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Resynthesis of *Brassica carinata* by protoplast fusion and recovery of a novel cytoplasmic hybrid

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Abstract

Brassica carinata (2n=34, BBCC), was synthesized by fusing dark grown etiolated hypocotyl protoplasts of *B. nigra* (2n=16, BB) with green mesophyll protoplasts of *B. oleracea* (2n=18,CC) using polyethylene glycol. Heterokaryons could be microscopically distinguished from the parental types by their dark green chloroplasts in the colourless hypocotyl protoplast background. The mean heterokaryotic fusion frequency estimated on the basis of this morphological distinction was about 16%. A total of 626 calli were obtained, of which 92 regenerated shoots after transfer to zeatin (2 mg/l) supplemented MS medium. Of these, 81 calli differentiated into plants morphologically similar to naturally occurring *B. carinata* and 11 calli yielded plants resembling parental types. Meiosis in seven hybrid plants showed the chromosome number to be 2n=34 - the sum of *B. nigra* and *B. oleracea* chromosomes. Molecular confirmation of the amphidiploid nature of hybrids was obtained by probing with a *B. juncea* derived genomic clone. The use of chloroplast and mitochondrial specific gene probes, revealed that one plant was a cytoplasmic hybrid having cp DNA sequences of both *B. oleracea* and *B. nigra* and mt DNA sequences of *B. nigra*.

Key words : *Brassica nigra* - *B. oleracea* - *B. carinata* - Protoplast fusion - Cytoplasmic hybrid

Abbreviations : PEG, Polyethylene glycol; 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, α Naphthaleneacetic acid; IBA, Indole-3-butyric acid; BAP, 6-Benzylaminopurine; MS, Murashige and Skoog (1962)

Introduction

Brassica carinata, an alloploid of *B. nigra* and *B. oleracea*, is cultivated for oil and fodder in Ethiopia and Kenya, where it occurs naturally. This species is reported to possess high degree of resistance to the fungal disease white rust caused by *Albugo candida*, and grows exceedingly well under moisture stress conditions (Malik, 1990). Because of these desirable attributes, there is considerable interest to breed cultivars of *B. carinata*. However, the restricted amount of genetic variability in natural *B. carinata* accessions is a constraint to breeding programmes (Song *et al* 1988, Prakash and Chopra 1991). *B. carinata* has been synthesized by sexual crossing to establish its evolutionary route (Frandsen 1947, Mizushima 1950) and for use as a bridging species for developing alloplasmic male sterile *B. oleracea* (Pearson 1972). Based on rDNA analysis, Quiros *et al* (1985) suggested that *B. carinata* is an amphidiploid of recent origin. Resynthesis of *B. carinata* through somatic cell hybridization allows the exploitation of the vast variability available in its constituent parents thus enlarging the genetic base of the species besides generating novel synthetic forms that recombine cytoplasmic organelles. It is interesting that all natural *B. carinata* accessions derive their cytoplasm from *B. nigra* as has been revealed by comparative study of chloroplast DNA restriction patterns of *B. carinata* and its diploid parents (Erickson *et al* 1983, Palmer *et al* 1983, Song *et al* 1988).

We report here the synthesis of *B. carinata* through protoplast fusion and morphological, cytological and molecular characterization of the synthesized products.

Materials and Methods

Protoplast isolation

The two parents used in protoplast fusion are i) *B. oleracea* var. *italica*, line **207**, (2n=18, CC) maintained at the Division of Horticulture, Indian Agricultural Research Institute and ii) *B. nigra* cv. **IC 257** (2n=16, BB), a tall type with a height exceeding 2 meters. Hypocotyl protoplasts of *B. nigra* were prepared according to the procedure of Kirti and Chopra (1990) with the minor modification that the enzyme solution contained 7.2% mannitol as osmoticum in place of sucrose. The protoplasts were purified by flotation on 21% sucrose.

Seeds of *B. oleracea* var. *italica* were surface sterilized with 0.1% mercuric chloride solution and germinated on half strength MS basal medium at 25 ± 1°C and a photoperiod of 16 hrs light of approximately 2500 lux intensity. Four weeks old leaves of aseptically grown seedlings were cut into 2 to 3 mm segments in a plasmolysing solution of 7.2% mannitol and 0.5% calcium chloride and incubated in an enzyme mixture of 0.5% cellulase (Onozuka R-10), 0.5% driselase (Kyo Hakko Kogyo) and 0.2% macerozyme (Onozuka R-10). In contrast to hypocotyls which required incubation in the enzyme mixture for 14 to 16 hrs, mesophyll tissue released protoplasts in 5 to 6

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hrs.

Protoplast fusion and culture

Protoplast fusion was performed following the method of Menczel *et al* (1987). The solution for fusion was a modification of the one used by Menczel and Wolfe (1984) and consisted of 20% polyethylene glycol (MW 8000, Sigma), 10% dimethyl sulfoxide, 3.6% glucose, 0.17% calcium chloride and 0.0095% potassium dihydrogen phosphate adjusted to pH 5.8 and sterilized by filtration. Hypocotyl and leaf protoplasts were mixed in a 1:1 ratio, washed twice and resuspended in W_5 solution (Menczel *et al* 1981) at pH 5.8 at a density of 2 x 10^6 protoplasts/ml. Three drops of approximately 50 µl each of the protoplast mixture were placed in 5 cm Petri dishes to which an equal volume of PEG solution was added. Five minutes later, the PEG solution was removed and replaced with an equal volume of Kao's (Kao 1977) culture medium supplemented with 7.2% glucose and 1 mg/l 2,4-D (Sigma), 0.1 mg/l NAA (Sigma) and 0.5 mg/l zeatin riboside (Sigma). After another 30 min, 2 ml of the culture medium mentioned above was added to each Petri dish giving a final plating density of 2 x 10^5 protoplasts/ml. Protoplast culture, dilution, proliferation and plant regeneration were performed according to the method of Kirti and Chopra (1990) except that plant regeneration medium contained 2 mg/l zeatin (Sigma) only.

Meiosis was studied in anthers of seven plants (Table 1) fixed in Carnoy's solution and squashed in 2% acetocarmine. Pollen fertility was determined by staining with 2% acetocarmine solution. Seed fertility was computed as percentage of well filled seeds from the total number of ovules in a siliqua.

DNA analysis

For molecular analysis, total DNA was extracted from fresh, expanding young leaves of the first four hybrid plants listed in table 1 along with diploid parental species following the method of Saghai - Maroof *et al* (1984). The leaf samples were frozen in liquid nitrogen and ground to a fine powder. The DNA extraction buffer consisted of 100 mM Tris Cl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 2% CTAB and 0.2% mercaptoethanol. DNA was purified by cesium chloride density gradient centrifugation and fractionated on 0.8% agarose gel (ultrapure, BRL) after restriction with enzymes *Hind* III and *Hpa* II (Promega). The DNAs were blotted on a gene screen plus^R membrane (NEN products, DuPont) using a vacugene (LKB) vacuum blotting system. The membranes were hybridized with nick translated ^{32}P labelled probes at 65°C. Nick translation was carried out with nick translation kit of NEN (DuPont) as per the manufacturer's instructions. The stringency of washing included 30 min cycles in each of the following three solutions at 65°C i) 2X SSC-0.1% SDS ii) 1X SSC and iii) 0.25X SSC. The membranes were exposed to X-ray film (Amersham) in a casette with intensifying screen at -70°C.

The probes used are i) a random genomic clone BJG 425 of *B. juncea* which binds to the diploid parents differentially (Mohapatra 1991) ii) mitochondrial gene for maize cytochrome C oxidase subunit I (Issack *et al* 1985) and iii) chloroplast gene for large subunit of ribulose bisphosphate carboxylase (Gateby *et al* 1981).

Results and Discussion

Heterokaryotic fusion products of *B. nigra* hypocotyl and *B. oleracea* mesophyll protoplasts were identified 24 hrs after the event by the presence of dark green chloroplasts in the colourless hypocotyl protoplast background. As expected, the parental types were either totally colourless (*B. nigra*) or dark green (*B. oleracea*). The frequency of heterokaryons estimated on the basis of the protoplast colour was approximately 16%. The morphological distinction of the hybrid products from the parental types, however, was lost by the third cell division. Unlike hypocotyl protoplasts, most of the mesophyll protoplasts not involved in fusions collapsed within 24 hrs of fusion. The dividing cell cultures were diluted with 0.5 ml aliqots of MS medium containing 2,4-D and BAP at 0.1 and 1 mg/l respectively, on the seventh and tenth day. The dividing cell clusters were transferred to MS agarose containing the same growth regulators, in composition and concentration, as in the dilution medium for proliferation.

Calli of 2 to 3 mm size produced on agarose proliferation medium were transferred to a regeneration medium which contained zeatin at 2 mg/l. Zeatin had been found to be effective for regenerating protoplast calli of natural *B. carinata* accessions (Narasimhulu *et al* 1992). Out of 626 calli obtained, 92 regenerated, each having 1-3 shoots. Plants regenerated from 81 calli were morphologically like natural *B. carinata*, three calli produced plants resembling *B. nigra* and eight calli gave plants similar to *B. oleracea*. The shoots were allowed to elongate in MS basal medium and were rooted in the same medium but supplemented with 1 mg/l IBA (Sigma). The plants were hardened in a growth chamber and transferred to pots in green house for maturation.

The somatic hybrid plants resembled natural *B. carinata* in most traits, particularly in leaf shape, inflorescence, flower and pod characters. Lower leaves in *B. nigra* are petiolate, dissected and covered with dense hairs where as those of *B. oleracea* are petiolated and nearly glabrous (Fig. 1). Somatic hybrids and natural *B. carinata* possessed lyrate pinnate petiolated glabrous leaves. The inflorescence in the hybrids was very long, as in *B. oleracea*, while the flowers had intermediate characters. Sepals are spreading in *B. nigra* and fairly erect in *B. oleracea*. The hybrids had sepals that form an angle of 30-45° with the central axis. Pods of hybrid plants resembled closely the natural species.

Distinct variability was observed in hybrids for the three morphological traits i.e. plant height, days to 50% flowering and maturity. Plant height ranged from 90 to 195 cm, days to flowering from 45 to 75 days and maturity from 95 to 145 days. The flowers were yellow except in two cases where they were cream in colour. Since variability observed in the first generation plants could be physiological, progeny testing should give a better indication of the heritable component of this variation (Evans *et al* 1984).

Pollen fertility, measured by pollen stainability, varied widely among the hybrids and ranged from 36 to 87%. Seed fertility was low, centering around 19%. Cytological investigations on seven plants revealed that all had 34 chromosomes, the exact sum of *B. nigra* and *B. oleracea* chromosomes (Fig.2). No deviation in the chromosome number was observed. Majority of cells (91%) had regular meiosis with 17 bivalents. However, occasional cells had up to three quadrivalents or four univalents (Table 1). The low level of fertility, inspite of normal chromosomal complement, may be related to increased cryptic chromosomal alterations/genetic distrubances often observed in plants derived from protoplasts as has been reported in *B.*

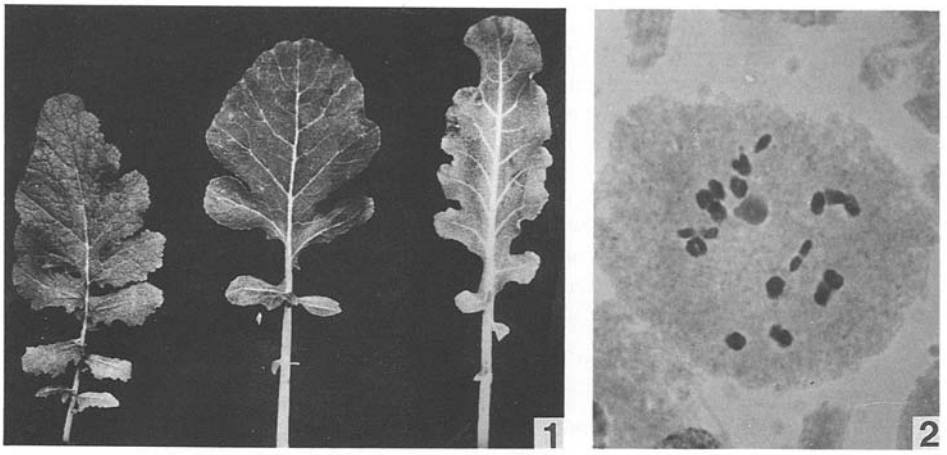
Table 1. Range of chromosome associations in seven randomly selected somatic hybrid *B. carinata* plants

Plant No.	Callus origin	Chromosome behaviour at metaphase I
1	3 - 1	17_{II} - $2_{IV} + 12_{II} + 2_{I}$
2	19 - 2	17_{II} - $3_{IV} + 11_{II}$
3	33 - 1	17_{II} - $1_{IV} + 15_{II}$
4	59 - 2	17_{II} - $2_{IV} + 11_{II} + 4_{I}$
5	68 - 1	17_{II} - $1_{IV} + 14_{II} + 2_{I}$
6	74 - 2	17_{II}
7	79 - 3	17_{II} - $15_{II} + 4_{I}$

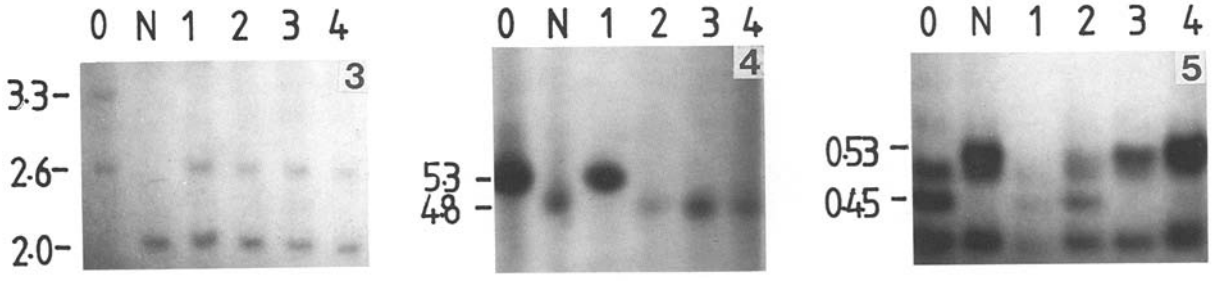
napus (Newell *et al* 1984) and in synthetic *B. napus* produced from protoplast fusion of monogenomic diploids (Sundberg *et al* 1987).

A genomic clone of *B. juncea* BJG 425 that binds differentially to *Hind* III fragments of digested DNAs of the two diploid parental species was useful for confirming hybridity of the four plants. The clone binds to two specific fragments of 3.3 and 2.6 kb in *B. oleracea* and to a single fragment of 2.0 kb in *B. nigra*. The four hybrid plants showed the occurrence of 2.6 kb *B. oleracea* and 2.0 kb *B. nigra* specific fragments thus confirming the hybrid nature of these plants at the molecular level (Fig. 3).

Molecular confirmation of organelle constitution of four hybrid plants, in relation to the parents, was made by chloroplast and mitochondrial specific gene probing. Total DNA restricted with *Hind* III and probed with a mitochondrial gene of maize



Figs. 1 Leaves of *B. nigra* (left) somatic hybrid *B. carinata* (middle) and *B. oleracea* var. *italica* (right) **2** Diakinesis in somatic hybrid showing 17 bivalents



Figs. 3 Southern showing total DNAs of parent and hybrid plants restricted with *Hind* III and probed with *Brassica juncea* genomic clone BJG 425. **4** Southern showing total DNA restricted with *Hind* III and probed with mitochondrial gene for cytochrome C oxidase subunit I **5** Southern showing total DNA restricted with *Hpa* II and probed with chloroplast gene for large subunit of ribulose bisphosphate carboxylase

The lanes are O : *B. oleracea*; N : *B. nigra*; 1, 2, 3 & 4 : somatic hybrids of *B. carinata*. Fragment sizes in kb are indicated in the left

cytochrome C oxidase subunit I hybridized to a 5.3 kb fragment of *B. oleracea* while it bound to a 4.8 kb fragment in *B. nigra* (Fig. 4). Of the four hybrid plants studied, plant no.1 had mitochondrial DNA sequences of *B. oleracea* origin while the other three plants had *B. nigra* mitochondrial DNA sequences.

Probing of total DNA digested with *Hpa* II enzyme, using a chloroplast gene for the large subunit of ribulose bisphosphate carboxylase, showed a specific *B. oleracea* fragment of about 0.45 kb not found in *B. nigra* (Fig. 5). Similarly *B. nigra* yielded a specific fragment of about 0.53 kb which was not found in *B. oleracea*. Hybrid plant no 1 had the chloroplast DNA sequences of *B. oleracea* and plants 3 and 4 had *B. nigra* chloroplast DNA sequences. Interestingly, hybrid plant no. 2 had a combination of *B. nigra* and *B. oleracea* chloroplast DNA sequences, the 0.53 kb *B. nigra* specific fragment and 0.45 kb *B. oleracea* chloroplast DNA specific fragment. This plant had the *B. nigra* mitochondrial DNA sequences. Therefore, it represents a novel cytoplasmic hybrid combination probably not available in nature.

B. carinata has been synthesized by sexual crosses using predominantly *B. nigra* as the female parent. The objective was limited to the identification of its putative progenitor (Frandsen 1947, Mizushima 1950) rather than the evaluation and utilization of variability. The *B. carinata* produced by Pearson (1972) was reportedly vigorous and fertile with a normal chromosomal complement of 17 pairs. The evaluation of the genetic variability in other sexually produced *B. carinata* was used to study the effect of cytoplasm on morphological traits including plant height, leaf size, maturity and harvest index (Prakash *et al* 1984).

Among alloploid Brassicas, *B. napus* has been synthesised by fusing protoplasts of monogenomic diploid progenitors by several investigators with the objective of developing a model system for somatic cell hybridization (Schenck and Robbelen 1982, Sundberg and Glimelius 1986, Terada *et al* 1987). However, synthesis of *B. carinata* by protoplast fusion has not been reported before. We attempted the synthesis of this species for developing a method of enlarging the genetic base of *B. carinata* by gaining access to the vast amount of available variability in the diploid gene pool. We used protoplast fusion to develop cytoplasmic hybrids which cannot be obtained by conventional hybridization. The recovery of novel cytoplasmic hybrids combining the mitochondria of *B. nigra* and chloroplasts of *B. oleracea* is a pointer to the potential of this approach for transferring cytoplasmically controlled traits of breeding importance in *Brassica* including male sterility.

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