

RESEARCH NOTE

Resynthesized *Brassica juncea* lines with novel organellar genome constitution obtained through protoplast fusion

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Introduction

Brassica juncea, a natural allotetraploid derived from *B. rapa* and *B. nigra*, is a major oilseed crop of the Indian sub-continent. Efforts to extend its cultivation to Australia and Canada as a replacement for *B. napus* are being made because of its desirable features such as high-temperature tolerance, disease resistance and non-shattering silique (Woods *et al.* 1991). Artificial resynthesis of *B. juncea* has been achieved through sexual hybridization but synthesis through somatic hybridization has not been well documented. Here we report the development of *B. juncea* lines through protoplast fusion. RFLP analysis revealed that the hybrids inherited their plastid genome from either of the parents while the mitochondrial genome was mostly derived from *B. nigra*. Hybrids with mixed or recombined mitochondrial and plastid genomes were also identified. A wide variation for various agronomic traits was recorded in progeny generations. These newly resynthesized *B. juncea* lines hold promise for *Brassica* variety improvement and in heterosis breeding.

Genetic diversity among *B. juncea* accessions is limited whereas there is a vast untapped diversity among the progenitor species. Therefore, resynthesis of *B. juncea* is highly relevant for further improvement of this crop. Since the proposal by Morinaga (1934) and U (1935) on the origins of the three cultivated allotetraploid *Brassica* species from the three diploid species, namely, *B. rapa* ($2n = 20$, AA), *B. nigra* ($2n = 16$, BB) and *B. oleracea* ($2n = 18$, CC), artificial resynthesis of the three allotetraploid species *B. juncea* (AABB), *B. napus* (AACC) and *B. carinata* (BBCC) has been reported by a number of investigators (see Prakash *et al.* 2009). Among them, *B. napus* and *B. carinata* have also been synthesized through protoplast fusion (Schenck and Röbbelen 1982; Narasimhulu *et al.* 1992). However, except for an

abstract (Campbell *et al.* 1990), there is no report of resynthesis of *B. juncea* through somatic hybridization. *B. napus* and *B. carinata* have been obtained through sexual hybridization using either of the diploid parent species as the cytoplasm donor. But synthesis of *B. juncea* using *B. nigra* as the maternal parent has proved difficult due to operation of strong unilateral incompatibility barriers. Plastid DNA RFLPs have revealed that natural accessions of *B. juncea* carry *B. rapa* organelle genomes (Erickson *et al.* 1983; Palmer *et al.* 1983). Hence, the significance of *B. nigra* organellar genome towards agronomic performance of *B. juncea* has not been studied so far. Protoplast fusion can overcome unilateral incompatibility and can also generate novel organellar combinations. Besides, the nuclear genomes of both the parents can be brought together unaltered in somatic hybrids, thus maintaining the heterozygosity present in the parents. In contrast, amphidiploids obtained through chromosome doubling of sexual hybrids would be homozygous and carry only a part of the total genetic diversity available in the parents. Thus a single somatic hybridization event can generate more genetic variation than sexual hybridization. We have resynthesized *B. juncea* through somatic hybridization and report here the variability generated for organellar genome constitution and agronomical traits.

Materials and methods

Three *B. rapa* varieties representing yellow sarson (cv. YID1), brown sarson (cv. Pusa Kalyani) and toria (cv. T9), and three *B. nigra* accessions viz. IC257 (designated as NT), Alaska (EC 426392, designated as NA) and a local dwarf collection (designated as ND) were used for somatic hybridization. Seeds were surface sterilized in 0.1% mercuric chloride +0.1% sodium laurylsulphate solution for 10 min, thoroughly washed with sterile distilled water and cultured

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on half-strength Murashige and Skoog (1962) medium (MS medium) at $25 \pm 2^\circ\text{C}$ in dark. To obtain shoot cultures, shoot apices were excised from four-day-old seedlings and cultured on the same medium under 18-h photoperiod ($30 \mu\text{M m}^{-2} \text{s}^{-1}$). Protoplasts were isolated from seven-day-old hypocotyl explants (*B. rapa*) or from leaf tissue of four-week-old shoot cultures (*B. nigra*). Details of protoplast isolation, fusion and culture, plant regeneration and hardening etc. are as described by Kirti *et al.* (1995). Primary regenerants were raised in pots till maturity in a net house. Progeny generations from selfed seeds of somatic hybrids were raised in the field during normal crop season (October–March).

Observations on various morphological and agronomic traits were recorded on fully grown plants. At flowering, pollen viability was tested by staining with 1% acetocarmine. Further, male and female fertility were tested by crossing with natural *B. juncea* and by selfing.

DNA was isolated from leaf tissues of hybrids and parents. Gene probes *atpA* (for mitochondria), *psbA*, *psbD* and *rbcL* (for plastid) were used for Southern hybridization to determine the organellar genome constitution of the somatic hybrids. Details of DNA isolation and Southern analysis have been described previously (Kirti *et al.* 1995).

Results

All nine possible combinations of 3×3 *B. nigra* and *B. rapa* parents were attempted for somatic hybridization. However, calli were obtained only in four combinations; namely YID + NA, YID + NT, YID + ND and T9 + NA. Initially plant regeneration was tested on MS medium with 1% sucrose and $0.1 \text{ (mg l}^{-1}\text{) IAA}$, $2.0 \text{ (mg l}^{-1}\text{) BAP}$, $2.0 \text{ (mg l}^{-1}\text{) zeatin riboside}$ and $3.5 \text{ (mg l}^{-1}\text{) AgNO}_3$. This medium has been used in our laboratory for recovering various somatic hybrids involving *B. juncea* (Kirti *et al.* 1995). However, of the four parental combinations, only the combination YID1 + NT showed plant regeneration on this medium (16/463 calli). Hence two other media were tested for plant regeneration. On a medium supplemented with 3% sucrose, the combination T9 + NA gave two shoots (from 76 calli) whereas the medium containing 0.1 mg l^{-1} zeatin and 2.0 mg l^{-1} 2-isopentyladenine supported shoot regeneration from calli of YID + NA (8/220) and T9 + NA (2/125). Shoots of the somatic hybrid T9 + NA turned hyperhydric and failed to root whereas other shoots could be readily rooted. Out of the 30 plants transferred to soil only 10 grew to maturity under net house conditions.

Morphology

Somatic hybrids were vigorous, green and comparable to natural *B. juncea* for general morphology. However, most of the hybrids were male-sterile. Among the three plants that showed some pollen, acetocarmine stainability of the pollen was highly variable (5%, 20% and 90%). Female sterility was also very high in primary hybrids as evident from poor

seed set upon hand pollination or open pollination with natural *B. juncea*. Similarly, natural *B. juncea* crossed with pollen from somatic hybrids gave low seed set (1–3 seeds per silique). Seeds obtained from self-fertilization or inter-mating among somatic hybrids were used for further stabilization of synthetic *B. juncea* lines. Only 10–15 seeds were recovered per plant from selfing or intercrossing of more than 100 flowers. Even under open pollination, seed set was very low. Only four plants of the combination YID1 + NT gave seeds whereas the other six plants gave no progeny.

Performance of the progeny generations

Plants of the synthetic 1 (S_1) generation showed a marked improvement in pollen fertility with most of the plants showing more than 80% pollen stainability in 1% acetocarmine. Similarly, seed set also improved and ranged from 3 to 12 per silique in different plants. Prevalence of self-incompatibility was also indicated in some plants as flowers showed extorse anthers and failed to set seed upon self-pollination. Variation for traits such as plant height, days to flowering, branching characteristics, silique length, seed size etc. was observed among the progeny plants. Progenies of YNT-13 and YNT-14 had angular stem, purple tinge on the petiole, broad leaves, appressed silique and flowered late, and thus resembled the *B. nigra* parent. By the S_3 generation, both male and female fertility improved almost to the level of natural *B. juncea*. However, segregation for morphological traits was observed even after four generations of selfing. Yellow-seeded plants were obtained in two lines of the S_3 generation. A wide range of variation for different morphological traits was recorded among S_5 lines (table 1).

Table 1. Variation for important agronomic traits among progeny lines of synthetic *Brassica juncea*.

Trait	Range of variation
Days to flowering	55–92
Plant height (m)	1.63–2.88
No. of primary branches	5–10
Silique length (cm)	2–5
No. of seeds per silique	8–18
1000-seed weight (g)	3.3–7.1

Organellar genome constitution of hybrids

A total of 10 somatic hybrids were analysed for organellar genome constitution along with the respective parents. The parental species *B. nigra* and *B. rapa* gave polymorphic RFLP patterns with mitochondrial (*atpA*) and plastid (*psbA* and *psbD*) gene probes. The organelle genome constitution of the somatic hybrids is summarized in table 2. Majority (8/10) of the hybrids showed a 3.8-kb *atpA* fragment corresponding to *B. nigra*. One hybrid, YNT-14 showed 3.95 kb fragment indicating mt-genome recombination (fig-

ure 1,a). Plastid genome constitution of the hybrids appeared to be randomly inherited from either of the parents. The hybrid YNA-5 inherited chloroplasts from both the parents whereas YNT-14 displayed a recombined plastid genome (figure 1,b). This was further confirmed through *rbcL* RFLP pattern where YNT-14 showed 4.0 kb fragment, unlike the parents which gave 3.7 kb fragment. Thus YNT-14 contained recombinant mitochondrial and plastid genomes.

Table 2. Mitochondrial and chloroplast genome constitution of *Brassica juncea* somatic hybrids based on RFLP analysis.

Hybrid	mt-Genome		Plastid genome		
	(<i>atpA</i>)	<i>psbA</i>	<i>psbD</i>	<i>rbcL</i>	
YND-1	<i>B. rapa</i>	<i>B. rapa</i>	<i>B. rapa</i>	–	–
YND-9	<i>B. nigra</i>	<i>B. nigra</i>	<i>B. nigra</i>	–	–
YND-10	<i>B. nigra</i>	<i>B. nigra</i>	<i>B. nigra</i>	–	–
YNT-1	<i>B. nigra</i>	<i>B. rapa</i>	<i>B. rapa</i>	–	–
YNT-3	<i>B. nigra</i>	<i>B. rapa</i>	<i>B. rapa</i>	–	–
YNT-13	<i>B. nigra</i>	<i>B. rapa</i>	<i>B. rapa</i>	–	–
YNT-14	Recombined	Recombined	Recombined	Recombined	Recombined
YNA-4	<i>B. nigra</i>	<i>B. nigra</i>	–	–	–
YNA-5	<i>B. nigra</i>	Mixed	–	–	–
YNA-7	<i>B. nigra</i>	<i>B. nigra</i>	–	–	–

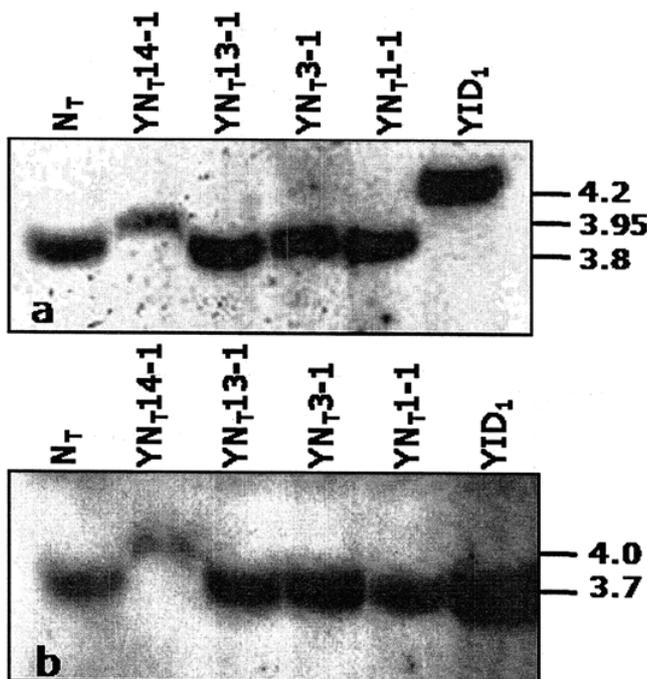


Figure 1. Southern blots of *B. nigra* (left lane), *B. rapa* (right lane) and somatic hybrids (inner lanes) prepared using total DNA (a) digested with *EcoRI* and probed with the mitochondrial gene *atpA*, and (b) digested with *BamHI* and probed with chloroplast gene *psbD* (size of the fragments in kb is shown on the right).

Discussion

Resynthesis of allopolyploids of *Brassica* is currently pursued for understanding basic aspects of polyploid evolution (Albertin *et al.* 2006) and for widening germplasm pool for plant breeding (Udall *et al.* 2004). The protoplast fusion and regeneration strategy employed here promoted growth and multiplication of fusion products and thus only hybrid plants were recovered. The frequency of plant regeneration was low but comparable to previous reports on synthesis of allopolyploid *B. carinata* and *B. napus* (Sundberg *et al.* 1987; Narasimhulu *et al.* 1992; Jourdan and Salazar 1993).

Synthetic *B. juncea* plants obtained via somatic hybridization were quite indistinguishable from natural *B. juncea* lines in gross morphology. However, primary hybrids exhibited high degree of male and female sterility. Low seed set in initial generations is common in synthetic allopolyploids obtained by sexual or somatic hybridization (see Prakash *et al.* 2009), and is attributed to genome incompatibility in newly synthesized polyploids. Further, aneuploidy may also contribute to sterility of somatic hybrids (Sundberg *et al.* 1987). Rapid genome reorganization involving homeologous recombination and stochastic as well as systematic gene silencing have been observed in newly synthesized polyploids of *Brassica* (Pires *et al.* 2004; Lukens *et al.* 2006). Appearance of yellow-seeded segregants in S₃ generation of synthetics supports occurrence of homeologous recombination.

Most of the hybrids contained mitochondrial genomes of *B. nigra*, whereas plastid genome appeared to be randomly inherited from either of the parents. In one instance, both mitochondrial and plastid genomes were found to be recombined. Mitochondrial genome recombination is frequently encountered in somatic hybrids (Kirti *et al.* 1995) but plastid recombination is rare. In Brassiceae, so far plastid recombination has been reported only once (Baldev *et al.* 1998). Based on studies with sexually synthesized *Brassica* allopolyploids, Song *et al.* (1995) have reported that organelle genomes exert a strong influence on reorganization of nuclear genomes and favour retention of genes from the maternal parent. Among the stable lines obtained after five generations many show *B. nigra*-like features such as broad leaves, tall stature, late flowering etc. Presence of *B. nigra* mt-genome might have favoured retention of *B. nigra* genes/alleles during stabilization of these synthetic lines leading to predominance of *nigra*-like features in the progeny lines.

Somatic hybrids inherit full chromosome complements of the parents and hence a large variability is expected in progeny generations. Indeed in the present study we found highly segregating families in each of the four independent hybrids. Thus although somatic hybridization is technically more demanding, it generates more variability by bringing together heterozygous parental genomes. Synthetic allopolyploids, in general, are not found to be highly productive. However, one progeny line showed seed yield (ca. 24 Q/ha) comparable to commercial varieties (ca. 26 Q/ha). Seyis *et*

al. (2006) demonstrated the potential of synthetic *B. napus* for developing heterotic hybrids. Synthetic *B. juncea* lines developed in this study could prove useful for both heterosis breeding and for varietal improvement.

In conclusion, this study demonstrated that stable lines of *B. juncea* with organellar genomes from either of the parents could be established following somatic hybridization. Such novel synthetic *Brassica* allopolyploids are useful for enlarging gene pool and for obtaining highly heterotic hybrids.

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