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Cytoplasmic male sterility in rapeseed (*Brassica napus* L.)

1. Restriction patterns of chloroplast and mitochondrial DNA

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Summary. Restriction patterns of chloroplast (cp) and mitochondrial (mt) DNA in *Brassica napus* rapeseed reveal the alloplasmic nature of cytoplasmic male sterility in this crop. Both the Shiga and Bronowski systems probably exploit cytoplasmic diversity in *B. napus* cultivars arising from introgression of cytoplasm from the other rapeseed species, *B. campestris*. Nuclear genes specific to these systems do not cause sterility in maintainers (Bronowski and Isuzu-natane) because they have a *campestris* cytoplasm, but give rise to sterility in *napus* cytoplasms. In the course of hybridization to *napus* cultivars a line with the triazine resistant cytoplasm (a *campestris* cytoplasm) has undergone an alteration in the mt genome rendering its restriction pattern more similar than previously to that of *napus*. The alteration may be an inversion between 7.2 and 3.4 kb in length.

Key words: *Brassica napus* – Cytoplasmic male sterility – Chloroplast DNA – Mitochondrial DNA – Restriction patterns

Introduction

Cytoplasmic male sterility is a widespread phenomenon among plant species (Edwardson 1970). Researchers have used a variety of approaches to determine the mechanism of cytoplasmic male sterility (reviewd by Pearson 1981) and many believe that the cytoplasmic mutation responsible resides in mitochondrial (mt) DNA although virus-like elements are implicated in some instances.

Restriction endonucleases have become a useful tool for examining cytoplasmic mutants. Comparisons of organelle DNA from normal and cytoplasmic male sterile (cms) mutants by means of restriction patterns have provided information in corn (Levings and Pring 1976; Pring and Levings 1978; Thompson et al. 1980), wheat (Quetier and Vedel 1977), tobacco (Frankel et al. 1979), faba beans (Boutry and Briquet 1982), sugarbeet (Powling 1982) and sorghum (Conde et al. 1982; Pring et al. 1982). More recently, altered restriction patterns of mt DNA following fusion of fertile and cms *Petunia* have allowed Boeshore et al. (1985) to locate a sequence of mt DNA unique to cms regenerants.

In rapeseed (*Brassica napus, B. campestris* L., hereafter referred to as *napus* rapeseed and *campestris* rapeseed), cms systems have not been developed to the point of commercial application and to date publication of data at the molecular level has focussed largely on the *Raphanus* (Bannerot et al. 1974) system. This cytoplasm, derived from radish, differs from that of a normal, fertile *napus* cultivar with respect to the location of chloroplast (cp) genes and restriction sites (Vedel and Mathieu 1983), thylakoid proteins (Rémy and Ambard-Bretteville 1983), mt DNA restriction patterns and mt in vitro translation products (Vedel et al. 1982). Regenerants from protoplast fusion experiments demonstrate that in this cytoplasm sterility is not associated with the chloroplast (Pelletier et al. 1983) and that recombination of mt genomes also occurs in *Brassica* (Chétrit et al. 1985). Palmer et al. (1983) have reported a mitochondrial plasmid in this cytoplasm and in one other described only as being from Korea.

We herein report a comparison, on the basis of restriction patterns, of cp and mt DNA from five cms lines in *napus* rapeseed, their maintainers (where available) and normal rapeseed plants.

Materials and methods

Plant material

A summary of some cms systems in *napus* is presented in Table 1. Seed for the normal cultivars, Westar (*napus*) and Torch (*campestris*) was provided by Dr. K. Downey, Agri-

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Table 1. Cytoplasmic male sterile system in rapeseed

Designation of male sterile systems	Male sterile cytoplasms	Maintainer(s)	Maintainer cytoplasm	Restorer(s)	Restorer cytoplasm
Shiga; nap;[a]	*napus* (nap)[a] (+)[b]	Isuzu-natane	*campestris* (cam)[a] (+)	most *napus* cultivars	*napus*
Bronowski; nap[a]	*napus* (nap) (+/–)	Bronowski	*campestris* (cam)[a] (+/–)	most *napus* cultivars	*napus*
Raphanus;[c] radish; ogu	*Raphanus*[c] *sativus* (+/–)	all *napus* cultivars	*napus* (+/–)	some *napus* lines	not yet examined
Diplotaxis[c]	*Diplotaxis muralis* (–)	Karat	not yet examined	many *napus* cultivars	*napus*
polima	(polima) (*juncea?*) (+)	most *napus* lines	*napus* (+/–)	some *napus* lines	polima
ctr	*campestris* triazine resistant (+)	Bronowski (?)	*campestris* (+/–)	all *napus* cultivars	*napus*

[a] See "Results and discussion" for explanation of nap system and nap (*napus*) and cam (*campestris*) cytoplasms

[b] (+), (–), (+/–) indicate presence, absence and variable presence of 11.3 kb mitochondrial plasmid within a line or between lines within a species

[c] Both *Raphanus* and *Diplotaxis* are genera of the Crucifera family as is *Brassica*

culture Canada, Saskatoon, Saskatchewan. Dr. T. Shiga (National Institute of Agricultural Sciences, Division of Genetics, Kannondai, Yatabe, Tsukuba, Ibaraki 30021, Japan) provided seed for his nap cms line, as well as the maintainer, *Isuzu-natane.* Dr. M. Renard (I.N.R.A., Station d'Amélioration des Plantes, Domaine de la Motte au Vicomte, BP 29, 35650 Le Rheu, France) was the source for the *napus* rapeseed line with the *Raphanus* (radish) or ogu cytoplasm. No restorers for this cytoplasm were available for this study and all *napus* cultivars are maintainers although restorer *napus* lines have apparently been developed in France (Heyn 1978). A *napus* rapeseed line with the polima cytoplasm was provided by Dr. L. Sernyk, Dept. of Plant Science, University of Manitoba (presently at Allelix Inc., Mississauga, Ontario). Most *napus* cultivars tested to date maintain the sterility of the cytoplasm. Male-sterile *napus* rapeseed plants with the *Diplotaxis* cytoplasm were from a population of F_1's segregating for sterility and derived from backcrossing this cytoplasm to the *napus* cultivar Karat. *Diplotaxis* is a genus of the Cruciferae family and the *Diplotaxis* cytoplasm was introduced to *napus* rapeseed by backcrossing to a wild accession of *D. muralis.* This segregating population was also provided by Dr. L. Sernyk. The development of a cms system in *napus* with the triazine resistant cytoplasm is described elsewhere (Grant and Beversdorf 1985). Commercial utilization of cytoplasmically-linked triazine resistance and male sterility for the purpose of producing F_1 hybrids is protected by patent applications in United States and elsewhere (Beversdorf et al. 1985). Seed of the *napus* variety, *Bronowski,* was obtained from Dr. Shiga in Japan and an unidentified source in Poland.

For purposes of extracting organelle DNA, multiplication of most lines was accomplished by uncontrolled pollination.

Extraction of chloroplast and mitochondrial DNA

Cp DNA was extracted as previously described (Erickson et al. 1983) either from flowering plants or seedlings grown in flats. Mt DNA was extracted from the same leaf tissue as the cp DNA by a method already described (Erickson et al. 1985). Since the mt plasmid is associated with a terminal protein, a proteinase K step is required during the extraction to prevent loss of the plasmid in the phenol phase (Erickson et al. 1985).

Results and discussion

Figure 1 A (cp DNA digested with Eco RI) and 1 B (mt DNA digested with PstI) illustrate the alloplasmic nature of cms systems in *napus* rapeseed. Table 1 presents a summary of those cms systems which were examined in this study. Each cms line can be distinguished on the basis of cp as well as mt DNA restriction patterns. Almost any enzyme can be used to distinguish the mt genomes of these cytoplasms but fewer of the commonly available enzymes can be used to distinguish the cp genomes. Comparisons were also made within these systems between sterile and maintainer (fertile) lines.

The cytoplasmic origins of the *Diplotaxis* line as well as the ogu line are already known (*Diplotaxis muralis* and *Raphanus sativus*) and Vedel et al. (1982) have shown that the restriction patterns of cp and mt DNA of the latter are still those of radish. It is not surprising then that organelle-

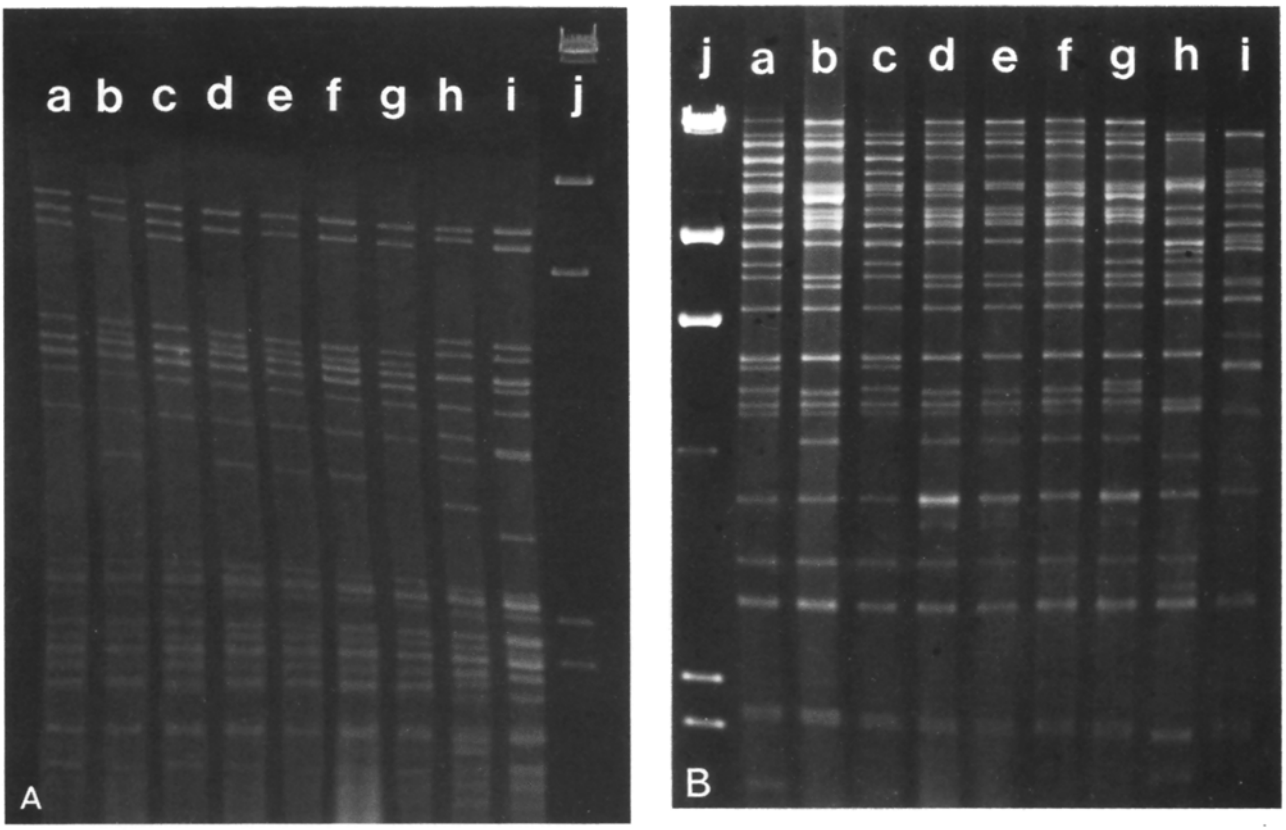

Fig. 1. Eco RI digests of cpDNA (**A**) and Pst I digests of mtDNA (**B**) from normal, fertile cultivars: *Westar* (*a*), *Torch* (*b*) and *Triton* (*f*); Shiga's nap cms line (*c*) and the two maintainers for this system: Isuzu-natane (*d*) and *Bronowski* (*e*); other cms lines: polima (*g*), *Diplotaxis* (*h*) ogu (*i*) (see Table 1). *Lane j* in **A** and **B** contains a Hind III digest of lambda DNA. *Westar* and *Torch* are *napus* and *campestris* cultivars respectively and each has an organelle restriction pattern typical of its species. *Triton* is a *napus* cultivar incorporating the *campestris* triazine resistant cytoplasm on which the ctr system is based

encoded proteins (Remy and Ambard-Bretteville 1983) and mt and cp DNA restriction patterns of these lines are distinct and different from those of *napus* and *campestris* (Fig. 1). It has not yet been determined whether the differences in organelle-encoded proteins or organelle DNA restriction patterns between these alien cytoplasms and those of *napus* and *campestris* are related to sterility. However, the differences in restriction patterns and protein electrophoregrams are useful as organelle markers to determine sorting out and recombination of organelle genomes following fusion experiments (Pelletier et al. 1983; Chétrit et al. 1985).

The origin of the polima cytoplasm is unknown and the Pst I pattern of polima mt DNA is different from all others examined, but is most similar to that of *campestris* (Fig. 1 B). This may indicate that the polima cytoplasm is from *juncea*, an amphiploid thought to have acquired its cytoplasm from *campestris* (Erickson et al. 1983; Palmer et al. 1983; Banga et al. 1983). Support for this hypothesis comes from recent evidence that lines from *juncea* carry genes that restore fertility to material with this cytoplasm (Dr. L. Sernyk, personal communication).

The cp DNA restriction patterns of Shiga's nap cms line were identical to those of typical *napus* cultivars such as *Tower* or *Westar* with every enzyme used to date (Sph I, Sst I, Sal I, Xho I, Sma I, Kpn I, Pvu II, Xba I, Eco R1, Bam H1, Hind III, Pst I, Bgl II, Cla I, Bcl I). Although the largest *napus* fragment is very faint in Pst digests of mt DNA from Shiga's nap cms line (Fig. 1 B), the rest of the pattern is the same as *napus*. However, the cp patterns of Shiga's maintainer (Isuzu-natane) were identical to those of *campestris* with all the above enzymes (Eco R1 for example, Fig. 1 A) and the same was true for the mt patterns with Pst I (Fig. 1 B).

Observations of sterility in progeny from many crosses have led Shiga (1980) to conclude that most *napus* cultivars from Europe and Japan have an S (sterility-inducing) or "nap" cytoplasm. Some Japanese cultivars have N (non-sterile-inducing) cytoplasms, derived apparently from two interspecific (*campestris* × *napus*) crosses, and designated as "cam" cytoplasms (Shiga 1980). Thus, his cms line has a nap cytoplasm and *Isuzu-natane*, a cam cytoplasm (Table 1) and certain nuclear genes involved in pollen development which function normally in *campestris* cytoplasm give rise to male sterility in *napus* cytoplasms; most *napus* cultivars possess the dominant nuclear alleles for these genes and are, therefore, fertile despite a sterile cytoplasm.

Restriction patterns of organelle DNA support Shiga's classification of cytoplasms in *napus* as well as the proposed origin of the cam cytoplasm. They also explain why the European *napus* cultivar, *Bronowski*, can replace *Isuzu-natane* as a maintainer for Shiga's nap cms line as well as induce (and subsequently maintain) sterility when used as a male in crosses with European and Canadian *napus* cultivars (Thomp-

son 1972; Shiga 1980; Rousselle and Renard 1982). With every enzyme used to date the cp patterns of *Bronowski* are the same as those of *Isuzu-natane* and the *campestris* species (Erickson et al. 1983; Eco R1, for example, Fig. 1 A). The same is true of the Pst I pattern of mt DNA (Fig. 1 B).

Confirmation of this general model for the Shiga-*Bronowski* cms system was recently provided by crossing experiments of Dr. L. Sernyk (personal communication). Occasionally the male sterility in Shiga's nap cms line breaks down and small amounts of pollen are produced. Such pollen was used to transfer the recessive nuclear alleles responsible for sterility to the *napus* cultivar *Regent* and to *Bronowski*. Plants which are homozygous for these alleles and contain the *napus* cytoplasm will be sterile. Regent is derived from *Tower*, a cultivar known to have the *napus* cytoplasm (Erickson et al. 1983) and stable sterile lines were produced from the cross with Shiga's nap cms line; the lines from the cross, *Bronowski* × Shiga's nap cms line, maintain this sterility.

A desire to combine triazine resistance and cytoplasmic male sterility prompted attempts to sexually hybridize a *napus* line containing the triazine-resistant cytoplasm and *Bronowski* with the latter as male (Grant and Beversdorf 1985). Although male-sterile plants were produced, maintainers have not yet been found. Cp DNA restriction patterns indicate that the triazine resistant cytoplasm is of the *campestris* type (Fig. 1 A; Erickson et al. 1983) as does the Pst I pattern of mt DNA (Fig. 1 B).

The above data suggests a general model for current cms systems in rapeseed: the use of cytoplasmic diversity in conjunction with nuclear genes more or less adapted to these cytoplasms to the extent that certain nuclear-cytoplasmic combinations give rise to more or less male sterility.

Alterations in the mitochondrial genome

Although restriction data indicate that Shiga's nap cms line has a *napus* cytoplasm, the largest Pst I fragment of the mt genome is very faint. Since the nuclear genes for sterility from this line also caused sterility in Regent (see above), a cultivar with typical *napus* mt restriction patterns, this feature is probably not involved in sterility.

A more interesting alteration has occurred in the mt DNA from two male-sterile F_1's (ctr/Bron(st)), generated by crossing a triazine resistant male-sterile *napus* (ctr) by *Bronowski* as male. The 4.4 kb Pst I fragment is absent (position bracketed by negative sign in lane a, Fig. 2) and a new 13.5 kb fragment has appeared (bracketed by a positive sign in lane a, Fig. 2). Also the 3.8 and 17 kb Sal I fragments are reduced in intensity (bracketed by negative sign in lane d, Fig. 2) and two new fragments of 5 and 15.8 kb have appeared (bracketed by positive sign in lane d, Fig. 2). These alterations occur in the same region of the mt genome according to the restriction map of Palmer and Shields (1984). It is to be noted that no other alterations are apparent from the restriction patterns and that this alteration is not in the region of the direct repeat thought to be involved in recombination of the mt genome in *Brassica* (Palmer and Shields 1984). As well, this restriction pattern is repeatable with several fold higher enzyme concentrations than normal and the size of the new fragments preclude the possibility of partial digestion. However, fragments of 4.4 kb (Pst I) and 17 kb (Sal I) do appear but very faintly in the digests from these altered ctr/Bron(st) plants. These observations suggest mitochondrial heterogeneity (for the alteration) between plants (one plant was large and one was small) or within plants. A 13.5 kb Pst 1 fragment was also faintly visible for perhaps the same reason in the digest of ctr/Bron(f) which was extracted from several fertile F_1 plants of the same cross (data not shown).

These alterations in restriction patterns could be due to an inversion in this region of the mt genome. Such an inversion (Fig. 3) and concomitant movement of restriction sites would account for the increase in size of the 3.8 kb fragment to 5.0 kb and corresponding decrease in size of the 17.0 kb fragment to 15.8 kb in the Sal digest. The same inversion in the same region of the genome would also account for the disappearance of the 4.4 kb fragment and the appearance of the

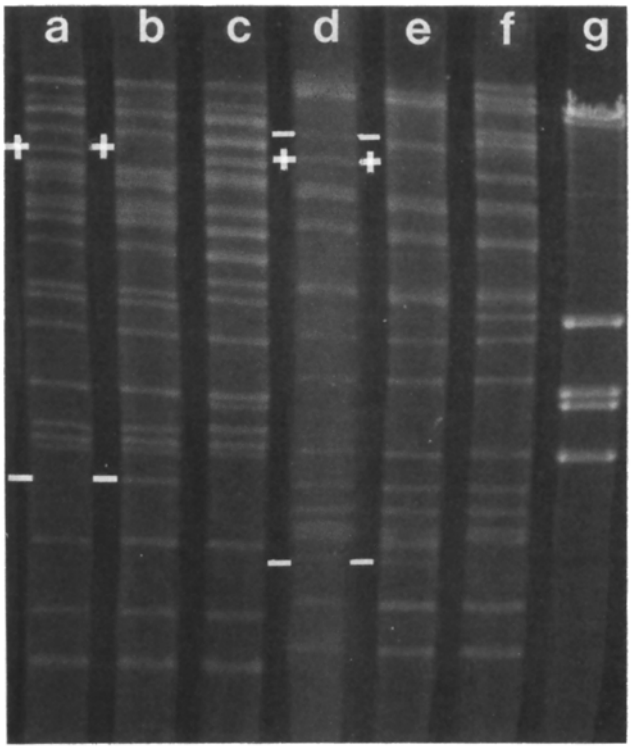

Fig. 2. Mt DNA from ctr/Bron (st) plants (*a, d*), *Triton* (*b, e*), and *Westar* (*c, f*) digested with Pst I (*a* to *c*) and Sal I (*d* to *f*). Note the new bands in ctr/Bron (st) (bracketed by a positive sign in *lanes a* and *d*) that do not occur in the original *campestris* cytoplasm (*lanes b* and *e*) but which do exist in the *napus* cytoplasm (*lanes c* and *f*). Note also the bands which have been lost in the ctr/Bron (st) plants (bracketed by a negative sign) and which are also missing in the *napus* cytoplasm

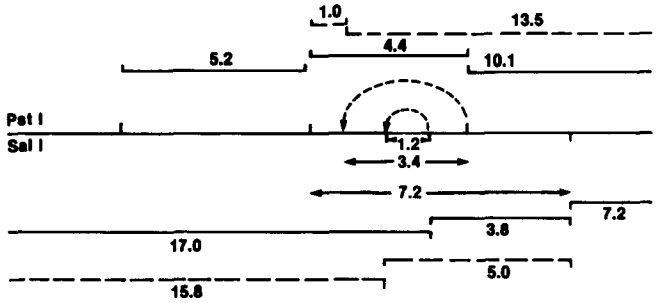

Fig. 3. Alteration in mitochondrial genome. Center line is restriction map of segment of normal *campestris* mitochondrial genome with Pst I sites marked above the line and Sal I sites below (from Palmer and Shields 1984). The solid lines above and below the center line are the normal Pst and Sal fragments (respectively) and the numbers represent kilobase pairs. The curved broken arrows show the displacement of the Pst and Sal sites resulting in the new Pst and Sal fragments represented by the broken lines. The presence of the 1.0 kb Pst fragment is inferred since the photo does not include that region of the gel

13.5 kb fragment in the Pst digest. An inversion would explain the displacement of the Sal site (1.2 kb) and of the Pst site (3.4 kb), both in the same direction with no net loss in fragment size. The continued presence of the 10.1 Pst fragment adjacent to the 4.4 kb Pst fragment could be explained by the presence of a 10.1 kb Pst fragment on the smaller of the two sub-circles of the mt genome postulated by Palmer and Shields (1984). The hypothesized inversion would eliminate the 10.1 kb fragment in the master circle or larger sub-circle resulting from recombination within the master circle, but the 10.1 kb Pst fragment in the smaller sub-circle would continue to exist.

Alignment of the centres for the Sal and Pst alterations, assuming that the axis of the inversion occupies the midpoint between the old and new restriction sites, requires an adjustment of about 1% in the restriction map of Palmer and Shields (1984). Such an alignment reveals that the restriction sites nearest to but unaffected by the putative inversion are Pst I and Sal I sites, approximately 7.2 kb apart (Fig. 3). The length of inverted DNA would thus be less than 7.2 kb and greater than 3.4 kb (the difference between the 10.1 and the 13.5 Pst fragments).

The most interesting observation, however, is that the presence of a 13.5 kb fragment and absence of a 4.4 kb fragment is a distinguishing feature of Pst I digests of *napus* mt DNA compared to that of *campestris*. An alteration has apparently occurred in the triazine resistant cytoplasm of these plants, a *campestris* cytoplasm undergoing repeated backcrossing to *napus* cultivars, such that its Pst I and Sal I restriction patterns of mt DNA have become more similar to that of *napus* than they were originally (Fig. 2).

Such an alteration could be due to: a) recombination with mt DNA in *napus* mitochondria transmitted through the pollen; b) a nuclear-directed mutation or; c) recombination/reorganization events that seem to occur frequently in the mt genome of plants. Sequencing data should provide some insight into the nature and extent of this modification.

Although this alteration in mt DNA occurred in male-sterile plants, it is probably not associated with male sterility. Single plant extractions from three other male-sterile F_1 plants in this cross revealed typical *campestris* patterns.

Restriction digests have been employed to characterize cp and mt DNA in cms lines in rapeseed. They have provided insights into the operation of these systems, revealed alterations in the mt genome, and given us valuable markers for following the fate of organelles and organelle DNA in fusion and transformation experiments.

References

Banga SS, Banga SK, Labana KS (1983) Nucleo-cytoplasmic interactions in *Brassica* In: Proc 6th Int Rapeseed Conf. Paris, pp 602–606

Bannerot H, Loulidard L, Cauderon Y, Tempe T (1974) Transfer of cytoplasmic male sterility from *Raphanus sativus* to *Brassica oleracea.* Proc Eucarpia Meeting-Crucifera Crop Sect 25:52–54

Beversdorf W, Erickson L, Grant I (1985) Hybridization process utilizing a combination of cytoplasmic male sterility and herbicide tolerance. US Patent No 4, 517, 763

Boeshore ML, Hanson MR, Izhar S (1985) A variant mitochondrial DNA arrangement specific to *Petunia* stable, sterile somatic hybrids. Plant Mol Biol 4:125–132

Boutry M, Briquet M (1982) Mitochondrial modification associated with cytoplasmic male sterility in faba beans. Eur J Biochem 127:129–135

Chétrit P, Mathieu C, Vedel F, Primard C (1985) Mitochondrial DNA polymorphism induced by protoplast fusion in *Cruciferae.* Theor Appl Genet 69:361–366

Conde MF, Pring DR, Schertz KF, Ross W (1982) Correlation of mitochondrial DNA restriction endonuclease patterns with sterility expression in six male-sterile sorghum cytoplasms. Crop Sci 22:536–539

Edwardson JR (1970) Cytoplasmic male sterility. Bot Rev 36:341–420

Erickson LR, Straus NA, Beversdorf WD (1983) Restriction patterns reveal origins of chlorplast genomes in *Brassica* amphiploids. Theor Appl Genet 65:201–206

Erickson L, Beversdorf WD, Pauls KP (1985) Linear mitochondrial plasmid in *Brassica* has terminal protein. Curr Genet 9:679–682

Frankel R, Scowcroft WR, Whitfeld PR (1979) Chloroplast DNA variation in isonuclear male sterile lines of *Nicotiana.* Mol Gen Genet 169:129–135

Grant I, Beversdorf W (1985) A new cms system (ctr) in spring-planted oilseed rape (*Brassica napus* (L.) (submitted)

Heyn FW (1978) Introgression of restorer genes from *Raphanus sativus* into cytoplasmic male sterile *Brassica napus* and

the genetics of fertility restoration. In: Proc 5th Int Rapeseed Conf Malmoe, pp 82–83

Levings III CS, Pring DR (1976) Restriction endonuclease analysis of mitochondrial DNA from normal and Texas male sterile maize. Science 193:158–160

Palmer JD, Shields CR, Cohen DB, Orton TJ (1983) An unusual mitochondrial DNA plasmid in the genus *Brassica*. Nature 301:725–728

Palmer JD, Shields CR (1984) Tripartite structure of the *Brassica campestris* mitochondrial genome. Nature 307: 437–440

Pearson OH (1981) Nature and mechanism of cytoplasmic male sterility in plants (a review). Hort Science 16:482–487

Pelletier G, Primard C, Vedel F, Chétrit P, Rémy R, Rousselle P, Renard M (1983) Intergeneric cytoplasmic hybridization in *Cruciferae* by protoplast fusion. Mol Gen Genet 191:244–250

Powling A (1982) Restriction endonuclease analysis of mitochondrial DNA from sugarbeet with normal and male-sterile cytoplasms. Heredity 49:117–120

Pring DR, Levings CS (1978) Heterogeneity of maize cytoplasmic genomes among male sterile cytoplasms. Genetics 89:121–136

Pring DR, Conde MF, Schertz KF (1982) Organelle genome diversity in sorghum: male-sterile cytoplasms. Crop Sci 22:414–421

Quetier F, Vedel F (1977) Heterogeneous population of mitochondrial DNA molecules in higher plants. Nature 168:365–368

Rémy R, Ambard-Bretteville F (1983) Two dimensional analysis of chloroplast proteins from normal and cytoplasmic male sterile *Brassica napus*. Theor Appl Genet 64:249–253

Rousselle P, Renard M (1982) Intérêt du cultivar *Bronowski* pour l'obtention de plantes mâle-stériles cytoplasmiques chez le colza (*Brassica napus* L.). Agronomie 2:951–956

Shiga T (1980) Male sterility and cytoplasmic differentiation. In: Tsunoda S (ed) *Brassica* crops and wild allies, biology and breeding. Jpn Sci Soc Press, Tokyo, pp 205–221

Thompson KF (1972) Cytoplasmic male sterility in oilseed rape. Heredity 29:253–257

Thompson RD, Kemble RJ, Flavell RB (1980) Variations in mitochondrial organization between normal and male-sterile cytoplasms of maize. Nucleic Acids Res 8:1999–2008

Vedel F, Mathieu C, Lebacq P, Ambard-Bretteville F, Rémy R, Pelletier G, Renard M, Rousselle P (1982) Comparative macromolecular analysis of the cytoplasms of normal and cytoplasmic male sterile *Brassica napus*. Theor Appl Genet 62:255–262

Vedel F, Mathieu C (1983) Physical and gene mapping of chloroplast DNA from normal and cytoplasmic male sterile (radish cytoplasm) lines of *Brassica napus*. Curr Genet 7:13–20