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Hybridizing *Brassica rapa* with wild crucifers *Diplotaxis erucoides* and *Brassica maurorum*

Harsh Garg · Shashi Banga · Payal Bansal · Chhaya Atri · S. S. Banga

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Abstract Two wide hybrids, *Diplotaxis erucoides* ($2n = 14$) × *Brassica rapa* ($2n = 20$) and *B. maurorum* ($2n = 20$) × *B. rapa*, were developed using the sequential ovary–ovule culture. Reciprocal crosses failed, possibly as a consequence of strong unilateral incompatibility. The F_1 hybrids in each combination were completely male sterile and morphologically intermediate to the respective parents. DNA marker polymorphism and chromosome counts confirmed their hybrid nature. High frequency of bivalents in the F_1 and the presence of trivalents/quadrivalents in the derived amphiploids suggested genomic duplications and homoeology of the parental genomes. Up to three homoeologous pairs between the *D. erucoides* (D^eD^e) and *B. rapa* (AA) genomes, and one between *B. maurorum* (B^mB^m) and *B. rapa* genomes were observed. Successful synthesis of the F_1 hybrids and amphiploids of *B. rapa* with *D. erucoides* and *B. maurorum*, and allosyndetic chromosome pairing are expected to permit introgressions of desirable loci into the cultivated Brassica germplasm, especially for resistance to *Alternaria brassicae* and *Albugo candida*.

H. Garg · S. Banga · P. Bansal · C. Atri · S. S. Banga (✉)
Department of Plant Breeding, Genetics and Biotechnology, Punjab Agricultural University, Ludhiana 141004 Punjab, India
e-mail: surin11@rediffmail.com

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Introduction

Harnessing alien genetic diversity is an important crop improvement activity. Wild *Brassiceae*, having evolved in diverse ecogeographical habitats (Tsunoda 1980), comprises a rich repository of variability especially for the defensive traits. Establishment of the phylogenetic relationships between cultivated Brassicas and contemporary crucifer genomes, through the interpretation of the chromosome pairing patterns or through genetic linkage maps anchored to the *Arabidopsis* genome, has underlined immense possibilities of directed gene exchange across taxonomic domains. Though somatic hybridization is the technique of choice in overcoming the fertilization barriers, various modifications of the embryo rescue techniques continue to be employed due to their simplicity and operational ease. Examples for successful gene introgressions abound, and include resistance to diseases (Hagimori et al. 1992), novel fatty acid composition (Fahlesson et al. 1994) and fertility restorer genes for a number of cytoplasmic male sterility (CMS) systems (Banga 2003). Commercialization of at least two alloplasmic CMS systems (*ogu, mori)* for hybrid seed production in *Brassica* oilseeds is a classic example of the efficacy of the introgression route for achieving plant breeding goals.

In this communication, we report on the development of hybrids of *B. rapa* with *Diplotaxis erucoides* and *Brassica maurorum*. Genetic homoeology between *B. rapa* and *D. erucoides/ B. maurorum* genomes has been reported in the past (Vyas et al. 1995; Chrungu et al. 1999). Both these reports, however, relied solely on the meiotic chromosome pairing data of the F_1 hybrids to draw inferences on the intergenomic affinities. Such data have a limited value for genome analysis (Jauhar and Crane 1989) as they fail to differentiate between the allo- and auto-syndetic pairing; hence they tend to inflate the affinity relationships. In the absence of competition for pairing partners in interspecific F_1 hybrids, even distantly related homoeologues may frequently engage in pairing. Only higher ploidy hybrids, such as triploids and higher, or amphiploids provide a realistic test of genome affinity. Here we use tetraploid amphiploids to provide a more objective assessment of the genetic relationships between the A genome of *B. rapa* and the D^e and B^m genomes of *D. erucoides* and *B. maurorum*, respectively.

Materials and methods

Field grown plants of the cultivated *B. rapa* L. ssp. *oleifera* cv TH 68 ($2n = 20$, AA), and two wild accessions, *B. maurorum* ($2n = 16$, B^mB^m) and *D. erucoides* ($2n = 14$, D^eD^e) were used to produce interspecific hybrids between cultivated and wild species. Direct as well as reciprocal crosses were attempted in both the cross combinations. Flower buds of the three parental species were emasculated and pollinated with freshly collected pollen from the desired pollinator species. Some of the pollinated buds were left on the plant while others were excised and used for the *in vitro* experiments. For the ovary culture, pistils were excised 2–3 days after pollination (DAP), surface sterilized with mercuric chloride (0.01%) for 8 min and cultured on the Murashige and Skoog's (MS) medium containing 5% sucrose, 0.8% agar and 500 mg/l of casein hydrolysate. Cultures were maintained at 25 ± 2°C under a 16-h light (2,000 lux)/8-h dark cycle as described earlier (Bhaskar et al. 2002). For the sequential culture, pollinated ovaries 8–9 days after the initial culture were dissected and enlarged ovules were recultured on a fresh MS medium containing gibberellic acid. Shoot tips from hybrid seedlings were multiplied in vitro through the shoot tip culture and the culture of nodal segments on the MS medium supplemented with benzyl amino purine (BAP) at 0.5 mg/l. The axillary shoots were then rooted on the half-strength MS or MS + IBA (0.5 mg/l) media and transferred to the field after 7–10 days of hardening under controlled environmental conditions. Chromosome doubling was induced by placing cotton swabs saturated with 0.1% colchicine on the meristematic sectors for 72 h. The hybrid nature of the recovered plants was verified by the Randomly Amplified Polymorphic DNA (RAPD) analysis. For this purpose, the total DNA was extracted from the young leaves of the parents and hybrids according to Doyle and Doyle (1990). Amplifications were performed in a MJ Research PTC 200 thermal cycler (MJ Research, Waltham, USA) using the method described by Bhaskar et al. (2002). The amplified products were separated on a 1.5% agarose gel in 1% TBE buffer, stained with 1% ethidium bromide and visualized under the UV light in a Gene Genius Gel documentation system. For cytological studies, young buds were fixed in the Carnoy's solution II (ethanol:chloroform:acetic acid in the ratio of 6:3:1). Anthers from appropriate buds were squashed in 2% acetocarmine, and pollen mother cells (PMC's) were viewed under Olympus microscope (XL-70) for meiotic configurations at diakinesis/metaphase. Mean bivalent, trivalent and quadrivalent frequencies were calculated as sum total of respective configurations divided by total number of PMC's observed. Parental species, hybrids and amphiploids were tested for reaction to *Alternaria brassicae* and *Albugo candida* infestation under aided epiphytotic conditions of high humidity and repeated inoculum applications.

Results and discussion

In the present study, two diploid wild crucifers were selected for hybridization with diploid *B. rapa*. Only in the crosses involving wild crucifers as female, 2–3% of the pollinated pistils formed pods, with the bulk of the pollinated pistils drying 5–7 days after pollination. Seed set in the surviving pods was low (<1%) and the seed was shriveled and failed to germinate. The failure to produce hybrid seeds from field pollinations appears to be largely due to post fertilization barriers, which cause embryo abortion (Shivanna 1996). The sequential ovary–ovule culture overcame post fertilization barriers in crosses involving *B. rapa* as the pollen parent. In the combination *D. erucoides* × *B. rapa*, 230 cultured ovaries yielded 34 ovules which upon subsequent culturing developed into 34 seeds. However, most of these seeds were shriveled and malformed and only three germinated, producing F_1 hybrids. In the *B. maurorum* × *B. rapa* cross, 144 cultured ovaries yielded 22 ovules, three of which germinated. Of the three, two were F_1 hybrids and remaining one maternal. All produced F_1 hybrids were multiplied through in vitro culture of shoot tips and nodal segments.

Attempts at genetic enrichment through interspecific hybridization in cultivated *Brassica* species date back to early nineteenth century when Sageret (1826) synthesized hybrids of *B. oleracea* with *B. rapa*. However, the intergeneric barriers were breached only in the early XX century with the synthesis of a hybrid between *Raphanus sativus* and *Brassica oleracea* (Karpechanko 1924). The development of the embryo rescue techniques, by overcoming the post-fertilization barriers, greatly expanded the scope of wide hybridization attempts. They provided the Brassica breeders an access to a range of potentially beneficial nuclear or cytoplasmic encoded traits present in the wild relatives. It also allowed the establishment of intergenomic relationships (Chandra et al. 2004). Because of the application ease, in vivo fertilization followed by in vitro culturing of fertilized ovaries has been especially rewarding. Alternatively, the fertilized ovules can be dissected out from the ovary after few days of culturing and recultured in a defined medium as discussed earlier.

Morphologically, both the F_1 hybrids were intermediate to the parents. Male fertility, as indicated by pollen stainability in 2% acetocarmine was very low (<2%). Induced amphiploids had normal-sized anthers and near normal pollen stainability (≈75%). While the cultivated *B. rapa* parent was susceptible to the *Alternaria brassicae* and *Albugo candida*, the two wild species, the F_1 hybrids with *B. rapa* and the induced amphiploids were resistant. In the RAPD analysis, primers OPA 16, OPF 01, OPW 19, and OPW 13 produced polymorphic DNA fragments between *D. erucoides* and *B. rapa* (Fig. 1A) while primers OPW 19, OPW 13, OPA 16, OPN 10, and PPF 01 generated polymorphic fragments between *B. maurorum* and *B. rapa* (Fig. 1B). Hybrid plants possessed many of the fragments specific to each of the parents, thus confirming their hybrid nature.

Somatic chromosome numbers (2*n*) of *D. erucoides, B. maurorum* and *B. rapa* were confirmed at 14, 16 and 20, respectively, with normal bivalent formation in the metaphase I of meiosis.

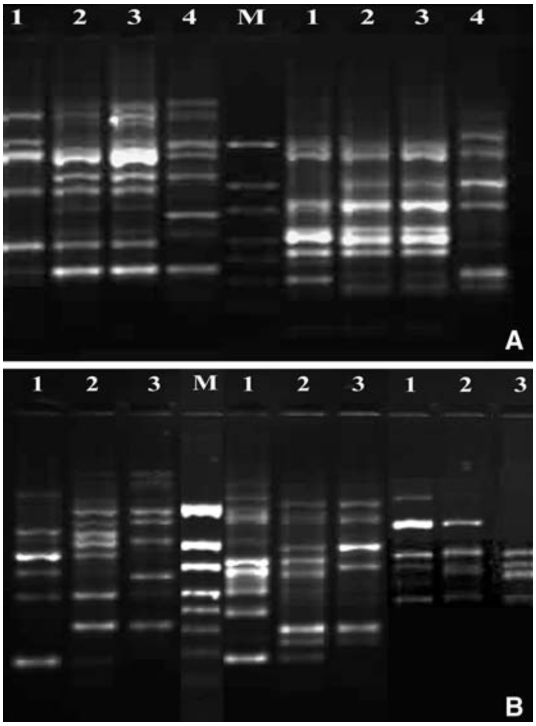

Fig. 1 (**A**) Molecular characterization of *D. erucoides* × *B. rapa* hybrid using primers OPW 19 and OPW 13. Lanes 1: female parent, 2: F_1 hybrid, 3: amphiploid, 4: male parent. (**B**) Molecular characterization of *B. maurorum* × *B. rapa* hybrid using primers OPW 19, OPW 13 and OPA 16. Lanes 1: female parent, 2: F_1 hybrid, 3: male parent

The *D. erucoides* × *B. rapa* F_1 hybrid had the expected $2n = 17$ chromosomes. In the pollen mother cells (PMC's) of the F_1 hybrids (Fig. 2a–f), a range of chromosome configurations was observed in diakinesis/metaphase I with 5II + 7I as the predominant configuration (Fig. 2d) present in about 24% of the PMC's (Table 1). A maximum of 8II were observed in 5% of PMC's whereas 16% of PMC's showed more than five bivalents. The mean bivalent frequency was 3.75. A single trivalent was also observed in about 4% of the PMC's. The *B. maurorum* × *B. rapa* F_1 hybrid also had the expected somatic chromosome number ($2n = 18$). 18 I was the predominant meiotic configuration in the F_1 hybrid and it occurred in about 24% of PMC's (Table 2, Fig. 3a). One to seven bivalents were observed per PMC, with mean bivalent frequency of 3.41 % (Table 2, Fig. 3b–f). Higher than expected frequency of bivalent formation in the two wide hybrids may not be construed as a reflection of high affinity of the D^e/B^m and A genomes as chromosome pairing in the absence of preferential pairing is not necessarily a function of homology. The archetype of the sub tribe *Brassicinae* is believed to have had the basic chromosome number of $x = 5$ or $x = 6$ (Quiros 1999). The increase in the chromosome number possibly occurred by whole genome duplications and subsequent divergence. Past cytogenetic investigations with *B. rapa* haploids (Armstrong and Keller 1981; Truco et al. 1996), have shown

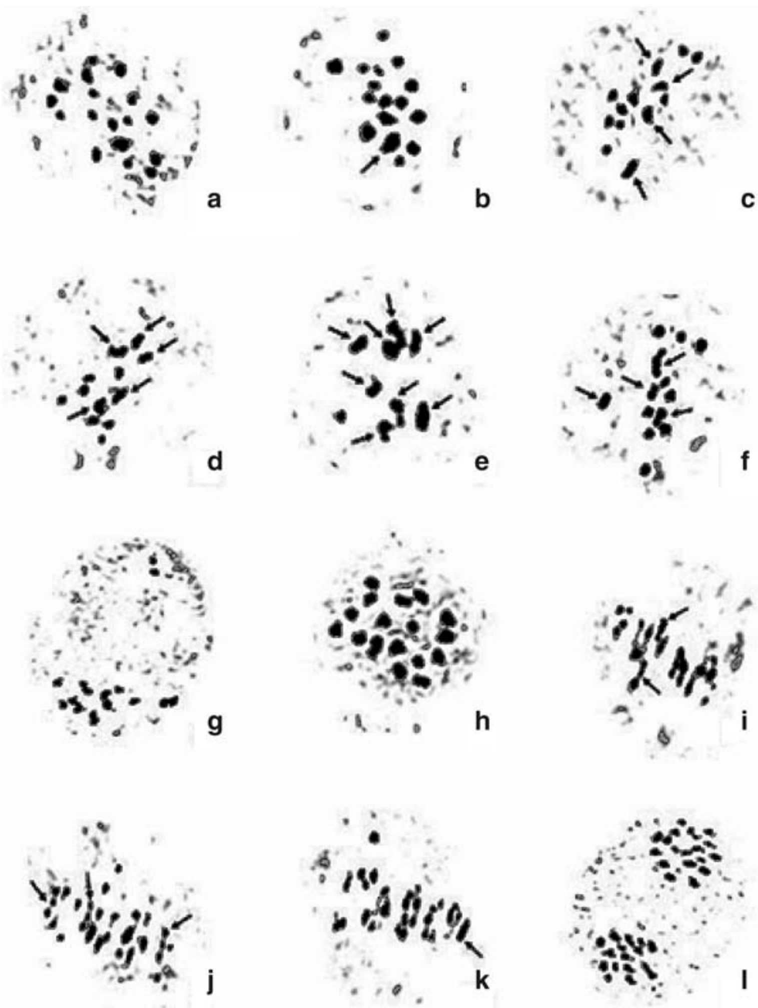

Fig. 2 Meiotic studies in the hybrid *D. erucoides* × *B. rapa* (**a–g**) and the induced amphiploid (**h–l**), (**a**) 17I, (**b**) 1II + 15I, (**c**) 4II + 9I, (**d**) 5II + 7I, (**e**) 8II + 1I, (**f**) 1III + 3II + 8I, (**g**) 2–15 distribution at anaphase I, (**h**) 17II, (**i**) 2III + 14II, (**j**) 3III + 12II + 1I, (**k**) 1IV + 15II, (**l**) 19–15 distribution at anaphase I

Table 1 Meiotic analysis in the intergeneric cross *D. erucoides* × *B. rapa*

Somatic chromosome number	PMC's observed	17 I	1II + 15I	2II + 13I	3II + 11I	4II + 9I	5II + 7I	6II + 5I	7II + 3I	8II + 1I	1III + 2II + 10I	I III + 3 II + 8 I	Mean bivalent frequency	Mean trivalent frequency
17	131	10 (0.08)	8 (0.06)	12 (0.09)	28 (0.21)	14 (0.11)	32 (0.24)	7 (0.05)	8 (0.06)	6 (0.05)	4 (0.03)	2 (0.01)	3.75	0.04

Figure in paranthesis indicate respective frequency

Table 2 Meiotic analysis in intergeneric hybrid *B. maurorum* × *B. rapa*

Somatic chromosome number	PMC's observed	18I	1II + 16I	2II + 14I	3II + 12I	4II + 12I	5II + 8I	6II + 6I	7II + 4I	Mean bivalent frequency
18	41	10 (0.24)	2 (0.05)	2 (0.05)	8 (0.20)	1 (0.02)	7 (0.17)	6 (0.15)	5 (0.12)	3.41

Figure in paranthesis indicate respective frequency

Table 3 Meiotic analysis in the amphiploid *D. erucoides* × *B. rapa*

Somatic chromosome number	PMC's observed	17II	1III + 15II + 1I	2III + 14II	3III + 1I + 12II	1IV + 15II	Mean bivalent frequency	Mean trivalent frequency	Mean quadrivalent frequency
34	80	64 (0.8)	6 (.08)	4 (.05)	2 (0.02)	4 (.05)	16.48	0.25	0.05

Figure in paranthesis indicate respective frequency

Table 4 Meiotic analysis in amphiploid *B. maurorum* × *B. rapa*

Somatic chromosome number	PMC's observed	18II	1III + 16II + 1I	1IV + 16II	1IV + 4I + 14II	Mean bivalent frequency	Mean trivalent frequency	Mean quadrivalent frequency
36	70	52 (0.74)	8 (0.11)	4 (0.06)	6 (0.09)	17.31	0.11	0.14

Figure in paranthesis indicate respective frequency

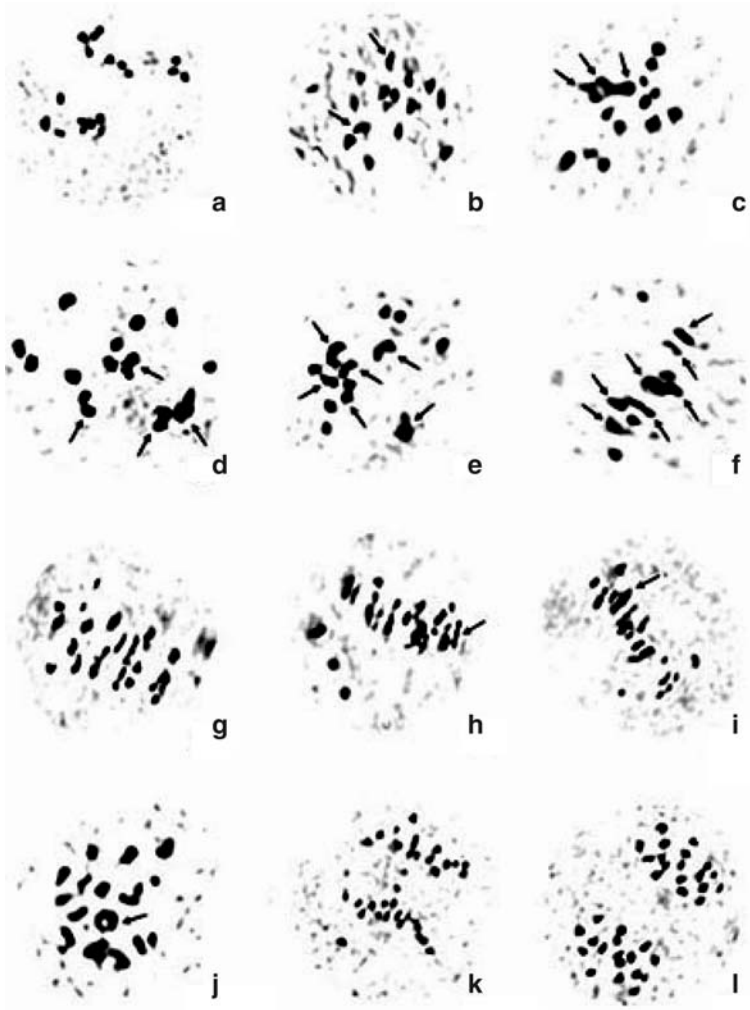

Fig. 3 Meiotic studies in the hybrid *B. maurum* × *B. rapa* (**a–f**) and induced amphiploid (**g–l**), (**a**) 18I, (**b**) 2II + 14I, (**c**) 3II + 12I, (**d**) 4II + 10I, (**e**) 6II + 6I, (**f**) 7II + 4I, (**g**) 18II, (**h**) 1III + 14II + 1I, (**i**) 1IV + 16II, (**j**) 1IV + 14II + 4I, (**k**) 16–2–18 distribution at anaphase I, (**l**) 19–17 distribution at anaphase I

occurrence of two bivalents and one trivalent resulting from autosyndetic pairing. Similarly, *D. erucoides* has also been reported to form up to three bivalents in haploid state (Delourme et al. 1989). Thus from purely theoretical considerations, 5II + 1 III configuration in the *D. erucoides* × *B. rapa* hybrid would be expected due to autosyndetic pairing within the parental genomes alone.

The amphiploid (AAD^eD^e; $2n = 34$) of *D. erucoides* × *B. rapa* had 17II as the predominant meiotic configuration, although PMC's with varying numbers of univalents, trivalents and, occasional quadrivalent were also observed (Table 3, Fig. 2h–k). Presuming normal pairing control mechanism in the newly developed amphiploid, the occurrence of up to three trivalents (Fig. 2j) or one quadrivalent (Fig. 2k), under the conditions of preferential pairing available in tetraploid amphiploid, can be considered as an indicative of allosyndetic pairing between the A and D^e genomes. This is likely since both *B. rapa* and *D. erucoides* belong to *Brassica* lineage (Song et al. 1990; Warwick and Black 1991; Lysak et al. 2005) and the genus *Diplotaxis* is considered to be the closest wild relative of crop *Brassica* species. Unequal distribution was observed during anaphase in the amphiploid (Fig. 2l). The *B. maurorum* × *B. rapa* amphiploid (AAB^mB^m; $2n = 36$) had 18II as predominant meiotic configuration (Table 4, Fig. 3g) occurring in 74% of the PMC's investigated, whereas the remaining 26% had either one trivalent (Fig. 3h) or one quadrivalent (Fig. 3i–j). Occurring under rigid regime of tetraploid test, these configurations were supportive of limited homology between A and B^m genomes as suggested by Takahata and Hinata (1983) in the interspecific hybrid they developed. Although the majority of the anaphase-I cells in the amphiploid showed normal 18–18 distribution of chromosomes, there was some evidence of unequal separation at anaphase I (Fig. 3k, l). Both the amphiploids showed no self-seed setting, however few open pollinated and cross seeds could be harvested.

The successful synthesis of F_1 hybrids/amphiploids of *B. rapa* with *D. erucoides* and *B. maurorum* can be viewed as a significant step towards the development of alternaria resistant *B. juncea*/*B. napus* as both the amphiploids showed resistance to *Alternaria brassicae* and *Albugo candida*. Availability of amphiploids with genomic constitutions of AAD^eD^e and AAB^mB^m is expected to allow exchange of genetic information between wild and crop Brassica genomes via pairing of B/C genome of *B. juncea/B. napus* with B^m or D^e genomes of the two amphiploids. Backcross introgression of gene(s) for alternaria resistance from *D. erucoides* and *B. maurorum* into *B. juncea* and *B. napus* has already been initiated using the synthesized amphiploids as bridging species.

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