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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

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 F₁ 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. (Edwardsen 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 were grown on MS plate supplemented with 0.1 M sucrose, 0.25% agarose, hormones as in PC1 medium and 0.1M sucrose, 0.25% agarose, 20mg/l hygromycin and 10mg/l phosphinothricin for the selection of hybrids. For each fusion experiment the following were kept as controls: (1) AABB Hm− protoplasts on 10mg/l phosphinothricin; (2) CC Ppt+ protoplasts on 20mg/l hygromycin; (3) AABB Hm− on hygromycin; (4) CC Ppt+ on phosphinothricin 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 20µM silver nitrate along with hormones and selection agents as in the SL1 medium). Shoots regenerated on MS supplemented with BAP (1.0mg/l) and 2,4-D (0.05mg/l) and MS with BAP (1.0mg/l) and NAA (1.0mg/l) in the presence of 20µM AgNO3. Colonies obtained on MS with BAP (1.0mg/l) and 2,4-D (1.0mg/l) were induced to regenerate by transfer to MS with BAP (1.0mg/l) and NAA (1.0mg/l).

The regenerated shoots were transferred to SM medium for further growth and rooted on RI medium containing 20mg/l hygromycin and 10mg/l phosphinothricin 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 EcoRI and hybridized to two heterologous probes of chloroplast origin, namely *rbcL* and *psbD*.

For mitochondrial genome analysis, total DNAs were digested with EcoRI and HindIII and hybridized to 11 mitochondrial gene probes, *atpA*, *atp6*, *atp9*, *cox1*, *coxII*, *coxIII*, *cob*, *rrn5-18*, *rrn26*, *nad3* and *nad4*, and eight overlapping cosmid clones of the B. *oxyrhina* mtDNA library covering about 190kb 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. oxyrhina* mtDNA cosmids clones were generated in our laboratory.

**Results**

**Protoplast isolation, fusion and regeneration of somatic hybrids**

The shoots of Hm− CMS AABB and Ppt+ normal CC were grown on RI and RI 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 (1.0mg/l) and 2,4-D (0.05mg/l). Protoplasts were plated at a density of 4 x 10⁴/ml in liquid PC1 medium (Mukhopadhyay et al. 1991) supplemented with either BAP (1.0mg/l) and 2,4-D (0.05mg/l), BAP (1.0mg/l) and 2,4-D (1.0mg/l) or BAP (1.0mg/l) and NAA (1.0mg/l). After 10 days of culture the media were diluted with PC2 medium (PC2 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 SL1 medium [K₁ medium (Nagy and Maliga 1976) supplemented with hormones as in PC1 medium and 0.1 M sucrose, 0.25% agarose, 20mg/l hygromycin and 10mg/l phosphinothricin] for the selection of hybrids. For each fusion experiment the following were kept as controls: (1) AABB Hm− protoplasts on 10mg/l phosphinothricin; (2) CC Ppt+ protoplasts on 20mg/l hygromycin; (3) AABB Hm− on hygromycin; (4) CC Ppt+ on phosphinothricin 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 20µM silver nitrate along with hormones and selection agents as in the SL1 medium). Shoots regenerated on MS supplemented with BAP (1.0mg/l) and 2,4-D (0.05mg/l) and MS with BAP (1.0mg/l) and NAA (1.0mg/l) in the presence of 20µM AgNO3. Colonies obtained on MS with BAP (1.0mg/l) and 2,4-D (1.0mg/l) were induced to regenerate by transfer to MS with BAP (1.0mg/l) and NAA (1.0mg/l).

The regenerated shoots were transferred to SM medium for further growth and rooted on RI medium containing 20mg/l hygromycin and 10mg/l phosphinothricin 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.

**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