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ORIGINAL PAPER

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Intergeneric hybridization between *Erucastrum cardaminoides* and two diploid crop *Brassica* species

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Abstract Two intergeneric hybrids involving wild species *Erucastrum cardaminoides* (2*n*=18, E^{cd} E^{cd}) and two crop brassica species, *Brassica rapa* (2*n*=20, AA) and *B. nigra* (2*n*=16, BB), were synthesized through in vitro sequential ovary culture. Morphological, molecular and cytological studies were conducted to establish their hybridity. Both hybrids, though morphologically distinct, were intermediate phenotypically between their respective parents. Cytological analysis of the *E. cardaminoides* × *B. rapa* hybrid (2*n*=19), revealed the occurrence of 17 I+1 II at diakinesis/metaphase in the majority (28%) of the pollen mother cells (PMCs), whereas in *E. cardaminoides* × *B. nigra* hybrid (2*n*=17), 13 I+2 II was the predominant (32%) meiotic configuration. A maximum of 5 II was recorded in both hybrids, indicating homoeologous pairing in the respective combined genomes. Chromosome doubling by colchicine application gave rise to two new amphiploids (AA $E^{cd}E^{cd}$ and BB $E^{cd}E^{cd}$) having normal chromosome pairing and pollen fertility. The occasional occurrence of one quadrivalent in the amphiploids confirmed partial homoeology between the E^{c} and A/B genomes. The *E. cardaminoides* ×*B. nigra* hybrid and amphiploid appeared to be tolerant to alternaria blight under field conditions.

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Introduction

Despite recent advances in genetic engineering and molecular biology techniques, wide hybridization remains a potent tool in crop breeding programmes for creating novel plant forms and introgressing the genes of interest from related wild species. The family *Brassicaceae* comprises a large number of wild and weedy plants which are excellent repositories of genes for many economically important traits, especially for tolerance to environmental stresses, and novel seed storage products. Therefore, the bringing together of the diverse genomes in wide crucifer hybrids (Bhaskar et al. 2002; Banga et al. 2003a) has not only provided an access to a range of potentially beneficial genetic or cytoplasmic variation but has also allowed the establishment of genomic affinities through study of homoeologous pairing between two sets of differentiated genomes. Many primitive species have been used for synthesizing new intergeneric hybrids/amphiploids (Sareen et al. 1992; Bhaskar et al. 2002, Banga et al. 2003a) following hybridization with crop *Brassica* species. A number of alloplasmic male sterility systems have also been bred by exploiting the cytoplasmic diversity available in these primitive species (Prakash 2001; Banga et al. 2003b).

Erucastrum cardaminoides is an annual to biennial crucifer which is endemic to rocky places, fields, volcanic rocks and soil, especially in the Micronesian region (Warwick et al. 2000). Due to its evolutionary history, this species is expected to be a source of gene(s) for many biotic and abiotic stresses (especially salt tolerance). We report here the synthesis of two new intergeneric hybrids between *E. cardaminoides* with *B. rapa* and *B. nigra* utilizing ovary-ovule culture technique. To date, this is the first published evidence on these hybrids.

Materials and methods

Pistils of field-grown plants of *Erucastrum cardaminoides* (Webb ex H. Christ) O.E. Schulz (2*n*=18, $E^{cd}E^{cd}$) were pollinated with pollen from two crop *Brassica* species, namely *B. rapa* L. (2*n*=20,

AA) and *B. nigra* (L.) Koch (2*n*=18, BB). Some of the pollinated pistils were excised 2–3 days after pollination, surface sterilized with 0.1% mercuric chloride and cultured on MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose, 0.8% agar and 500 mg/l casein hydrolysate. The cultures were maintained at 25±2°C under a 16/8-h (light/dark) photoperiod with light supplied at an intensity of 2,000 lux. The cultured ovaries were dissected 10–12 days following initial culture in order to extract the growing ovules therein. These ovules were re-cultured on fresh medium. The hybrid seedlings obtained from the cultured ovules were multiplied in vitro through shoot-tip culture and the culture of nodal segments on MS medium supplemented with 0.5% benzylaminopurine. The axillary shoots were rooted on half-strength MS medium. The seedlings were hardened for 7–10 days in culture tubes containing water to prevent desiccation before being transferred first to pots in the greenhouse and finally to the field. Amphiploidy was induced in the hybrid plants by placing cotton swabs saturated with 0.1% colchicine on the meristematic sectors for 48 h. For the cytological investigations, young buds were fixed in Carnoy's solution and the anthers then squashed in 2% acetocarmine to study pollen grain fertility and chromosome pairing.

Random amplified polymorphic DNA (RAPD) analysis was carried out to establish the hybridity of the hybrid plants. Genomic DNA was extracted from the young leaves of the parents and hybrids according to the protocol of Doyle and Doyle (1990). Amplifications were performed in a MJ Research PTC 200 thermal cycler (MJ Research, Waltham, Mass.) using the method described by Bhaskar et al. (2002). The amplified products were separated by electrophoresis on a 1.5% agarose gel in 1× TBE buffer and stained with 10 ppm ethidium bromide. The resulting gels were visualized under UV light in the Gene Genius gel documentation system.

Results

While nearly 2–3% of the in vivo-pollinated pistils resulted in pod development in both intergeneric crosses, the pods were devoid of seeds. This failure to produce hybrid seeds was largely attributed to post-fertilization barriers that caused embryo abortion. As the field pollinations were not effective in producing hybrid seeds, in vitro embryo rescue techniques like ovary and sequential ovule culture were explored to produce the intended interspecific hybrids. In the *E. cardaminoides* × *B. rapa* cross, 297 ovaries were cultured, yielding a total of 41 ovules that were cultured further on fresh medium. Of these 41 ovules only six germinated, and only four of these produced hybrid seedlings. In the *E. cardaminoides* × *B. nigra* cross, 212 cultured ovaries yielded 25 ovules,

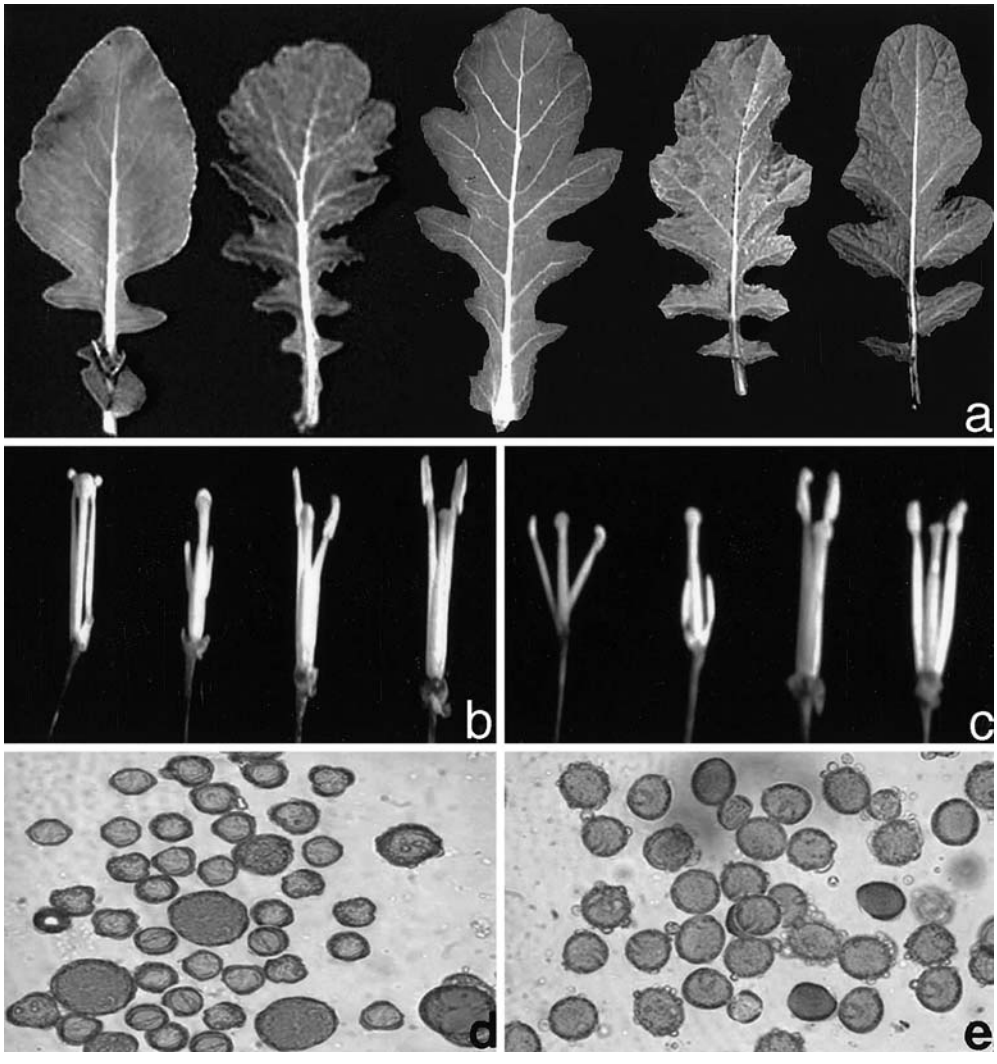

Fig. 1 **a** Leaf morphology of (*left to right*) *Brassica rapa*, *Erucastrum cardaminoides* × *B. rapa*, *E. cardaminoides*, *E. cardaminoides* × *B. nigra* and *B. nigra*. **b** Anther morphology in (*left to right*) *E. cardaminoides*, the F_1 hybrid, the amphiploid and *B. rapa*. **c** Anther morphology in (*left to right*) *E. cardaminoides*, the F_1 hybrid, the amphiploid and *B. nigra*. **d**, **e** Pollen grain stainability of the F_1 hybrid and the amphiploid, respectively

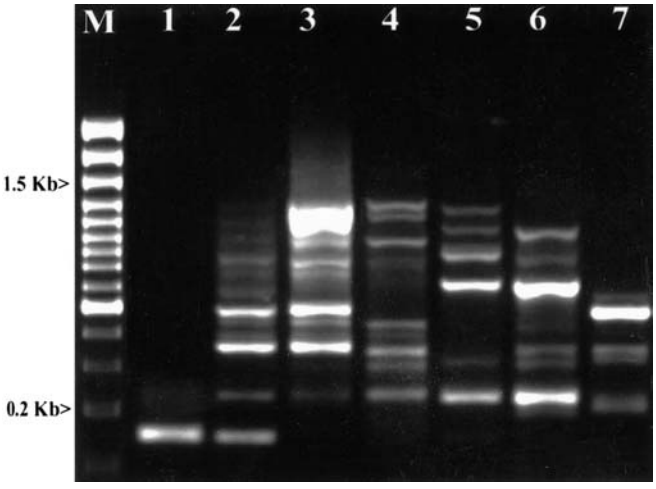

Fig. 2 RAPD analysis. *Lanes*: *M* Marker, *1 B. rapa*; *2* the F_1 hybrid (*E. cardaminoides* × *B. rapa*), *3* amphiploid, *4 E. cardaminoides*, *5* F_1 hybrid (*E. cardaminoides* × *B. nigra*), *6* amphiploid, *7 B. nigra*

four of which germinated to produce three hybrid seedlings. It was apparent that the excision and reculturing of the enlarged ovules on a fresh medium resulted in an improved rate of recovery of hybrid seedlings. The plantlets obtained from the cultured ovules were multiplied through in vitro shoot-tip culture and the culture of nodal segments and finally transferred to the field.

Both hybrids (*E. cardaminoides* × *B. rapa* and *E. cardaminoides* × *B. nigra*) were morphologically intermediate between their respective parents, especially with respect to leaf morphology (Fig. 1a), and were slow-growing. *E. cardaminoides* plants are characterized by dark-green, non-clasping, small sessile leaves, a gluscent stem, compact inflorescences and pale-yellow flowers. *B. rapa* plants have a pale-green, non-gluscent stem, thin petiolate leaves, loose inflorescences and deep-yellow flowers. *B. nigra* has deep-green, non clasping leaves with medium serration and pigmented veins. The hybrid, *E. cardaminoides* × *B. rapa*, possessed small, almost entire, thin, pale-green and semi-clasping leaves. Its flowers were small and bright yellow with reduced anthers and semi-sterile (38%) pollen grains. In contrast, the induced amphiploid plant showed vigorous growth, a larger bud size and flowers with normal anthers and fertile (75%) pollen grains (Fig. 1b, d, e). The leaves were bigger and serrated. The hybrid plants between *E. cardaminoides* × *B. nigra* had pale-green, fleshy, non-clasping leaves, with pale-yellow flowers with small anthers that had a with low pollen grain stainability (32%). The amphiploid plants on the other hand had thicker, deeply serrated, semi-clasping leaves with normal flowers and improved (67%) pollen grain stainability (Fig. 1c–e). Both the hybrid and the synthetic amphiploid, *E. cardaminoides* × *B. nigra*, were relatively more tolerant to alternaria blight than the cultivated *B. rapa*, as indicated by slow blighting.

Establishment of hybridity using RAPD markers

RAPD analyses of genomic DNA were carried out to establish the hybrid nature of the F_1s and their amphiploids using 10-mer arbitrary primers. The primer OPV 07 generated polymorphic bands between *E. cardaminoides* and *B. rapa*, whereas OPA 16 showed polymorphism between *E. cardaminoides* and *B. nigra* (Fig. 2). The hybrids revealed bands specific to the wild parent, *E. cardaminoides*, as well as *B. rapa* or *B. nigra* (Fig. 2).

Intergenomic affinity

Cytological investigations of the intergeneric hybrids and the induced amphiploids were carried out to establish the extent of genomic relatedness between the E^{cd} and A/B genomes.

E. cardaminoides x B. rapa

The somatic chromosome number of the parental species—*E. cardaminoides* and *B. rapa*—was 18 and 20, respectively with regular bivalent formation. The F_1 hybrid and the induced amphiploid harboured, as expected, $2n$=19 and $2n$=38 chromosomes respectively. Meiotic investigations (Table 1, Fig. 3a-f) in the F_1 revealed the occurrence of 19 univalents in 22% of the PMCs sampled. The maximum number of bivalents—five— was observed in 5% of the PMCs, whereas four bivalents were recorded in 16% of the PMCs. Meiotic configurations of 13 I+3 II and 15 I+2 II occurred in nearly 30% of the PMCs. Bivalents, in general were monochiasmatic and rod-shaped. The mean bivalent frequency was 1.87 (Table 1). Chromosome separation during anaphase was irregular, with frequent anaphase bridges (Fig. 3g). Induced amphiploid ($2n$=38) showed 19 II (Fig. 3h) as the most predominant meiotic configuration (Table 2), although

Table 1 Pollen fertility and chromosome pairing in the intergeneric hybrid, *Erucastrum cardaminoides* × *Brassica rapa* (*PMC* post-meiotic cell)

Somatic chromosome number	Pollen fertility (%)	Number of PMCs observed	Meiotic configuration						
			19 I	1 II+17 I	2 II+15 I	3 II+13 I	4 II+11 I	5 II+9 I	Mean bivalent frequency
19	38.0	102	22 (0.22)[a]	29 (0.28)	17 (0.17)	13 (0.13)	16 (0.16)	5 (0.05)	1.87

[a] Values in parenthesis are percentage frequencies

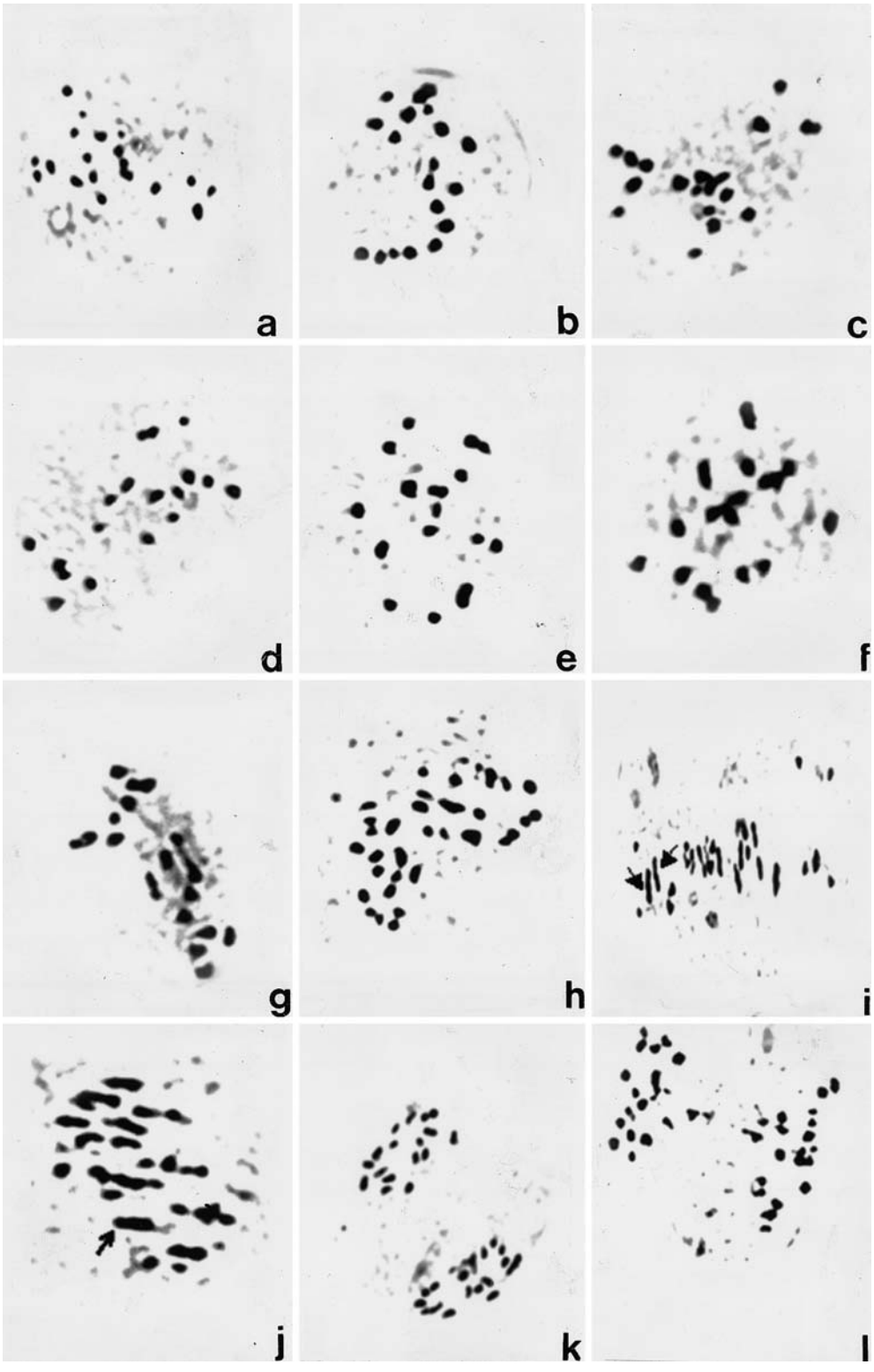

Fig. 3 Meiotic studies in the F_1 hybrid *E. cardaminoides* × *B. rapa* (**a–g**) and the induced amphiploid (**h–l**). **a** 19 I, **b** 17 I+1 II, **c** 15 I+2 II, **d** 13 I+3 II, **e** 11 I+4 II, **f** 9 I+5 II, **g** anaphase bridges, **h** 19 II, **i** 16 II+2I II, **j** 17 II+1 IV, **k** 19-19 distribution at anaphase, **l** 18-20 distribution anaphase

Table 2 Pollen fertility and chromosome pairing in an amphiploid between *E. cardaminoides* × *B. rapa* (*PMC* post-meiotic cell)

Somatic chromosome number	Pollen fertility (%)	Number of PMCs observed	Meiotic configuration					
			19 II	17 II+1 IV	16 II+2 III	Mean bivalent frequency	Mean trivalent frequency	Mean quadrivalent frequency
38	75.0	45	40 (0.89)[a]	2 (0.05)	3 (0.07)	18.7	0.13	0.04

[a] Values in parenthesis are percentage frequencies

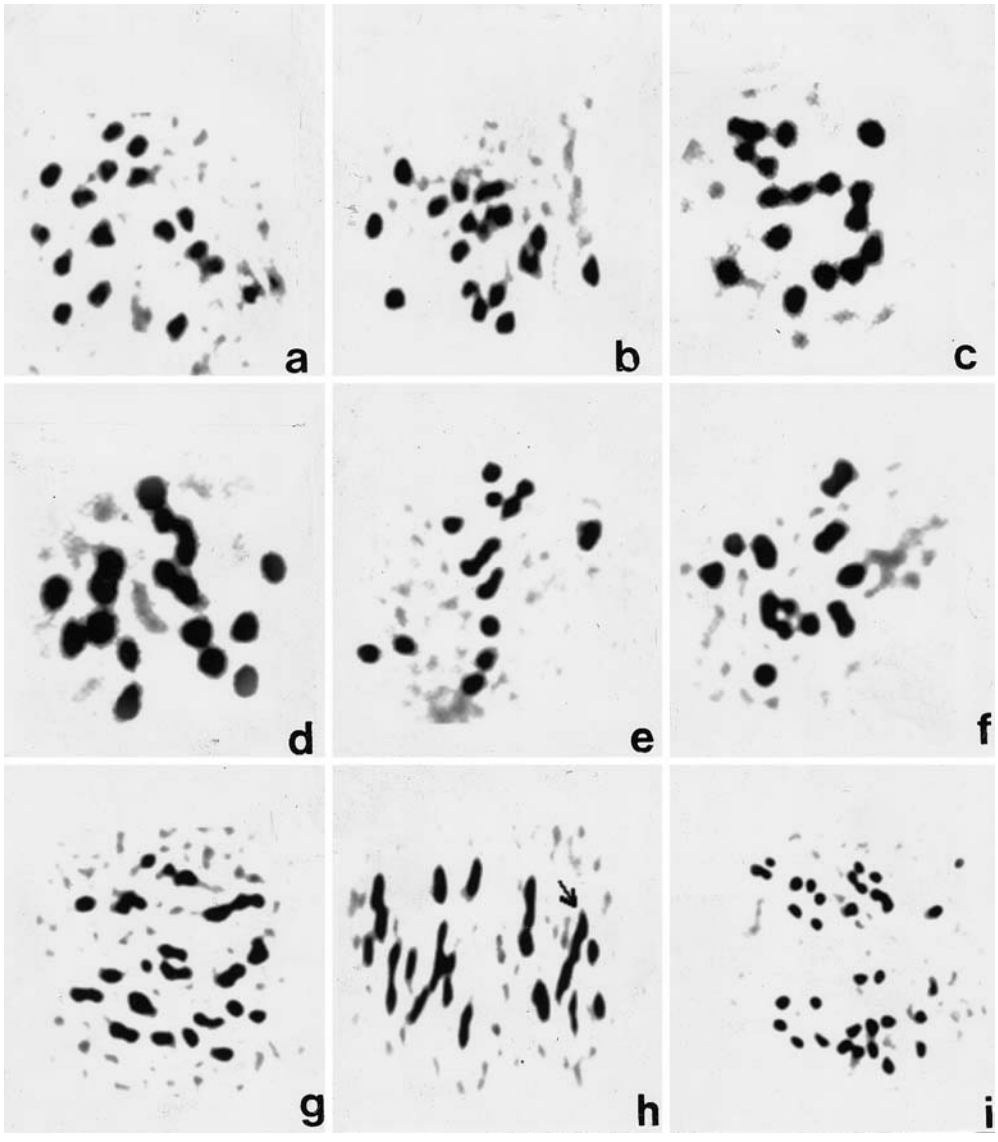

Fig. 4 Meiotic studies in the F_2 hybrid *E. cardaminoides* × *B. nigra* (**a–f**) and the induced amphiploid (**g–i**). **a** 17 I, **b** 15 I+1 II, **c** 13 I+2 II, **d** 11 I+3 II, **e** 9 I+4 II, **f** 7 I+5 II, **g** 17 II, **h** 15 II+1I V, **i** 17-17 anaphase distribution

Table 3 Pollen fertility and chromosome pairing in the intergeneric hybrid, *E. cardaminoides* × *B. nigra* (*PMC* post-meiotic cell)

Somatic chromosome number	Pollen fertility (%)	Number of PMCs observed	Meiotic configuration						
			17 I	1 II+15 I	2 II+13 I	3 II+11 I	4 II+9 I	5 II+7 I	Mean bivalent frequency
17	32.0	112	26 (0.23)[a]	21 (0.19)	36 (0.32)	16 (0.14)	7 (0.06)	6 (0.05)	1.78

[a] Values in parenthesis are percentage frequencies

two trivalents or one quadrivalent (Fig. 3i, j) were also observed in varying frequencies. Anaphase distribution was mostly normal (Fig. 3k), but irregular distribution (Fig. 3l) was also seen.

E. cardaminoides × *B. nigra*

The cytological data is presented in Table 3 and Fig. 4a-i. The most prevalent meiotic configuration in the F_1 hybrid ($2n$=17) was 13 I+2 II, which occurred in about 32% of the cells. This was followed by 17 I (23%) and 15 I+1 II (19%). PMCs with three, four or five bivalents, respectively, were also seen at varying frequencies. Mean bivalent frequency was 1.78. The induced amphiploid ($2n$=34) generally showed 17 II (Table 4, Fig. 4g) or a quadrivalent (Fig. 4h). The mean quadrivalent frequency was 0.08 and the mean bivalent frequency was 16.8. Although the majority of anaphase 1 cells showed a normal 17–17 distribution of chromosomes (Fig. 4i), there

Table 4 Pollen fertility and chromosome pairing in an amphiploid between *E. cardaminoides* × *B. nigra* (*PMC* post-meiotic cell)

Somatic chromosome number	Pollen fertility (%)	Number of PMCs observed	Meiotic configuration			
			17 II	15 II+1 IV	Mean bivalent frequency	Mean quadrivalent frequency
34	67	52	48 (0.92)[a]	4 (0.08)	16.8	0.08

[a] Values in parenthesis are percentage frequencies

was evidence of a precocious separation of chromosomes at the completion of metaphase. Despite regular bivalent formation and good pollen grain fertility (67%), the amphiploid showed poor seed set on selfing and crossing with crop brassica species.

Discussion

Among the members of subtribe *Brassicineae*, genus *Erucastrum* has been postulated to be closest to and capable of gene exchange with the *Brassicas*. This became apparent from the demonstration of homology between the A/C *Brassica* genomes with the E^c genome of *E. canariense* (Harberd and McArthur 1980; Bhaskar et al. 2002) and with the E^a genome of *E. abyssinicum* (Harberd and McArthur 1980). To further enlarge the scope of gene introgression from *Erucastrum*, we synthesized two intergeneric hybrids between *B. rapa*/*B. nigra* and *E. cardaminoides*. The hybrids had poor pollen and seed fertility and were intermediate between the parents with respect to key morphological traits. Cytological analysis revealed up to five bivalents in the hybrids. However, in the absence of preferential pairing, the bivalents may not be a true reflection of the allosyndetic pairing between the two well-differentiated genomes (A vs. E^{cd} and B vs. E^{cd}). Moreover, at least two bivalents each are expected due to intra-genome duplications in A or B genomes alone (Röbbelen 1966; Truco et al. 1996). Autopairs are also likely in the E^{cd} genome, as *Brassicaceae* members are considered to be paleoploids with a basic chromosome number of x=5 or 6 (Quiros 1999). Despite the inherent duplications, allosyndetic pairing between the A/B and E^{cd} genomes was indicated by the frequent occurrence of anaphase bridges, which are the manifestation of genetic exchange in the region of homology between structurally differentiated genomes. In addition to bridges and laggards, the anaphase chromosome distribution was also irregular. The induction of amphiploidy restored chromosomal balance and pollen grain fertility in both combinations, although seed fertility did remain low. The amphiploid of *E. cardaminoides* × *B. nigra* was more vigorous than that of *E. cardaminoides* × *B. rapa*. Recognition of at least one quadrivalent or two trivalents in the induced amphiploids provided a realistic test of genomic affinity as it allowed a good measure of segment-by-segment homology and possibly of nucleotide sequence homology along the length of paired chromosome. A high fidelity of homologous recombination results specifically from the involvement of perfectly homologous sequences of significant length in effective recombination. Gene targeting experiments in mice have shown that as low as 0.1–0.5% sequence divergence is sufficient to act as a recombination barrier (Radman and Wagner 1993). The demonstration of partial genome homologies between *Erucastrum* and *Brassica* was confirmative of the species classification based on conventional taxonomy where both *Erucastrum* and *Brassica* have been grouped together. That both the A and B genomes were partially homoeologous with the E^{cd} genome appeared interesting due to the established evolutionary diversion between the A and B genomes (Quiros 1999). Successful synthesis of two intergeneric hybrids between *E. cardaminoides* × *B. rapa* and *E. cardaminoides* × *B. nigra* has now opened up the possibility of genetic enrichment of crop *Brassica* species through gene introgression from this genera. In addition, the hybrids synthesized in the present study are being utilized for the development of a new CMS-fertility system.

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