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Stability of mitochondrial DNA in tissue-cultured cells of rice

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Summary. Restriction analysis of mitochondrial (mt) DNA from 3-month-old callus cultures of the cytoplasmic male sterile rice, V41A, which contains S_2 or "wild abortive" cytoplasm, and its fertile maintainer, V41B, showed the same BamHI restriction profiles as mtDNA from the corresponding leaf material. Similarly, mtDNA of rice (var. Taipei 309) from leaves, a 2-month-old cell suspension (T3MS2/A), a totipotent suspension (T3MS) and a 19-month-old suspension, which had lost its protoplast regeneration ability (LB3), showed indistinguishable BamHI restriction profiles. However, clear differences in mtDNA restriction profiles were observed between LB3 and a 30-month-old suspension culture of Taipei 309 (LB1), which appeared to reflect substantial changes in the relative abundance of specific DNA sequences. Hybridisation of a maize *coxII* gene probe to blots of restricted mtDNA confirmed that, while the relative abundance of certain mtDNA sequences was preserved during long-term tissue culture of rice, major changes in abundance were observed with other sequences.

Key words: Mitochondrial DNA – Rice – S_2 cytoplasmic male sterility – Tissue culture

Introduction

Cytoplasmic male sterility (CMS) in higher plants is a maternally inherited, mitochondrially encoded trait, which is exploited in the production of hybrid seed of many crops (Levings and Brown 1989). CMS in rice (*Oryza sativa* L.) has been classified into four main types: S_1 (or "Chinsurah Boro II"), S_2 ("wild abortive" or "WA"), S_3 ("Gambiaca") and S_4 (Virmani et al. 1986; Young et al. 1983).

The molecular basis of CMS in rice is as yet poorly understood. Small plasmid-like mitochondrial DNA (mtDNA) molecules are present in mitochondria of plants carrying S_1 (Yamaguchi and Kakiuchi 1983; Kadowaki et al. 1986; Shikanai et al. 1987) and S_2 cytoplasms (Mignouna et al. 1987). However, in the case of S_2 cytoplasm, at least, there is no simple correlation between the presence or absence of small mitochondrial DNA molecules and the expression of CMS (Saleh et al. 1989). As in other higher plant species, specific alterations in the organisation of the main, high-molecular-weight mitochondrial genome may be associated with CMS in rice (Saleh et al. 1989). However, it is possible that the four classes of CMS in rice reflect different underlying molecular causes. For example, double-stranded mtRNA may be involved in S_1 CMS (Wang et al. 1989b).

The recent development of protoplast fusion and regeneration methods (Yang et al. 1988b, 1989; Akagi et al. 1989) for the production of cybrids of certain varieties of rice containing S_1 cytoplasm in different nuclear backgrounds has provided another approach to studying the molecular basis of CMS in this species. However, the identification of specific alterations in mtDNA associated with CMS may be complicated in cybrids, since tissue-culture-induced changes in mtDNA restriction profile have been reported in a dihaploid cell line of the *Indica* variety PI353705 (Chowdhury et al. 1988).

This report presents an analysis of the stability in tissue culture of mtDNA from a prospective protoplast donor and recipient for transfer and study of S_2-CMS in rice. The mtDNA of the S_2-CMS line, V41A, has been described previously (Saleh et al. 1989). Methods for the regeneration of plants from protoplasts of the prospective fertile recipient, a *Japonica* variety Taipei 309, have been described (Finch et al. 1989), and efficient methods for the genetic transformation of this variety have been developed (Yang et al. 1988a; Zhang et al. 1989).

Materials and methods

Plant material

The *Indica* rice CMS line V41A was developed from a wild rice, *Oryza sativa* f. *spontanea* L., carrying wild abortive cytoplasm (Lin and Yuan 1980). The nuclear source was the Chinese rice variety V41, which was also present in the fertile maintainer rice V41B. The fertile *Japonica* variety Taipei 309 has been described (Abdullah et al. 1986). The latter contains no nuclear restorer genes for S_2-CMS (Dr. S. S. Virmani, unpublished results). Seeds of V41A and V41B lines were provided by Dr. S. S. Virmani and seeds of Taipei 309, by Dr. T. T. Chang, IRRI, Manila/The Philippines.

Culture methods

Leaf material. Leaves were obtained from rice plants micropropagated in vitro according to Kumari et al. (1988) with modifications. Seeds were dehusked and surface sterilised with 30% Domestos bleach (Lever Brothers, UK) for 45 min and rinsed with sterile water. The seeds were germinated on hormone-free MS medium (MSO; Murashige and Skoog 1962) solidified with 0.8% (w/v) agar (Sigma). Seven-day-old seedlings were excised to remove the endosperm and the radicle, and were implanted into MS agar containing 2 mg l^{-1} 6-benzylaminopurine (BAP). After 4 weeks, multiple shoots had formed. These were separated and replanted into MSO agar. The micropropagated materials were maintained under continuous light (2,000 lx, Thorn 36W Pluslux 3,500 fluorescent tubes) at 28° ±2 °C.

Callus cultures. Callus cultures of V41A and V41B were initiated from mature seed scutellum. Dehusked and surface-sterilised seeds were placed onto Linsmaier and Skoog (1965) medium (LS) containing 2.5 mg l^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D), 3% (w/v) sucrose and 1 mg l^{-1} thiamine-HCl, and solidified with 0.8% (w/v) agarose (Sigma, type I) at pH 5.8 (LS2.5). The callus culture were initiated and maintained in the dark at 28° ±2 °C. After 28 days the callus cultures were transferred to fresh LS2.5 for further proliferation.

Suspension cultures. Suspension cultures (cell lines LB1 and LB3) of Taipei 309 were initiated from leaf base (Abdullah et al. 1986) and mature seed scutellum (cultures T3MS and T3MS2/A; Finch et al. 1989).

Isolation of mitochondrial DNA and restriction endonuclease analysis

Mitochondrial DNA from micropropagated leaf material was isolated as described (Saleh et al. 1989). For isolation of mitochondria from callus and suspension culture, 15 g fresh weight of material was gently homogenised for 5 min using a mortar and pestle. MtDNA was then isolated as described (Saleh et al. 1989). DNA samples were then analysed by electrophoresis on 0.8% agarose gels using TBE buffer (Saleh et al. 1989).

DNA probe and isolation of plasmid DNA

Plasmid pZmE1 containing the maize cytochrome c oxidase subunit II gene (*coxII*), cloned as a 2.4-kb fragment of maize mtDNA in pBR322 (Fox and Leaver 1981), was provided by Prof. C. Leaver, University of Edinburgh, Scotland. Plasmid DNA was isolated from *E. coli* HB101 (Birnboim and Doly 1979) and digested with EcoRI. The EcoRI fragment containing the *coxII* gene was isolated by preparative gel electrophoresis on low melting temperature agarose and labelled with ^{32}P by random priming, using a MultiPrime kit following the manufacturer's instructions (Amersham).

Southern blotting and hybridisation of DNA

MtDNAs from Taipei 309 cell lines LB1 and LB3 were digested with HindIII, BamHI and EcoRI, and the restriction fragments were separated by electrophoresis on a 0.8% agarose gel. Blotting of DNA fragments onto Gene Screen filters (DuPont) was carried out as described by Maniatis et al. (1982). Hybridisation of the filters with ^{32}P-labelled *coxII* insert of pZmE1 was carried out 16–24 h at 37 °C in 40% formamide, 5 × SSPE (1 × SSPE = 180 m*M* NaCl, 10 m*M* NaH_2PO_4, 1 m*M* Na_2EDTA, pH 7.0), 0.3% (w/v) sodium dodecyl sulphate, 100 μg ml^{-1} sheared denatured salmon sperm DNA. After hybridisation, filters were washed three times for 15 min at 65 °C in 5 × SSPE, 0.1% SDS and once in 2 × SSPE, 0.1% SDS. Autoradiography of dried filters was carried out at −70 °C using Fuji RX X-ray film with an intensifying screen.

Results and discussion

Digestion of mtDNA from 3-month-old callus cultures of V41A and V41B with BamHI produced restriction patterns (Fig. 1 a) that were indistinguishable from the corresponding BamHI patterns previously reported for mtDNA from leaves of these rice lines (Saleh et al. 1989) and another S_2-CMS containing *Indica* variety IR54752 (Fig. 1 b).

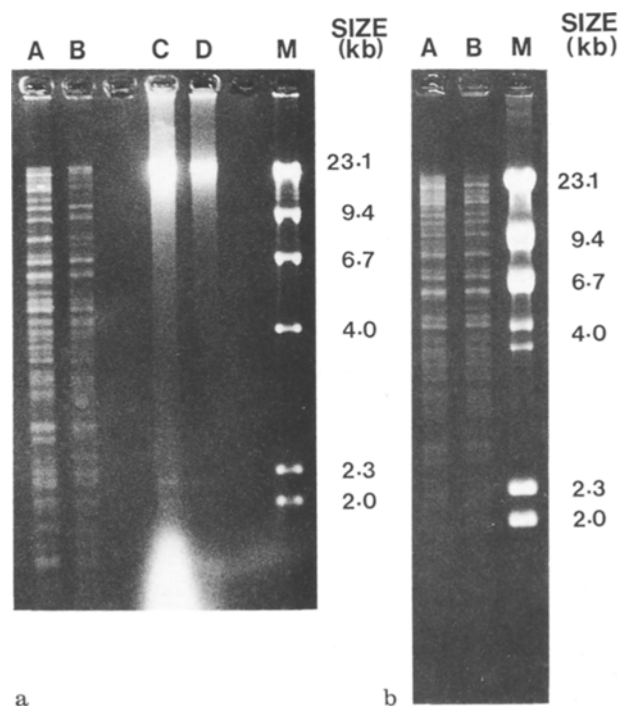

Fig. 1. **a** Agarose gel electrophoretic patterns of (i) BamHI-digested total mitochondrial DNA from CMS (*A*), fertile maintainer lines (*B*), and (ii) native mitochondrial DNA of CMS (*C*), fertile maintainer lines (*D*) of *Indica* rice variety V41. Molecular size markers (*M*) are given by a HindIII digest of phage lambda DNA. **b** Agarose gel electrophoretic patterns of BamHI-digested total mitochondrial DNA of IR54752A (*A*) and V41A (*B*), cytoplasmic male sterile lines of *Indica* rice variety carrying "wild abortive" cytoplasm (S_2-CMS). Molecular size markers (*M*) are given by a HindIII digest of phage lambda DNA

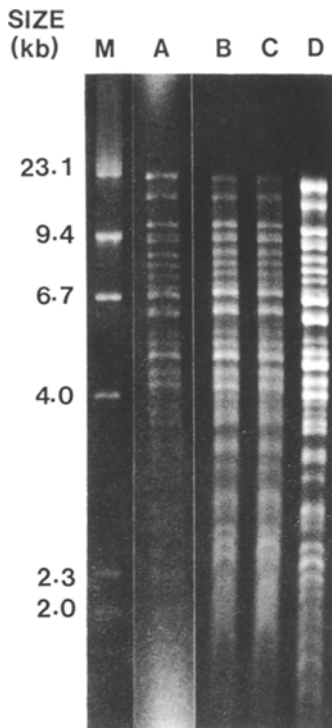

Fig. 2. Agarose gel electrophoretic patterns of BamHI-digested total mitochondrial DNA of Taipei 309 isolated from leaves (*A*), totipotent cell suspension, T3MS (*B*), 2-month-old cell suspension, T3MS2/A (*C*), and cell suspension which had lost its protoplast regeneration capability, LB3 (*D*). Molecular size markers (*M*) are given by a HindIII digest of phage lambda DNA

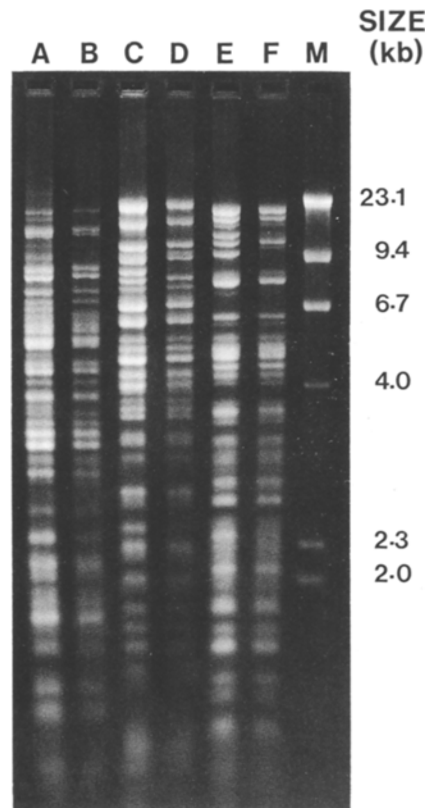

Fig. 3. Agarose gel electrophoretic patterns of total mitochondrial DNA of Taipei 309 leaf base cell suspensions (LB1 and LB3), which had lost their protoplasts regeneration capabilities. *Lanes A, C* and *E* show LB3 digested with HindIII, BamHI and EcoRI, respectively. *Lanes B, D* and *F* show LB1 digested with HindIII, BamHI and EcoRI, respectively. Molecular size markers (*M*) are given by a HindIII digest of phage lambda DNA

Similarly, mtDNA from leaves and the cell suspension culture LB3 of the fertile *Japonica* rice variety Taipei 309 showed indistinguishable BamHI restriction profiles (Fig. 2). During the last 2 years, a number of cell suspensions of Taipei 309 have been examined for their mtDNA restriction profiles. These include young (2-month-old) suspension cultures such as T309MS2/A, totipotent cell suspensions (8-month-old) capable of plant regeneration from protoplasts at high frequency, such as T3MS (from which 12% of protoplasts derived microcolonies differentiated to produce an average of four plants each; R. P. Finch, unpublished results) and cell suspensions in which the protoplast regeneration capability had been lost. The latter group includes the cell lines LB1 (19-month-old) and LB3 (30-month-old) described above. With the exception of mtDNA from line LB1, mtDNAs from all other suspension cultures showed restriction profiles identical to that of mtDNA from leaves of the parental plant (Fig. 2).

These results suggest that during short-term callus or cell suspension culture, the mtDNA of S_2-CMS rice V41A remains unaltered. This is also true for totipotent, regenerable suspension culture of the intended recipient of S_2-CMS, Taipei 309. Thus, these varieties are suitable parental lines for protoplast fusion approaches to the transfer of S_2-CMS.

Clear differences were observed between the restriction patterns of mtDNA from the LB1 cell suspension culture and those of the LB3 cell suspension culture, where the latter BamHI restriction mtDNA profiles were identical to those of Taipei 309 leaves and the cell suspensions T3MS and T309MS2/A. These differences were apparent after digestion with the three enzymes tested, namely HindIII, BamHI and EcoRI (Fig. 3). Inspection of ethidium-bromide-stained gels revealed that the different restriction profiles reflected differences in stoichiometry of specific fragments, rather than the presence or absence of a given fragment. This interpretation was supported by hybridisation of the maize *coxII* gene probe to blots of mtDNA cut with HindIII, BamHI or EcoRI (Fig. 4). While an identical set of prominent hybridisation signals was observed for each digest of the two mtDNAs, the relative intensities of several minor signals were clearly different. For example, a signal at ca. 9.4 kb in the HindIII pattern of LB3 mtDNA was more promi-

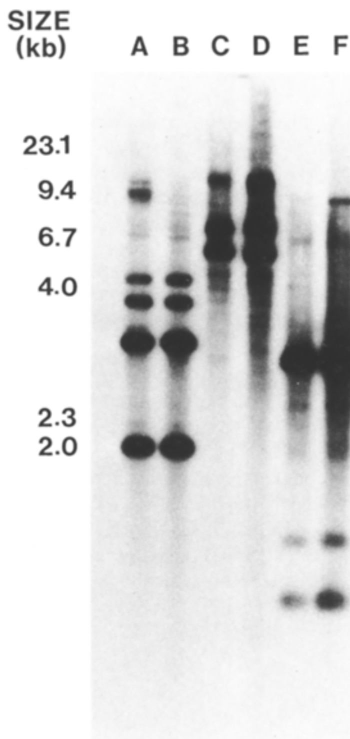

Fig. 4. Hybridisation of maize cytochrome oxidase subunit II (*coxII*) gene probe to Southern blot of HindIII-, BamHI- and EcoRI-digested mtDNAs of Taipei 309 (as in Fig. 3). *Lanes A, C* and *E* show hybridisation to LB3 mtDNAs digested with HindIII, BamHI and EcoRI, respectively. *Lanes B, D* and *F* show hybridisation to LB1 mtDNA digested with HindIII, BamHI and EcoRI, respectively

nent than the corresponding fragment in the LB1 sample. Similarly, a ca. 9 kb signal in the EcoRI digest of LB1 mtDNA was clearly more intense than its counterpart in LB3 mtDNA. Thus, both major changes in stoichiometry of certain mtDNA sequences, as seen on ethidium-bromide-stained gels and alterations in the relative levels of less abundant DNA fragments, as demonstrated by hybridisation experiments, may occur during long-term suspension culture of Taipei 309.

Minor changes (presence or absence of restriction fragments) in mtDNA organisation during suspension culture of a 7-year-old dihaploid cell line of the *Indica* variety PI353705 (similar to Assam 5) have recently been reported (Chowdhury et al. 1988). These changes reflect the probable existence of repeated sequences in the rice mt genome similar to those reported in other higher plant mtDNA (Lonsdale 1984; Palmer and Shields 1984; Stern and Palmer 1984). Such sequences act as sites of inter- and intra-molecular recombination, and this process results in the observed complex organisation of higher plant mtDNA (Lonsdale 1984; Levings and Brown 1989). The sequence rearrangements in mtDNA observed in tissue cultures may mimic those occurring in plantae that might determine CMS/fertility, e.g. in the rice varieties V41A/B (Saleh et al. 1989). The molecular weight of the rice mtDNA has been estimated as 300 kb; this genome appeared in the electron microscope as a collection of mainly linear molecules of about 60–105 kb (Wang et al. 1989a). These observations lend further support to the existence of recombination sites in rice mtDNA.

In summary, the results presented in this paper indicate that tissue culture of the rice lines V41A and Taipei 309 produces no significant alteration in the organisation of mtDNA in the short term. Thus, these lines would represent a suitable donor and recipient, respectively, for CMS transfer via protoplast fusion, and their use would simplify the interpretation of any mtDNA rearrangements in resulting male sterile cybrids.

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