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Diploid *Brassica napus* somatic hybrids: Characterization of nuclear and organellar DNA

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Summary. Five somatic hybrids between *Brassica campestris* and *B. oleracea* were obtained. Molecular, morphological and cytological information all suggest that the resynthesized *B. napus* plants were hybrids. All five plants were diploid ($2n = 38$) and had mainly bivalents at meiosis. Seedset was low after selfing but normal after crossing with *B. napus*. Molecular proof of the hybrid nature of these plants was obtained by hybridization of a rDNA repeat to total DNA. Analysis of chloroplast DNA restriction patterns revealed that all hybrids had chloroplasts identical to the *B. oleracea* parent. The analysis of mitochondrial DNA indicated that three hybrids had restriction patterns identical to those of *B. campestris*, and the other two had restriction patterns similar to those of *B. oleracea*. The 11.3 kb plasmid present in mitochondria of the *B. campestris* parent was also found in mitochondria of all five hybrids. This suggests that the plasmid from a *B. campestris* type of mitochondria was transferred into mitochondria of a *B. oleracea* type.

Key words: *Brassica* – Protoplast fusion – Chloroplast DNA – Mitochondrial DNA – Mitochondrial plasmid

Introduction

The resynthesis of the allopolyploid *Brassica napus* (oilseed rape, $2n = 38$) from its progenitors, *B. campestris* ($2n = 20$) and *B. oleracea* ($2n = 18$), is regarded as a powerful tool for introducing new genetic variation into this crop. Traditionally, this is done with sexual crosses between the parental species (e.g. U 1935; Olsson 1960; Akbar 1987). Another alternative that has recently received attention is somatic hybridization, which also allows parental cytoplasms to combine. Chloroplast and mitochondrial segregation and/or recombination often follow (Galun 1982; Hanson 1984; Bravo and Evans 1985) so that novel cytoplasmic constitutions are obtained in somatic hybrids and cybrids. Such new cytoplasmic constellations could be of value for plantbreeding, e.g. in oilseed rape, chloroplasts encoding atrazine resistance were combined with mitochondria encoding cytoplasmic male sterility and chlorophyll deficiency caused by the presence of *Raphanus* chloroplasts was corrected (Pelletier et al. 1983; Jarl and Bornman 1988).

Resynthesis of *B. napus* through somatic hybridization was first reported by Schenk and Röbbelen (1982). They, as well as Taguchi and Kameya (1986), depended on the comparatively vigorous growth of hybrid calli, as compared with parental ones, for hybrid plant regeneration. Other systems used for selection of hybrid cells or calli comprised mechanical isolation of hybrid cells (Sundberg and Glimelius 1986), iodoacetamide inactivation of *B. oleracea* protoplasts (Terada et al. 1987), atrazine resistance of hybrid cells (Jourdan et al. 1986; Robertson et al. 1987) and flow cytometry (Sundberg et al. 1987). The low regeneration capacity of *B. campestris* (Glimelius 1984) is used in many of these systems. Characterization of the hybrid plants obtained in these studies was based on analysis of plant morphology, chromosome number, DNA content and isozyme pattern. Molecular characterization of nuclear and organellar DNA was restricted to one hybrid plant (Robertson et al. 1987). The type of chloroplasts was determined in the somatic hybrids obtained by Sundberg et al. (1987).

The work presented here on somatically resynthesized *B. napus* was initiated in a plant breeding context where emphasis was mainly on earliness and winter hardiness. We investigated the possibility of obtaining hy-

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brid plants without applying any particular hybrid selection method (Schenk and Röbbelen 1982; Taguchi and Kameya 1986). In the present study, diploid hybrid plants were produced and their chloroplast and mitochondrial DNA were investigated.

Materials and methods

Plant material

Brassica oleracea var. *alboglabra* (summer type Chinese kale) was used as one parent and one of three different breeding lines of *B. campestris* ssp. *oleifera* (summer type turnip rape), Sv 03242, Sv 03401, Sv 03261, was used as the other parent. Another combination was *B. oleracea* var. *acephala* (winter type kale) and the breeding line Sv 03242. All seeds were supplied by Svalöf AB.

Protoplast isolation, fusion and culture

Protoplasts were isolated according to Vamling and Glimelius (1988). Hypocotyl protoplasts were obtained from 5-day-old dark grown seedlings. The hypocotyls were finely cut and treated with an enzymatic mixture containing 1% cellulase and 0.1% macerozyme dissolved in medium A (Kao and Michayluk 1981). Mesophyll protoplasts were obtained from the leaves of 4–5 week old plants grown on MS-medium (Murashige and Skoog 1962) without hormones. Leaves were cut into small pieces and treated with an enzymatic mixture containing 0.5% cellulase and 0.5% macerozyme (Yakult Pharmaceutical, Japan). Polyethylene glycol 4000-mediated fusion of mesophyll and hypocotyl protoplasts (ratio 2:1, titer $5 \times 10^5 - 1 \times 10^6$ ml^{-1}) was carried out in concanavalin A-treated Petri dishes according to the method of Glimelius et al. (1978). The crude mixture of treated protoplasts was cultured according to Glimelius (1984). The hormone levels applied were in medium A; 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.54 μM 1-naphthylacetic acid (NAA) and 0.88 μM 6-benzylaminopurine (BAP); and in K_3-medium (solidified with 0.25% agarose), 4.5 μM 2,4-D and 0.5 μM BAP. K_3-medium containing 4.6 μM zeatine, 0.57 μM indolacetic acid (IAA) and 4.4 μM BAP was used for plant regeneration. Calli were recultured on this medium every 2 weeks. Regenerated shoots were transferred to hormone-free MS-medium. After obtaining a normal growth pattern, the shoots were dipped in a rooting mixture (0.1% alpha-naphtyl acetic acid) and potted in the greenhouse.

Chromosome analysis and crosses

Mitotic chromosome counts were made on root tip cells fixed in Carnoy and stained with Feulgen and Giemsa. Meiotic analyses were made on pollen mother cells prepared as follows. Buds were fixed in ethanol:chloroform:acetic acid (4:3:1). After 2 h the buds were transferred to Snow's carmine and kept at 60 °C for 2 days. Before microscopic evaluation the buds were washed in 70% alcohol.

The regenerated plants were self-pollinated, sib-crossed and crossed with summer type *B. napus* (cv Sv Topas and breeding line Sv 02326 from Svalöf AB, Sweden).

Molecular characterization

The vegetative parts of greenhouse grown plants were used for the isolation of total DNA. The DNA extraction method was as previously described (Halldén et al. 1987). Chloroplast (cp)DNA and mitochondrial (mt)DNA were isolated as described by Brears et al. (1986) and Erickson et al. (1985), respectively. The restriction endonucleases Eco RI, Bam HI, Xba I, Pst I and Hind III (Boehringer, Mannheim) were used as recommended by the manufacturer. Gel electrophoresis was on 0.7% or 0.8% horizontal agarose gels in 0.04 M Tris (pH 7.6), 0.02 M sodium acetate and 0.002 M EDTA (TEA) run at 2.0 V/cm over night. After denaturation the DNA fragments separated by electrophoresis were transferred to Gene Screen filters (New England Nuclear) by electroblotting in TEA at 0.40 A for 16 h. The recombinant plasmid (pHV294) used as probe to the total DNA contained a cloned barley rDNA repeat unit (Gerlach and Bedbrook 1979). The recombinant plasmids (pBcm H1, pBcm H2, pBcm H3) used to probe for the mitochondrial plasmid were kindly supplied by Dr. L. Erickson (Erickson et al. 1986). The plasmids were labelled with ^{32}p-dCTP to a specific activity of 5×10^7 cpm/μg according to Maniatis et al. (1982). The hybridization to Gene Screen filters was carried out as described by Halldén et al. (1988). Autoradiography was performed at −80 °C with intensifying screens for 4–24 h.

Results

Plant regeneration

Five plants numbered 1–5 were regenerated from the combination *B. oleracea* var. *acephala* and *B. campestris* Sv 03242. Two plants (plants 1 and 3) originated from one callus and three plants (plants 2, 4 and 5) from another callus. The regeneration frequency in this combination was 1.2% (2 out of 165 calli). In the 3 other combinations, no plants were obtained (1815 calli). Also, the parental genotypes were tested for regeneration capacity under identical culture conditions, and no plants were obtained.

Morphology

The morphology of the regenerated plants (Fig. 1) appeared to be intermediate to both parents, with more resemblance to the *B. oleracea* parent. The five plants had a thick main stem, good vegetative growth and undulated leaves. They also required vernalization to induce flowering. The leaves were, however, densely covered with trichomes as were the leaves of the *B. campestris* parent. One character that distinguished the regenerated plants from both parents was strong anthocyanin coloration. This appeared at the petiole bases of the leaves of old *B. campestris* plants, and was absent in the *B. oleracea* parents.

Chromosome analysis and crossings

Roottip chromosomes were counted in each plant. The diploid number, 38 chromosomes, was found in all five plants. At metaphase I of pollen mother cells, 18–19 bivalents were often observed (Fig. 2). This suggests that the plants were true hybrids.

Seedset was recorded after selfing, reciprocal sib-crosses and crosses with commercial or breeding *B. napus*

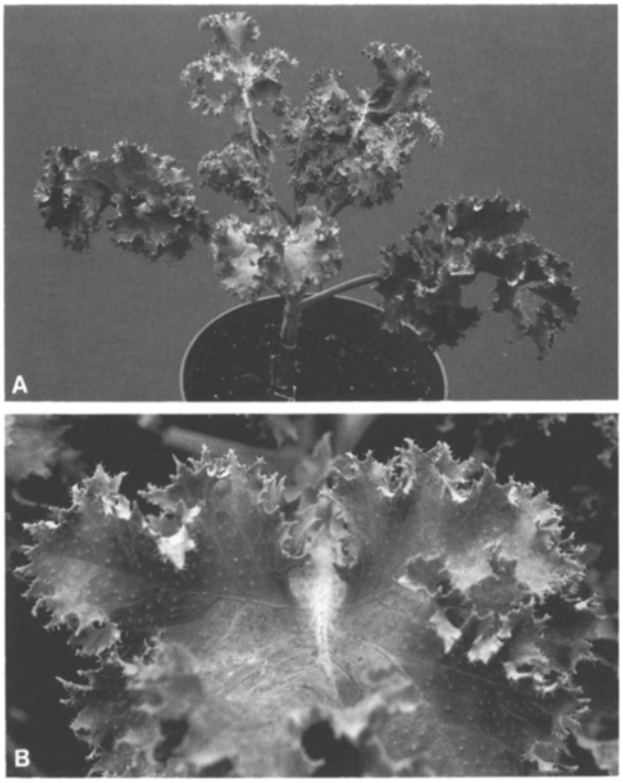

Fig. 1. **A** A somatic hybrid with similarity to the *B. oleracea* parent; **B** the high magnification of the leaf shows the trichomes characteristic of the *B. campestris* parent

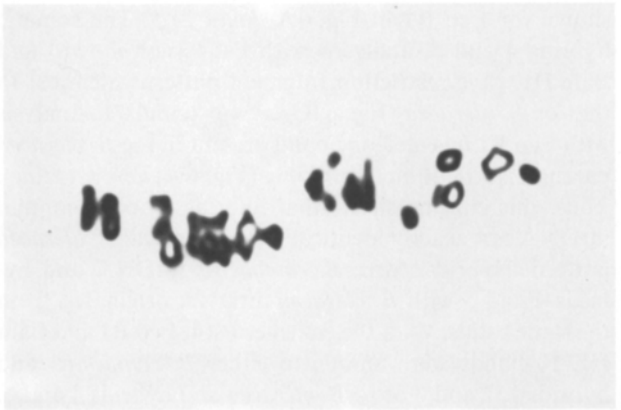

Fig. 2. Meiotic metaphase I from hybrid 1 showing 19 bivalents

material (Table 1). After selfing, there was low (hybrids 2–5) or no (hybrid 1) seedset. All sib-crosses, except two with a low seedset, were unsuccessful, while crosses with other *B. napus* material generally resulted in a high seedset. The lack of, or low, seedset in the reciprocal crosses between Sv 02326 and plants 1 or 3 needs to be further elucidated. Both plants 1 and 3 originated from the same callus and may thus have similar genotypes.

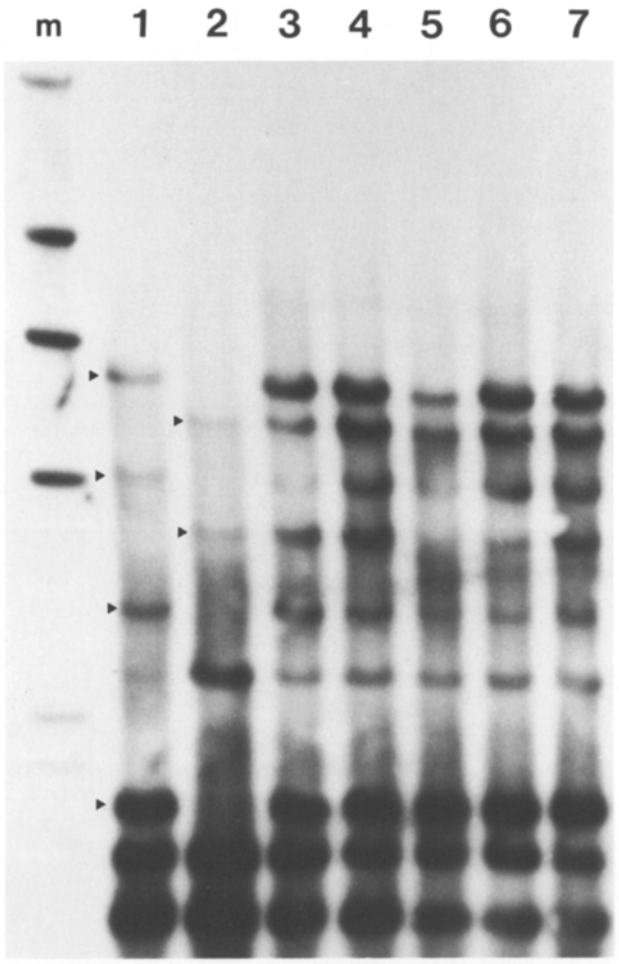

Fig. 3. Analysis of nuclear rDNA sequences in the somatic hybrids and their parents. Total DNAs from the *B. oleracea* parent (*lane 1*), the *B. campestris* parent (*lane 2*) and somatic hybrids 1–5 (*lanes 3–7*) were digested with Bam H1, electrophoresed on a 0.8% agarose gel and hybridized with pHV 294 as in "Materials and methods". *Arrowheads* indicate bands specific for the parental species and repeatedly found in the somatic hybrids. *m* represents size marker fragments produced by Hind III digestion of lambda DNA

Molecular analysis

Analysis of nuclear ribosomal DNA sequences revealed that the regenerated plants do indeed contain hybrid nuclei. Hybridization of the barley rDNA repeat unit (Gerlach and Bedbrook 1979) to Southern blots of total DNA from the regenerants and their parents showed the full complement of both parental sets of bands in the regenerants (Fig. 3).

Restriction enzyme analysis of the cpDNA was carried out with the restriction endonucleases Bam H1, Hind III, Xba I and Eco RI, all of which have been shown to distinguish the cpDNA of *B. campestris* from that of *B. oleracea* (Erickson et al. 1983). The five so-

Table 1. Hybrid fertility estimated as mean number of seeds per pod: $-=0$ seeds/pod; $+=$more than 0 and less than 1 seed/pod; $++=1-4$ seeds/pod; $+++=>4$ seeds/pod, as for natural oilseed rape (analyses of 10 inflorescences in each combination); *ND* = not determined

	Somatic hybrids					*B. napus*	
	1	2	3	4	5	Sv Topas	Sv 02326
1	−	−	−	−	−	+	−
2	−	+	−	−	−	+++	+++
3	−	−	++	+	−	+++	−
4	−	−	−	++	−	+++	+++
5	−	−	−	+	+	+++	+++
Sv Topas	+++	++	+	+++	++	ND	ND
Sv 02326	−	++	+	++	++	ND	ND

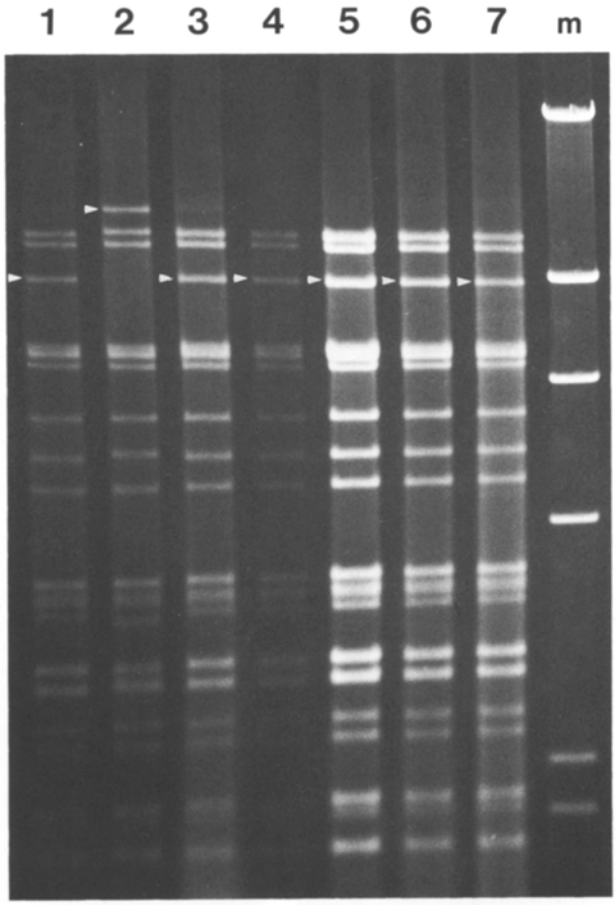

Fig. 4. Restriction patterns of cpDNA after digestion with Bam H1. Lanes and labelling as in Fig. 3. *Arrowheads* indicate bands specific to the parental species

matic hybrids showed restriction patterns identical to the pattern of the *B. oleracea* parent for all four enzymes, of which the result using Bam H1 is seen in Fig. 4. Thus, the initial mixture of the two cytoplasms was followed by the elimination of the chloroplasts from *B. campestris*.

Analysis of mtDNA on agarose gels without prior restriction enzyme treatment revealed the presence of the 11.3 kb plasmid in mitochondria of the *B. campestris* parent and in mitochondria of the five somatic hybrids (Fig. 5A). The plasmid could not be detected in mitochondria of the *B. oleracea* parent as confirmed by Southern hybridizations of clone pBcm H1 shown in Fig. 5B (Erickson et al. 1986). Even strong overexposure of the autoradiograph failed to reveal the presence of the plasmid in the mitochondria from the *B. oleracea* parent (not shown).

In three of the regenerated plants (hybrids 1, 2 and 3), restriction endonuclease analysis of mtDNA with Pst I, Eco RI and Bam HI, all of which distinguish between mtDNA from *B. oleracea* and *B. campestris*, revealed restriction fragment patterns without any detectable differences from those of the *B. campestris* parent. This is shown for Eco R1 in Fig. 6A, *lanes 2–5*. The somatic hybrids 4 and 5, analysed with Pst I (not shown) and Bam HI, gave restriction fragment patterns identical to that of *B. oleracea* (Fig. 6B, *lanes 1, 6* and *7*). Analysis with Eco RI revealed one band present in the *B. oleracea* parental restriction pattern only (Fig. 6A, *lane 1, arrow*). Thus, this enzyme shows that hybrids 4 and 5 contain mtDNA not exactly identical to the parental *B. oleracea* pattern. Hybrid 2 with *B. campestris* mtDNA and hybrids 4 and 5 with *B. oleracea* mtDNA originated from the same callus. With the enzymes Pst I, Eco RI and Bam HI, 10 bands were unique to either *B. campestris* and hybrids 1, 2 and 3 or to *B. oleracea* and hybrids 4 and 5. In addition to this, a series of bands marked by *dots* in Fig. 6 are seen in *B. campestris* and the five hybrids. As reported by Palmer et al. (1983), digestion of the 11.3 kb plasmid gives rise to restriction fragments of molecular weights 7.8 kb (Eco RI) and 6.8 kb, 4.5 kb (Bam HI). The presence of these plasmid specific bands was confirmed by Southern hybridizations of clones pBcm H1, pBcm H2 and pBcm H3 (Erickson et al. 1986).

Although we have not checked for the presence of mitochondria from the *B. campestris* parent in hybrids 4 and 5 in low numbers, we suspect that the plasmid has been physically transferred from the *B. campestris* type of

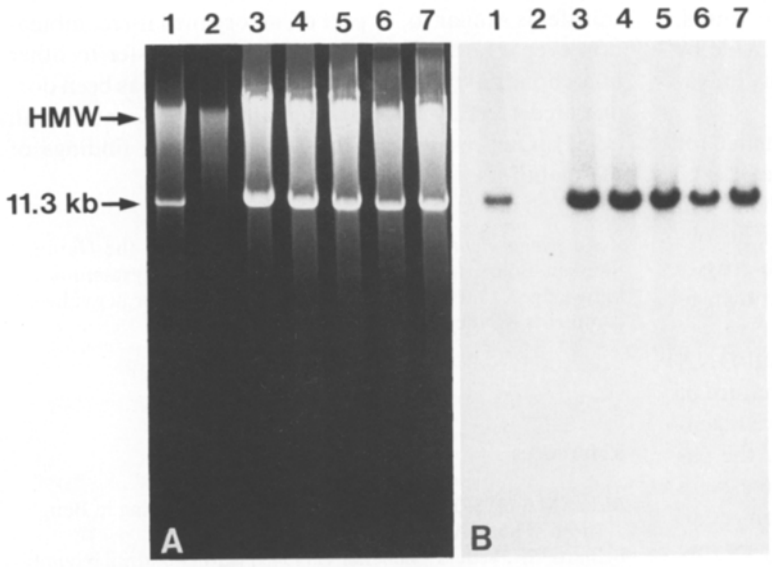

Fig. 5. **A** Analysis of undigested mtDNA on a 0.7% agarose gel. *B. campestris* (*lane 1*), *B. oleracea* (*lane 2*) and hybrids 1–5 (*lanes 3–7*). *HMW* refers to the position of the high molecular weight mitochondrial genome. The 11.3 kb plasmid is indicated. **B** Autoradiograph of a gel as in **A** after hybridization with the recombinant plasmid pBcm H1

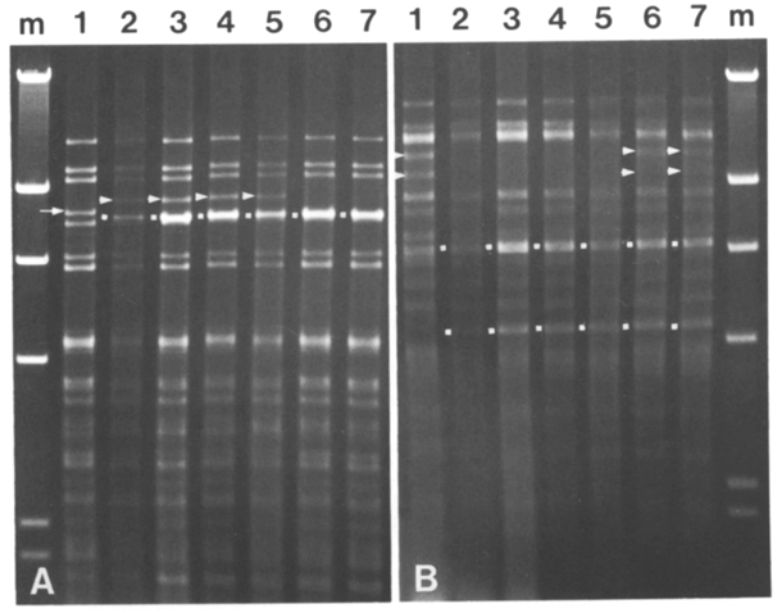

Fig. 6 A and B. Restriction patterns of mtDNA after digestion with Eco RI (**A**) and Bam H1 (**B**). Lanes and labelling as in Fig. 3. *Arrow* in **A**, *lane 1*, indicate a band found in *B. oleracea* but not in the hybrids. *Dots* indicate fragments generated by restriction enzyme digestion of the 11.3 kb plasmid

mitochondria to mitochondria with the basic mt genome from *B. oleracea*.

Discussion

Our results indicate that the five plants are true hybrids, i.e. resynthesized *B. napus*. This is based on the morphological, chromosomal and molecular data presented. Only hybrids and no parental plants were obtained in the experiments. Thus, in addition to the expected and observed low regeneration capacity of *B. campestris*, no plants were regenerated from the two *B. oleracea* genotypes tested. Success in regenerating several *B. oleracea* genotypes (e.g. Bidney et al. 1983; Glimelius 1984; Terada et al. 1987), however, argues for considering the use of a suitable hybrid selection system. More important is the need to raise the regeneration potential of hybrid calli. In all the studies made so far on the resynthesis of *B. napus* through somatic hybridization, only a limited number of diploid hybrid plants were produced. That all five regenerated plants appear to have 38 chromosomes with 19 or 18 bivalents at meiosis suggests their amphidiploid nature and favors their immediate use in a *B. napus* breeding program. Diploid somatic hybrids have also been reported by Schenk and Röbbelen (1982), Taguchi and Kameya (1986), Terada et al. (1987) and Sundberg et al. (1987). In these studies high frequencies of aneu-

ploid and polyploid somatic hybrids were also found. Meiotic analyses of somatic hybrids were only made by Schenk and Röbbelen (1982), who observed many univalents and multivalents at metaphase I.

Self-fertilization and test crosses are also valuable for characterization of somatic hybrids in breeding contexts. Our observations are comparable to those of Schenk and Röbbelen (1982) and Sundberg et al. (1987). The observed low seedset after self-fertilization and sib-crosses could be due to self-incompatibility, which is known to occur in both parents.

Hybridization of a rDNA repeat unit to total DNA is a suitable method for confirming the hybrid nature of regenerated plants (Uchimiya et al. 1983). All five regenerated plants were shown to contain the sum of the parental sets of rDNA repeats, which was also the case in the regenerated plant analyzed by Robertson et al. (1987). The advantage with this method over isozyme analysis is that it works on DNA directly. Isozyme analysis, dealing with gene products, may be sensitive to differences in plant tissues, plant age, physiological state, etc.

The restriction patterns of chloroplast genomes in all five regenerated plants were identical to those of *B. oleracea* and are probably the result of parental chloroplast segregation. In the diploid somatic hybrids obtained by Sundberg et al. (1987) the cpDNA was of *B. campestris* type in four plants and of *B. oleracea* type in one plant. Chloroplast segregation has been shown to be a rapid process in *B. napus* × *B. napus* cybrids, in which segregation was completed 19–22 cell divisions after fusion (Morgan and Maliga 1987). Chloroplast genomes are usually stable and in rare cases may be the result of intermolecular recombination (Medgyesy et al. 1985).

However, intra- and intergenomic mitochondrial recombinations in somatic hybrids and cybrids are more common (e.g. Belliard et al. 1979; Chétrit et al. 1985; Vedel et al. 1987). In a resynthesized *B. napus* somatic hybrid, Robertson et al. (1987) were able to trace the likely crossover point of recombination in the mitochondrial genome. The analysis of mitochondrial genomes in our five plants leads to the conclusion that three plants had *B. campestris* mtDNA and two plants had a slightly modified *B. oleracea* mtDNA. These more or less parental mitochondrial genomes were found in plants originating from the same callus (hybrid 2 with *B. campestris* type and hybrids 4 and 5 with *B. oleracea* type). This implies that different segregational pathways of parental mitochondria can take place in the same callus that might have a mono- or multicellular origin. Another significant observation is the finding of the *B. campestris* 11.3 kb plasmid in mitochondria of all five hybrids. This implies that the plasmid was transferred to mitochondria largely of the *B. oleracea* type in two of the hybrid plants. It remains to be elucidated whether the mechanism of this transfer is similar to, or part of, an organellar recombination event. Loss of this plasmid or its transfer to other mitochondrial types after protoplast fusion has been documented recently in a larger material by Kemble et al. (1987). Our results are consistent with the findings of these authors

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