Theor Appl Genet (1987) 74:666–669

Mitochondrial DNA modifications associated with cytoplasmic male sterility in rice

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Received March 23, 1987; Accepted April 28, 1987
Communicated by R. Hagemann

Summary. Mitochondrial DNA was isolated from fertile and cytoplasmic male sterile lines of rice. Restriction analysis showed specific modifications in the male sterile cytoplasm. In addition to the major mitochondrial DNA, three small plasmid-like DNA molecules were detected by agarose gel electrophoresis in both cytoplasms. An additional molecule was specifically found in the sterile cytoplasm. These mitochondrial DNA modifications support the hypothesis of the mitochondrial inheritance of the cytoplasmic male sterility in rice.

Key words: Cytoplasmic male sterility – Mitochondrial DNA – Plasmids – Rice

Introduction

Rice breeding has been very successful during the last two decades and yields have been raised in many parts of the world. During recent years, even F_1 hybrids have been successfully developed and utilized in this self-pollinated crop to increase its varietal productivity by about 20%. China already grows hybrid rices commercially (Lin and Yuan 1980) and attempts are underway outside China (Virmani and Edward 1983) to develop and utilize this technology. The identification and development of cytoplasmic male sterility (CMS) and restorer lines were a major step in the success of this technology.

Little is known of the molecular basis of the interactions between the nuclear and the cytoplasmic genetic information. However several biochemical observations support a mitochondrial inheritance of CMS. Restriction analysis of the isolated mitochondrial DNA showed specific modifications in male sterile cytoplasm of maize (Borck and Walbot 1982), wheat (Quetier and Vedel 1977), tobacco (Belliard et al. 1979), sorghum (Pring et al. 1979), brassica (Vedel et al. 1982), sugarbeet (Powling 1982), petunia (Boeshore et al. 1983), and faba beans (Boutry and Briquet 1982). In addition to the main mitochondrial DNA, small linear and circular DNA molecules have been observed in mitochondria (for review see Lonsdale 1984). Variant molecules have been found in male sterile cytoplasms of maize (Pring et al. 1977; Kembel and Bedbrook 1980), sorghum (Dixon and Leaver 1982), brassica (Palmer et al. 1983), sugarbeet (Powling 1981), faba bean (Boutry and Briquet 1982; Negruk et al. 1982; Goblet et al. 1983; Goblet et al. 1985), rice (Yamaguschi and Kakiuchi 1983) and Oenothera (Brennieke and Blanz 1982). Fertile and sterile cytoplasms have been distinguished by the presence of variant polypeptides synthesized by isolated mitochondria in maize (Forde and Leaver 1980), sorghum (Dixon and Leaver 1982), faba bean (Boutry and Briquet 1982), wheat, sugarbeet and tobacco (Boutry et al. 1984).

In *Japonica* rice CMS lines (BT-type), it was found that mtDNA isolated from callus tissue culture of CMS BT-type contains two DNA species, in addition to the main mtDNA; in contrast the normal cytoplasm Taichung 65 does not contain these DNA species (Yamaguchi and Kakiuchi 1983).

In this paper, we report the preliminary molecular analysis of cytoplasmic male sterility in an *Indica* rice CMS line developed in China. This line was developed from a wild rice: *Oryza sativa* f. *spontanea* L., cytoplasmic source designated as WA (Lin and Yuan 1980), and the nuclear genotype of the Chinese rice variety Zhen Shan 97.

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Materials and methods

Plant materials

The *Indica* rice CMS line Zhen Shan 97A (WA cytoplasm) and its maintainer line Zhen Shan 97B with normal cytoplasm were chosen for the study. Seeds of these lines were provided by the International Rice Research Institute (IRRI), Manila, Philippines.

Extraction and purification of mitochondria for DNA analysis

Seeds were soaked overnight in water containing 100 U/ml penicillin, streptomycin and fungizone and germinated in humid soil in the dark for 7 days at 30 °C.

Mitochondria were isolated at 4 °C by a modification of methods previously described (Boutry and Briquet 1982; Kemble et al. 1980). Then 10 g of germs were washed and homogenized twice for 10 s in a cold Waring blendor in 60 ml homogenization medium containing 0.5 M mannitol, 10 mM Tris, 1 mM EGTA, 1 mM dithiothreitol, 100 mM diethyldithiocarbamate sodium salt and 0.2% bovine serum albumin (BSA) at pH 7.2 (HCl). The homogenate was filtered through one layer of cheesecloth and centrifuged twice for 2 s at 5,000 rev/min in a Sorvall GSA rotor. The resulting supernatant was centrifuged for 10 min at 12,500 rev/min. The crude mitochondrial pellet was resuspended in 4 ml of suspension medium containing 0.5 M mannitol, 10 mM Tris at pH 7.2 (HCl), 0.1% BSA, 10 mM $MgCl_2$ and 10 µg DNAse/g tissue fresh weight and incubated for 30 min at 25 °C. After this, 7 ml of washing medium containing 0.5 M mannitol, 10 mM KH_2PO_4, 0.15 M NaCl, 50 mM EDTA and 0.1% BSA at pH 7.2 (KOH) were added to the mixture and the mitochondria were collected by centrifugation for 10 min at 18,000 rpm in a Sorvall SS34 rotor. The pellet was resuspended in 2 ml washing medium and layered on a discontinuous sucrose gradient formed by 15 ml of 1.45 M sucrose; 10 ml of 1.2 M sucrose containing 10 mM Tris, 20 mM EDTA and 0.1% BSA at pH 7.2 (HCl) and centrifuged for 90 min at 20,000 rpm in a Beckman SW28 rotor. Mitochondria banding at the interface of the two layers were removed, diluted by 4 volumes of the washing medium and centrifuged for 10 min at 18,000 rpm in the Sorvall SS34 rotor. Purified mitochondria were suspended in 4 ml of lysis buffer containing 2% sodium sarkosylate, 0.1 M NaCl, 10 mM EDTA at pH 8.0 (NaOH) and 0.1 mg/ml of proteinase K and lysed for 30 min at 37 °C. Lysate was phenol extracted 3 times and ethanol precipited. After resolubilisation, RNA were removed by precipitation with 50 mM $CaCl_2$ at 0 °C for 2 h. The supernatant was further digested by RNAse I for 1 h at 37 °C.

Electrophoretic analysis of native mitochondrial DNA

The mitochondrial DNA was electrophoresed overnight at 60 V in 1.5% agarose gel containing 2 mM EDTA and 20 mM sodium acetate or 90 mM sodium borate in 50 mM Tris at pH 8.0. After the electrophoresis, the gel was stained for 45 min in 1.5 µg/ml ethidium bromide. Molecular weight markers were obtained from a digest with ECO RI and Hind III restriction digest of phage λ DNA.

For S_1 nuclease treatment, the DNA was diluted in 3 mM sodium acetate, 1 mM $ZnSO_4$, 50 mM NaCl and 5% (v/v) glycerol at pH 4.6 (acetic acid) and treated with 20 units nuclease S_1 for 30 min at room temperature or 40 units' nuclease S_1 for 45 min at 37 °C.

Restriction endonuclease analysis

The mitochondrial DNA was incubated with the indicated restriction enzyme for 2 h at 37 °C in 30 µl reaction medium, as described by Boehringer. The reaction was stopped by adding 4 µl of 0.35 M EDTA, 15% Ficoll, 0.1% bromophenol blue at pH 7.5 (NaOH). DNA fragments were electrophoresed on 1% agarose gel containing 2 mM EDTA, 20 mM sodium acetate, 50 mM Tris at pH 8.0 (acetic acid) and 0.5 µg/ml of ethidium bromide.

Results and discussion

Mitochondrial DNA was prepared from a fertile and a sterile cytoplasm of *Indica* WA-type rice. When electrophoresed without previous restriction, the mitochondrial DNA migrated into two regions: a broad band of large molecular weight corresponding to the main mitochondrial DNA and, in addition, discrete bands of low molecular weight, which were reminiscent of mito-

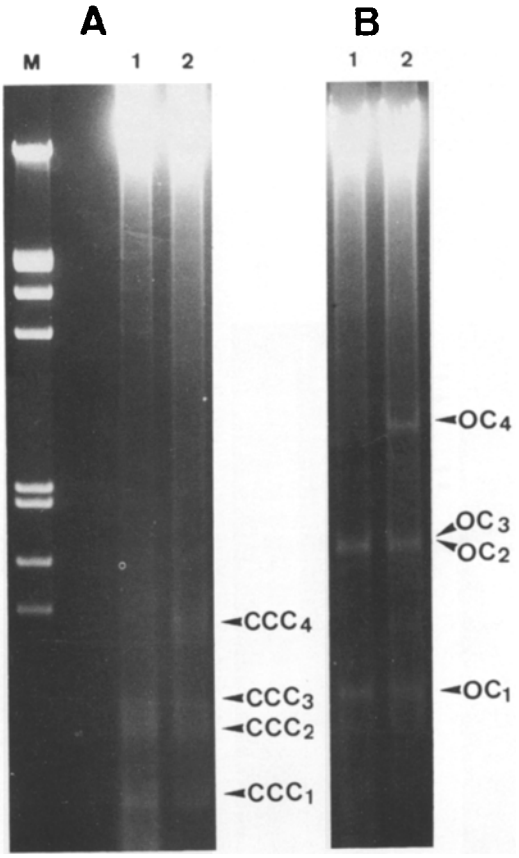
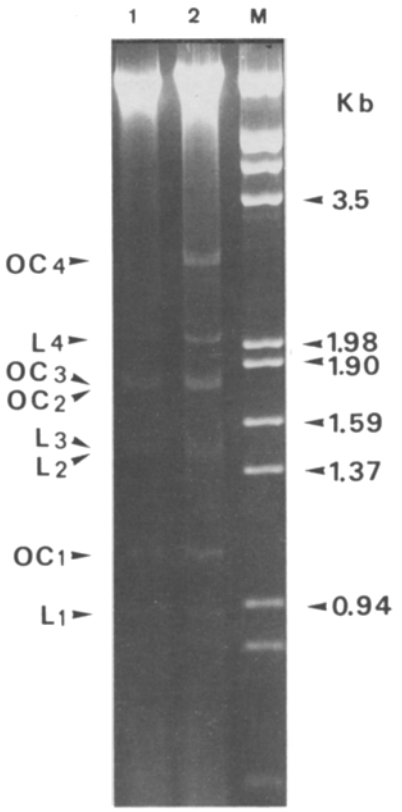


Fig. 1 A, B. Agarose gel electrophoretic patterns of mitochondrial DNA extracted from fertile (*lane 1*) and male sterile (*lane 2*) lines of *Oryza sativa* L. Mitochondrial DNA was obtained and analysed by electrophoresis on a 1.5% agarose gel, as in "Materials and methods". **A** Mitochondrial DNA analysed on a Tris-acetate agarose gel without digestion with nuclease S_1. **B** Mitochondrial DNA digested with nuclease S_1 after 30 min of incubation and analysed as in **A**



chondrial plasmid-like molecules identified in other species. These bands were observed with fertile cytoplasm (Fig. 1 A, line 1). The same bands and an additional one were found in the sterile cytoplasm (Fig. 1 A, line 2). The results contrast with those reported for the *Japonica* BT-type rice for which no small mitochondrial DNA molecules were found in the fertile cytoplasm and only two molecules were seen in the male sterile cytoplasm (Yamaguchi and Kakiuchi 1983). Linear and supercoiled mitochondrial plasmids have been reported in several plant species (Lonsdale 1984; Goblet et al. 1983). In order to identify the structure of the mitochondrial plasmide-like molecules found in rice, we analysed the mitochondrial DNA after S_1 nuclease treatment which convert supercoiled DNA molecules into open circular and linear forms (Wiegand et al. 1975; Goblet et al. 1983). Indeed, after S_1 nuclease treatment, the three (in the fertile cyto-

Fig. 2. Mitochondrial DNA of male fertile (*lane 1*) and male sterile (*lane 2*) lines digested with nuclease S_1 after incubation for 45 min, and analysed in a tris-borate agarose gel. *CCC, OC* and *L* indicate the supercoiled, open circular and linear DNA forms, respectively, of the mitochondrial plasmid-like molecules. *M:* Eco RI-Hind III digested bacteriophage lambda DNA

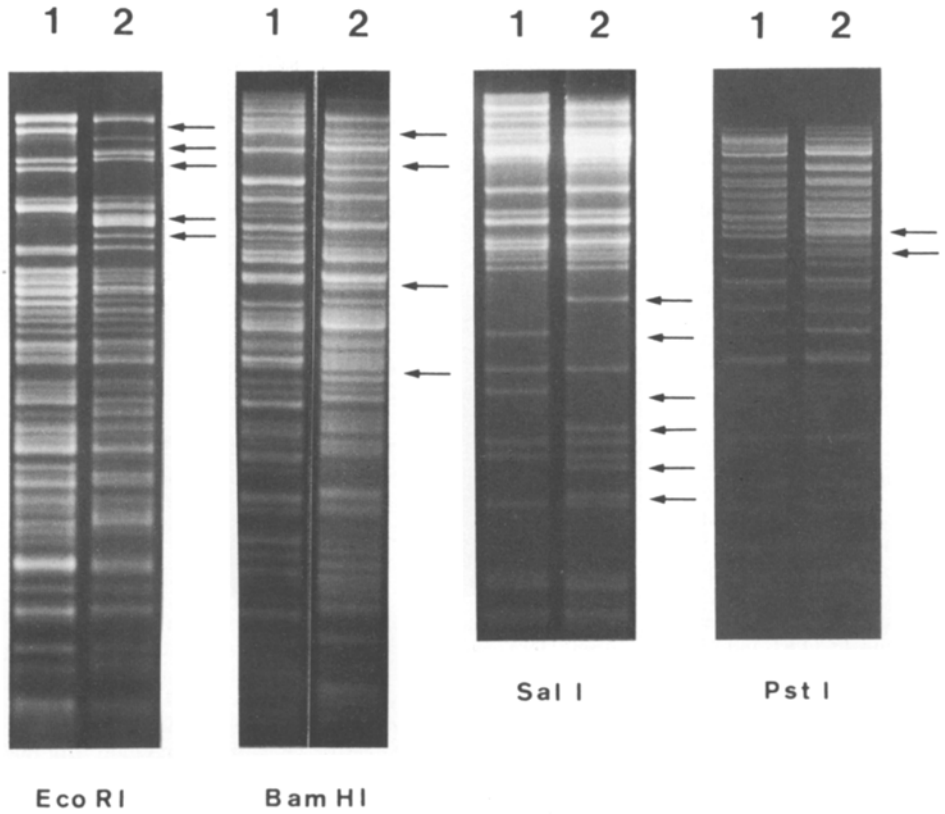


Fig. 3. Agarose gel electrophoretic patterns of restriction endonuclease digest of total mitochondrial DNA from male fertile (*lane 1*) and male sterile (*lane 2*) lines of rice. Mitochondrial DNA was digested and analysed by the restriction endonucleases Eco RI, Bam HI, Sal I, Pst I and electrophoresed as in "Materials and methods". *Arrows* indicate the modified DNA fragments in the mitochondria of the male sterile cytoplasm

plasm) or four (in the male sterile cytoplasm) plasmid-like molecules observed in the native DNA gave rise to new forms of apparently high molecular weight (Fig. 1 B). That these molecules were in a closed circular form was confirmed by their not being altered by an SDS alkaline treatment which eliminated the main mitochondrial DNA (results not shown). The S_1 nuclease treatments carried out at different times of incubation allowed us to tentatively identify the different forms indicated in Fig. 2.

From measurements of the electrophoretic mobility, the size of the linear forms would be approximately 1,000 bp for the L1, 1,350 bp for the L2 and L3, which are the three common molecules found in all the lines, and 2,100 bp for the L4 which is the linear form of the plasmid specifically observed in the sterile line. Electron microscopy analysis and/or cloning of the different forms will give the definitive structure and sizes of these plasmids. We have also analysed the mitochondrial DNA of both rice cytoplasms after restriction with Eco RI, Bam HI, Sal I and Pst I (Fig. 3). While most restriction fragments were common to both cytoplasms, several bands were specifically identified in the fertile or the sterile cytoplasm, suggesting mutations and/or rearrangements of the mitochondrial DNA.

In conclusion, our results indicate that sterile and fertile cytoplasms from *Indica* WA-type rice contain small plasmid-like DNA molecules. They can be distinguished by their mitochondrial DNA restriction patterns and by the additional mitochondrial plasmid-like molecule in the sterile cytoplasm. For other species, the indication of an eventual relationship between mitochondrial DNA modifications, the presence of specific plasmids and the fertile/sterile trait awaits further investigations. However, these variant bands can already provide a useful cytoplasm marker.

Acknowledgements. We thank Dr. M. Boutry for helpful discussion and critical reading of the manuscript. This work was supported by the research programme "Science et Technique au Service du Développement", Sub-programme "Agriculture Tropicale", of the Commission of the European Communities (contract n° TDS-A-119(B)) and by the "Fonds National de la Recherche Sciencitique (FNRS-Belgium).

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