Theor Appl Genet (1993) 86:567–572

THEORETICAL AND APPLIED GENETICS

Brassica carinata resynthesized by protoplast fusion*

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Received: 24 September 1992 / Accepted: 3 November 1992

Abstract. *Brassica carinata* (bbcc) was resynthesized by protoplast fusion between *B. nigra* (bb) and *B. oleracea* (cc). In two fusion experiments 64 hybrid plants were obtained and identified to be true hybrids by isoenzyme analysis, nuclear DNA content, chromosome number, and intermediate morphology. Of these plants 56% were normal amphidiploids with 2n = 34 chromosomes and a DNA content equivalent to that of natural *B. carinata*. The remaining plants were polyploid, morphologically abnormal, and infertile. The majority of the hybrids contained both chloroplasts and mitochondria from *B. nigra*, but some plants combined chloroplast and mitochondria from the different progenitors. Hybrids with a DNA content equivalent to that of *B. carinata* had a wide range of male fertility (4–98%), but consistently low female fertility. Only a few selfed seed were produced, but these germinated and grew into vigorous plants.

Key words: Amphidiplids – *Brassica carinata* – Interspecific hybridization – Protoplast fusion

Introduction

Brassica carinata Braun, the natural amphidiploid between *B. nigra* and *B. oleracea* (Prakash and Hinata 1980), is an important oilseed and vegetable crop in parts of East Africa. It is being developed as a potential oilseed crop in regions of rainfed agriculture, particularly in India, because of its tolerance of drought and resistance to pests and diseases such as *Alternaria* blight, white rust, and blackleg (Anand et al. 1985; Kolte 1985).

One approach to germ plasm enhancement in *B. carinata* is artificial resynthesis from interspecific hybridization of the progenitor species. Such synthetic plants have been produced by crossing *B. oleracea* and *B. nigra* for cytogenetic analysis (Mizushima and Katsuo 1953) and for crop improvement (Prakash et al. 1984; Sarla and Raut 1988). Somatic hybridization offers another method for resynthesis. The somatic hybrids would be instant amphidiploids that would not require chromosome doubling of either the parents before hybridization or the interspecific F_1 hybrids to obtain fertile plants. A principal objective of our research is to broaden the genetic base of amphidiploid *Brassica*. In the present article we report on the production and characterization of *B. carinata* amphidiploids obtained by protoplast fusion.

Materials and methods

Plant material, protoplast fusion, and isozyme analysis

B. oleracea ssp acephala cv 'Vates Blue Curled' scotch kale (lot no. 11102) was purchased from Harris Moran Seed Co, Rochester, N.Y. *B. nigra* PI no. 180416, an introduction from India, was obtained from the USDA North Central Region Plant Introduction Station at Ames, Iowa. *B. carinata* line Cr-GC 80 was obtained from the Crucifer Genetics Cooperative, University of Wisconsin, Madison. Protoplast isolation, fusion, and culture were as described previously (Robertson et al. 1987; Jourdan et al. 1989). Isoenzyme analysis was done on crude extracts of approximately 200 mg of leaf tissue utilizing buffers and assay systems described by Thorpe et al. (1987).

Chromosome counts and DNA quantification

Chromosomes were observed in squashes of fixed, immature anthers after staining with 2% acetocarmine. DNA quantifica-

Communicated by G. Wenzel

* Salaries and research support provided by State and Federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. Journal Article No. 296-92

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tion was performed on propidium iodide-stained nuclei obtained from immature leaves following a modified procedure of Arumuganathan and Earle (1991) with a Coulter EPICS Elite Flow cytometer equipped with an argon laser of 15 mW output at 488 nm to give a final window output of 635–725 nm. Briefly, approximately 1 g of leaf tissue was chopped in 10 ml of $MgSO_4$ buffer lacking propidium iodide (Boehringer), filtered through four layers of cheese cloth and one layer of Miracloth, and centrifuged at 1000 *g* for 10 min; the pellet was then resuspended in 1 ml of $MgSO_4$ buffer. To each sample was added 0.1 µg RNAse-A (Sigma) and 75 µg propidium iodide in 15 µl water. The samples were then analyzed in the flow cytometer. Chicken red blood cells (2.33 pg DNA/nucleus) or *B. napus* cv 'Westar' nuclei (2.4 pg DNA/nucleus) were used as standards. The coefficient of variation for replicated samples was 10–15%.

Organelle RFLP analyses

Total DNA was isolated from leaves by the method of Doyle and Dickson (1987), digested with restriction enzymes as recommended by the manufacturer (Boehringer), electrophoresed in 0.8% agarose gels, transferred to Zetabind nylon membranes as described by the manufacturer (Cuno), and hybridized to specific probes. Plasmids containing chloroplast (cp), or mitochondrial (mt) sequences were radiolabelled by random primer extension as described by the manufacturer (Boehringer). The chloroplast probe (p8a) originates from orchid cpDNA and was provided by K. Song, University of Wisconsin; the mitochondrial probes (p5.2, s10.1, p7.7) were derived from *B. rapa* mtDNA and were provided by C. Makaroff, Miami University, Oxford, Ohio.

Results

Characterization of somatic hybrids

Two fusion experiments were carried out: one used green cotyledons of *B. oleracea* and etiolated hypocotyls of *B. nigra*; the other used green leaf tissue of both species. In each experiment the frequency of fusion products was 5–10%. Over 2000 small calluses were obtained after fusion; from these, 96 plants were regenerated. All plants contained abundant trichomes, a characteristic of *B. nigra*. No kale-like plants were obtained, which indicated that iodoacetate was very effective in inactivating the *B. oleracea* protoplasts. *B. nigra* plants are only rarely regenerated from protoplasts in our culture system, and 30% of the plantlets can be rooted. Among the 75 regenerated plants containing trichomes that could be transferred to soil, 64 were tentatively identified as being possible somatic hybrids by their vigorous growth, dark leaf color and ease of rooting (in contrast to the light-green color and difficulty of rooting that is typical of *B. nigra*).

Morphology

The kale plants used in these experiments had deeply ruffled leaves and were morphologically uniform (Fig. 1, *cc*). The *B. nigra* population was heterogeneous for both leaf shape (Fig. 1, *bb*) and stature, varying from 10–30 cm in length at flowering. The hybrids displayed a morphology generally intermediate to that of the parents. They were characterized by an erect habit with distinct internodes, dark blue-green leaves, frequent purple pigmentation along the midveins and nodes, and the presence of trichomes throughout the plant. However, the population was heterogeneous and consisted of three distinct morphotypes: (a) 6 plants (9%) with a rather bushy appearance, short stature, small leaves (Fig. 1, $bbcc_2$) and racemes, and a lightgreen pigmentation on the leaves and stems; (b) 30 plants (47%) with an erect habit, high stature, intermediately ruffled leaves (Fig. 1, $bbcc_1$, larger racemes with symmetrical flowers that displayed the typical *Brassicaceae* arrangement, and deep blue-green leaves with purple coloration on the veins and nodes; (c) 28 plants (44%) with a variable habit but distinguished by thick, rugose, waxy and irregularly-shaped leaves (Fig. 1 bc_p), short and thickened racemes that frequently aborted, and deep blue-green pigmentation with irregular purple coloration on the midveins and nodes. The first two plant types paralleled the general morphology of the parents and can be considered to be 'normal', whereas the latter group had noticeable 'abnormal' characteristics. Additional characterization was subsequently done primarily on plants of the first two groups.

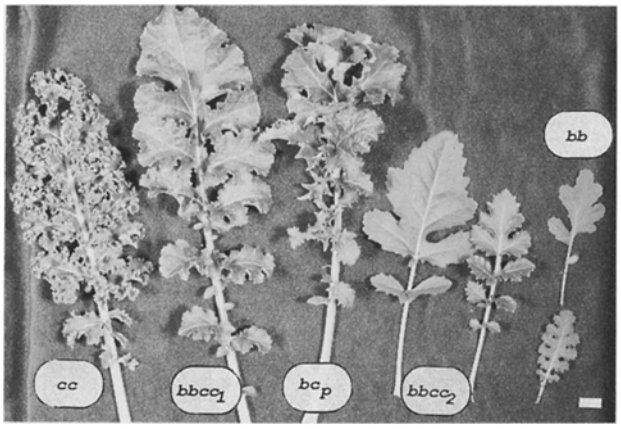

Fig. 1. Leaf morphology of synthetic *B. carinata* and its progenitors. *bb B. nigra* PI no. 180416 regenerated from protoplasts, *cc B. oleracea* ssp *acephala* grown from seed, $bbcc_1$ normal, large somatic hybrids, $bbcc_2$ normal, petite somatic hybrids, bc_p abnormal, polyploid somatic hybrids. *Bar*: 1 cm

Isoenzymes

Analysis of the dimeric enzyme, glucose phosphate isomerase (GPI, EC 5.3.1.9), confirmed the morphological data that all 64 regenerated plants were hybrids. Although the hybrids varied in the alleles for *gpi-2*, all plants displayed a zymogram distinct from that of the parents (Fig. 2). The *gpi-1* locus codes for the plastidic form of GPI and has no polymorphisms in our preparations. In contrast, the cytosolic form encoded by the *gpi-2* locus may have up to six alleles (Chevre et al. 1990), which we labelled *a-f* (from the most anodal to the most cathodal form) for ease of identification. The variability in *gpi-2* isoenzymes among the hybrids probably originates from heterogeneity in the original parent population. A survey of 24 random plants from each of the parental lines showed that the kale plants segregated 11*a*:12*abd*:1*d* for *gpi-2* alleles, whereas the *B. nigra* plants were all uniform for allele *f*. Isoenzymes of shikimate

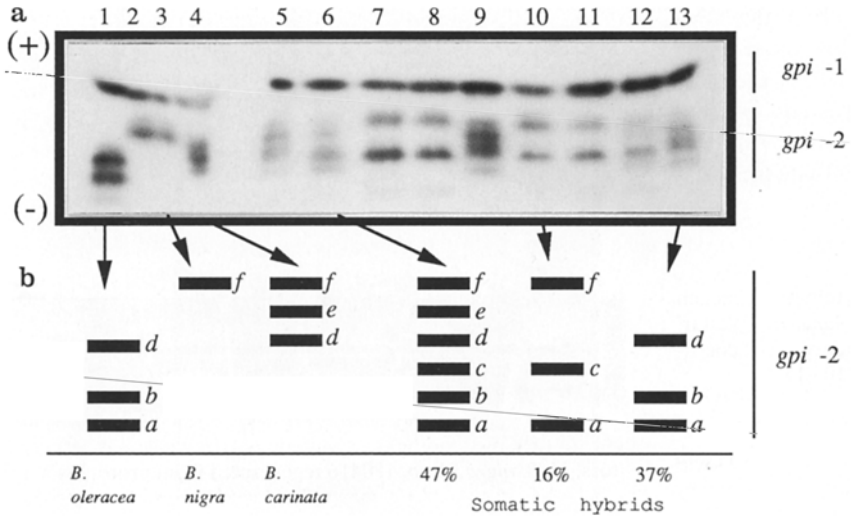

Fig. 2 A, B. Glucose phosphate isomerase isoenzymes in parents and somatic hybrids. **A** Starch gel electrophoretic pattern. *Lane 1 B. oleracea* parent, *lanes 2 and 3 B. nigra* parent, *lane 4* natural *B. carinata* CrGC80, *lanes 5–13* somatic hybrids. **B** Interpretive diagram for *gpi-2*. *Italicized* letters represent individual alleles; three general patterns were recognized for the somatic hybrids, and the distribution of each in the population is given in percentages

Table 1. Selected characteristics of parent, somatic hybrids, and natural *B. carinata*

Characteristic	*B. oleracea* VATES kale	*B. nigra* PI#180416	Somatic hybrids	*B. carinata* CrGC80
Chromosome number (n)	9	8	17–28	17
DNA content (pg/nucleus)	1.19	0.98	2.40–3.83	2.48
Trichomes[a]	+	+++	++	++
Days to flower[b]	>250	30–40	40–80	nd
Plant stature at flowering (cm)	~130	10–30	25–180	~50
Pollen stainability (percentage)	94	93	80–98 (7, 29%)[f] 40–80 (7, 29%) 4–40 (10, 42%)	95
Number of ovules/pistil[c]	25–32	6–8	13–16	14–15
Seed set[d]	+++	+++	+	+++
Organelle composition[e] (cp/mt)	ole/ole	nig/nig	ole/ole (3, 12.5%)[f] nig/nig (15, 62.5%) ole/nig (3, 12.5%) nig/ole (2, 6.3%) nig/novel (1, 4.2%)	nig/nig

nd, Not determined
[a] +, Presence of few trichomes only at seedling stage; ++, presence of few trichomes throughout the plant; +++, abundant trichomes throughout the plant
[b] Determined without vernalization and under 16-h photoperiod
[c] Evaluated in 20–35 pistils per plant
[d] +++, Seed set per fruit approximates the number of ovules per silique; +, <1 seed per silique
[e] Based on organelle (*cp* chloroplast; *mt* mitochondria) RFLP analyses; ole, *B. oleracea* pattern; nig, *B. nigra* pattern, novel, pattern distinguishable from either parent
[f] (Number of individuals; percentage of the population)

dehydrogenase and phosphoglucomutase also confirmed the hybridity of the plants.

Chromosome numbers and nuclear DNA content

Somatic hybrids of 'normal' morphology had 17 haploid chromosomes, whereas hybrids of 'abnormal' morphology had 20 or more chromosomes (Table 1). The normal hybrids are, therefore, true amphidiploids with the expected 2n=34 chromosomes characteristic of *B. carinata*. Examination of the nuclear DNA content of selected hybrids by flow cytometry confirmed that the normal hybrids generally have a DNA content equivalent to that of natural *B. carinata* (±15%) while the abnormal hybrids have up to twice the DNA content of the natural species (3.83 to ca. 5 pg DNA/nucleus). Thus, the abnor-

mal hybrids appear to be of higher ploidy than the natural *B. carinata*. Some of these plants are most likely aneuploids, but we could not ascertain whether the extra chromosomes originate from multiple fusions or from karyological abnormalities that may arise during the callus phase nor can we determine if they originate preferentially from one progenitor species.

Flowering and pollen viability

The kale cultivar had an absolute requirement for vernalization, whereas the *B. nigra* line flowered within 40 days of being transplanted to soil without a chilling treatment. None of the hybrids required vernalization for flowering. Most of the polyploid hybrids produced flowers much later than the normal plants (Table 1). The higher ploidy hybrids produced large pollen grains of low viability (<50%; Table 1) that were not readily shed. Although the somatic amphidiploids generally produced pollen of an appearance similar to that of natural *B. carinata*, there was a significant range in viability (4–98%) among the former population. Nevertheless, all hybrids with a pollen viability greater than 80% were normal in appearance. Pollen viability, however, does not necessarily correlate with male fertility. Pollen from the somatic hybrids was ineffective in producing seed either on the other somatic hybrids or on *B. carinata* CrGC80.

Seed set

Female fertility in all of the hybrids was low, as reflected by very poor seed set after selfings, crosses among hybrids, and crosses with natural *B. carinata*. We were unable to obtain any seed in the higher ploidy hybrids. Seed production in the remaining hybrids was variable. In general, self-pollinations were unsuccessful with either open flowers or buds. Ten hybrids initially developed normal siliques after selfing and crosses with CrGC80, but later most of these siliques ceased development before reaching maturity; only occasionally did they produce seeds, and these were mainly shrivelled. After more than 500 pollinations, 23 seeds were produced on 1 plant (3 from selfings and 20 from crosses). Examination of 35 siliques 20 days after pollination with CrGC80 revealed the presence of 42 aborted seeds (shrivelled, brown seed coat) and 3 plump seeds containing normal embryos at the 'walking stick' phase.

Organelle composition

Characterization of the chloroplast and mitochondrial composition of 24 somatic hybrids utiizing one cpDNA-specific probe and three mtDNA-specific probes revealed that most plants inherited the *B. nigra* complement of organelles (Fig. 3; Table 1). However, 3 plants inherited the *B. oleracea* chloroplasts and mitochondria while 5 plants had chloroplasts from one parent and mitochondria from another.

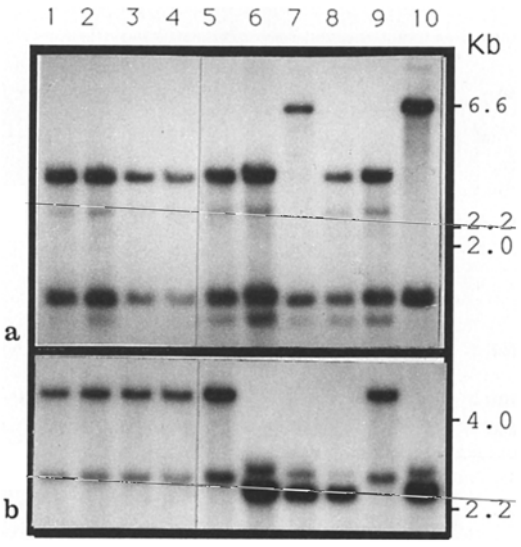

Fig. 3A, B. Organelle RFLPs detected in total DNA samples digested with *Eco*RI and hybridized with chloroplast-specific probe p8a (**A**) and mitochondria-specific probe p 5.2 (**B**). *Lanes 1–8* somatic hybrids, *lane 9 B. nigra*, *lane 10 B. oleracea*. *Numbers at right* indicate molecular weight sizes in kilobase pairs (kb)

Discussion

We have demonstrated that *B. carinata* can be synthesized by somatic hybridization of the progenitor species and have shown that protoplast fusion can be used to generate cytoplasmic and nuclear variability for possible improvement of this crop. All of the three major amphidiploids of *Brassica* have now been synthesized by protoplast fusion (*B. napus*: Sundberg and Glimelius 1986; Jourdan et al. 1989; etc; *B. juncea*: Campbell et al. 1990). The *Brassica* group thus provides one of the best examples of the application of somatic hybridization in germ plasm enhancement.

The somatic resynthesis of *B. carinata* did not result in a uniform population of hybrids. Some of the variability observed among the hybrids probably depends as much on pre-existing heterogeneity within the population of seedlings (and protoplasts) in the fusion partners (e.g., GPI isozyme patterns in *B. oleracea* and leaf morphology in *B. nigra*) as on culture-induced changes (e.g., somaclonal variation and possible karyological changes). Pre-existing heterogeneity is likely to be more important when wild species are used as one of the hybridization parents because of plant-to-plant variability in the population. A majority (56%) of the somatic hybrids display a blend of characteristics derived from each of the parents. The *B. nigra* characteristics of annual habit, abundance of trichomes, and purple pigmentation appear to be dominant or semidominant over the biennial habit, general absence of trichomes, and lack of purple pigmentation of *B. oleracea* since none of the hybrids required vernalization for induction of flowering, all contained

trichomes, and most had extensive purple pigmentation. These plants represent the only potentially useful material for further breeding because of balanced chromosomes and some fertility.

Reduced fertility is not an uncommon feature of resynthesized amphidiploids obtained by both somatic and sexual hybridization (Kräling 1987; Sundberg et al. 1987; Rosén et al. 1988) and has been primarily attributed to meiotic irregularities. However, in somatic hybrids additional factors (mutations?) introduced by the cell culture process may play a significant role. Self-incompatibility has also been implicated (Rosén et al. 1987), but this aspect has only recently been examined in some detail in synthetic *B. napus* where self-incompatible plants were obtained after somatic hybridization of the progenitors (Ozminkowski and Jourdan, 1993). The parental genotypes used for hybridization are the key determinants of fertility in resynthesized amphidiploids. The extremely low fertility of our synthetic *B. carinata* probably arises from incongruity, a condition whereby well-regulated physiological and biochemical interactions fail to function in a coordinated way because of non-matching information between the genomes of two species brought together by hybridization (Hogenboom 1984). Among the many possible uncoordinated interactions are meiotic irregularities. These irregularities may arise from the wide divergence between the two progenitor species. Phylogenetic studies of *Brassica* suggest that *B. nigra* and *B. oleracea*/*B. rapa* are part of two divergent evolutionary lineages (Warwick and Black 1991) so their combination into *B. carinata* is likely to cause disturbances in fertility. The comparatively higher fertility typically associated with newly synthesized *B. napus* is consistent with the closer evolutionary relationship between *B. oleracea* and *B. rapa*.

At the level of cytoplasmic traits, *B. nigra* organelles predominate; the majority of hybrids inherited both chloroplasts and mitochondria from this parent. However, some of the plants contain combinations of *nig* chloroplasts and *ole* mitochondria and vice-versa; these combinations are not possible through sexual hybridization. Because we have only examined a small portion of the mtDNA, we cannot preclude the possibility of recombination between the genomes. This type of recombination has been variable, from quite frequent (Jourdan et al. 1989) to absent (Kemble and Barsby 1988). This discrepancy may be due to physiological, genotypical and technical factors that are not known at present.

The transmission of organelles during vegetative development following protoplast fusion is also not clearly understood. Both the biased and non-biased assortment of organelles have been observed in somatic hybrids (Kemble and Barsby 1988; Landgren and Glimelius 1990; Ozminkowski 1992). However, because these studies have usually dealt with small populations of hybrids and with important differences in procedure, it is not yet possible to separate with confidence the biological from the technical factors that influence organelle sorting out. For example, different cell types (leaf, hypocotyl, cotyledon, cell culture), pretreatments (iodoacetate, irradiation), fusion procedures (PEG, electrical current), and culture methods (selective and non-selective conditions) have been employed. Within a given experimental system where no selectable markers are used, it appears that general biological factors (e.g., ploidy level and genetic divergence) may play a more important role in determining the composition of organelles in the regenerated plants (Sundberg and Glimelius 1991).

Because so few sexual hybrids have been described in the literature only limited comparisons between somatic and sexually synthesized *B. carinata* are possible. Sarla and Raut (1988) described two hybrids obtained by ovary culture. Chromosome doubling by colchicine application was successful in only one hybrid. Though pollen stainability with acetocarmine was only 36.5%, the plant produced two seeds per pod. The higher female fertility of this hybrid may be due to greater compatibility between the two genotypes used for hybridization.

Acknowledgements. We thank K. Song and C. Makaroff for the kind gift of cloned organelle DNA, H. Laufman for assistance with flow cytometry, and S. Swain for care of the plants in the greenhouse.

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