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Interspecific somatic hybridization between *Brassica napus* and *Brassica hirta* (*Sinapis alba* L.)

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Summary. Somatic hybridization between *Brassica napus* and *B. hirta* (or *Sinapis alba*) is described. No cybrid plant with *B. napus* nucleus exhibiting cytoplasmic male sterility was recovered. Somatic hybrids were identified morphologically and, for some of them, by cytological observations. They were also characterised by Southern hybridization of nuclear rDNA. Chloroplast and mitochondrial DNA restriction analysis showed that 2 plants out of 14 have *B. hirta* ctDNA, one the *B. napus* mtDNA and the other a hybrid. Nine possess *B. napus* ctDNA with a hybrid mtDNA. For six of them, mtDNA patterns present novel bands, suggesting intergenomic recombination during fusion. These hybrids will be included in the breeding program.

Key words: *Brassica napus* – *Brassica hirta* – Somatic hybridization – mtDNA – ctDNA – rDNA

Introduction

Interspecific crosses are commonly used in plant breeding to elucidate genome relationships within a family and to enlarge genetic variability of cultivated species. The oleiferous crop rapeseed (*Brassica napus*) is a natural amphiploid whose variability is relatively reduced compared to its progenitor species *B. oleracea* and *B. campestris*. Crosses were made either to synthesize a new amphiploid (the "Hakuran"; Nishi 1980; Zwierzykowska 1983) or, with the related species, to obtain the diverse genomic combinations at different ploidy levels (Morice 1963; Labana 1983).

In addition to nuclear characters, several cytoplasmic traits are also of great interest. One of these is cytoplasmic male sterility (cms) because it may provide an economic means of creating advantageous F1 hybrids of *B. napus* (Shiga 1980; Lefort-Buson and Dattee 1985). A stable cms already exists in *B. napus*; it was introduced by an intergeneric cross between the cms *Raphanus sativus* line Ogura with *B. napus* (Bannerot et al. 1974). At present, another intergeneric cross with *Diplotaxis muralis* is being studied (Pellan-Delourme 1986). New combinations may be possible through further crosses involving other related species: *B. hirta* and oleiferous crop (white mustard) may be one such species.

The nomenclature of the *Brassiceae* is confusing in this case. The name *B. hirta* was given by Moench (1802); Rabenhorst (1839) named this species *B. alba*. However, Prakash and Hinata (1980) and Baillargeon (1985) selected the usual name in Europe of *Sinapis alba* L., thus relegating it to an alien genus. A cross with *B. oleracea* gives completely sterile hybrids (Harberd and McArthur 1980). *B. hirta* bears tolerance traits to drought and to some diseases. *Alternaria brassicae*, which only occasionally causes severe yield losses in Europe (Mridha 1983), can be very devasting in India (Verma and Rai 1980). Tolerance to this disease is missing in *B. napus*. In *B. hirta*, the cv Carine bears the *Alternaria* tolerance (H. Brun, personal communication). Interspecific hybrids would permit the introduction of this trait in *B. napus* and would show whether the combination *B. napus* nucleus/*B. hirta* cytoplasm could induce a stable cms.

Brassica are among the few important crops whose in vitro culture has been mastered, particularly protoplast fusion and plant regeneration (Barsby et al. 1986; Glimelius et al. 1986; Robertson and Earle 1986). This tool is now being used for breeding purposes, as illustrated by Schenck and Röbbelen (1982) and

Sundberg and Glimelius (1986) with the artificial synthesis of *B. napus,* as well as by Pelletier et al. (1983) with the re-matching of characters having a cytoplasmic inheritance. It may shorten the required time to obtain alloplasmic lines, provided the regeneration rate is adequate to recover all possible products from a somatic fusion. In the present paper, protoplast fusion was used to obtain somatic hybrids and cybrids between *B. napus* and *B. hirta.* They were characterized by molecular analysis of their nuclear and organelle DNAs and by cytological observations.

Material and methods

Plant material

For *B. napus,* the pure line Brutor has the highest regeneration capacity from protoplasts under our conditions. For *B. hirta,* the cv Carine is tolerant to *Alternaria.* Both can be distinguished by numerous morphological traits. Their 2 *n* chromosome numbers are 38 and 24, respectively.

Somatic fusion and plant regeneration

Protoplasts were isolated from leaves of plants grown in vitro. Fusion procedure and in vitro culture of protoplasts and plants have been previously described (Pelletier et al. 1983), with the exception of the following modifications: medium A contains the B5 mineral salts and is solidified by agar 2 g/l plus agarose 3 g/l; gentamicine was omitted; medium E contains glucose, sucrose, mannitol and agarose, 10 g/l each, the cytokinins are BAP 0,5 mg/l and IPA 0,5 mg/l. Buds appeared within 2–4 weeks.

Three different fusion experiments were performed. Each included control cultures of both parental protoplasts and of a mixture of them without fusion treatment.

Growth of regenerated plants

Once regenerated and rooted, plants were transferred into pots and stored in a cold room (8 °C). This allowed the transplantation of the whole set of regenerated plants into the field at the same favorable time (April 1984) and at almost the same stage of development. They flowered simultaneously and were tested for male sterility.

DNA preparation and restriction digestion

The methods have been described elsewhere for mitochondrial (mt) DNA (Vedel et al. 1982) and for chloroplast (ct) DNA (Pelletier et al. 1983). Total plant DNA was prepared from 1 g of leaf tissue as reported by Dellaporta et al. (1983) and further purified by CsCl banding. DNA was digested with restriction enzymes according to the manufacturer's instructions (Boehringer Mannheim) and the digests were run on vertical 0.7% agarose gels.

DNA hybridization

A ^{32}P labelled probe coding for ribosomal RNA sequences from *Raphanus sativus* was isolated and characterized (Tremousaygue et al. 1988). We hybridized this probe to total plant DNA digests (Southern 1975).

Cytology

Meiosis were analysed on pollen mother cells using the standard aceto-carmin method.

Results

Fusion experiments

The parental control showed that *B. hirta* protoplasts regenerate colonies under standard conditions but not shoots (one-third of the colonies regenerated roots alone). Similarly, in the mixture controls, all regenerated plants were of *B. napus* type; therefore after a fusion treatment, only one set of parental plants was recovered.

Three types of regenerated plants were expected: 1* *B. napus* parental type, 2* cybrid type with a *B. napus* nucleus and a hybrid cytoplasm and 3* hybrid type arising from fusion product (if only one parent able to regenerate is sufficient to recover hybrid plants).

The plant regeneration rate was variable among the three fusion experiments. Frequency of plant regeneration depended on the initial density of surviving protoplasts after fusion, growth rate of the colonies and particularly on the length of exposure to the D medium: optimum regeneration rate was observed after 2 weeks of subculture in this medium (case of the 3rd experiment, Table 1). The modifications of A and E media improved the maximum rate of regeneration up to 60% of the calli of the control *B. napus* (data not shown).

Phenotype of regenerated plants

Beginning with the in vitro step, it was possible to distinguish plants having very hairy deep green leaves with an intermediate shape between parental ones.

Table 1. Frequency of plant regeneration from protoplasts derived calli

Experiment	No. of calli tested	No. of calli regenerating[a]	%	No. of flowering plants[c]
B. napus	160	55	34.3	22
B. hirta	780	0	0	0
Mixture	360	64	17.7	16
1st fusion	3,820	245	6.4	208
2nd fusion	5,400	184	3.4	159
3rd fusion	6,000	1,164[b]	19.4	1,055
	15,420	1,603	10.3	1,422

[a] Only one shoot was recovered per callus
[b] Underestimated since not all the buds were effectively transplanted
[c] Not all plants were conducted till flowering

Table 2. Silica beak length (mm) of *B. napus, B. hirta* and 5 of their somatic hybrids and first offspring (mean of 10 silica)

	Mean ± SE length (mm)
B. napus	6.2 ± 1.6
B. hirta	16.4 ± 0.9
C	10.7 ± 1.1
H	14.2 ± 1.0
F	12. ± 2.1
U	16 ± 2.2
V	11.8 ± 2.1
F♀ × *B. nap.*♂	9.4 ± 1.9
H self 1	15.6 ± 1.1
H self 2	13.6 ± 2.2

Among 1,500 regenerated plants, 14 were selected and kept in the greenhouse where they flowered; two more were found later. They were named A, B, C, D, E, F, G, H, I, J, K, L, M, P, R, U, V,.

All other plants had a *B. napus* phenotype and were transplanted into the field.

Analysis of B. napus type regenerated plants. All of these plants displayed a highly uniform morphology with the exception of 23 plants which looked polyploid. Nevertheless, 5 plants demonstrated a more or less reduced pollen shedding but none was completely male sterile. The plant most nearly male sterile was studied over two generations of backcrosses with *B. napus.* We observed segregation of the male sterility trait. It is of nuclear origin and due to the effect of only one gene. Its mtDNA analysis showed a pattern identical to that of *B. napus.*

Identification of somatic hybrids. Their vegetative traits, inflorescence type and silica shape remained intermediate (Table 2). However, none had identical morphology, with leaves ranging from little (E) to much more denticulate (M) than those of *B. napus.*

All but one plant, whose flowers did not open, produced pollen. Pollen viability was tested by germination on a solidified medium (P. Guerche, personal communication). About one-fifth of the pollen grains germinated. Female fertility was also quite low: it differed among the hybrid-like plants from zero to a maximum of eight viable seeds per pod in backcrosses with *B. napus.* For example, for some of these plants, the mean numbers of seeds per fruit bearing pod was 1.3 (D), 2.6 (C), 3.3 (F) and 3.7 (H) (the parent *B. hirta* gives 6 seeds per pod and the *B. napus* gives around 25 seeds).

So far, nine plants had set seeds after hand pollination, either by crossing with *B. napus* or by selfing or both (C, D, F, G, H, L, P, U, V). Some gave mature seeds (C, D, F, G, H), the others needed in vitro culture of the ovaries to rescue the immature embryos.

Cytological studies

These are still incomplete. Nevertheless, 5 plants (B, C, U, V, P) contained 62 chromosomes. This number could result from the addition of the parental chromosome stocks, but not necessarily. However, if this is the case, the expected genomic structure of the plants from the first backcross with *B. napus* should be AACCSa ($2n = 50$). The observed chromosome number of such plants ranges from 42–53. After the second backcross, 10%–30% of the progenies had 38 chromosomes. It seems that the chromosomes of *B. hirta* are transmitted at a low rate through the second backcross. The somatic hybrid U was most studied: it demonstrated meiotic irregularities, having systematically one quadrivalent at meiosis. In its first backcross generation, the plants had 45–53 chromosomes and there was frequently one hexavalent at meiosis. The second backcross gave 50% of plants containing 38 chromosomes. Further studies of all the progenies are currently underway.

Southern hybridization of nuclear rDNA

This method permits distinction between the two parents. In the EcoRI pattern (Fig. 1), rDNA appears to be distributed among six main bands (5.8, 4.9, 4.6, 4.0, 2.9 and 2.6 kb) for *B. napus* and only three (4.9, 3.2 and 3.1 kb) for *B. hirta.* Thirteen hybrid-like plants were analyzed. It appears that their patterns contained bands specific to each parent. Each of the 13 plants possessed both the 2.6 kb fragment from *B. napus* and the 3.1 kb fragment from *B. hirta* and was a nuclear somatic hybrid. The examination of hybridization patterns showed that they are not all the same and that they don't have a similar relative band intensity. This doesn't seem to be due to technical problems of transfer of DNA from the gel to the filter. Only in two cases (E, J) were the patterns clear enough to show that they possessed the sum of the two parental patterns. Others lacked either the 2.9 kb from *B. napus* (P, U) or the 3.2 kb from *B. hirta* (C, H). The plant M exhibited a new band of 3.5 kb.

Characterization of cytoplasmic organelle DNAs in the hybrid plants

Chloroplast (ct) DNA. The restriction enzyme *Sal*I allows for discrimination between the patterns of the two parental ctDNA (Fig. 2). A physical map of *B. hirta* ctDNA with reference to *Sal*I and *Xho*I recognition sites was constructed by Link et al. (1981).

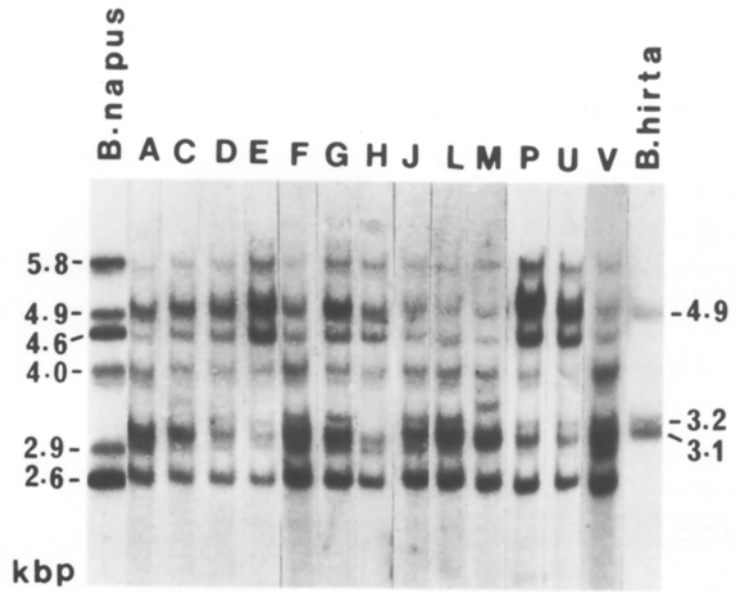

Fig. 1. Length variation of nuclear rDNA Southern blots of *EcoR*I digests from hybrids and parents, hybridized with nick-translated rDNA probe from radish. Hybrid B (not shown) presented hybrid rDNA patterns like those from the other 13 hybrids

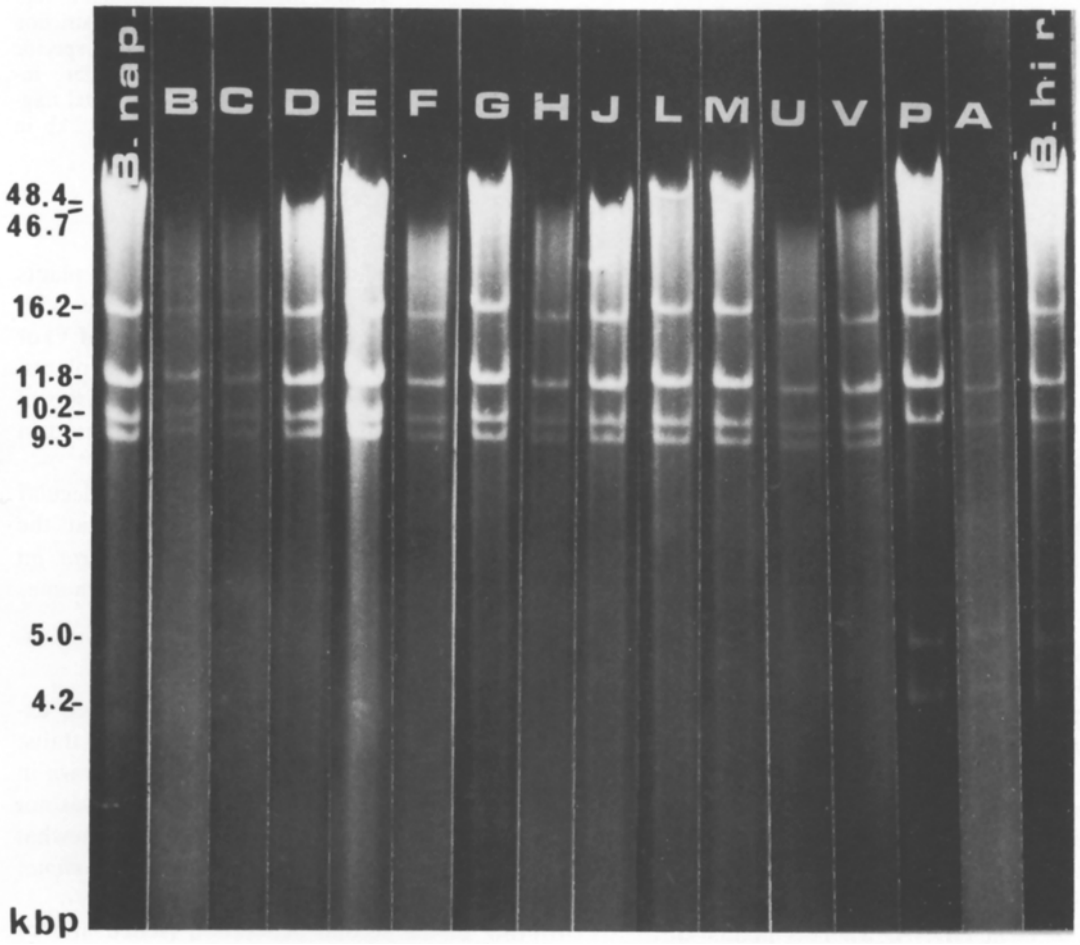

Fig. 2. Identification of chloroplasts by 0.7% agarose gel electrophoresis of *Sal*I digests of ctDNA isolated from parents and hybrids

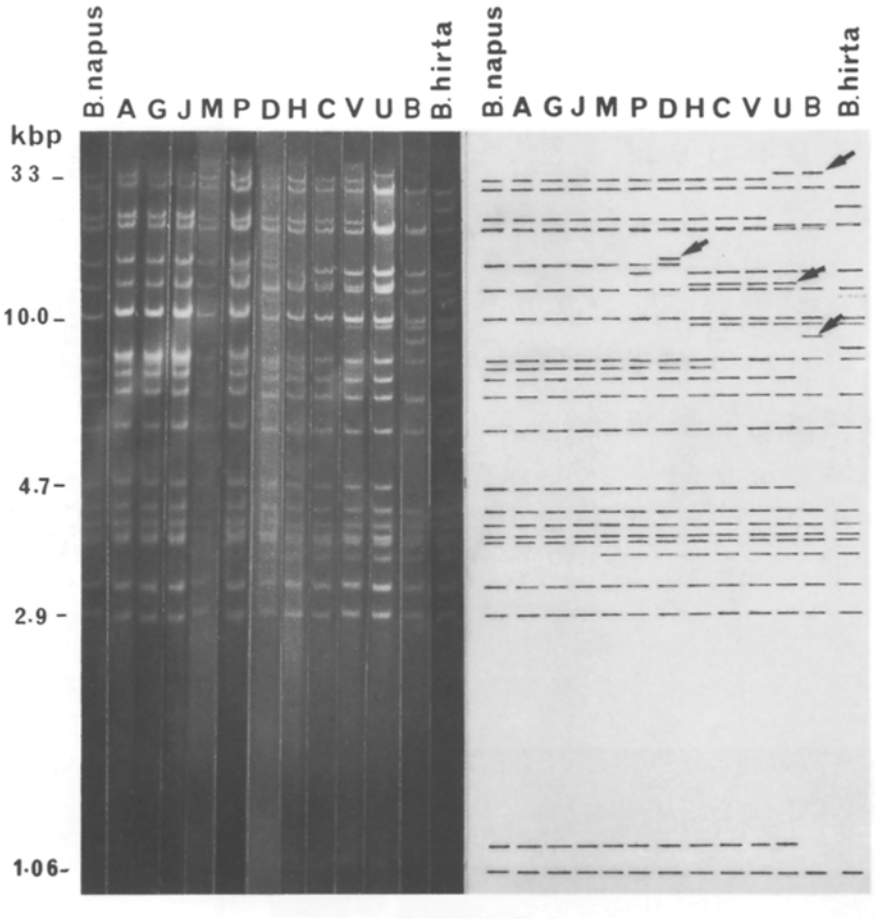

Fig. 3. *Left panel:* 0.7% agarose gel electrophoresis of *Sal*I digests of mtDNA isolated from parents and hybrids. *Right panel:* schematic representation of different *Sal*I restriction patterns. Novel fragments with specific molecular weights in hybrids are indicated by *arrows*. The four novel fragments are 40, 14.9, 12.5 and 9.2 kb in size, respectively

B. hirta ctDNA is characterized by seven *Sal*I restriction fragments of 48.8, 46.7, 16.2, 11.8, 10.2, 5.0 and 4.2 kb in size, respectively. A physical map of *B. napus* ctDNA was established by Vedel and Mathieu (1983) by using *Sal*I, *Bgl*I, *Sma*I, *Kpn*I and *Pst*I enzymes. *B. napus* ctDNA consists of six *Sal*I restriction fragments: 48.4, 46.7, 16.2, 11.8, 10.2 and 9.3 kb, respectively.

Fourteen somatic hybrids were analyzed. All but two exhibited the *B. napus* pattern. The two others (A and P) showed the *B. hirta* ctDNA.

Mitochondrial (mt)DNA. The mtDNA of the parental lines diverged in restriction site markers to the extent that restriction fragments unique to each parent could be identified. Figure 3 shows the *Sal*I restriction patterns for mtDNAs from parents and from the 11 hybrids analyzed (the other plants did not give enough material to be studied). Three cases were observed: 1) hybrids having the *B. napus* mtDNA pattern without apparent modification: A, G and J. As shown above, plant A possessed *B. hirta* ctDNA, whereas plants G and J possessed *B. napus* ctDNA; 2) hybrids having a pattern with bands belonging to both parents: plants M and P; and 3) hybrids having new patterns with parental bands and either one (plants D, H, C and V) or two (plant U and B) novel bands. Among these plants, C and V present identical patterns. Only four classes of *Sal*I novel bands 40, 14.9, 12.5 and 9.2 kb occurred in these somatic hybrids.

Size estimates obtained by adding up the molecular weights of all the *Sal*I fragments showed that the hybrid mt genomes are larger than the *B. hirta* mt genome and, except B, larger than *B. napus* mt genome.

Offspring studies

Offspring in the greenhouse, except L×*B. napus,* exhibited great vigour, at least in their vegetative traits: they were twice as high and strong as parents grown in the same conditions. However, in the field, this was not as apparent. Seed set of some of them was somewhat restored compared to their hybrid parents (C×*B. napus,* D selfed and D×*B. napus*).

Offspring of the second generation (selfed and/or backcrossed) exhibited very heterogeneous phenotypes,

some kept intermediate traits, the others were very close to *B. napus* and had the highest seed set; this indicates a quick sorting out of *B. hirta* chromosomes.

Discussion

Nuclear DNA analysis showed that all the tested hybrid-like plants, regenerated after fusion between *B. napus* and *B. hirta,* were somatic hybrids. However, differences were observed between some of the rDNA hybridization patterns. These could result from chromosome losses or from rearrangements involving the probed rDNA region in the hybrid genome. Rearrangements in the nucleolar chromosomes have been reported earlier for regenerated plants from in vitro culture and more recently for somatic hybrids between *Solanum tuberosum* and *S. phureja* (Pijnacker et al. 1987). In this case, not only rearrangements but also elimination of nucleolar chromosomes were shown. Southern hybridization of nuclear rDNA may be an indication of such phenomena in *Brassica* somatic hybrids.

Different cytoplasms were observed among these plants. In particular, six hybrids (D, H, C, V, U and B) associate *B. napus* ctDNA with hybrid mtDNA. The presence of novel bands in these six plants suggests that the novel mt genomes occurred through intergenomic recombination during protoplast fusion. Intergenomic mt recombination was demonstrated previously in *Petunia* cybrid (Rothenberg et al. 1985) and in *Brassica* cybrids (Chetrit et al. 1985; Vedel et al. 1986). In the latter case, we have shown that some events occur at regions homologous to regions presumed to play a role in natural recombination. The number of *Sal*I mt novel bands appears to be reduced in *B. napus*/*B. hirta* hybrids compared with *B. napus*/*R. sativus* cybrids.

These lines can be used to observe the influence of a hybrid cytoplasm on the phenotypic expression of *B. napus* nucleus and, in particular, on male fertility (after all *B. hirta* chromosomes have been eliminated by crossing). The choice of *B. hirta* as a potential species to induce an interspecific cms in *B. napus* was suggested by their genetic distance. Palmer et al. (1983) established their relationship on the basis of ctDNA divergence: *B. hirta* shows a 1.94 per 100 bp sequence divergence from *B. napus,* which is greater than that between *R. sativus* and *B. napus* (1.08%). Although there is no correlation between this distance (calculated on ctDNA) and the possibility of induction of cms (having a mtDNA basis), the remoteness of the two species could be a source of cms. On the other hand, the normal *R. sativus* cytoplasm is known to induce at least a partial cms in *B. oleracea* (McCollum 1981).

No male sterile cybrids appeared in the entire set of regenerated plants having the *B. napus* phenotype. This means that either no cybrids were recovered, or that the *B. napus* nucleus in the hybrid cytoplasm does not result in cms. Taking into account that 1% of the regenerated plants were hybrids, it is highly probable that some cybrids with napus nucleus were also regenerated. In *Nicotiana,* Gleba et al. (1984) demonstrated that 63% of the intraspecific and 26% of the interspecific somatic fusion products are cybrids. In *B. napus,* some intraspecific fusion experiments conducted in our laboratory indicated that 50% of the fusion products are cybrids (unpublished results). Glimelius et al. (1986) mentioned that cybrids may be recovered after interspecific fusion in *Brassiceae.* Unless there is an incompatibility between the cytoplasm and nucleus of the parental species, it seems that cybrids should have been present. As none were male sterile, this combination seems unable to induce cms in cybrids, at least with these varieties. However, we observed that mitochondrial patterns of the somatic hybrids were more often of the *B. napus* type or very similar; thus, perhaps no cybrid having the required part of the *B. hirta* mtDNA was recovered. The different alloplasmic lines resulting from backcrosses of the nine somatic hybrids having set seeds with *B. napus* are now under study.

All of the hybrids displayed a low fertility; this was also observed in the artificial amphiploids made with *B. napus,* such as *Raphanobrassica.* This has been explained by a genetic imbalance between the parental genomes. Selection improved the fertility of the amphiploids but not by enough to be of practical use; they were therefore included in backcrossing programs with *B. napus* to introduce growth habit or clubroot resistance (McNaughton 1973). Similar research is being conducted on these "naphir" hybrids.

Concerning *Alternaria* tolerance, preliminary results (D Martin, personal communication) showed that none of the somatic hybrids was as tolerant to *Alternaria brassicae* as the *B. hirta* parent. This trait appears to be semi-dominant and thus difficult to introduce into *B. napus.* Nevertheless, offspring of the first and second generation are under investigation. Research is ongoing to improve the pathologic test and, in particular, to study the pathotoxins released in culture filtrates of this fungus and their effect on the host plants.

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