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Mitochondrial DNA polymorphism induced by protoplast fusion in Cruciferae

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Summary. The mitochondrial genomes of five rapeseed somatic hybrid plants, which combine in a first experiment *Brassica napus* chloroplasts and a cytoplasmic male sterility trait coming from *Raphanus sativus,* and in a second experiment chloroplasts of a triazine resistant *B. compestris* and a cytoplasmic male sterility trait from *R. sativus,* were analyzed by restriction endonucleases. Restriction fragment patterns indicate that these genomes differ from each other and from both parents. The presence of new bands in the somatic hybrid mitochondrial DNA restriction patterns is evidence of mitochondrial recombination in somatic hybrid cells. In both parental and somatic hybrid plants large quantitative variations in a mitochondrial plasmid-like DNA have been observed. Our results suggest that the cytoplasmic support for male sterility is located in the chromosomal mitochondrial DNA instead of the plasmid-like DNA.

Key words: Mitochondrial DNA – Cytoplasmic male sterility – Cybrids – *Brassica* – Mitochondrial plasmid-like DNA

Introduction

Belliard et al. (1978, 1979) characterized interspecific cytoplasmic hybrids of *Nicotiana* using restriction enzymes. Analysis of the chloroplast DNA cleavage patterns indicated that only one or the other parental chloroplast DNA was present in the progeny of the cybrids. Recombination was not observed between chloroplast DNAs and no correlation could be found between chloroplast type and cytoplasmic male sterility or fertility. In contrast, the mitochondrial DNAs of progeny of cybrids were different from those of the partners of fusion and there appeared to exist a correlation between mitochondrial DNA patterns and the different level of expression of male sterility (cms) found between different cybrids.

New cytoplasmically inherited and stable flower morphologies on one hand and novel fragments detected in mt DNA patterns, on the other hand, were interpreted as convergent evidence of molecular recombination in the mitochondrial genome. Several groups of investigators working on cytoplasmic hybrids from Solanaceous species reported data in agreement with the above findings (Nagy et al. 1981; Galun et al. 1982; Schiller et al. 1982; Boeshore et al. 1983; Fluhr et al. 1983). Nagy et al. (1983) confirmed that alterations in tobacco mt DNA were not induced by either components of the culture media used for protoplast culture and plant regeneration or homoplasmic fusions, and they provided evidence that rearrangements could be detected only in heteroplasmic fusion combinations.

The mitochondrial genomes of *Cruciferae* appeared to be smaller than all the ones previously found in higher plants (Lebacq and Vedel 1981; Vedel et al. 1982). This fact, associated with the simplicity of the mt DNA restriction patterns, appeared very valuable in studying this mt genome organization in higher plants (Palmer and Shields 1984; Vedel et al. 1984) and the fate of parental mt DNA in cybrids. Furthermore, Palmer et al. (1983) described an unusual mitochondrial DNA plasmid, 11.3 kb in size, in some *Brassica* species. These authors reported a strong association between the presence and abundance of the plasmid and cytoplasmic male sterility.

Recently we have regenerated cybrids, combining in a first experiment, *Brassica napus* chloroplasts and a cytoplasmic male sterility trait from *Raphanus sativus* and, in a second experiment, chloroplasts of a triazine resistant *Brassica campestris* and a cms trait from *Raphanus sativus* (Pelletier et al. 1983). Transfer of chloroplasts has been confirmed by restriction cp DNA analysis and two dimensional protein electrophoresis. The purpose of this study was (a) to confirm that cms

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cybrids actually retained the male sterility character of the cms parent, (b) to determine the mt DNA constitution of *Brassica* cybrids, (c) to correlate the cms trait with a given cytoplasmic element.

Materials and methods

Plant material

The different *B. napus* lines used in fusion experiments and their cybrids have been described by Pelletier et al. (1983) and are summarized here in Table 1. Cms 0 lines of *B. napus* were obtained by sexual crosses involving a cms Japanese radish variety (Ogura 1968; Bannerot et al. 1974). Each cybrid was crossed to a 'Brutor' variety to obtain the first and successive progenies. The restorer line RF was derived from the material obtained by Heyn (1978), who selected it from a cross between a cms 0 (see legend of Table 1) *B. napus* as female and a *B. napus* × *R. sativus* amphidiploid as male. The RF line possessed 38 chromosomes and white fertile flowers. Cms 0 *B. napus* pollinated by this line segregated fertile and sterile plants. The exact genetic constitution of this material and the determinism of restoration are not fully elucidated. Plants were grown in a green-house, either at the CNRA in Versailles or at the Phytotron in Gif-sur-Yvette, at 22 °C and 16 h day-length.

Isolation of mt DNAs

Mt DNAs were isolated from leaves as previously described by using CsCl-ethidium bromide gradients (Vedel et al. 1982).

DNA restriction and agarose gel electrophoresis

Two to 4 µg of mt DNA were digested in 30 µl reactions with sufficient enzyme to give complete digestion. The restriction enzymes used were Sal I, Bgl I (Boehringer Mannheim) and Kpn I (Bethesda Research Laboratories). The restriction fragments were separated by electrophoresis in 0.7% agarose vertical slab gels. A mixture of DNA fragments generated from λ DNA by Hind III and from λ DNA by Hind III and EcoRI (Boehringer Mannheim) was used as a molecular weight standard. The mitochondrial DNA plasmid was isolated after preparative electrophoresis on 0.7% agarose gels of the native mt DNA from cms lines, as described previously (Vedel and Mathieu 1983).

Southern transfer and hybridization

After denaturation, the mt DNA fragments in gels were transferred to nitrocellulose (Schleicher and Schüll BA 85) or to Gene Screen filters (New England Nuclear) by the method of Southern (1975). The mt DNA plasmid was nick-translated using the Amersham nick-translation kit. The probe was hybridized to the prehybridized filters (Vedel and Mathieu 1983). After hybridization, the filters were washed before being dried and autoradiographed for 1 to 7 days using Ilford X-ray films (rapid R, type S) as previously described (Vedel et al. 1982).

Results and discussion

Fertility restoration of rapeseed cybrids

A cms character is qualitatively defined by a specific genetic system of fertility restoration. The cms 0 used in this study is restored by genes existing in radish (Ogura 1969; Bonnet 1975). Pollination by the restorer material (RF, described in Materials and methods) was performed on three cybrids and the cms line C. Results described in Table 2 show clearly that cybrids are restored by RF, although with different segregation ratios (an observation which will not be discussed here).

The fact that cybrids are restored by this material confirms that they actually retained the male sterility character present in the cms parent of fusion and rules out two other possibilities: in vitro induced variation leading to cms and a new cms character created by recombination between parental cytoplasmic (mt) DNAs.

Analysis of parental and cybrids mt DNAs

a) Occurence of mt plasmid-like DNA in the parents of the fusion. A mt plasmid like DNA 1·1.3 kb in size was described to be genetically distributed across a group of 20 *Brassica* cytoplasms (Palmer et al. 1983). In a previous work on the comparison of the cytoplasms of normal and cms 0 *B. napus* we did not find this plasmid

Table 1. Cytoplasmic genotypes and phenotypes of parent and cybrid plants

		Organelle DNA		Cytoplasmic traits			
		Chloroplast	Mitochondria	Atrazine	Chloro-phyll	Nectary	cms/F
Parents	'Brutor'	*B. napus*	*B. napus*	S	normal	+	F
	C	*R. sativus*	*R. sativus*	S	deficient	–	cms 0[a]
	'Tower'	*B. campestris*	*B. campestris*	R	normal	+	F
Cybrids	27	*B. napus*	described	S	normal	+	cms
	58	*B. napus*	in this	S	normal	+	cms
	85	*B. napus*	paper	S	normal	+	cms
	118	*B. napus*		S	normal	+	cms
	77	*B. campestris*		R	normal	+	cms

[a] cms 0: A cytoplasmic male sterility discovered in radish by Ogura (1968) and transfered into *Brassica* by Bannerot *et al.* (1974)
S = susceptible; R = resistant; F = male fertile; (+) = normal nectaries, (–) = underdeveloped nectaries

(Vedel et al. 1982). Figure 1 shows the Sal I, Kpn I and Bgl I restriction patterns for mt DNA from cybrid 58 and from normal (n) and cms 0 (s) lines of *B. napus*, the parents used in the fusion experiments ('Brutor' and C lines, respectively). It is noticeable that the cms 0 line C (not previously analyzed by us) contains the mitochondrial plasmid, 11.3 kb in length, (Fig. 1, lane S1) described by Palmer et al. (1983). Digestions with the restriction endonucleases Sal I, Kpn I, and Bgl I convert the main mitochondrial genome into a series of discrete bands without affecting the mitochondrial plasmid. Figure 1 also indicates that the 11.3 kb molecule occurs in the mitochondria from cybrid 58 but is absent in the n parental line 'Brutor'.

On the other hand, we found the mt DNA plasmid in several cms 0 spring lines, such as the C line, but not in n spring lines nor non vernalized n and cms 0 winter lines of rapeseed. An hypothetical link between the occurence of the mt DNA plasmid and the flowering phenomenon in *B. napus* was investigated. Plantlets of n and cms 0 winter lines were grown at 5 °C and 9 h day length for 6 weeks. After the cold treatment, the plantlets were grown as indicated in the 'Materials and methods' and their mt DNA analyzed every two weeks until complete flowering. The mt DNA plasmid was absent in vernalized n and cms 0 winter lines as shown both in the Sal I restriction patterns and in the autoradiographs obtained after hybridization between a blot of these patterns and the ^{32}P nick-translated plasmid (Fig. 2). Clearly, the environmental variations involved in the flowering process do not lead to the presence of the 11.3 kb element.

Table 2. Fertility restoration of three rapeseed cybrids

Crosses		Progenies		More probable ratio
Female	Male	Fully fertile plants	Entirely sterile plants or sporadically fertile plants	
C	× RF	10	31	1/3
Cybrid 27	× RF	22	28	1/1
Cybrid 58	× RF	21	26	1/1
Cybrid 118	× RF	17	30	1/3 or 1/1
RF selfed		23	13	9/7 or 3/1

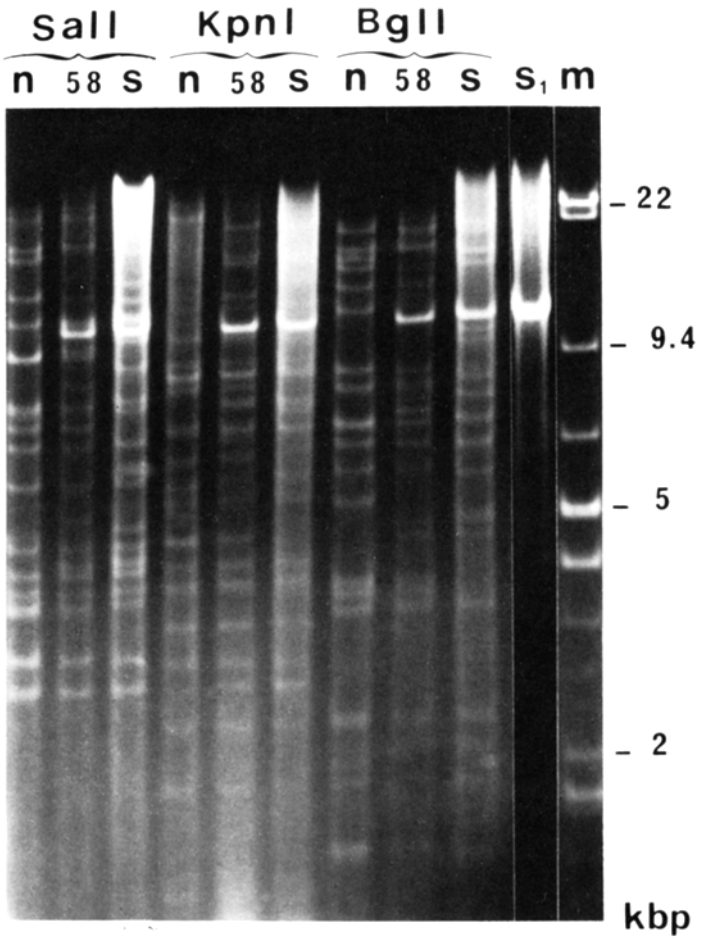

Fig. 1. Sal I, Kpn I and Bgl I restriction patterns of mt DNAs from (n) 'Brutor'; (*s*) C; (*58*) cybrid from 'Brutor' and C; (s_1) native mtDNA from C; (*m*) molecular weight standard (marker II + Marker III, Boehringer Mannheim). The restriction fragments were separated by electrophoresis on vertical slab gels containing 0.7% agarose

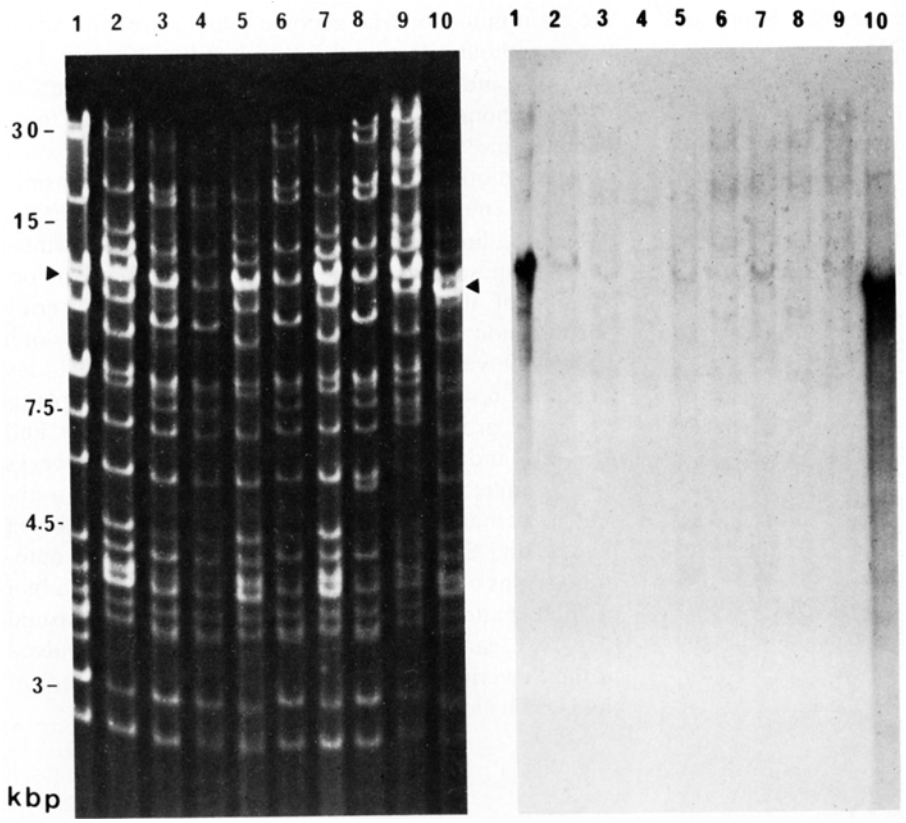

Fig. 2. Absence of the 11.3 kb mitochondrial plasmid-like DNA in normal (line 'Jet 9') and cms (line S 82) *B. napus* of the winter type, before and after a 6 weeks cold treatment at 5 °C. *On the left,* agarose slab gel electrophoresis of mt DNA Sal I digests: (*1*) and (*10*) mt DNAs from spring lines 'Tower' and C, respectively, used as proofs; (*2*) and (*3*) mt DNAs from winter cms and n *B. napus* respectively, before the cold treatment; (*4*) and (*5*) mt DNAs from winter n and cms *B. napus* respectively, 5 days after the end of the cold treatment; (*6*) and (*7*) mt DNAs from winter n and cms *B. napus* respectively 20 days after the end of the cold treatment; (*8*) and (*9*) mt DNAs from winter n and cms *B. napus* respectively, during flowering, 40 days after the end of the cold treatment. Triangles locate the 11.3 kb mt plasmid on Tower and C Sal I patterns. *On the right,* hybridization of ^{32}P nick-translated mt plasmid-like DNA (purified by preparative gel electrophoresis from cybrid 27) to a Gene Screen blot of the gel shown on the left

Of the three spring lines used in the two fusion experiments, 'Tower' (n *B. napus* with *B. campestris* cytoplasm) and C (cms 0 *B. napus*) contain the mt DNA plasmid (Fig. 2, lanes 1 and 10, respectively) and 'Brutor' does not. We have found the plasmid in atrazine-resistant *B. campestris* (the female parent of 'Tower' line) but not in the cms 0 radish (the female parent of C). However, as pointed out by Palmer et al. (1983) the failure to find the plasmid in one or more accessions of a given species does not signify that other accessions of the same species will not be found to contain the plasmid.

b) Mitochondrial DNAs of cybrids. Figure 3 shows parental and five cybrid restriction patterns. It is clear that each cybrid possesses a new pattern different from the parents and from each other. Cybrids 27, 58, 85, 77^{II} possess respectively 3, 2, 2 and 1 fragments with a new molecular weight, absent in parents. It is also clear that these four cybrids possess the plasmid-like molecule. Mt DNA of cybrid 118 is characterized by a new Sal I pattern, although apparently without both new restriction fragment and plasmid-like molecule. The patterns presented in Fig. 3 correspond to cybrid progenies at the second generation and no differences have been found with plants of the first generation.

These results proved that in cruciferae protoplast fusion leads to plants with new mitochondrial DNA – this is in agreement with previous findings in the Solanaceae (Belliard et al. 1979; Nagy et al. 1981; Galun et al. 1982; Boeshore et al. 1983). The hypothesis of mitochondrial recombination is in accordance with physical maps of *Brassica* mt DNA which need such events to be explained (Palmer et al. 1984; Vedel et al. 1984). Cloning studies on cybrid mt DNAs are in progress to elucidate the role of homologous intramolecular recombination sequences, already detected by physical mapping (Palmer et al. 1984; Vedel et al. 1984), in recombination occuring after protoplast fusion.

Which cytoplasmic DNA is involved in the cms character?

Previous results (Pelletier et al. 1983) allow us to discard the chloroplast genome as the cytoplasmic element re-

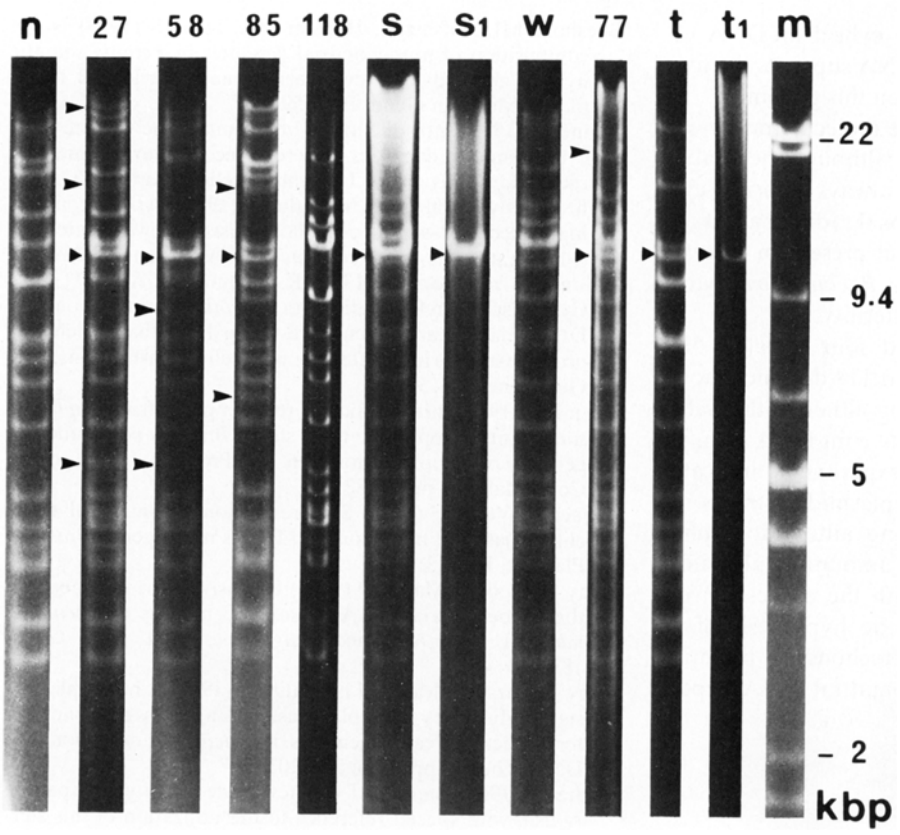

Fig. 3. Sal I restriction patterns of mt DNAs from parents of the two fusion experiments and from their cybrids. (*n*) 'Brutor', (*s*) C; (*27*), (*58*), (*85*), (*118*) cybrids from 'Brutor' and C; (*t*) 'Tower'; (77^{II}) cybrid from C and 'Tower'; (s_1) and (t_1) native mt DNA from C and 'Tower' respectively; (*w*) cms *B. napus* of the winter type (S 82); (*m*) molecular weight standard as in Fig. 1. *Arrows* indicate the new Sal I fragments; *triangles* locate the 11.3 kb mt plasmid like DNA

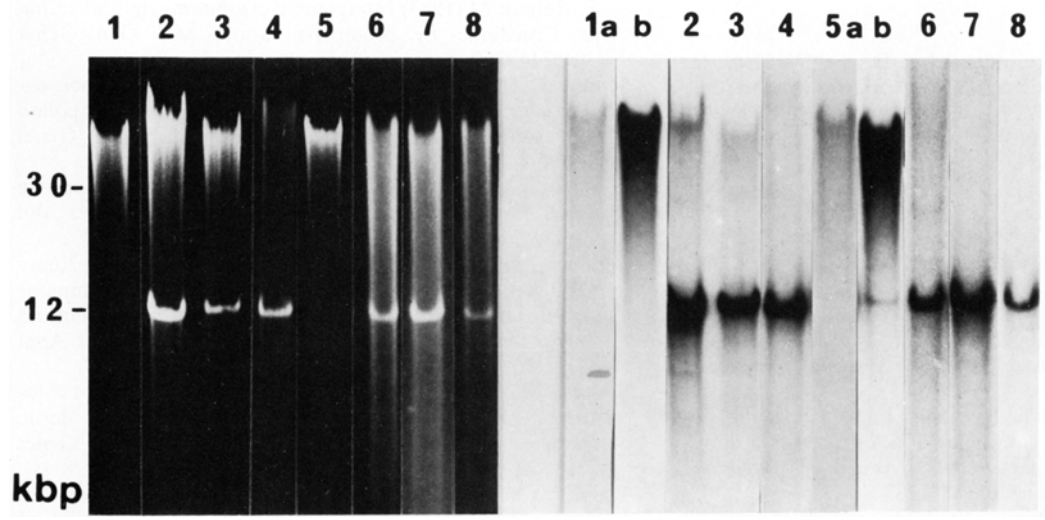

Fig. 4. Distribution of the 11.3 kb mt plasmid-like DNA in the parents of the two fusion experiments and in their cybrids. *On the left,* fractionation by electrophoresis on 0.7% agarose gel of native mt DNAs from (*1*) 'Brutor'; (*2*) cybrid 27; (*3*) cybrid 58; (*4*) cybrid 85; (*5*) cybrid 118: (*6*) C; (*7*) cybrid 77^{II}; (*8*) 'Tower'. *On the right,* hybridization of ^{32}P nick-translated mt plasmid-like DNA isolated from cybrid 27 to a Gene Screen blot of the gel shown on the left. In the case of *1b* and *5b,* the autoradiographs correspond to an exposure 20 times longer than in *1a* and *1b*

sponsible for cms. The presence in cybrid mt DNA of fragments belonging to radish mt DNA supports the hypothesis that cms genes are located on this genome.

The presence of the plasmid-like molecule may render the interpretation more difficult although the analysis of cms 0 lines shows that it is not always inherited cytoplasmically. It is absent in the cms 0 radish parent as well as in cms 0 winter rapeseed, but present in several spring varieties. It is also present in *B. campestris* cytoplasm which does not lead to male sterility.

Among the five cybrids analysed, four lines (27, 58, 85, 77^{II}) possess this plasmid. Cybrid 118 does not show it on the u.v. patterns corresponding either to the Sal I mt DNA digest (Fig. 3) or to the native mt DNA (Fig. 4, lane 5 on the left). Hybridization experiments indicate the presence of the mitochondrial plasmid as traces in 118 native mt DNA only after long autoradiography (Fig. 4, lane 5b on the right). The amount of plasmid-like molecules is not correlated with the expression of cms. These observations weaken the hypothesis of a causal relationship between the mitochondrial plasmid and cms, comforting the chromosomal mt DNA hypothesis.

Conclusion

Cybrids obtained in *Brassica* genus confirm previous data obtained in Solanaceae – alloplasmic male sterility is the result of mitochondria-nucleus interactions; protoplast fusion is followed by interparental recombination of mt DNA leading to several new mitochondrial genomes. This material is the most suitable to determine the mechanisms of recombination and the location of "male sterile genes".

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