Theor Appl Genet (2002) 105:754–758
DOI 10.1007/s00122-002-0915-3

P.B. Bhaskar · I. Ahuja · H.S. Janeja · S.S. Banga

Intergeneric hybridization between *Erucastrum canariense* and *Brassica rapa*. Genetic relatedness between E^C and A genomes

Received: 20 October 2001 / Accepted: 7 January 2002 / Published online: 18 May 2002

Abstract An intergeneric hybrid between a wild species, *Erucastrum canariense* (2n = 18; E^CE^C), and a cultivated oilseed brassica species, *Brassica rapa* (2n = 20; AA), was synthesized through ovary culture in White's basal medium supplemented with casein hydrolysate. Morphological, cytological and DNA-based analysis helped to establish the hybrid nature of the derived plants. Hybrid plants were morphologically intermediate between the two parents and were completely male, as well as female sterile. Cytological analysis revealed the occurrence of 19 I in about 38% of the PMCs investigated. However 1–8 bivalents/PMC were also observed, indicating a significant level of homology between the two genomes. Normal chromosome pairing and pollen fertility was restored following colchiploidy. The intergeneric amphiploid developed during the investigation can be used as a bridging species for the transfer of desirable genes from E^C to cultivated genomes (especially A and C), and for resistance to Alternaria blight and mustard aphid. Under field conditions, the *E. canariense* intergeneric hybrid and the amphiploid appeared to be moderately resistant to Alternaria blight and also harboured a significantly lower population of mustard aphid than the cultivated *B. rapa*.

Keywords Wide hybridization · *Brassica rapa* · *Erucastrum canariense* · Ovary culture · Genome relatedness

Introduction

The family Brassicaceae comprises a large number of wild and weedy species which are excellent gene sources for many economically important traits such as resistance to biotic and abiotic stresses, photosynthetic efficiency and novel fatty acid composition. Systematic introgression of desirable genes from wild crucifers into cultivated species has been applied extensively via the backcrossing procedure. Wild and primitive species have also been used for the synthesis of allopolyploids (Sareen et al. 1992) and alloplasmic male-sterile lines (Banga 1993, 1997; Prakash et al. 1995) in brassica oilseeds. Ovary-ovule-embryo culture (Inomata 1993; Shivanna 1996) or protoplast fusion (Prakash et al. 1999) have been widely used to overcome species barriers to the gene flow.

Erucastrum canariense, a wild crucifer, is endemic to rocky places, fields, volcanoic rock and soil, especially in the Canary Island of the Macronessian region in the Atlantic (Warwick et al. 2000). Although no elaborate studies have been conducted regarding its agronomic attributes, the species is expected to be a source of genes for various abiotic stresses due to its history of evolution under salt and water stress-conditions. The present paper describes the synthesis of a new intergeneric hybrid between *E. canariense* and *Brassica rapa* through ovary culture. Also described is the phylogenetic relatedness between the E^C and A genomes of *E. canariense* and *B. rapa* respectively.

Communicated by H.F. Linskens

P.B. Bhaskar · I. Ahuja · H.S. Janeja · S.S. Banga (✉)
Department of Plant Breeding, Punjab Agricultural University, Ludhiana – 141 004, India
e-mail: surin11@rediffmail.com

Materials and methods

Field-grown plants of *B. rapa* L. ssp. *oleifera* var. *toria* (2n = 20, AA), and *E. canariense* Webb and Berthal (E^CE^C, 2n = 18), were used as the basic plant material. To study the cross-compatibility status, reciprocal pollinations between *E. canariense* and *B. rapa* were attempted. Pollinated pistils were excised 24 h after pollination to study pollen germination and pollen-tube growth using the aniline blue flourescence technique (Shivanna and Johri 1985).

For ovary culture, pollinated pistils were excised 2–3 days after pollination (DAP), surface-sterilized with 0.01% mercuric chloride and cultured on White's basal medium containing 5% sucrose, 0.7% agar and 500 mg/l of casein hydrolysate. After about 30 days of culturing, the almost-mature seeds were taken out and cultured on fresh medium with 2% sucrose. In another set of experiments, cultured ovaries were dissected 8–10 days after culturing, and enlarged ovules, if any, were excised and re-cultured on fresh medium. The cultures were maintained at 25 ± 2 °C and a 16 h light (2,000 lux)/8 h dark cycle.

The seedlings obtained from cultured ovaries/ovules were multiplied in vitro through the culture of shoot tips and single node segments on Murashige and Skoogs (MS) medium supplemented with 0.5 mg/l of BAP. The axillary shoots were rooted on half-MS medium. The seedlings thus developed were transferred to pots containing coco peat, hardened for a few days in the green house and then shifted to the field. To induce amphiploidy in these probable hybrid plants, cotton swabs saturated with 0.1% colchicine were placed on each node for 48 h. For cytological investigations young buds were fixed in Carnoy's solution, and anthers were squashed in 2% acetocarmine. Pollen fertility was also studied using acetocarmine staining.

For RAPD analysis, nuclear DNA was extracted from the leaves of parents and their hybrids according to protocol given by Doyle and Doyle (1990). Amplifications were performed in a MJ Research PTC 200 thermal cycler. The reaction mixture contained 20 µg of DNA, 25 mM of each primer (Genemed), 200 mM each of dATP, dCTP, dGTP and dTTP (Labware, USA), 1.5 mM of $MgCl_2$, 50 mM of KCl, 10 mM of Tris HCl (pH 9.0 at 25 °C), 0.1% Triton X-100 and 1 unit of *Taq* DNA polymerase (Labware, USA). The final volume of the reaction mixture was 20 µl. The cycler was programmed for one cycle at 94 °C for 1 min, 37 °C for 37 s, 72 °C for 1 min followed by 40 cycles of 94 °C for 5 s, 37 °C for 15 s and 72 °C for 1 min for denaturation, annealing and primer extension respectively. A final 7-min extension was carried out at 72 °C after the cycles were completed. The amplified products were separated by electrophoresis on 1.5% agarose (Sigma ultrapure) gels in 1 × TBE buffer, stained with 10 ppm of ethidium bromide.

Results and discussions

Flourescence microscopic studies of pollinated pistils revealed the absence of pre-fertilization barriers between *E. canariense* as female parent and *B. rapa* as male. As field pollinations were not effective in the realization of hybrid seeds, the barrier in this cross appeared to operate at post-fertilization stages. In the reciprocal cross, however, pollen germination and pollen-tube growth was lacking and the pre-fertilization barriers seem to be important in this cross. Such unilateral crossincompatibility is of frequent occurrence in crucifer wide crosses (Shivanna 1996). Apparantly the barriers to hybridization in these two species were operative largely at post-fertilization stages when *E. canariense* was used as the female parent. Pre-fertilization barriers seemed to be important in the reciprocal cross. As field pollinations were not effective in the realization of hybrid seeds, ovary and sequential culture was used to obtain the intergeneric hybrid, *E. canariense* and *B. rapa.* Ovaries were cultured 2–3 days after pollination (DAP) on White's basal medium containing sucrose (50 g/l) and casein hydrolysate (500 mg/l). After 3 weeks of culturing, the development of seeds was visible and a total of 43 seeds were obtained after 30 days of culturing. These seeds were allowed to germinate on fresh White's medium where only one seed germinated. In another experiment, enlarged hybrid ovules were excised and cultured on a fresh medium 8–10 days after initial culturing. Out of the four seeds/ovules germinated, two produced hybrid seedlings. The remaining seeds showed enlargement and fasciation of the cotyledons. They failed to grow further when left on the same medium. However, when these were transferred to MS basal medium supplemented with kinetin (0.5 mg/l), the cotyledons produced embryoids and shoot buds. In general the sequential culture proved more effective than ovary culture. The over-all culture efficiency being 1.54% for sequential ovary–ovule culture (5 plants/259 ovaries cultured) as compared to 0.83% (2/240 cultures) recorded for ovary culture alone. The superiority of the sequential culture over the simple ovary culture was also observed in several other studies (Delourme et al. 1989; Gundimeda et al. 1992). The culture of shoot tips and single node segments on MS + 0.5 mg/l of BAP promoted extensive shoot multiplication. Rooting was obtained on half-MS medium with a lower concentration of agar (0.5%).

Hybrid plants were morphologically intermediate between the two parents and were vigorous in their growth. Leaf, flower and pod characteristics of the parents were distinct enough to be diagnostic for hybrids (Fig. 1a–d). Leaves of *E. canariense* were hispid, fleshy and deeply serrated, while those of *B. rapa* were thin, petiolate and pale green. All the hybrid plants showed intermediate leaf and inflorescence morphology. The hybrid plants had small flowers with shrivelled anthers containing sterile and irregular pollen grains (Fig. 1e). They were also female sterile, as was apparent from absence of the seed set following open- as well as hand-pollination. In contrast, the amphiploid plants had big flowers (larger than the parents) and well-developed anthers (Fig. 1f) with fertile (75–80%) pollen grains. Under field conditions, *E. canariense*, the hybrid and the amphiploid appeared to be moderately resistant to alternaria blight and harboured a significantly lower population of mustard aphid than the male parent *B. rapa*.

RAPD analysis of nuclear DNA

RAPD analysis of parents and hybrids was carried out using 20 decamer arbitrary primers. The primers G 660, G 677 generated polymorphic bands between the parents. All the four F_1 hybrid plants studied consistently showed *E. canariense* specific bands at 625 bp and 485 bp (Fig. 2) confirming their hybridity when amplified using the G 660 primer. Interestingly two bands (1.4 and 1.0 kb) present in both the parents were absent in one (D) of the four hybrids.

Cytological investigations

Cytological analysis of PMCs in the parental species indicated regular meiosis, which was reflected in excellent pollen fertility. The sporopytic chromosome number of *E. canariense* and *B. rapa* was confirmed to be 18 and 20 respectively. Analysis of metaphase-1/diakinesis-1 of the intergeneric hybrid plants revealed the occurrence of 19 I in about 38% of the PMCs studied, whereas one to as many as eight bivalents also occurred in varying frequencies (Table 1, Fig. 3a–j). The mean bivalent frequency was 1.86. Most of the bivalents were rod shaped

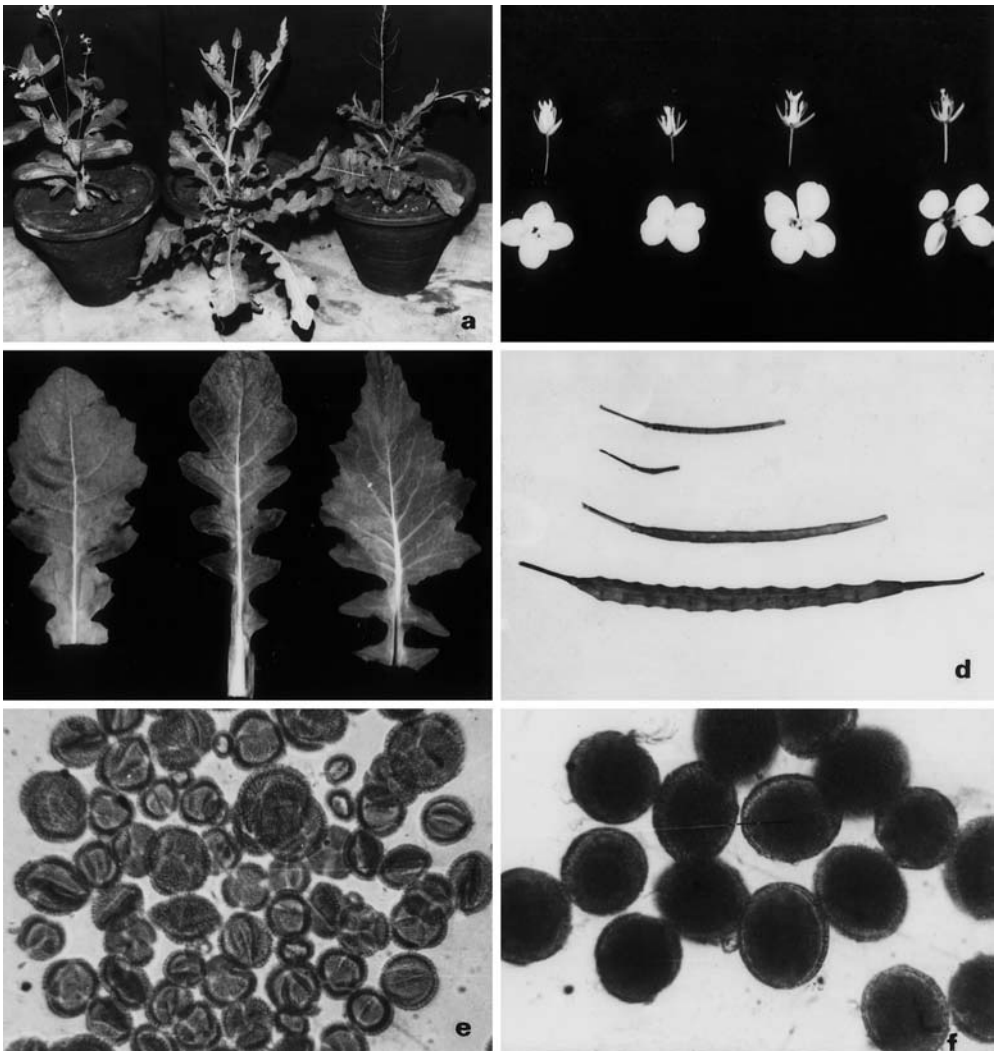

Fig. 1a–f Morphological and palaenological characterization of *E. canariense*, *B. rapa*, their F_1 hybrid and the induced amphiploid. **a** Plant morphology of *B. rapa*, the F_1 hybrid and *E. canariense*; **b** floral morphology of *B. rapa*, the F_1 hybrid, the amphiploid and *E. canariense*; **c** leaf morphology of *E. canariense*, the hybrid and *B. rapa*; **d** Siliqua of *E. canariense*, the F_1 hybrid, amphiploid and *B. rapa*; **e** unstained sterile pollen grains in the F_1 hybrid; **f** fully fertile and stained pollen grains of the amphiploid plant

Table 1 Chromosome pairing in the F_1 of the intergeneric hybrid, *E. canariense* × *B. rapa* (2n = 19). Figures in parentheses indicate percent frequencies

No. of PMCs observed	Meiotic configurations								Mean bivalent frequency
	19 I	17 I+1 II	15 I+2 II	13 I+3 II	11 I+4 II	9 I + 5 II	7 I + 6 II	3 I + 8 II	
152	58 (38.1)	24 (15.8)	16 (10.5)	26 (17.1)	8 (5.3)	12 (7.9)	4 (2.6)	4 (2.6)	1.86

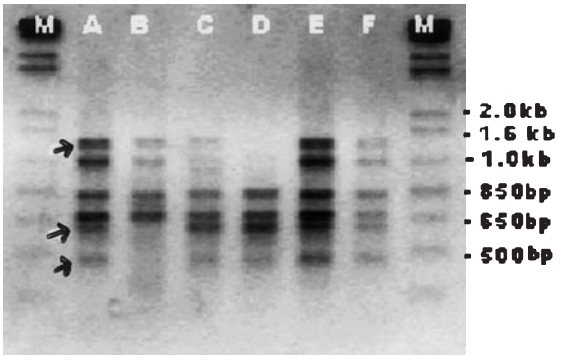

Fig. 2 RAPD analysis of **A** *E. canariense*; **B** *B. rapa*; **C–F** F_1 hybrid plants

and mono-chiasmate. Higher associations were not recorded. All the bivalents may not be reflective of the allosyndetic pairing between these two well-differentiated genomes as at least two bivalents and one trivalent have been previously ascribed to autosyndetic pairing within the A-genome-chromosomes alone (Armstrong and Keller 1981). No information exists regarding autosyndetic pairing within the E^C genome of *E. canariense*, although autopairing is expected, keeping in view the suggested basic chromosome number ($x = 5$) in the Brassicaceae. Inspite of this, and taking into account the possibility of allosyndetic pairing within the E^C genome, the occurrence of as many as eight bivalents is indicative of substantial intergenomic affinity between the two spe-

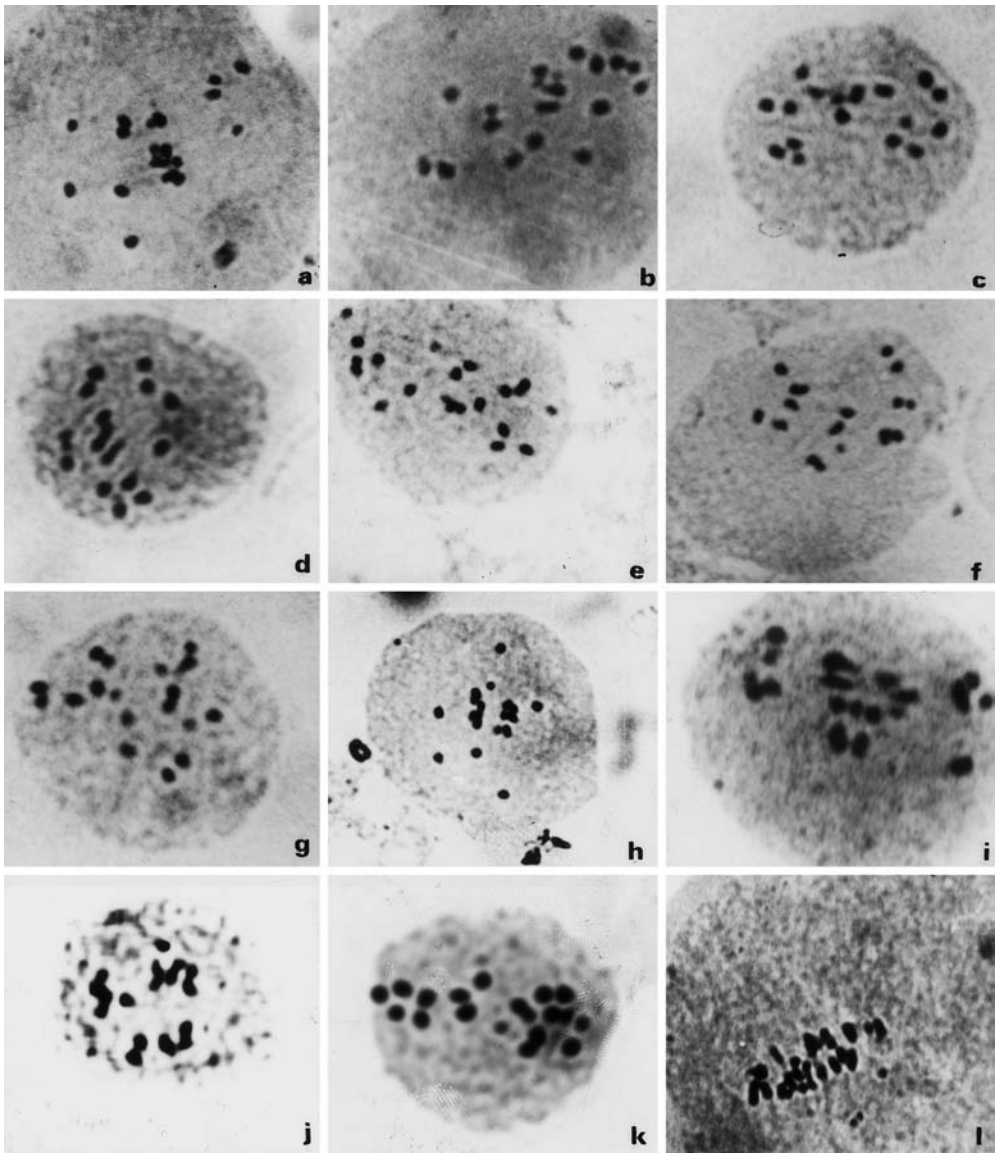

Fig. 3 Meiotic studies in the F_1 hybrid *E. canariense* × *B. rapa* (**a**–**k**) and the induced amphiploid (**l**). **a, b, c** 2 II + 15 I; **d, e** 3 II + 13 I; **f, g** 4 II + 11 I; **h** 5 II + 9 I; **i** 6 II + 7 I; **j** 8 II + 3 I; **k** 8–11 anaphase-1 distribution; **l** 19 II in amphiploid

cies. As per the published evidence no cytogenetic investigation describing homology between *E. canariense* and *B. rapa* genome is available. But the hybrid between *E. canariense* (n = 9) and *B. oleracea* (n = 9) has been reported to have up to eight bivalents (Harberd and McArthur 1980) with a mean bivalent frequency of 4.03. It may be mentioned here that the C-genome of *B. oleracea* is very closely related to the A-genome of *B. rapa* (Truco et al. 1996). The unequal distribution of chromosomes (Fig. 3k) and anaphase bridges were fairly common during meiotic anaphase-I.

The induced amphiploid had the expected 2n = 38 chromosome number, and 19 II was the predominant meiotic configuration (Fig. 3L). The occurrence of 1 III in a low frequency of PMCs was suggestive of allosyndetic pairing between the two genomes. The majority of the anaphase-1 cells in the amphiploid had a normal 19–19 distribution of chromosomes. Inspite of generally regular bivalent formation and excellent pollen fertility (65 to 80%), the amphiploid showed a poor seed set on selfing and crossing with *B. rapa*, *B. juncea* and *B. napus*. The very low seed setting on selfing or crossing with the *B. rapa* parent might be due to the similar 'S' allele for self-incompatibility operative in the hybrid and *B. rapa*. The highest cross seed set was obtained following hybridization with *B. napus* (2n = 38; AACC) suggesting again some affinity between the E^C and A/C genome species.

The synthesis of a stable amphiploid (*E. canariense* × *B. rapa*) is of significance as a conduit for gene transfer between *E. canariense* and crop brassicas, for the synthesis of a complete set of alien addition lines and also to develop a *E. canariense*-based CMS fertility restorer system for brassica oilseeds.

Acknowledgements The funds provided by the National Agricultural Technology Project "*Development of Hybrids in Rapeseed-mustard*" for this study are duly acknowledged. The experiments described in this manuscript comply with the current laws of India.

References

Armstrong KC, Keller WA (1981) Chromosome pairing in haploids of *Brassica campestris*. Theor Appl Genet 59:49–52

Banga SS (1993) Heterosis and its utilization. In: Labana KS, Banga SS, Banga SK (eds) Breeding oilseed Brassicas. Monogr Theor Appl Genet 19:21–43

Banga SS (1997) Genetics and breeding in Brassica oil seed crops. Acta Hort 59:389–96

Delourme R, Eber F, Chevre AM (1989) Intergeneric hybridization of *Diplotaxis erucoides* with *Brassica napus*. Cytogenetic analysis of F_1 and BC_1 progeny. Euphytica 41:223–228

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Gundimeda HR, Prakash S, Shivanna KR (1992) Intergeneric hybrid between *Enarthocarpus lyratus*, a wild species, and crop Brassicas. Theor Appl Genet 83:655–662

Harberd DJ, McArthur ED (1980) Meiotic analysis of some species and genus hybrids in the *Brassiceae*. In: Tsunoda S, Hinata K, G'omez C (eds) Brassica crops and wild allies. Japan Scientific Societies Press, Tokyo, pp 65–87

Inomata N (1993) Embryo rescue techniques for wide hybridization. In: Labana KS, Banga SS, Banga SK (eds) Breeding oilseed Brassicas. Monogr Theor Appl Genet 19:94–105

Prakash S, Kirti PB, Chopra VL (1995) Cytoplasmic male sterility (CMS) systems other than *ogu* and *polima* in *Brassica*: current status. Proc 9th Int Rapeseed Conf, Vol. I, pp 44–48

Prakash S, Takahata Y, Kirti PB, Chopra VL (1999) Cytogenetics. In: Gomez Campo (ed) Biology of Brassica coenospecies. Elsevier Science, Amsterdam, pp 59–106

Sareen PK, Chowdhury JB, Chowdhury VK (1992) Amphidiploids/synthetic crop species. In: Kalloo G, Chowdhury JB (eds) Distant hybridization of crop plants. Springer, Berlin Heidelberg New York, pp 62–69

Shivanna KR (1996) Incompatibility and wide hybridization. In: Chopra VL, Prakash S (eds) Oilseed and vegetable Brassicas: Indian perspective. Oxford and IBH Publishing Co, New Delhi, pp 77–102

Shivanna KR, Johri BM (1985) The angiosperm pollen: structure and function. Wiley Eastern, New Delhi

Truco MJ, Hu J, Sadowski J, Quiros CF (1996) Inter- and intragenome homology of the *Brassica* genomes: implications for their origin and evolution. Theor Appl Genet 93:1225–1233

Warwick SI, Francis A, La Fleche J (2000) Guide to wild germplasm of *Brassica* and allied crops (tribe Brassiceae, Brassicaceae). AAFC-ECORC Contribution no. XXXX