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Synthesis of intergeneric hybrids and establishment of genomic affinity between *Diplotaxis catholica* and crop *Brassica* species

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Abstract Intergeneric hybrids of the wild crucifer *Diplotaxis catholica* (2n = 18, D^CD^C) as female with two crop *Brassica* species, namely *Brassica rapa* (2n = 20; AA) and *Brassica juncea* (2n = 36; AABB) as male, were developed, using ovary and sequential culture. Reciprocal crosses were not successful, suggesting unilateral cross incompatibility. Morphologically, the hybrid plants resembled the crop brassica parents, but were nearly male- as well as female-sterile. Induction of amphiploidy helped to improve pollen fertility for the *D. catholica* × *B. rapa* cross (73%), but less so for the *D. catholica* × *B. juncea* cross (35–40%). Female fertility was also higher in both the amphiploids. Cytological analysis of the F_1 hybrids revealed aberrant meiosis with predominant occurrence of the univalents. Partial genomic homoeology between the A genome of *B. rapa* and the D^C genome of *D. catholica* was indicated by the presence of up to five bivalents in 14.7% of the PMCs in the *D. catholica* × *B. rapa* hybrid, and 1–2 trivalents or a quadrivalent in nearly 44% of the PMCs in the derived amphiploid. In the second cross, *D. catholica* × *B. juncea*, up to six bivalents and one trivalent were observed indicating homoeology between the A/B genomes of *B. juncea* and the D^C genome of *D. catholica*. The possibility of introgression of desirable genes from *D. catholica* into crop *Brassica* species exists in view of significant affinity between the D^C and A/B genomes.

Keywords *Brassica rapa* · *Brassica juncea* · *Diplotaxis catholica* · Intergeneric hybrid · Genomic affinity

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Introduction

Attempts at distant hybridization in the Brassicaceae date back to the synthesis of intergeneric hybrids between *Raphanus sativus* and *Brassica oleracea* (Sageret 1826; Karpechenko 1924). During this earlier phase, the recovery of many wide hybrids was limited due to the failure of fertilization and/or faulty endosperm development. Introduction of embryo-rescue techniques helped to significantly enhance the pace and scope of wide hybridization (Shivanna 1996). Bringing together well-differentiated genomes, and the subsequent occurrence of homoeologous pairing, have now allowed *Brassica* breeders the access to a range of potentially beneficial nuclear/cytoplasmic-encoded traits. Notable examples are the introduction of the beet cyst nematode resistance from *R. sativus* to *Brassica napus* (Lelivelt and Krens 1992); the *alternaria* leaf spot resistance from *Sinapis alba* to *B. napus* (Primard et al. 1988), and a number of male-sterility inducing cytoplasms and fertility restorer genes from wild to crop brassica species (Prakash 2001).

The genus *Diplotaxis*, mainly distributed in central Europe and the Mediterranean region, is one of the nearest wild relatives of *Brassica*. It is capable of experimental hybridization with *Brassica* species, and its potential as a germplasm resource for both nuclear and cytoplasmic characteristics is well documented (Warwick et al. 2000). *Diplotaxis* has 31 different species of which *Diplotaxis catholica* is considered to be resistant to white rust and blackleg disease. It has also been reported to be a source of cytoplasmic male sterility for *Brassica juncea* (Mohapatra et al. 1998). Genetic homoeology between *D. catholica* and *B. juncea* genomes has been investigated (Kirti et al. 1995). However, the reported cross involved one monogenomic (*D. catholica*) and another digenomic (*B. juncea*) species, and hence the extent of homoeology between individual *juncea* genomes (A or B) and the *catholica* (D^C) genome could not be evaluated sufficiently due to the presence of three different genomes in the F_1 hybrid.

The work reported here is concerned with the development of intergeneric hybrids between *D. catholica* (2n = 18; D^CD^C) and two *Brassica* species namely *Brassica rapa* (2n=20; AA) and *B. juncea* (AABB; 2n = 36), and establishment of homoeology between the D^C and A/B genomes.

Materials and methods

Field grown plants of *B. rapa* L. ssp. *oleifera* cv TL-15 (2n = 20, AA), *B. juncea* (L.) Czern et Coss. cv Varuna (2n = 36, AABB) and *D. catholica* (L.) DC (2n = 18, D^CD^C) were used for making reciprocal crosses. Flower buds of selected female parent were emasculated and pollinated with freshly collected pollen from the male parent. Some of the pollinated buds were left on the plant and others were excised and used for in vitro experiments. For ovary culture, pistils were excised 2–3 days after pollination (DAP), surface sterilized with mercuric chloride (0.01%) and cultured on 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). The seeds were excised aseptically, from 20 to 25 day-old cultured ovaries and cultured on the MS medium. For sequential culture, pollinated ovaries 8–9 days after initial culture were dissected and ovules showing enlargement were re-cultured on fresh MS medium. Shoot tips from hybrid seedlings were multiplied on MS medium supplemented with benzyl amino purine (BAP) at 0.5 mg/l. The axillary shoots were then rooted on half MS medium and transferred to field after 7–10 days of hardening under controlled environmental conditions. Flower buds of F_1 hybrids and amphiploid plants were fixed in Carnoy's solution and stained with 2% acetocarmine for cytological investigations.

Results and discussion

Ovary and sequential culture was used to rescue the young embryos, as field pollinations failed to yield any hybrid seed in both the combinations. Out of 263 cultured ovaries in the cross *D. catholica* × *B. rapa*, only eight well-developed ovules were obtained which gave rise to two successful hybrid seedlings. Similarly in the second cross, *D. catholica* × *B. juncea*, the culturing of 213 ovaries produced five enlarged ovules giving rise to only three successful hybrid seedlings. Young hybrid seedlings were multiplied from shoot segments on MS supplemented with BAP (0.5 mg/l). Hybrid seeds were obtained only when *D. catholica* was used as the female parent.

Hybrid and amphiploid plants resembled the cultivated male parents with regard to plant morphology. Leaf structure was intermediate but leaf serration was more like *D. catholica*. Flowers of F_1 hybrid plants were small, and contained shrivelled anthers having sterile pollen grains of variable size. Pollen fertility in *D. catholica* × *B. rapa* and *D. catholica* × *B. juncea* F_1 plants was 34% and 32% respectively. Female fertility was also low as indicated by the very low cross seed set. In contrast the amphiploid obtained from the cross between *D. catholica* × *B. rapa* produced turgid anthers and showed about 73% pollen fertility. On the other hand the cross between the *D. catholica* × *B. juncea* amphiploid had only low pollen fertility (35–40%). Under field conditions, the hybrid and the amphiploid plants were free from white rust as against the highly susceptible and moderately resistant reaction in *B. rapa* and *B. juncea* respectively. These observations could be considered as promising, justifying further work.

Meiotic behaviour

Studies of pollen mother cells (PMCs) during metaphase-I/diakinesis-I in the parental species revealed regular meiosis, which was reflected in >95% pollen fertility. The sporophytic chromosome number of parents, i.e. *D. catholica*, *B. rapa* and *B. juncea*, was confirmed to be 18, 20 and 36 respectively, and of the two derived hybrids it was 19 (*D. catholica* × *B. rapa*) and 27 (*D. catholica* × *B. juncea*). In the *D. catholica* × *B. rapa* hybrid (2n = 19, AD^C) none of the PMCs evaluated showed the expected 19 univalents. A majority of the PMCs (58.8%) had 15 I + 2 II and 13 I + 3 II meiotic configurations (Fig. 1a, b). A maximum of five bivalents were observed in 14.7% of the PMCs studied, whereas 4 II were observed in 26.4% PMCs (Fig. 1c). Mean bivalent frequency was 3.26 (Table 1). All the bivalents, however, may not be ascribed to allosyndetic pairing, since at least two autosyndetic bivalents and one trivalent are expected due to genomic duplication within the A genome (Truco et al. 1996). Though autosyndetic pairing within the D^C genome is possible, Kirti et al. (1995) did not find any pairing within the D^C genome in their somatic hybrid plant having the genomic constitution of $AABBD^C$ (2n = 45).

Meiotic analysis of the *D. catholica* × *B. juncea* hybrid (2n = 27, ABD^C) also revealed lower (14–25/PMCs) than the expected 27 univalents mainly due to the frequent occurrence of bivalents (Table 1). Mean bivalent frequency was 4.41% with 33.7% PMCs showing a 5 II + 17 I configuration (Fig. 1d, e). One trivalent was also observed in about 10% of the cells (Table 1, Fig. 1e). However, a low frequency of PMCs (15%) had upto 6 II (Fig. 1f). The occurrence of a higher bivalent frequency (4.41) in the trigenomic (ABD^C) hybrid than that (3.26) observed in the digenomic hybrid (AD^C) may be indicative of homoeology between the D^C and B genome chromosomes or of autopairing within the B genome chromosomes. However, the presence of a trivalent which was not observed in the AD^C hybrid might be due to affinity between the A, B and D^C genomes. Kirti et al. (1995) have previously observed the presence of two quadrivalents in their somatic hybrid ($AABBD^CD^C$; 2n = 54) between these two species, which reflected the intergenomic affinities between the *D. catholica* and *B. juncea* genomes. They, however, did not throw any light on the genomic specificity of *B. juncea* chromosomes having an affinity with *catholica* chromosomes.

The majority of the meiocytes (56.2%) in the digenomic amphiploid (2n = 38; AAD^CD^C) had 19 bivalents (Fig. 2a–c). This is consistent with the preferential pairing expected between duplicated chromosomes. One quadrivalent or one or two trivalents were observed in as many

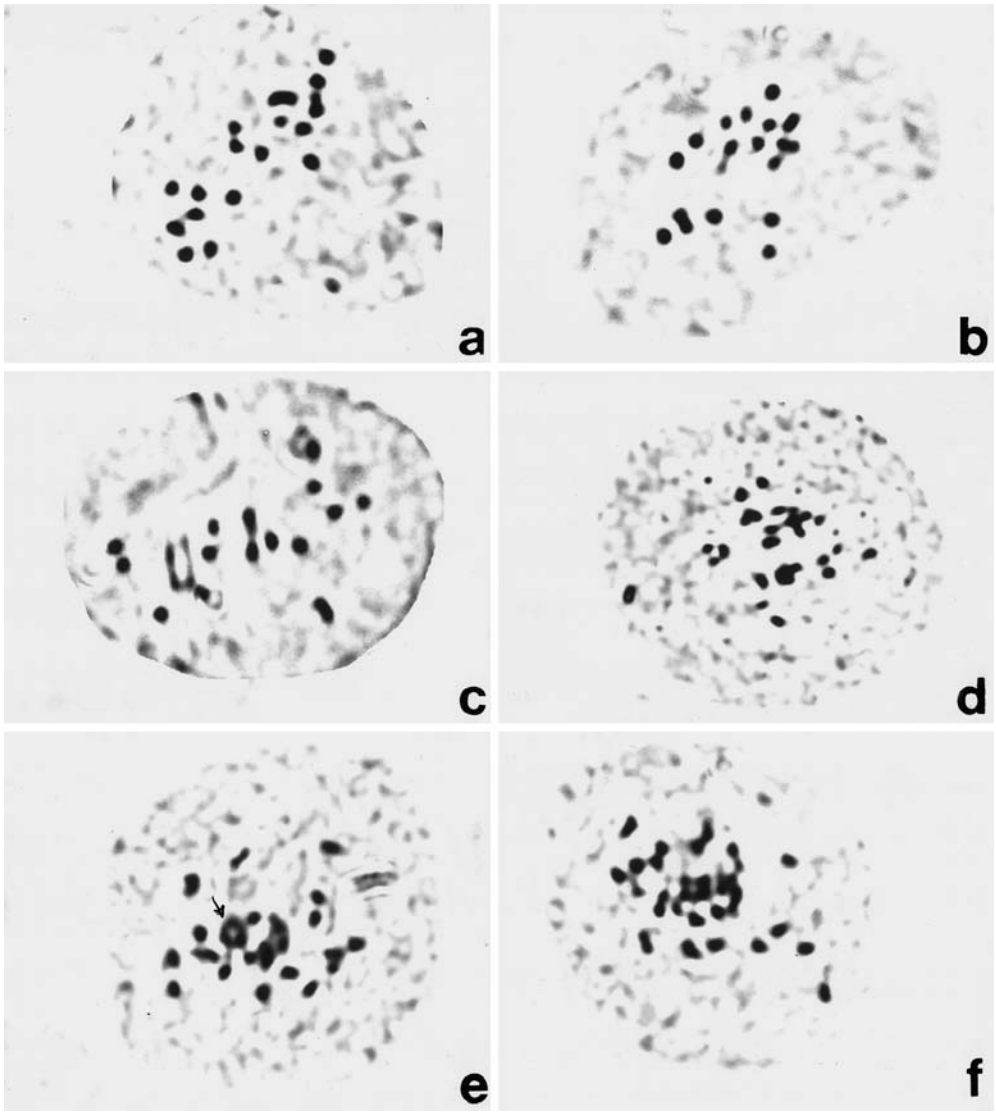

Fig. 1 Cytological analysis in the F_1 hybrids *D. catholica* × *B. rapa* (**a–c**) and *D. catholica* × *B. juncea* (**d–f**). **a** 2 II + 15 I; **b** 3 II + 13 I; **c** 4 II + 11 I; **d** 5 II + 17 I; **e** 5 II + 1 III + 14 I; **f** 6 II + 15 I

Table 1 Meiotic behaviour and pollen fertility of two hybrids and one amphiploid

Hybrid combination	Genomic complement	Somatic chr. number	PMCs studied	Mean frequency of				Pollen[a] fertility (%)
				Univalents	Bivalents	Trivalents	Quadrivalents	
D. catholica × *B. rapa* (F_1 hybrid)	AD^C	19	136	12.47 (9–15)	3.26 (2–5)	–	–	34
D. catholica × *B. rapa* (amphiploid)	AAD^CD^C	38	127	0.28 (0–1)	18.03 (16–19)	0.47 (1–2)	0.06 (0–1)	73
D. catholica × *B. juncea* (F_1 hybrid)	ABD^C	27	160	16.92 (14–25)	4.41 (1–6)	0.1 (0–1)	–	32

[a] Based on acetocarmine staining only, variable pollen size; figures in parentheses indicate range

as 6.25 and 37.47% of the cells with an overall frequency of 0.06 and 0.48 respectively, indicating a substantial homoeology between A and D^C genomes for which there is no published evidence. Inspite of the occurrence of 19 II in a majority of the PMCs, the amphiploid was not stable. Amphiploid plants had poor seed set on selfing, and on crossing with *B. rapa* and *B. juncea*. This may be due to the multivalent associations observed at diakinesis/metaphase-I leading to an unequal distribution, anaphase bridges and chromosome breakages during anaphase-I/II (Fig. 2d). Secondary association between pairs of bivalents was also observed. Attempts to induce amphiploidy in the trigenomic hybrid (ABD^C) between *D. catholica* and *B. juncea*, resulted in few sectors having normal

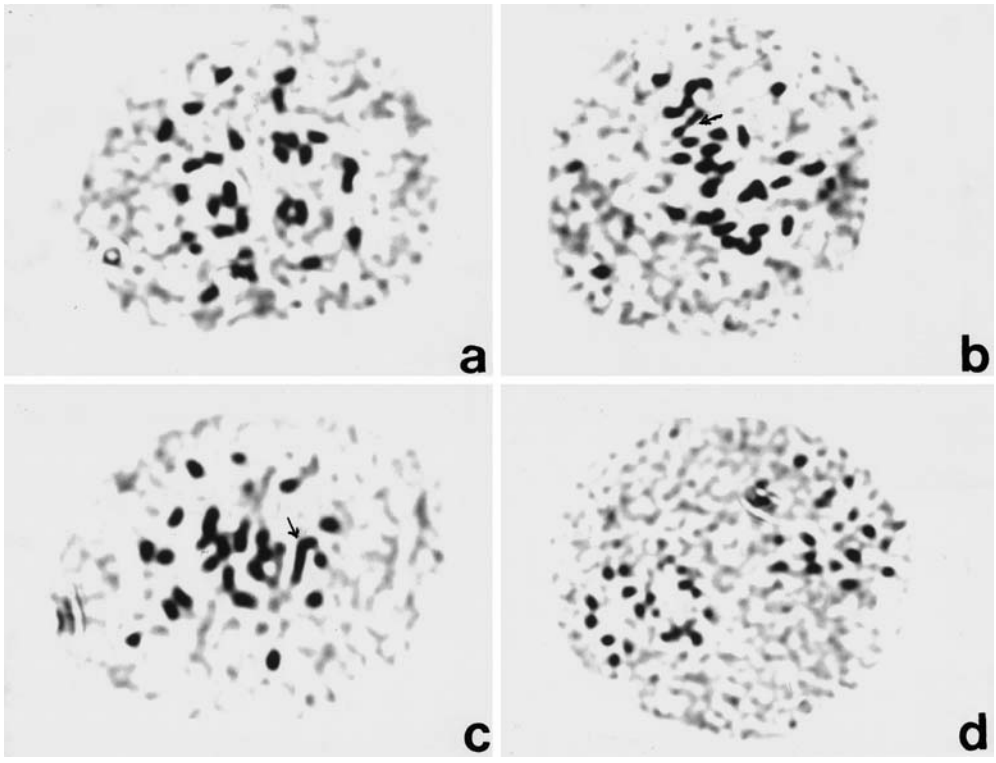

Fig. 2 Amphiploid, *D. catholica* × *B. rapa* (**a–d**). **a** 19 II, **b** 1 III + 1 I + 17 II; **c** 1 IV + 17 II; **d** 17–21 anaphase-1 distrubution. The *arrow* indicates a trivalent/quadrivalent

flowers with well-developed anthers and sporadic open-pollinated seed set. The ploidy status of these sectors could not be confirmed cytologically due to improper condensation of the chromosomes in the pollen mother cells.

Summarizing, ovary and sequential culture helped in the synthesis of the *D. catholica* × *B. rapa* and *D. catholica* × *B. juncea* hybrids. A partial homoeologous relationship of the A and B genomes with the D^C genome is proposed. The development of hybrids and the amphiploid, and the demonstration of an appreciable level of homoeology between the D^C and A/B genomes, suggests the possibility of intergenomic recombination and the introgression of desirable genes, especially for resistance to abiotic stresses, from *D. catholica* into crop brassica species.

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