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Differences between, and possible origins of, the cytoplasms found in fertile and male-sterile onions (*Allium cepa* L.)

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Summary. The DNA of the organellar genomes of *Allium cepa* has been examined to detect restriction fragment length polymorphisms. Differences can be shown between both the chloroplastal and mitochondrial genomes of the N and cms-S cytoplasms in their restriction fragment profiles. Southern blot analysis of the mtDNA profiles using probes containing defined mitochondrial genes also detected polymorphisms. No differences can be shown between the organellar genomes of the N and cms-T onions by either of these techniques. These data indicate different origins for the two sterility-conferring cytoplasms, suggesting autoplasmic and alloplasmic origins for the cms-T and cms-S cytoplasms, respectively. No evidence of the presence of virus-like particles was found in any of the cytoplasms.

Key words: *Allium cepa* – Chloroplastal DNA – Cytoplasmic male sterility – Mitochondrial DNA – Restriction fragment length polymorphism

Introduction

Cytoplasmic male-sterility (cms) is a commonly occurring trait in higher plants and has been found in 71 species and 271 interspecific crosses (Kaul 1988). Plants showing this trait either fail to produce or to shed viable pollen. Other developmental processes within the plant, including the production of the female gametes, appear to be unaffected allowing the plants to be used as females in the commercial production of F_1 hybrids. Two cytoplasms which confer male-sterility have been found in onion. The first identified was the cms-S cytoplasm discovered in the variety 'italian Red' (Jones and Emsweller 1936). The genetic control of fertility was characterised by Jones and Clarke (1943), showing that sterility was due to a cytoplasmic determinant and that fertility could be restored by a dominant nuclear gene, *Ms*. A second cytoplasm, cms-T, was identified by Berninger (1965) and characterised by Schweisguth (1973); the restoration system comprises an independent gene (*A*) and two complementary genes (*B* and *C*).

The events which result in the abortion of the microspores in male-sterile onion containing the cms-S cytoplasm are preceded by the abnormal development of the tapetum (Tatebe 1952, Monosmith 1926; Peterson and Foskett 1953; Yen 1959; Patil et al. 1973). The lesion in onion with the cms-T cytoplasm may have its effect at an earlier developmental stage with abnormalities occurring in microspore meiosis as well as in tapetal development (Dyki 1973a, b). The weight of evidence to date suggests that the cytoplasmic lesion causing these abnormal behaviour patterns in male-sterile lines of other species is encoded in their mitochondria. Correlations have been shown between the restriction fragment profiles of mitochondrial DNA (mtDNA) and the male-sterility phenotype in a wide range of species. The cms trait has also been shown to be graft transmissible in a number of other species (Cech and Podzena 1962; Corbett and Edwardson 1964; Curtis 1967; Thompson and Axtell 1978), and male sterility is amongst the symptoms caused by a range of viruses (Bennett 1969; Larsen 1981; Shephard 1972).

The principal aims of this work were to establish whether the characteristics of onion mtDNA could be correlated to the male fertility phenotype, and if so whether the onion cms-S and cms-T male sterility-conferring cytoplasms differed with respect to these characteristics. The characteristics of chloroplast DNA (cpDNA)

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were also examined; although previous literature does not suggest a functional role for chloroplasts in the cms trait, this information allows us to suggest mechanisms by which the cms-S amd cms-T cytoplasms may have arisen. The cytoplasms were also examined for the presence of mitochondrial plasmids and for virus-like particles that may act as potential cytoplasmic markers.

Materials and methods

Plant materials

mtDNA was extracted from sprouting onion bulbs and cpDNA from green leaf material from the varieties given in Table 1.

Isolation of mtDNA

One hundred and fifty grams of tissue was homogenised in 600 ml of buffer A (10 m*M* TES, 0.5 *M* mannitol, 10 m*M* EGTA, 0.2% BSA, 0.05% cysteine, pH 7.2) and filtered through Miracloth (Calbiochem). The high level of chelating agent in this buffer was necessary to prevent gel formation (presumably due to pectic substances) in the initial extract. The filtrate was centrifuged twice at 1,600 *g* (Sorvall GSA rotor) for 10 min, discarding the pellet each time, and then centrifuged at 12,000 *g* and the pellet resuspended in fresh buffer A. This suspension was then centrifuged at 1,000 *g* (Sorvall SS34 rotor) for 10 min and the supernatant retained. Mg^{2+} was added to a final concentration of 10 m*M* and DNase I to 10 mg per kg tissue homogenised. The mixture was incubated at 4 °C for 1 h, and the samples under-layered with 20 ml shelf buffer (10 m*M* TES, 20 m*M* EDTA, 0.6 *M* sucrose, pH 7.2) and centrifuged at 12,000 *g* for 20 min. The pellet was resuspended in 1 ml lysis buffer (10 m*M* TRIS.HCl, 10 m*M* EDTA, 2% *N*-laurylsarcosine, 0.0012% proteinase (Type XI, Sigma)) and incubated at 37 °C for 1 h. The lysate was then deproteinised using three cycles of phenol-chloroform extraction, and the DNA collected by ethanol precipitation (after Kemble et al. 1980). The DNA was then resuspended and loaded onto the top of a seven-step CsCl gradient (1.19, 1.24, 1.34, 1.39, 1.44 and 1.70 g ml^{-1}) in a 5-ml tube and centrifuged at 130,000 *g* for 1 h (MSE 6 × 4.2 ml rotor). Fractions of 400 µl were sampled from the tube by puncturing the base of the tube; these were collected in the wells of a microtitre plate. Aliquots of 1 µl were taken from each well and subjected to agarose gel electrophoresis to visualise the DNA. The solution in those wells containing mtDNA substantially free of contaminating DNA fragments were combined, dialysed and the DNA recovered by ethanol precipitation. mtDNA yields were measured spectrophotometrically and were typically 5–10 µg per 150 g fresh weight.

Isolation of chloroplast DNA

A 20-g sample of onion tissue was homogenised in 100 ml of extraction buffer B (0.35 *M* sorbitol, 50 m*M* TRIS.HCl, 10 m*M* EGTA, 2% BSA, 0.05% cysteine, pH 8.0). The homogenate was centrifuged at 100 *g* for 10 min and the superntant retained. This was then centrifuged at 1,500 *g* for 10 min to pellet the chloroplasts. These were then resuspended in extraction buffer, re-pelleted and again resuspended in extraction buffer. This was then treated with DNase I as above. The suspension was layered onto the top of a two-phase sucrose gradient consisting of 7 ml 50% and 4 ml 30% (w/v) sucrose in 50 m*M* TRIS. HCl (pH 8.0) and 10 m*M* EDTA. The gradient was centrifuged at 80,000 *g* for 30 min (MSE 6 × 16.5 ml rotor). After centrifugation, the chloroplasts were collected from the 50%/30% interface and the suspension washed twice by pelleting at 1,500 *g* in extraction buffer. The chloroplasts were lysed and the cpDNA extracted and purified as for mtDNA, but omitting the CsCl centrifugation.

Table 1. Onion lines and varieties

Name	Contry of origin	Cytoplasm
Copra[a]	Netherland	cms-S
Crossbow[b]	United Kingdom	cms-S
Imai[a]	Japan	N
Jaune Paille de Rilleux[a]	France	cms-T
Jaune Paille des Vertus[a]	France	cms-T
Kaizuka[a]	Japan	N
Prospero[a]	United Kingdom	cms-S
Rawska[a]	Poland	N
Rijnsberger[a]	Netherlands	N
Robusta[a]	Netherlands	N
Royal Oak[c]	United Kingdom	cms-S
Senshyu[a]	Japan	N
Tropic Red[a]	New Zealand	N
I/1ms × I/10[a]	United Kingdom	cms-S
I/19[a]	United Kingdom	N
II/3[a]	United Kingdom	N
II/3ms[a]	United Kingdom	cms-S
Kz1[a]	United Kingdom	N
Kz1/ms[a]	United Kingdom	?
315 × Z2-5-2[d]	France	cms-T
315 × SR2-1-3[d]	France	cms-T
A78 × B96[b]	United Kingdom	cms-S
SR2-1-3[d]	France	N
Z2-5-2[d]	France	N

Source: [a] Genetic Resources Unit, Inst. for Horticultural Research, UK; [b] Elsoms Seeds Ltd, [c] A.L. Tozer Ltd, [d] INRA, France

cp and mtDNA restriction analysis and hybridisation studies

The digestion of organellar DNA was carried out using restriction enzymes supplied by either Pharmacia or Boehringer Mannheim at 20 units μg^{-1} of DNA for periods in excess of 3 h at 37 °C and the fragments separated by agarose gel (0.7% w/v) electrophoresis (Maniatis et al. 1982). Either HindIII-cut lambda DNA or a 1-kb ladder (Pharmacia) was used as a size marker. Gels were blotted onto Hybond-N (Amersham) using the method of Southern (1975). These blots were probed with DNA fragments containing the following mitochondrial genes from *Zea mays: cox1* (Isaac et al. 1985), *cox2* (Fox and Leaver 1981), *atp6* (D. Lonadale, personal gift), *atp9* (Dewey et al. 1985) and *cob* (Dawson et al. 1984) and *cox3* from *Oenothera berteriana* (Heisel et al. 1987); these fragments readily hybridised to onion mtDNA. For hybridisation studies mtDNA inserts were denatured for 10 min in a boiling water bath and radio-labelled using the method of Feinberg and Vogelstein (1983, 1984). Prehybridisation and hybridisation were carried out in a buffer containing 4 × SSC, 1 × Denhardt's solution, 40% (v/v) formamide and 1 µg ml^{-1} salmon sperm DNA at 42 °C. Autoradiography was carried out using Hyperfilm MP (Amersham) according to the manufacturer's instructions.

Analysis of mitochondria for the presence of mitochondrial plasmids

The mtDNA used in assays for the presence of mitochondrial plasmids was prepared as above with the omission of the CsCl centrifugation. Undigested samples were subjected to elec-

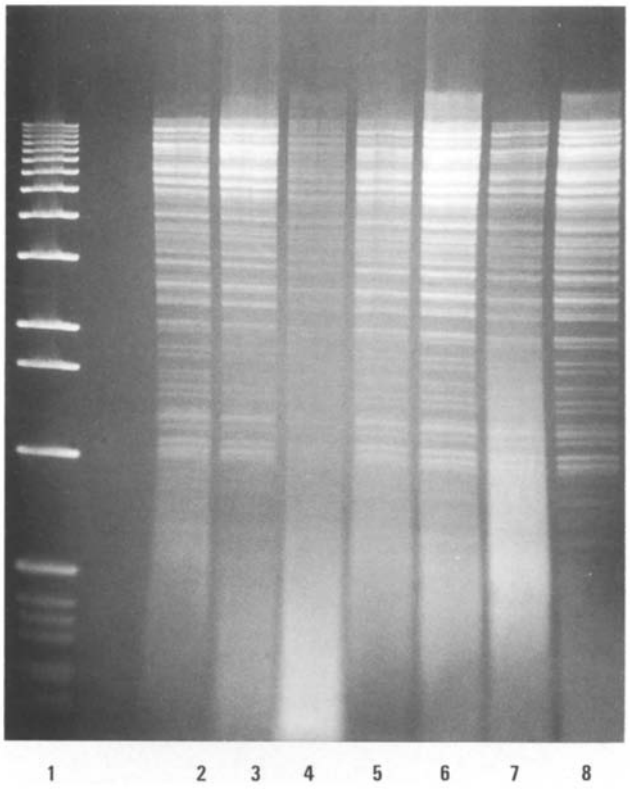

Fig. 1. Electrophoresis of mtDNA digested with *Hin*dIII from seven onion lines containing the fertile, N cytoplasm. *Lane 1* 1-kb ladder, *lane 2* Senshyu, *lane 3* Robusta, *lane 4* Rijnsberger, *lane 5* Imai, *lane 6* Rawska, *lane 7* Kaizuka, *lane 8* SR2-1-3

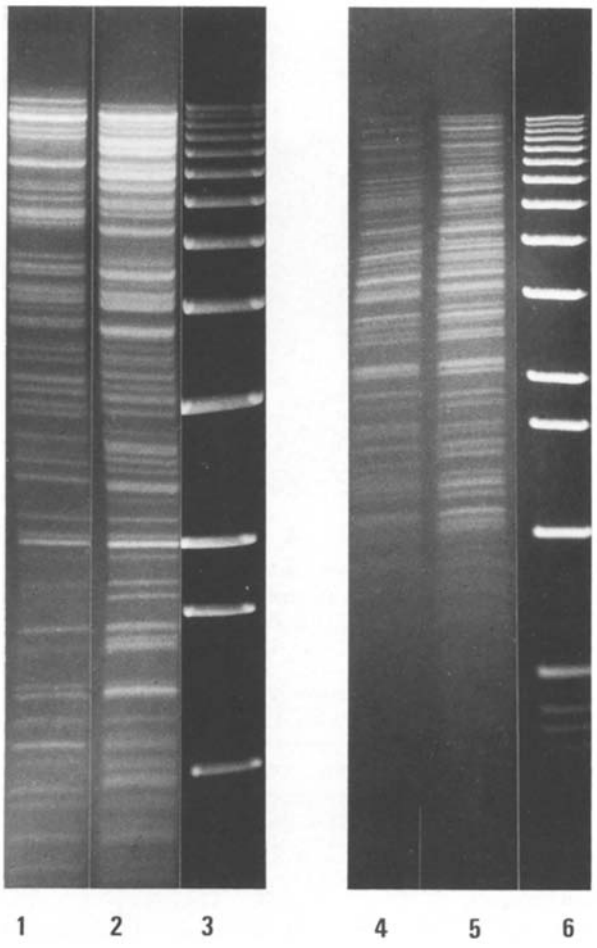

Fig. 2. Restriction digests of onion mtDNA from the lines II/3 ms (cms-S cytoplasm, *lanes 1 and 4*) and II/3 (N cytoplasm, *lanes 2 and 5*) generated with the enzyme *Bam*HI (*lanes 1 and 2*) and *Hin*dIII (*lanes 4 and 5*). *Lanes 3 and 6* contain 1-kb ladder (Pharmacia)

trophoresis in 0.7% w/v agarose gels, viewed and blotted. The mtDNA samples used to prepare such gels were radio-labelled and used to probe these blots.

Preparation of nucleic acid fractions using lithium chloride

For viral dsRNA evaluations, nucleic acids from 10 g of onion tissue were prepared and fractionated by the method of Diaz-Ruiz and Kapes (1978) and subjected to agarose gel (0.7% w/v) electrophoresis.

Preparation of material for electron microscopy

To assay for the presence of virus-like particles (VLPs) onion tissue or sap was prepared for electron-microscopy using the following techniques.

Method 1. Tissue from onion leaves and anthers was fixed in Karnovski's fixative (Karnovski 1965) overnight, followed by a secondary fixation in 1% (w/v) osmium tetroxide, and then dehydrated using an alcohol series. Infiltration with L. R. White resin proceeded over a 48–72 h period. Gold sections were placed onto formvar- and carbon-coated grids, stained with 1% (w/v) uranyl acetate and Reynolds' lead citrate (Reynolds 1963) and viewed using a Philips 301 electron-microscope.

Method 2. For the detection of virus-like particles (VLPs) in sap 0.5 g of onion tissue was macerated with 0.5 ml of extraction buffer A. The extract was centrifuged in a microfuge for 5 min and a serial dilution made with extraction buffer A. One drop of each dilution was placed on a formvar- and carbon-coated grid and left to settle for 30 s. One drop of 1% (w/v) sodium phosphotungstate was added and the grid left to stain for 30 s. Fifty random fields of view were then viewed as above.

Results

The mtDNA from various lines containing the fertile, N genome showed no RFLPs for either of the enzymes BamHI and HindIII. Figure 1 shows the fragment profiles produced by digesting the mtDNA of seven lines with HindIII. This fertile material represented a diverse range of genotypes with lines originating in France, the Netherlands, Japan and Poland. Identical results (not shown) were also obtained with five other lines from the UK, France and New Zealand. Digests of mtDNA from six lines known to contain the cms-S cytoplasm produced profiles that clearly distinguished this cytoplasm; examples of the differences between the profiles produced

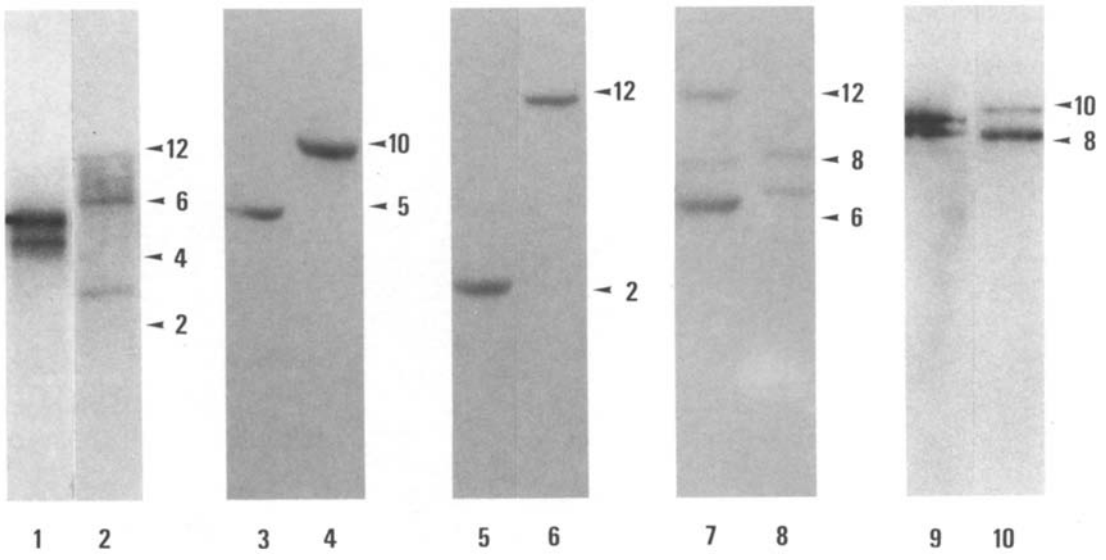

Fig. 3. Autoradiographs of onion mtDNA from the line II/3 (N genome, *lanes 1, 3, 5, 7, 9*) and the line II/3ms (cms−S genome, *lanes 2, 4, 6, 8, 10*) probed with plant mitochondrial genes. *Lanes 1 and 2 Hin*dIII/*cox1*, *3 and 4 Bam*HI/*cob*, *5 and 6 Sma*I/*cob*, *7 and 8 Sma*I/*atp6*, *9 and 10 Hin*dIII/*atp6*

Table 2. Combinations of enzymes and probes used to characterise the fertile (N) and cms-S (S) and cms-T (T) cytoplasms

	cob	*cox1*	*cox2*	*cox3*	*atp6*	*atp9*
*Bam*HI	NST	NST	NS	NT	NST	NST
*Hin*dIII	NST	NST	NST		NST	NS
*Sma*I	NS	NS			NS	
*Eco*RI			NS			
*Bsc*I	NT	NT				

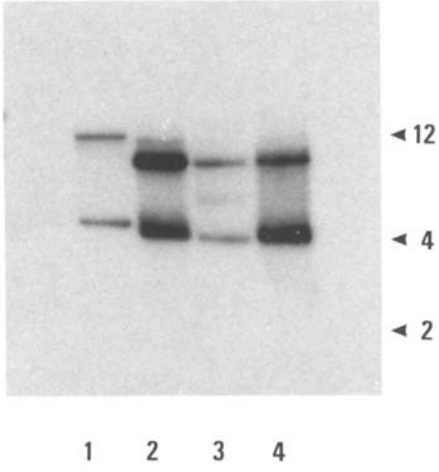

Fig. 4. Autoradiographs of *Hin*dIII-digested onion mtDNA probed with the maize *cox2* gene. *Lane 1* II/3 (N cytoplasm), *lane 2* Crossbow (cms-S cytoplasm), *lane 3* II/3ms (cms-S cytoplasm), *lane 4* Royal Oak (cms-S cytoplasm). Size markers in kbp

from N and cms-S cytoplasms are shown in Fig. 2. Of the combinations of restriction enzyme and probe shown in Table 2, only two, BamHI/*cox2* and HindIII/*cob*, failed to distinguish the two genomes; five such combinations are shown in Fig. 3. No RFLPs in mtDNA were found with mtDNA probes between lines containing the N genome; however, differences could be detected between the relative concentrations of fast and slow bands. These differences showed no pattern and may be related to individual genotypes.

In contrast to the fertile genotypes, minor differences could be detected in the profiles of mtDNA from material containing the cms-S genome. This variation was most easily seen in the Southern blots of these profiles where, in addition to the major bands, probes detected fainter bands that had variable positions (Fig. 4). This type of variation was never observed in any analysis of fertile material; however, this did not prevent these techniques from clearly distinguishing the cms-S from the N genome.

Restriction fragment profiles of lines known to contain the cms-T cytoplasm, generated with HindIII (Fig. 5) and also with BamHI and BscI (not shown), gave patterns that were apparently identical with those of the N genome. Similarly, no variation could be detected using Southern blot analysis of these mitochondrial genomes with any of the combinations of enzyme or probe used (see Table 2).

The analysis of the restriction fragment profiles of onion cpDNA showed similar results to those found with the mtDNA. No variation in the restriction fragment profiles could be detected amongst varieties known to contain the fertile, N cytoplasm; similarly, no differences were found between the chloroplast genomes of lines containing the cms-S cytoplasm. How-

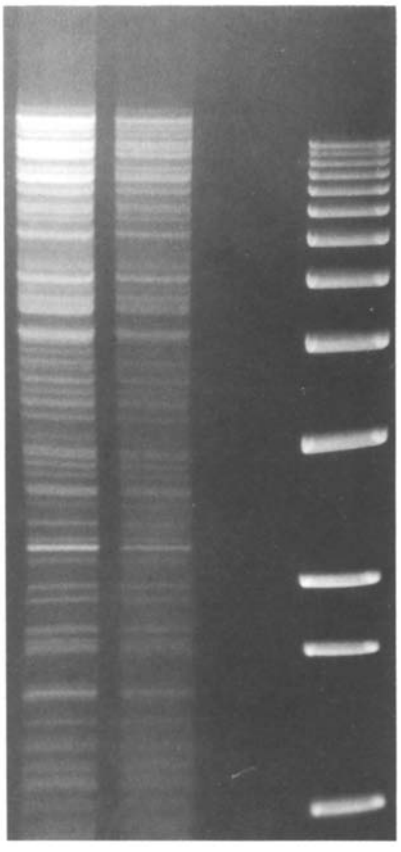

Fig. 5. MtDNA digests from onion line II/3 (N cytoplasm, *lane 1*) and line 315 × SR2-1-3 (cms-T cytoplasm, *lane 2*) produced with the enzyme *Bam*HI and separated in a 0.7% (w/v) agarose gel. (*Lane 3* contains a 1-kb ladder)

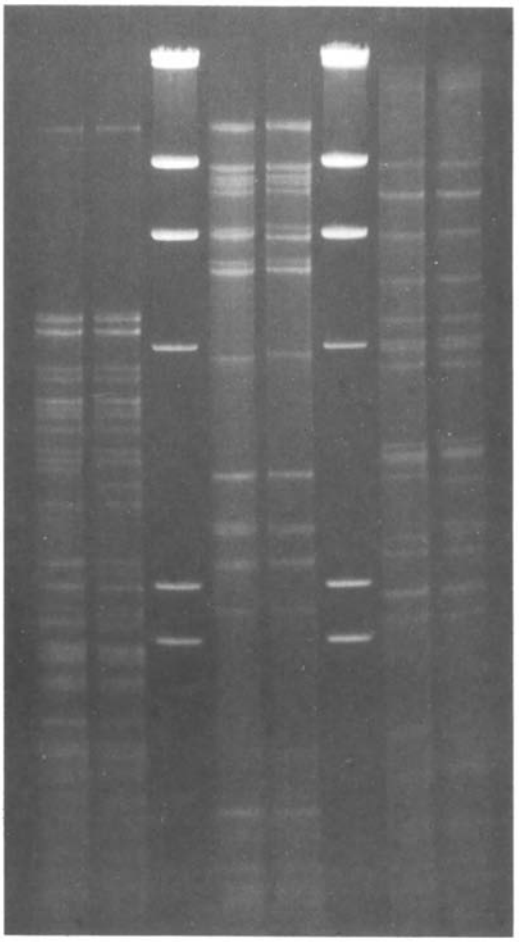

Fig. 6. Digests of onion cpDNA from the cv 'Crossbow' (cms-S cytoplasm, *lanes 2, 5, 8*) and line II/3 (N cytoplasm, *lanes 1, 4, 7*) digested with the enzymes *Eco*RI (*lanes 1 and 2*), *Hin*dIII (*lanes 4 and 5*) and *Xba*I (*lanes 7 and 8*) separated in a 0.7% (w/v) agarose gel. *Lanes 3 and 6* contain *Hin*dIII-digested lambda DNA markers

ever, HindIII, EcoRI or XbaI detected polymorphisms between the cpDNA of these two classes of cytoplasm (Fig. 6). Restriction fragment profiles of the chloroplastal genome of lines known to contain the cms-T cytoplasm gave banding patterns that were identical with those of the fertile cytoplasm (Fig. 7).

There was no evidence for the presence of mitochondrial plasmid-like DNA molecules in either the gels of unrestricted mtDNA nor in Southern blot analysis of these gels probed with onion mtDNA. Sectioned material and leaf extracts from the three cytoplasms were examined for the presence of virus-like particles by electron-microscopy. No evidence could be found for the presence of VLPs by either of these techniques nor could dsRNA be detected in the material.

Discussion

RFLP analysis of mtDNA from the range of material used in this study has allowed the identification of two groups of cytoplasms. Firstly, a group designated M by de Courcel et al. (1989), which contains the N and cms-T cytoplasms, and secondly a group that contains the cms-S cytoplasm. The RFLP profiles of mtDNA from eleven male-fertile onion lines containing the N-cytoplasm, generated with the enzymes BamHI and HindIII, showed no variation. Further, no differences could be detected between mtDNA RFLP profiles from varieties known to contain the cms-T cytoplasm and those containing the N-cytoplasm. Male-sterile lines containing cms-S types produced banding patterns that were similar to each other, but distinct from that of the M-group, thus allowing the cytoplasms to be identified. However, variation was found between the profiles of these cms-S types.

These results in part contrast those of de Courcel et al. (1989), who found no variation in the RFLP pro-

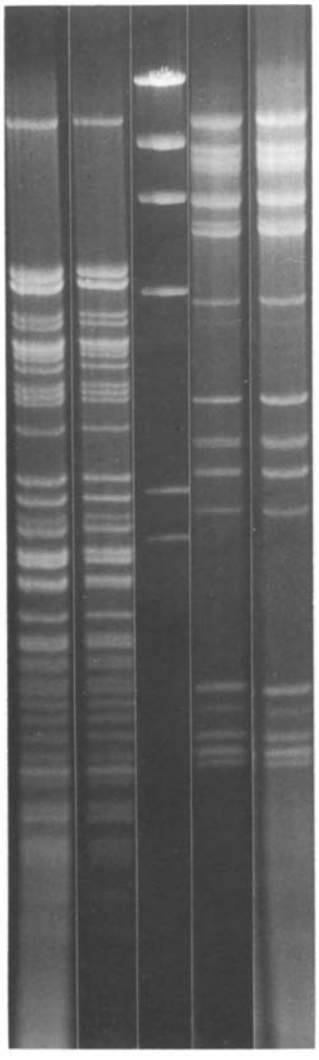

Fig. 7. Onion cpDNA from lines 315 × Z2-5-2 (cms-T cytoplasm, *lanes 1 and 4*), Z2-5-2 (N cytoplasm, *lanes 2 and 5*) digested with the enzymes *Eco*RI (*1 and 2*) and *Hin*dIII (*lanes 4 and 5*) separated in a 0.7% (w/v) agarose gel (*lane 3* contains *Hin*dIII-digested lambda DNA markers)

files generated with six restriction enzymes, including BamHI, of onion lines carrying the cms-S cytoplasm, but who did find sufficient variation in their M-group to split this group into four sub-sections. Despite the variation in the cms-S cytoplasms found in this work and the variation in the M group materials of de Courcel et al., onion mt-genomes can be unambiguously placed within their respective groups, i.e. M and cms-S. Variation of this type has been found within the N and cms-C groups of maize (Levings and Pring 1977; Pring et al. 1979). The detection of variation will depend on the range of lines used and the diversity of the material from which isolations of the cytoplasmic types have been made.

Although RFLP profiles can be used to distinguish the two cytoplasmic groups, the complex patterns of bands obtained can require careful interpretation to allow identification of cms-S and M genomes. The use of radio-labelled probes greatly facilitates this process. Cloned genes from *Zea* and *Oenothera* have been shown to hybridise at a small number of positions (usually two) within a profile, providing a very simple basis for the discrimination between the two groups. Of the fourteen combinations of restriction enzyme and mtDNA probe used, only two failed to separate cms-S types from M group material. As with the RFLP profiles, no variation in the position of those bands to which the various probes hybridized was found in M group material. The only variation was in the relative intensity of fast and slow bands between members of this group. These differences showed no pattern and were specific to individual genotypes. Within the cms-S group, in addition to the major bands, hybridisation also occurred to further minor bands. The ease with which heterologous probes can be used for hybridisation studies with the onion mitochondrial genome is probably related to the low rate of change of the primary sequence in the plant mitochondrial genomes (Palmer and Zamir 1982; Palmer and Herbon 1988; Fox and Leaver 1981; Chao et al. 1984; Bonen et al. 1984).

The RFLP profiles of the onion chloroplastal genomes also allow the two groups, M and cms-S, to be distinguished. As with the mtDNA profiles, no differences could be found between the cpDNA from N-cytoplasm and cms-T types. Thus, the cms-S group shows the co-inheritance of both a mitochondrial and a chloroplastal genome with differences from those of the M group. The cms-S chloroplastal genome has been shown to be slightly larger than that of the N genome. Both nucleotide substitutions and small insertions or deletions have been shown to occur in the cp-genomes of other species (Banks and Birky 1985; Palmer et al. 1985; Bowman et al. 1983; Palmer and Zamir 1982).

As the cms-T cytoplasm could not be separated from the N cytoplasm on the basis of the RFLP profiles of its organellar DNAs, the possible presence of VLPs was investigated. In *Vicia faba* the 447 cms line is characterised by the presence of VLPs in the cytoplasm and the possession of a 16.7-kbp linear dsRNA molecule (Edwardson et al. 1976; Grill et al. 1983; Grill and Garger 1981; Turpen et al. 1988). In onions, male-sterility is amongst the symptoms caused by viruses (Kaul 1988). However, neither VLPs nor dsRNA molecules could be detected in cms-T onion material, and the cms trait could not be transmitted by grafting amongst the material used by van der Meer and van Bennekom (1970). Thus, a marker of male-sterility has not been identified that allows the the cms-T cytoplasm to be identified and distinguished from the N cytoplasm. If the lesion causing male-sterility is indeed located in the mitochondrial genome, it must be caused by some small change such as a point mutation or

small insertion that cannot easily be detected by RFLP analysis. For the cms-S cytoplasm, onions can be added to the list of species in which cms is correlated with differences in the RFLP profile of mtDNA.

The RFLP analysis of the onion organellar genomes suggests different origins for the two sterility-conferring cytoplasms. Both the chloroplastal and mitochondrial genomes of cms-T onions appear identical to, or at least are closely related to, the organellar genomes of onions containing the normal, fertile N-cytoplasm. The male-sterile lines used in the Dutch cultivars, and containing the cms-T cytoplasm, have been isolated from lines containing the N-cytoplasm (Banga and Petiet 1958), and other male-sterile lines have been identified from fertile Polish material (van der Meer and van Bennekom 1969). Furthermore, male-sterile plants, denoted Kz1/ms, have been found in an accession, Kz1, of the male-fertile, Japanese variety 'Kaizuka' grown at Inst. for Horticultural Research: these have remained male-sterile over a number of seasons. RFLP analysis of the mtDNA of these lines (data not shown) shows both to be typical members of the M group. The cytoplasms found in the Dutch and Polish material and in the Kz1/ms line apparently represent independent evolutions of the cms-T cytoplasm. Thus, it seems that this cytoplasm may originate from a mutation within the N-cytoplasm and would be autoplasmic in origin.

The probability of mutations occurring in both the mitochondrial and chloroplastal DNA genomes of cms-S material and co-segregating with the cms phenotype is small: therefore, an autoplasmic origin for this cytoplasm is unlikely. It seems more probable that sterility caused by the cms-S cytoplasm could have resulted from an interspecific cross. Such a cross would result in the co-inheritance of the organellar genomes and any disharmony between the nucleus and a foreign cytoplasm could result in cms. Interspecific hybridization has been achieved between *A. cepa* and a number of other allium species. Crosses with *A. fistulosum* have been achieved by a number of workers. The seed set of these crosses is low, and the F_1's show varying degrees of infertility (Van der Meer and Van Bennekom 1976; Dolezel et al. 1980). However, the crosses of *A. cepa* to *A. galanthum, A drobovii* and *A. pskemense* all produce viable seed; also, the F_1 readily crosses with either parent (Saini and Davis 1969).

The location of this putative hybridisation event is difficult to establish. The onion is unknown in the wild and has its primary centre of origin in Vavilov's Central Asiatic region; secondary centres of diversity include the Near East and the Mediterranean (Vavilov 1951). de Courcel et al. (1989) suggest that one of the progenitor species had its origin in the Mediterranean region, but there is no direct evidence for this. The diversity found within *A. cepa* in the Mediterranean area is most likely due to introduction as a result of trade in the region rather than the evolution of new forms. Further work on the organellar genomes of these and other allium species may allow the source of the cms-S cytoplasm as well as origin of the cultivated onion to be found.

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