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Intergeneric protoplast fusion between *Brassica carinata* and *Camelina sativa*

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Abstract

Camelina sativa is a wild crucifer that is reported to be resistant to Alternaria blight. Polyethylene glycol mediated fusion was attempted between protoplasts from etiolated hypocotyls of Brassica carinata and mesophyll protoplasts of Camelina sativa. The mean frequency of heterokaryons was 6.8%. Three hybrid shoots were regenerated, each from a single fusion-derived callus. These shoots failed to produce roots capable of withstanding transplantation. Confirmation of hybridity was obtained from the morphology of in vitro produced leaves, somatic chromosome number in leaf tips, and restriction fragment length polymorphism for a nuclear rDNA probe. Analysis for organelle constitution using RFLPs indicated that the hybrid contained chrloroplasts derived from the wild species and mitochondria from the cultivated Brassica species.

Abbreviations: 2,4-D, 2,4-dichlorophenoxy--acetic acid; IAA, Indole-3-acetic acid; NAA, -Naphthaleneacetic acid; IBA, Indole-3-butyric acid; GA_3, gibberellic acid; BAP, 6-Benzylaminopurine; MS, Murashige and Skoog (1962) basal medium.

Introduction

Alternaria blight, caused by Alternaria brassicae, is among the most important biotic factors reducing yields of crop Brassicas. No source of resistance against this disease is so far available among the crop Brassicas.

Camelina sativa is a wild crucifer that occurs as an annual weed throughout Europe. It has been reported to possess a high degree of resistance to Alternaria blight (Tewari 1991). The antimicrobial component responsible for its resistance has been traced to two new phytoalexins, camelexin and methoxycamelexin. The molecular structure of these phytoalexins is similar to thiabendazole, a commercial fungicide (Browne et al 1991).

Hybridization attempts between cultivated Brassicas and Camelina sativa have been unsuccessful due to the occurrence of strong reproductive barriers. Because introgression of genes conferring resistance to Alternaria blight ranks high among breeding objectives for crop Brassicas, we have attempted somatic hybridization to combine the genomes of B.carinata with C.sativa. This paper reports results of these experiments.

Materials and Methods

Plant material: The two genotypes used in the protoplast fusion experiment are Brassica carinata (A. Br.) BCR 171 (2n=34) and Camelina sativa (L.) Crantz. (2n=40). Seed material of C.sativa was kindly provided by Dr S.S.Banga.

Protocols for the preparation of leaf protoplasts and protoplast fusion were described by Kirti et al (1992a). Protoplast fusion products were cultured according to the regeneration methodology described for B.carinata protoplasts (Narasimhulu et al. 1992).

For the induction of roots on the hybrid shoots a variety of factors were tested. These included: i) six different basal media, viz., MS, B.(Gamborg et al. 1968), SH (Schenk and Hildebrandt 1972), NN (Nitsch and Nitsch 1969), Blaydes (Blaydes 1966), and White's (White 1954); ii) growth regulators IAA, IBA, NAA and GA_3 in concentrations ranging from 0.1 to 10 mg/l; iii) modification in concentrations of inorganic nitrogen and sucrose; iv) modification of osmotic potential using mannitol or increased agar concentration; v) culture in liquid medium on filter paper bridges; vi) MS basal medium of varying strengths and vii) in vitro grafting onto B.carinata.

Cytology: Leaf apices from old shoot cultures were treated for 1.5 h in a saturated solution of p-dichlorobenzene and fixed for 24 h in Carnoy's solution. Mitotic squashes were prepared from leaf

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tips hydrolyzed in 1N HCl at 60°C for 7 min and stained with 1% aceto-orcein.

DNA analaysis was carried out following the protocols outlined in Kirti et al. (1992a). The probes used were:i) a full-length rDNA sequence (18S-25S) of wheat nuclear genome (Gerlach and Bedbrook 1979); ii) a mitochondrial maize gene for 5S-18S rRNA (Chao et al. 1983); and iii) chloroplast gene for large subunit of ribulose bisphosphate carboxylase-oxygenase (rbcL) (Gatenby et al. 1981). Because of contamination problems, only one of the three hybrids could be maintained in culture and was used in these analysis.

Results and Discussion

Recovery of hybrids: The mean frequency of heterokaryons among the cultured protoplasts of the two species was 6.8%. Morphological distinction of fusion products and cell colonies derived from hypocotyl protoplasts could not be determined beyond the third cell division. Mesophyll protoplasts not involved in fusion were unstable and collapsed within 24 h. Out of a total of 227 calli obtained from three independent fusion experiments, three individual calli regenerated shoots over a period of 6 months. Each callus piece produced a single shoot. Shoots transferred to hormone-free MS basal medium failed to grow further. A low concentration of BAP (0.1 mg/l) was found essential for shoot proliferation and maintenance.

Efforts in inducing roots from the somatic hybrid shoots initially in MS medium supplemented with 1 mg/l IBA failed. This is in contrast with the ease with which other somatic hybrids (Trachystoma ballii + B. juncea, Moricandia arvensis + B. juncea and B.spinescens + B.juncea) could be rooted and transplanted to soil (Kirti et al. 1991; 1992a, b). Growth regulators IAA, IBA, NAA and GA_3 were tested in concentrations ranging from 0.1 to 10 mg/l, either independently or in combination, on clonal derivatives of the hybrid shoots. Occasionally, 1 or 2 slender roots were observed in medium with 2 mg/l IBA/IAA and in 0.5 mg/l NAA. Concentrations exceeding 4 mg/l IAA or IBA, or 1 mg/l in the case of NAA, led to callus formation at the base of the shoot with simultaneous root formation in 8-15% of the cases. But these did not survive when transplanted in soil. Five other basal media as listed earlier with IBA at 2 or 4 mg/l also failed to induce rooting in the hybrid shoots.

Experiments attempting rhizogenesis by supplementation of mannitol, use of higher agar concentration, culture in liquid medium on filter paper bridges in MS basal medium of varying strengths, and grafting onto B.carinata stocks in culture did not yield plants capable of growth in soil after transplantation. To our knowledge, the only instance where somatic hybrids failed to produce transplantable grafts is a protoplast fusion derivative of Datura combining a herbaceous species with a tree species (Schieder 1980). Grafts in this case showed several abnormalities including tumorous outgrowths. While no such abnormalities were evident in the non-grafted hybrid shoots of C.sativa + B. carinata, failure to survive transplantation suggests incomplete graft union or prevalence of strong genetic incompatibility.

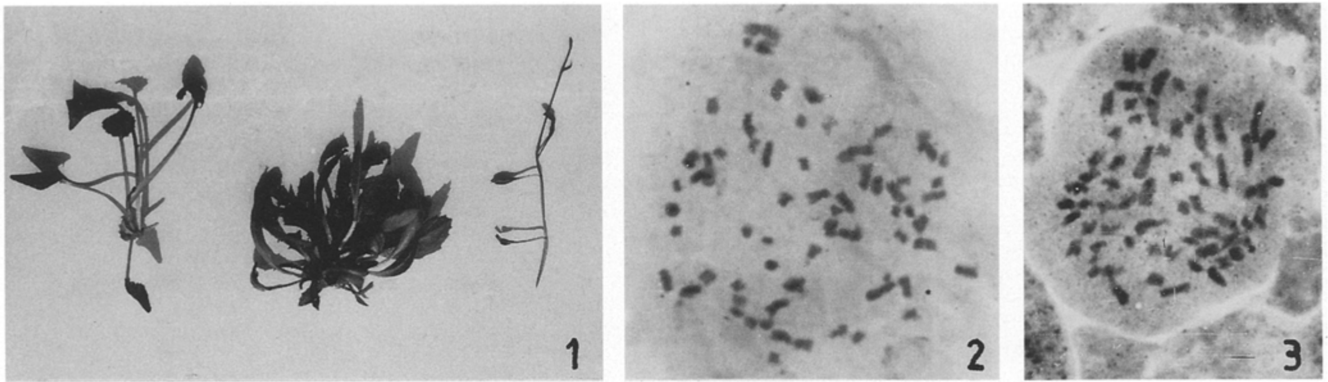
Hybridity of fusion products was established on the basis of morphology and karyology of regenerated shoots, and DNA analysis.

Morphology: Because whole plants could not be raised, our observations were restricted to morphological characteristics of in vitro produced leaves. The hybrid leaves combine features of both parental species. They were lanceolate with serrated margins, thick and brittle with an acute tip. Culture-derived seedling leaves of C.sativa are lanceolate having entire margins. They are thin when compared to the hybrid and the other parent B.carinata. B. carinata grown in culture produced broad, oval-shaped, thick and brittle leaves with serrated margins (Fig. 1).

Cytology: Leaf tip mitosis of two hybrid shoots was carried out to determine the chromosome number. The expected mitotic chromosome number of the hybrid is 74, the sum of its parental chromosomal constitution; viz., B.carinata (2n= 34) and C.sativa (2n = 40). The mitotic metaphase showed about 70 chromosomes in both cases (Figs 2, 3). Analysis revealed size difference between the chromosomes of the wild and crop species. Chromosomes of C.sativa were distinctly longer compared to B.carinata. While the 40 long chromosomes of C.sativa were clearly seen, it was difficult to account for all 34 small-sized B.carinata chromosomes in the hybrid cells.

Molecular analysis: Restriction fragment length polymorphism was used in the molecular confirmation of the hybrid nature of regenerated shoots as well as the origin of their organelles. Species-specific Southern hybridization patterns were established by comparing DNA samples of the parental species and of the hybrid following Eco RI and Hpa II enzyme digestion and hybridizing with appropriate probes. A heterologous nuclear probe of full-length nuclear rDNA of wheat was used for confirming the nuclear hybridity using Eco RI digested DNAs . It hybridized to a 3.7 kb fragment specific to C.sativa, and to 1.8 and 1.0 kb fragments specific to B.carinata. The hybrid had the 3.7 kb C.sativa specific fragment, and the 1.8 and 1.0 kb B.carinata specific fragments, thereby confirming its hybrid nature (Fig. 4)

The chloroplast composition of the somatic hybrid shoots was determined by probing the Hpa II digested total DNAs with



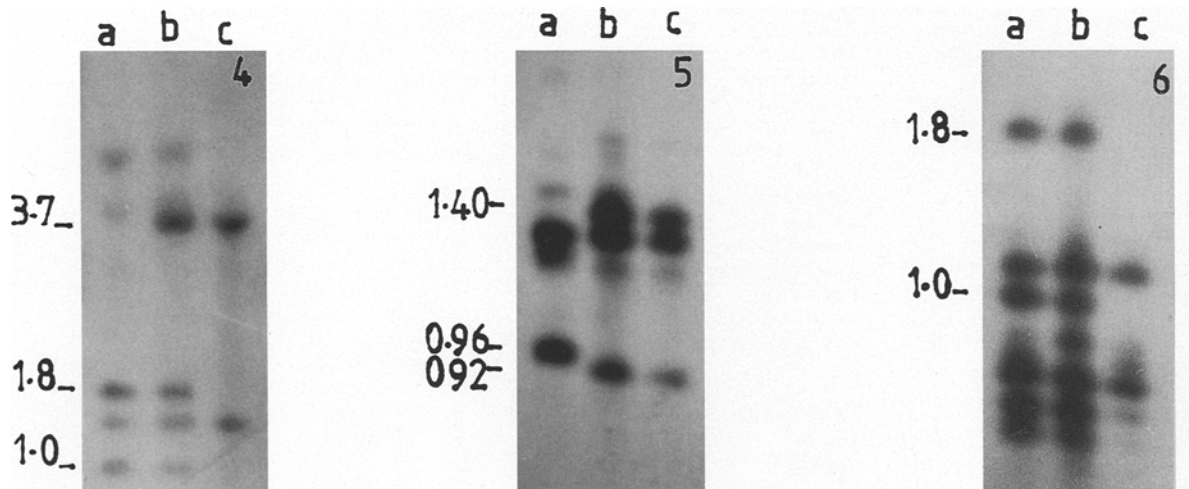


Fig.1 In vitro grown shoots of : a) B. carinata, b) somatic hybrid B. carinata + C. sativa (SH_1), and c) C. sativa. Fig.2 Chromosomal analysis of leaf tip of hybrid SH_1. Fig.3 Chromosomal analysis of leaf tip of hybrid SH_2. Fig.4-6 Southern hybridization of DNA. The lanes are : a) B. carinata, b) somatic hybrid B. carinata + C. sativa (SH_1), and c) C. sativa. 4. Eco RI digested DNA samples hybridized with 18S-25S rDNA probe (pTA71). 5. Hpa II digested DNA samples hybridized with chloroplast-encoded gene for the large subunit of ribulose biphosphate carboxylase-monoxygenase ($pZmB_1B$). 6. Hpa II digested DNA samples hybridized with mitochondrial-encoded 5S-18S rRNA gene probe.

a chloroplast-encoded gene, rbcL. The probe hybridized to two fragments, 1.5 and 0.96 kb, specific to B.carinata (Fig. 5). In C. sativa the probe hybridized to 1.4 and 0.92 kb specific fragments. The somatic hybrid shoots have the two Camelina specific fragments, suggesting that chloroplasts are of the wild species origin.

Origin of mitochondria in the somatic hybrid was determined by probing the total DNA with the mitochondrial-encoded gene for 5S-18S rRNA. Hpa II enzyme digest shows the probe binding to two fragments, 1.8 and 1.0 kb, specific to B.carinata. There is no fragment specific for C.sativa in this restriction (Fig.6). The hybrid had the B.carinata specific fragments, indicating the presence of mitochondria of B.carinata origin.

Somatic hybridization brings together three genetic systems, namely, the nuclear, montochondrial and chloroplast genomes of two genetically different parents in a heterokaryon (Eberhard 1981). This leads to complex somatic incompatibility reactions due to nuclear, inter-organellar and nucleo-organellar genomic interactions in subsequent cell divisions (Kumar and Cocking 1987). In the absence of selection pressure, random sorting of organelles eventually leads to new, stable organelle assortments as seen in the present study. The outcome of the present fusion event has led to a combination of parental nuclei, with mitochondria of the cultivated Brassica species and chloroplasts from the alien species. Efforts are being made to produce plants that can be used in further genetic studies.

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