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Correction of chlorophyll deficiency in alloplasmic male sterile *Brassica juncea* through recombination between chloroplast genomes

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Summary

Brassica juncea cv. Pusa Bold carrying *B. oxyrrhina* cytoplasm (*oxy* cytoplasm) was male sterile and chlorotic under field conditions at low temperature (Prakash & Chopra, 1990). Leaf protoplasts of the chlorotic male sterile alloplasmic line (2n = 36) were fused with hypocotyl protoplasts of green male fertile, *B. juncea* cv. RLM-198 (2n = 36) using polyethylene glycol. Of the 1043 plants regenerated from 10 fusion experiments, 123 had 'gigas' features and were identified as presumptive fusion products. Among field-grown population, one plant was dark green even at low temperatures and male sterile. It possessed 72 chromosomes which formed 36 bivalents at late diakinesis of meiosis-I. This plant was back-crossed to *B. juncea* cv. Pusa Bold (the maintainer line) for three successive generations. One male sterile, normal green BC_3 progeny plant with 2n = 36 was analyzed for organelle constitution. Probing its total DNA with the mitochondrial gene for cytochrome oxidase subunit I revealed that it possessed mitochondria of *B. oxyrrhina*. Southern hybridization pattern with the gene for ribulose bisphosphate carboxylase oxygenase-large subunit (rbcL) revealed that the chloroplast genome of the chlorophyll deficiency-corrected plant had characteristics of both *B. juncea* and *B. oxyrrhina*. The deficiency correction has been attributed to recombination between chloroplast genomes of the two species.

1. Introduction

Protoplast fusions allow gene transfer across sexual incompatibility barriers and realization of novel cytoplasmic hybrids for the exploitation of cytoplasmically inherited traits. In Cruciferae, transfer of nuclear genes encoding resistance to *Phoma lingam* (Sjödin & Glimelius, 1989) and antibiotic hygromycin (Sacristan *et al.* 1989) from *B. nigra* to *B. napus* has been reported. Pelletier *et al.* (1983) employed somatic cell fusion for manipulating cytoplasm-controlled traits. They developed a combination of pollen sterility inducing mitochondria of *Raphanus sativus*, herbicide-resistant chloroplasts of *B. campestris* and nuclear genome of *B. napus*. Menczel *et al.* (1987) and Jarl *et al.* (1988, 1989) corrected the chlorophyll deficiency of the alloplasmic male sterile lines of *B. napus* by replacing the chloroplasts of the alien species with chloroplasts of the cultivated species.

Diversity of male sterility systems is essential for sustained commercial exploitation of heterosis. Prakash & Chopra (1990) developed alloplasmic male steriles combining the nuclear genome of *B. juncea* with *oxy* cytoplasm. These male steriles were chlorotic at low temperature. Correction of chlorophyll deficiency through protoplast fusion and generation of a green male sterile line of *Brassica juncea* through chloroplast recombination are reported in this communication.

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2. Material and methods

Brassica juncea (L.) Czern & Coss cv. RLM-198, having normal cytoplasm and green leaves, was somatically hybridized with the alloplasmic chlorotic male sterile *B. juncea* carrying *oxy* cytoplasm. Hypocotyl protoplasts of the normal green line were isolated from 6- 7-day-old etiolated seedlings grown aseptically on half-strength MS agar medium containing 1% sucrose. Young expanded leaves of 3- to 4-week-old *in vitro*-grown plantlets were the source for protoplasts of the male sterile line. Protoplast isolation, culture, fusion and plantlet regeneration were carried out following the protocols described previously (Kirti & Chopra, 1990; Kirti *et al.* 1991).

For characterizing cytoplasmic organelles, total DNA was isolated following the method of Saghai–Maroof *et al.* (1984), purified on cesium chloride

density gradient, digested with restriction enzymes, *Hind* III, *Eco*R V, *Eco*R I (Bangalore Genei, India) and *Hpa* II (Promega), electrophoresed on 0·8% agarose gel and Southern-blotted onto Gene Screen Plus^{R} nylon membrane (DuPont) using a LKB vacuum blotting unit. All the digestions were made with the same batch of DNAs from the parents and the cybrid plants. The following ^{32}P-labelled probes were used in 'Southern' hybridization: (1) chloroplast gene coding for the large subunit of ribulose bisphosphate carboxylase-oxygenase (rbcL) (Gatenby *et al.* 1981); (2) mitochondrial gene coding for cytochrome oxidase subunit I (Cox I) (Isaac *et al.* 1985). For studying meiosis, buds were fixed in Carnoy's solution and anthers were smeared in 2% acetocarmine.

3. Results and discussion

The fusion products were identified, 24 h after plating, on the basis of morphological characteristics of protoplasts derived from leaves and hypocotyls. Within this period, leaf protoplasts collapsed leaving only hypotocyl-derived and hybrid protoplasts to develop further. The heterokaryotic fusion frequency, estimated on the basis of differential morphology of parental protoplasts, was about 10%.

From 10 fusion experiments, 1043 plantlets were regenerated from about 20000 calli. On the basis of 'gigas' features, typical of polyploids, 123 plants were identified as possible hybrids and were transferred to pots. These plants could be classified at flowering into three groups (i) green male fertile, (ii) chlorotic male sterile and (iii) green male sterile (Table 1). No chlorotic fertile plant was observed among these selected plants. Since our interest was in the correction of chlorosis, the single green male sterile plant was analyzed further. Cytological observations at diakinesis and metaphase I in pollen mother cells of this plant recorded 72 chromosomes. On pollination with cv. Pusa Bold for three successive generations male steriles with normal green leaves were obtained. These BC_3 plants had floral morphology (Table 2) similar to that of the chlorotic male sterile line of Prakash & Chopra (1990) except for the chlorosis-corrected character of the leaves. On random analysis, they were found to possess 36 chromosomes forming 18 bivalents at metaphase I. Meiosis proceeded normally but pollen aborted after tetrad formation. The transmission of male sterility without chlorosis, normal meiotic pairing resulting in 18 bivalents and subsequent normal chromosome disjunction suggested that chlorophyll deficiency has been corrected by alteration(s) in cytoplasmic organelle genome composition.

Table 1. *Phenotypic classification of hybrid plants based on 'gigas' features*

Total number of plants with 'gigas' features	123
(i) Green fertile	68
(ii) Green male sterile	1
(iii) Chlorotic male sterile	54

Using the mitochondrial gene Cox I and chloroplast encoded rbcL as probes the 'Southern' hybridization patterns of cytoplasmic organelle genomes of *B. juncea* cv. RLM-198, *B. juncea* with *oxy* cytoplasm and the selected BC_3 plant (CY-2) were studied. The Cox I probe hybridized with a 5·5 kb *Hind* III fragment in *B. juncea* cv. RLM-198 and with a 4·5 kb *Hind* III fragment in both *B. juncea* with *oxy* cytoplasm and the selected BC_3 progeny plant CY-2 (Fig. 1). Similarly, Cox I hybridized with a 7·0 kb *Eco*R V fragment in *B. juncea* cv. RLM-198 and a 23·0 kb *Eco*R V fragment in both *B. juncea* with *oxy* cytoplasm and the selected progeny CY-2 (Fig. 2). These observations indicate that CY-2 retained mitochondrial genome of *B. oxyrrhina* essential for male sterility.

Hybridization of the chloroplast gene probe rbcL with *Eco*R V fragments showed the probe binding to a 3·4 kb fragment in *B. juncea*, and a 1·6 kb fragment in *B. juncea* with *oxy* cytoplasm. The cybrid CY-2 and its progeny plant CY-2-1 had the characteristic *B. juncea* 3·4 kb fragment (Fig. 4). When *Eco*R I fragments were analyzed with the rbcL probe, *B. juncea* was characterized by a 7 kb fragment, whereas *B. juncea* with *oxy* cytoplasm had the characteristic 23 kb fragment. The progeny plant CY-2 had the 23 kb fragment of *B. juncea* with *oxy* cytoplasm (Fig. 3). When *Hind* III restricted DNAs were hybridized with rbcL, the probe bound to a specific 3·8 kb fragment of *B. juncea* and 3·5 and 1·3 kb fragments of

Table 2. *Morphological characteristics of cv. RLM-198, cybrid CY-2 and the alloplasmic male sterile*

	RLM-198	Cybrid CY-2	Pusa Bold with *oxy* cytoplasm
Leaves under low temperature	Dark green	Dark green	Chlorotic
Nectaries	Well developed	Well developed	Well developed
Flowers	With well-developed anthers	Reduced anther size	Reduced anther size
Pollen	Fertile, viable	Totally sterile	Totally sterile
Anther dehiscence	Normal dehiscence	No dehiscence	No dehiscence

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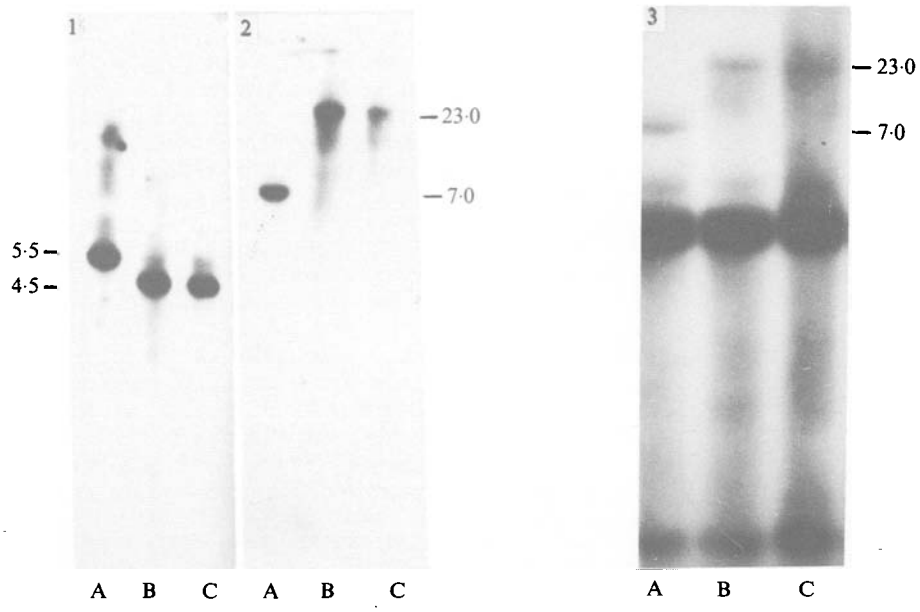

Figs 1–3. Total DNAs of *B. juncea* cv. RLM 198 (A), cybrid plant CY-2 (B) and *B. juncea* cv. Pusa Bold carrying *oxyrrhina* cytoplasm (C) were digested with different restriction enzymes and hybridized with nick-translated and ^{32}P-labelled probes for the mitochondrial gene cytochrome oxidase subunit I and chloroplast encoded gene for large subunit of ribulose bisphosphate carboxylase. Fig. 1. *Hin*d III-digested DNA probed with Cox I. Fig. 2. *Eco*R V restricted DNA probed with Cox I. Fig. 3. *Eco*R I restricted plant DNAs probed by 'Southern' hybridization with rbcL gene.

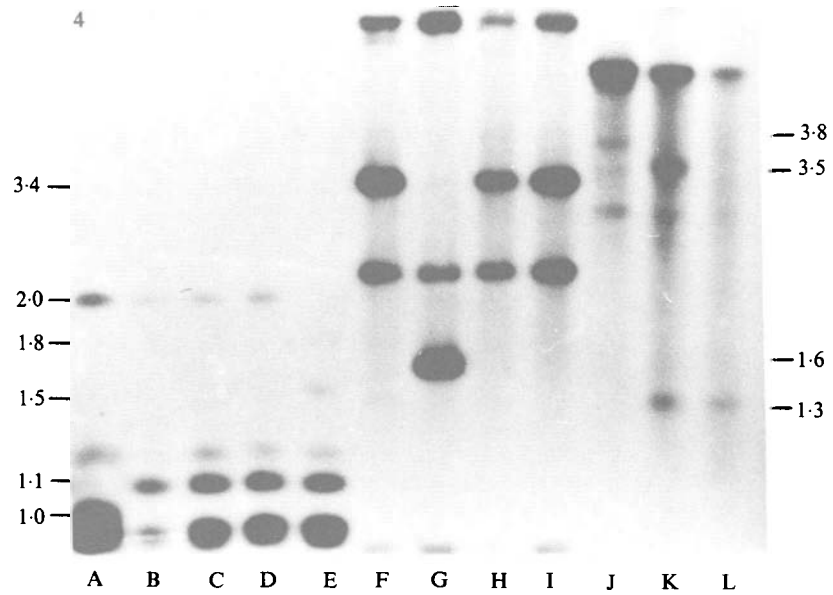

Fig. 4. Total DNAs of *B. juncea* with *oxyrrhina* cytoplasm (A), cybrids CY-2 (B), CY-2-1 (C), CY-2-2 (D) and *B. juncea* cv. RLM-198 (E), restricted with *Hpa* II; *B. juncea* cv. RLM-198 (F), *B. juncea* with *oxy* cytoplasm (G), CY-2 (H), and CY-2-1 (I), digested with *Eco*R V; *B. juncea* cv. RLM-198 (J), *B. juncea* with *oxy* cytoplasm (K) and CY-2 (L) restricted with *Hin*d III and probed with rbcL.

B. juncea with *oxy* cytoplasm. In the cybrid CY-2, the probe hybridized with the 1·3 kb fragment characteristic of the *oxy* cytoplasm, but the 3·5 kb fragment was missing (Fig. 4). Further analysis of *Hpa* II restriction fragments revealed that in *B. juncea* this probe specifically bound to the 1·8, 1·5, and 1·1 kb fragments whereas in the case of *B. juncea* with *oxy* cytoplasm the probe bound to 2·0 and 1·0 kb fragments. The cybrid CY-2, and its progeny plants CY-2-1 and CY-2-2 had the 1·1 kb fragment of *B. juncea* and the 2·0 kb fragment of *B. oxyrrhina*. The 1·8 and 1·5 kb fragments of *B. juncea* and the 1·0 kb fragment of *B. juncea* with *oxy* cytoplasm were missing in the cybrid plants (Fig. 4). All these observations along with this novel hybridization pattern in the cybrid suggested the occurrence of recombination

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between the chloroplasts of *B. juncea* and *B. oxyrrhina*. The correction of chlorophyll deficiency in the selected BC_3 progeny plant is probably due to chloroplast recombination rather than to simple chloroplast substitution.

DNA recombination in chloroplasts is a rare event (Fejes *et al.* 1990). Evidence for chloroplast DNA recombination was obtained in somatic hybrids of *Nicotiana tabacum* + *N. plumbaginifolia* and in *N. tabacum* + *Solanum tuberosum* (Medgyesy *et al.* 1985; Thanh & Medgyesy, 1989). Kemble *et al.* (1988) reported the absence of chloroplast DNA rearrangements in protoplast fusion products of *B. napus*. Specific selection for chlorosis correction (green colour) may have aided our detection of this rare recombination event. Chlorophyll deficiency in alloplasmic combination is generally a phenotypic manifestation of genome–plastome incompatibility. Formation of the recombined plastome in the present investigation corrected the chlorosis of the cybrid. The uniformity of normal chlorophyll development in progeny plants of the cybrid indicates the stability of the recombinant plastome over generations. Organelle recombination by protoplast fusion, as demonstrated in the present study, is a potential means of introducing new gene combinations in higher plants and for overcoming limitations of uniparental inheritance of organelles.

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