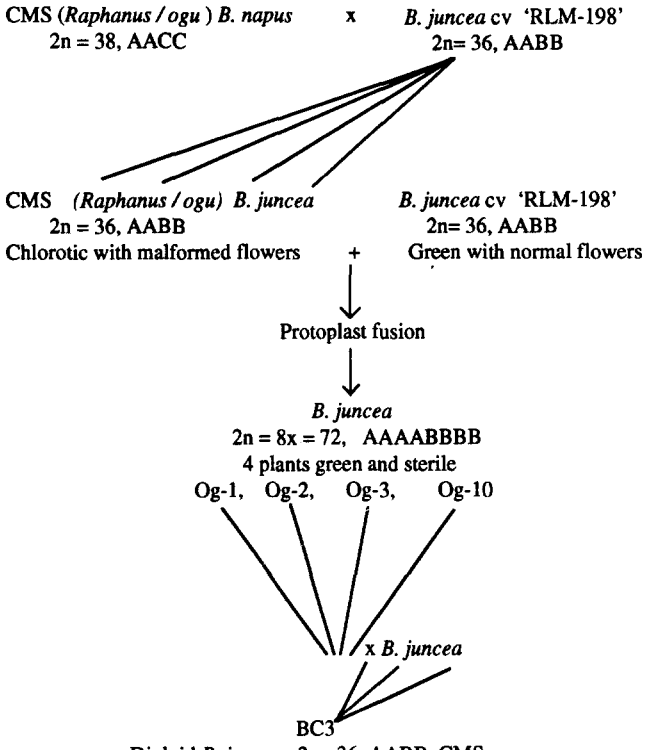


Fig. 1 Schematic representation of the methodology used for the transfer of *Raphanus ogu* CMS from *B. napus* to *B. juncea* and correction of chlorosis

Protoplast culture, fusion and regeneration of plants

Protocols employed for culture and plant regeneration from hypocotyl protoplasts of *B. juncea* were as per Kirti and Chopra (1990). Protoplasts were cultured in Kao's medium (Kao 1977) containing 1.0 mg/l 2, 4-dichlorophenoxy acetic acid (2, 4-D), 0.1 mg/l α-naphthalene acetic acid, 0.5 mg/l zeatin riboside and 7.2% glucose (pH 5.6) for 3 days in the dark and then transferred to light conditions (16-h light photoperiod). Dilutions of the protoplast cultures were carried out on the 7th, 10th and 13th day with Kao's medium containing 0.1 mg/l 2, 4-D, 1.0 mg/l 6-benzylamino purine (BAP) and 3.4% sucrose. Cell colonies were plated on the 15th day on MS medium containing 0.1 mg/l 2, 4-D, 1.0 mg/l BAP (pH 5.6) that was solidified with 0.5% agarose. Calli were regenerated on MS medium containing 0.1 mg/l indole-3-acetic acid, 2.0 mg/l BAP and 2.0 mg/l zeatin riboside (pH 5.6) that was solidified with 0.5% agarose.

Protoplast fusion was carried out essentially according to Kirti et al. (1992) using a modified fusion solution that consisted of 10% v/v dimethyl sulfoxide and 20% w/v polyethylene glycol (MW 8000). Fusion products were cultured at a density of 2×10^5 per milliliter.

Testing for chlorosis and male sterility

Rooted plants were screened for chlorosis development in a growth chamber at 8 °C under 16-h light / 8-h dark cycle. At this temperature plants with *ogu* chloroplasts exhibit a high degree of chlorosis and can be separated. Selected green plants were transferred to pots and grown in their natural environment. Male-sterile plants were identified from among them at flowering.

Chloroplast DNA analysis

Fresh leaves (25 g) were homogenized in 100 ml ice-cold isolation buffer consisting of 1.25 *M* NaCl, 50 m*M* Tris HCl (pH 8.0), 5 m*M* EDTA, 0.1% BSA and 0.1% βME. Sunsequent operations were all carried out at 4 °C. The suspension was filtered through two layers of cheese cloth and centrifuged at 1500 *g* for 10 min. The supernatant was poured off and the pellet was resuspended in 25 ml of isolation buffer using a clean artist's brush. The chloroplast suspension was centrifuged again at 1500 *g* and the supernatant was subsequently discarded. Chloroplasts were lysed in 100 m*M* Tris HCl (pH 8.0), 20 m*M* EDTA, 1.4 *M* NaCl, 2% CTAB and 2.0% βME for 1 h at 60 °C. Chloroplast DNA (cp DNA), was extracted with chloroform-isoamyl alcohol and precipitated with ice-cold isopropanol. It was then air-dried and dissolved in sterile water, treated with Dnase-free Rnase at 37 °C for 1 h, extracted with phenol-chloroform, precipitated with ethanol, air-dried once again, and finally dissolved in TE (10 m*M* Tris, pH 8.0, 1 m*M* EDTA). This purified cpDNA was restricted and run on 0.8% agarose gels.

Mitochondrial DNA (mt DNA) analysis

Samples of total cellular DNAs were prepared using the CTAB (cetyl trimethyl ammonium bromide) proceudre of Saghai-Marof et al. (1984) with modifications (Kirti et al. 1992). DNAs were purified by cesium chloride density gradient centrifugation. They were then restricted, run on 0.8% agarose gel, blotted onto Hybond$^+$ membranes and probed with mitochondrial probes *coxI* (Issac et al. 1985) and *atpA* of *Nicotiana plumbaginifolia* (Chaumont et al. 1988). Nick translations were carried out with the Dupont-NWN nick translation kit.

Results

CMS (*Raphanus/ogu*) *B. juncea*

Plants closely resembling normal, fertile *B. juncea* in general morphology had all features of the *ogu* CMS phenotype. They were late in flowering by 14–16 days, and their leaves were highly chlorotic. Many flower buds on the inflorescence did not open, and their stigmas protruded out. Those buds which did mature into flowers had petaloid anthers, a crooked style and an unusually broad stigma. Many of the pods were curved, and female fertility was low (59–74%).

Chlorosis correction

Two protoplast fusion experiments were carried out, and a total of 123 plants were regenerated. These plants were transferred to a growth chamber at 8 °C where 92 were identified as dark-green. At flowering, 4 plants, Og 1, 2, 3 and 10, were completely male-sterile and were identified as putative cytoplasmic hybrids. At metaphase 1 of meiosis they were found to be 2n = 72, confirming that they were fusion products. Meiosis was highly disturbed due to the frequent occurrence of quadrivalents, trivalents and univalents.

Morphology of chlorosis-corrected CMS (*Raphanus/ogu*) *B. juncea*

The 4 cytoplasmic hybrids Og 1, Og 2, Og 3 and Og 10 were backcrossed to 'RLM 198' three times. The BC_4

plants resembled normal fertile plants and were vigorous in growth, flowered earlier like normal 'RLM 198' plants and had completely green leaves. Anthers in the flowers were small and slender (in the progenies of Og 1, Og 3 and Og 10 plants) and contained sterile pollen grains. Anthers in the progeny of Og 2 were petaloid as in uncorrected CMS *ogu B. juncea* (Figs. 2 and 3). A desirable feature of the corrected male sterile was the well-developed nectaries as against the poorly developed nectaries of the original CMS flowers.

Meiosis was studied in 5 plants, each picked at random, of the Og 1, Og 3 and Og 10 populations. Of these 2, 4 and 2 plants, respectively, had *B. juncea*–like meiosis as revealed by the regular formation of 18 bivalents and subsequent 18:18 anaphase segregation. The other plants were hyperdiploids carrying extra chromosomes ranging in number from 2 to 6 thus having the chromosome constitution $2n = 36 + 2$ to $2n = 36 + 6$.

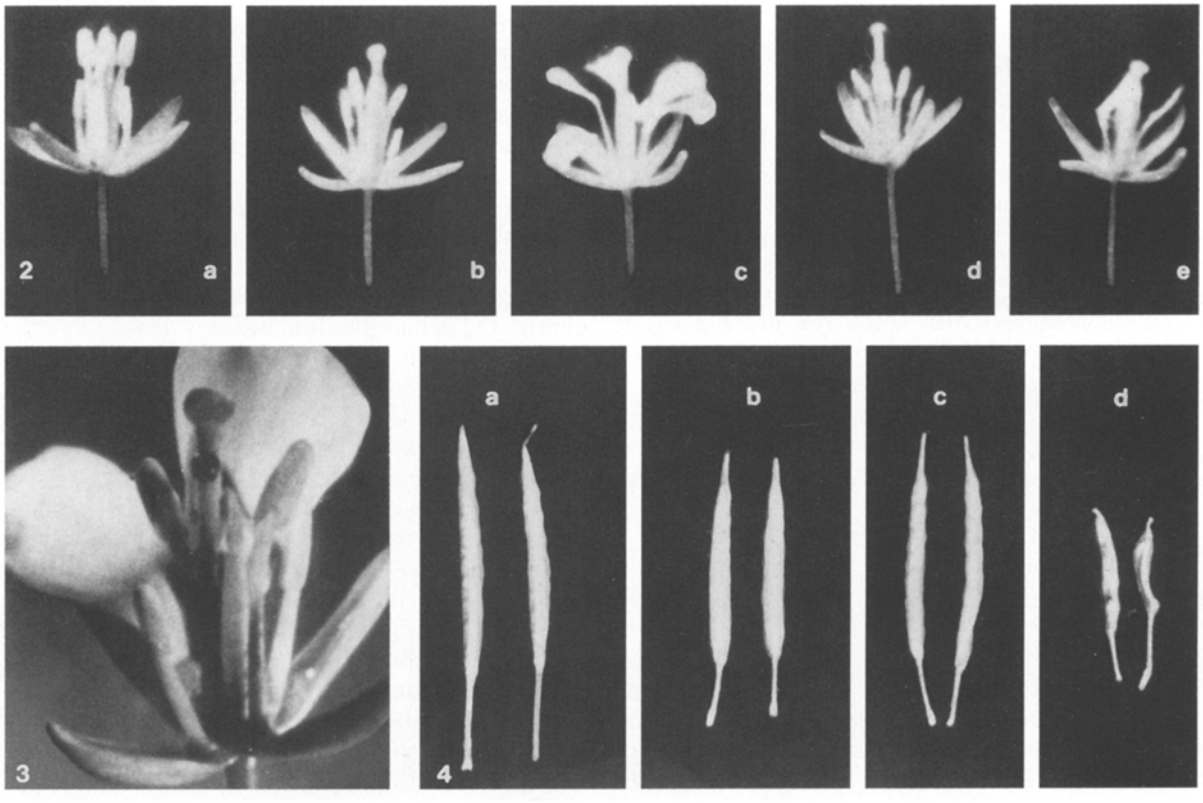
The chlorosis-corrected plants with $2n = 36$ chromosomes had remarkably improved female fertility (90%), and their pods were of a normal morphology (Fig. 4), though their size varied in different populations. The pods were long and slender, like those of normal 'RLM 198' plants in the progenies of Og 1, Og 3 and Og 10 while in the progeny of Og 2 they were small as in chlorotic *ogu B. juncea*. The chlorosis-corrected plants matured 15–20 days earlier than the chlorotic *ogu B. juncea*.

Molecular analysis of cytoplasmic hybrids

Chloroplast DNA isolated form cv 'RLM 198', carrying *ogu* cytoplasm and 3 cytoplasmic hybrids in BC_1 generations were restricted with *Hpa*II. The restriction patterns of *B. juncea* and *ogu* were different, and each was characterized by specific fragments. The cybrids (Og 1-1, Og 2-1 and Og 3-1 in the BC_1 generation) had all the specific fragments of *B. juncea* indicating that they contained *B. juncea* chloroplasts (Fig. 5). Chloroplast DNA from the Og 10 cybrid could not be analyzed.

When total cellular DNAs were digested with *Hin*dIII and probed with cytochrome oxidase subunit I (*coxI*, Issac et al. 1985) *B. juncea* was characterized by a 5.5-kb fragment, whereas *ogu* CMS had two specific 5.7-kb and 4.8-kb fragments. All 3 cybrids showed novel fragments hybridizing with the probe. In cybrid Og 1-1 and Og 3-1 *coxI* hybridized to a 5.0-kb fragment. Og 2-1 had two novel fragments of 5.0 and 4.4 kb that were distinct from the *ogu*-specific 4.8-kb fragment and *B. juncea*-specific 5.5-kb fragment (Fig. 6A). When probed with *atpA*, *B. juncea* was characterised by a 3.0-kb fragment and *ogu* CMS by a 3.2-kb fragment. Og 1-1 and Og 3-1 had 4.0-kb and 2.7-kb fragment, while

Figs. 2–4 Flowers (petals removed) of **a** *B. juncea* cv 'RLM 198', **b** Og 1-1, **c** Og 2-1, **d** Og 3-1, **e** *B. juncea* cv 'RLM 198' with *ogu* cytoplasm. **Fig. 3**. Close-up view of a flower of Og 1-1. **Fig. 4**. (a–d) Mature pods of **a** *B. juncea* cv 'RLM 198', **b** cybrid Og 1-1, **c** Og 3-1, **d** *B. juncea* cv 'RLM 198' with *ogu* cytoplasm



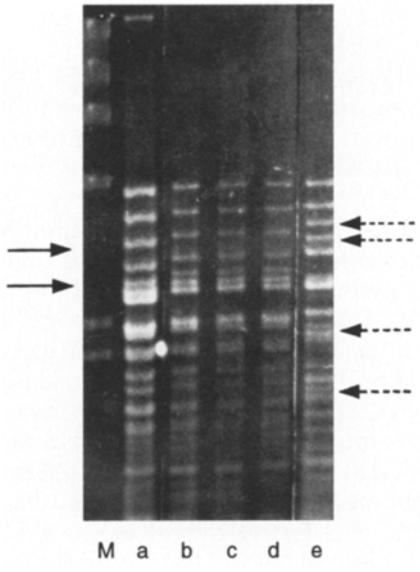


Fig. 5 Chloroplast DNAs restricted with *Hpa*II. *Arrows* indicate *B. juncea*-spcific cpDNA fragments, *broken arrows* indicate *ogu*-specific fragments. **a** *B. juncea* cv. 'RLM 198', **b** Og 1-1, **c** Og 2-1, **d** Og 3-1, **e** *B. juncea* cv RLM 198' with *ogu* cytoplasm, *M* marker (*Hin*dIII-digested λDNA). Fragments are 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0 kb

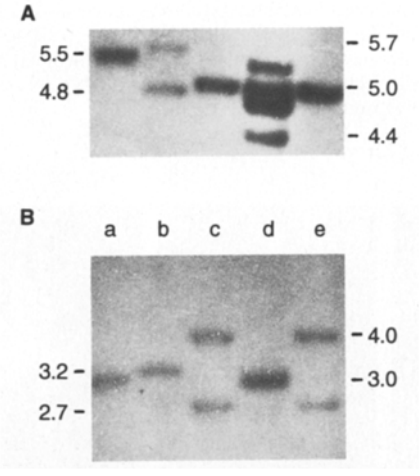


Fig. 6A,B Southern hybridization of total cellular DNA digested with *Hin*dIII and probed with *cox*I (**A**) and *atpA* (**B**). *a B. juncea* cv 'RLM 198', *b B. juncea* cv. 'RLM 198' with *ogu* cytoplasm, *c* Og 1-1, *d* Og 2-1, *e* Og 3-1

Og 2-1 had the *B. juncea*-specific 3.0-kb fragment (Fig. 6B).

Discussion

Leaf chlorosis, attributed to incompatibilities between alien chloroplasts and the nuclear genome, is of common occurrence in distant hybrids and has been documented for wheat (Mukai and Tsunewaki 1976) and *Brassica* spp. (Bannerot et al. 1974; Prakash and Chopra 1990). In *Brassica*, chlorosis associated with cytoplasmic male sterility has been observed when the *Raphanus*-derived, *ogu* cytoplasm is introduced into the species *B. campestris*, *B. napus* or *B. juncea*. Similar chlorosis and the associated male sterility is also caused by *B. oxyrrhina* cytoplasm in *B. campestris* and *B. juncea* (Prakash and Chopra 1990).

The correction of chlorosis in distant hybridization-derived male steriles has been achieved using two approaches: (1) the incorporation of nuclear genes that nullify the effect of the incompatible chloroplasts, and (2) substitution of the wild species chloroplasts with those of the agronomic cultivar. The transfer of nuclear genes from *Raphanus* to CMS *B. napus* for converting chlorotic male-sterile plants to normal green ones by Paulman and Robbelen (1988) serves as a good example of the first approach. As for the second approach, Pelletier et al. (1983) corrected the leaf chlorosis of *B. napus* resulting from *Raphanus/ogu* chloroplasts by producing a cybrid through somatic hybridization and selecting a green CMS in which *Raphanus* chloroplasts had been replaced by chloroplasts of of *B. napus*. Similar results by means of the protoplast fusion technique have been achieved by Jarl and Bornman (1988) in *B. napus*.

Concomitant with the correction of chlorosis of the CMS by substitution of *B. juncea* chloroplasts for those of *ogu*, the cybrid material displayed two advantageous agronomic features: (1) enhanced vigor of the corrected product as exhibited in such parameters as higher biomass and earliness in flowering, which can be ascribed to a greater photosynthetic efficiency imparted by the deep green color of the plants, and (2) a vastly improved female fertility and desirable pod characteristics due to removal of defects in floral morphology. The mitochondria of the chlorosis-corrected CMS lines appeared to be of recombinant origin. The appearance of novel fragemnts hybridizing to probes *coxI* and *atpA* points to intergenomic recombination between the mitochondria of *ogu* and normal *B. juncea*. Recombinant mitochondria in cytoplasmic hybrids in the present study may account for the improved floral morphology and nectary development. Of the 4 cybrids, 3 possessed normal-looking anthers but contained sterile pollen. Only 1 cybrid, Og 2, had petaloid anthers. Belliard et al. (1979) were of the view that flower morphology and male sterility in tobacco must be controlled by nucleo-mitochondrial interactions. For a workable CMS system, a functional fertility-restoring system is of paramount importance for hybrid seed production. Since the mtDNA of these cybrids has been altered, there may be a relatively easy route for constructing restorer systems. Pelletier et al. (1988) gave evidence for easier fertility restoration of the CMS trait that is associated with mtDNA recombination. Other studies have shown that the *ogu* mitochondrial genome undergoes rearrangements in parasexual hybridization (Vedel et al. 1986; Menczel et al. 1987; Morgan and Maliga 1987; Robertson et al. 1987; Jourdan et al. 1989a). On the other hand, Kemble et al. (1988) and Jourdan et al. (1989b) observed

no such rearrangements in parasexual hybrids involving *ogu* cytoplasm. However, both altered and unaltered *ogu* mitochondria in the two sets of fusion experiments involving *B. oleracea*, *B. napus* and *ogu* CMS were observed by Kao et al. (1992). Yarrow et al. (1990) reported an improvement of polima CMS system in *B. napus* through somatic cell fusion.

The present investigation is the first successful attempt in transferring *ogu* cytoplasm to *B. juncea* followed by corrections of a high degree of leaf chlorosis and other disadvantages associated with the *ogu* CMS system. Since 'RLM 198' the chloroplast donor, is fully self-fertile, the cytoplasmic male sterility of corrected *ogu* lines can be ascribed to nuclear mitochondrial interactions. Presently, attempts aimed at transferring this cytoplasm to Indian *B. napus* lines and constructing fertility restorer systems for them are underway. Studies on mitochondrial recombination are also being extended.

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