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RAPD analysis of isolated mitochondrial DNA reveals heterogeneity in elite *wild abortive* (*WA*) cytoplasm in rice

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Abstract RAPD profiles were generated using mitochondrial DNA (mtDNA) isolated from two cytoplasmic male-sterile lines, two restorer lines and four maintainer lines of rice. Of the 40 primers tested, 25 generated consistent and easily scoreable patterns that were used for the computation of pairwise similarities as well as UPGMA analyses. The different lines of rice, including lines IR58025A and IR62829A that contained the same *wild abortive* (*WA*) cytoplasm, were distinguishable on the basis of RAPD profiles. These latter two lines were not distinguishable from each other by mtDNA RFLP analyses with as many as 16 mtDNA probes. The data illustrate the utility of the RAPD technique as a powerful tool for distinguishing different cytoplasms that by other techniques appear to be similar. To our knowledge, this is the first report wherein RAPD profiles obtained with isolated mtDNA templates enable the distinction between two or more types of cytoplasms in rice.

Key words mtDNA · RAPD · Rice · *WA* cytoplasm

Introduction

Random amplification of polymorphic DNA (RAPD) is a recent and powerful technique for determining inter- and intra-specific DNA variations. First developed in 1990 independently by two groups (Williams et al. 1990; Welsh and McClelland 1990), the technique has since come a long way in its applications to plant genetic analyses. The real merit of the technique is in its simplicity, rapidity and reliability. The RAPD technique has been applied to an impressive list of both prokaryotic as well as eukaryotic genomes and has resulted in a high-resolution analysis in almost all cases (Ranade 1995; Ranade and Sane 1995, 1996).

In recent years, a few of the notable applications of RAPD technique include studies on genetic variability in both natural plant populations and the germplasms of important crop plants (Chalmers et al. 1994; Orozco-Castillo et al. 1994; Lu et al. 1996), in the identification and isolation of markers linked to a number of traits (Tanhuanpaa et al. 1995; Kutcher et al. 1996; Warburton et al. 1996), in the determination and saturation of molecular linkage maps exclusively using RAPDs as well as in combination with restriction fragment length polymorphisms (RFLPs), isozymes and DNA fingerprints (Bradshaw et al. 1994; Yazdani et al. 1995; Baudracco-Arnas and Pitrat 1996; Sondur et al. 1996). The technique has also been used for the determination of molecular phylogenetics and systematics (Cipriani et al. 1996; Marillia and Scoles 1996; Samec and Nasinec 1996) and for tagging and mapping of the *Rf-3* nuclear fertility-restoring gene in *WA* cytoplasm of rice (Zhang et al. 1997). An overwhelming majority of the above reports involve the isolated total plant DNA as a template, which means that these studies have analysed the data from the three types of genomes, namely, nuclear, chloroplastic and mitochondrial DNA templates, taken together. Surprisingly, with the exception of sugar beet (Lorenz et al. 1994, 1997), there are no reports, to our knowledge, of this technique being applied to the analysis of isolated and purified organelle DNA alone to distinguish different cytoplasms and their origins.

Organelle genome diversity has been studied extensively, and in several species, using the RFLP approach, especially for determination of the diversity in mitochondrial genomes and with particular reference to cytoplasmic male sterility (CMS). In *Allium cepa*,

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differences between the various cytoplasms as well as the origins of the cytoplasms found in male-sterile and fertile lines have been determined through the RFLP approach using isolated chloroplast (cp) and mitochondrial DNA (mtDNA) as templates (Holford et al. 1991). A similar study, but with only isolated mtDNA was carried out to identify the different subtypes of male-sterile cytoplasms in sorghum (Bailey-Serres et al. 1986) and sugar beet (Weihe et al. 1991). Also, we have earlier reported mtDNA RFLPs for different mitochondrial genes such as *atpA* and *orf156* in male-sterile lines, their maintainers and their restorers in both *Sorghum* and rice (Sane et al. 1994, 1996; Seth et al. 1996). In recent years, chloroplast, mitochondrial and nuclear genomes have been compared in RFLP analyses in plants such as rice (Ishii et al. 1993), onions (Satoh et al. 1993) and oil palms (Lack et al. 1995), to either determine molecular marker diversity or to differentiate CMS cytoplasms. The polymerase chain reaction (PCR) has also been employed to determine the inheritance of chloroplast and mitochondrial genomes in pedunculate oaks through the use of gene-specific primers (Dumolin et al. 1995).

However, one of the potential drawbacks of the RFLP approach in studying mitchondrial genome diversity is the fact that mitochondrial genes are highly conserved and hence may not often reveal much variation. In contrast, intergenic regions of the mitochondrial genes show tremendous variability due to the highly recombinative nature of the mitochondrial genome. This variability in the mitochondrial genomes is more likely to be detected by RAPD due to the random nature of the binding of the random primers, thereby screening a much larger portion of the genome and leading to a better characterization of the organelle genome diversity.

We have been interested in the molecular organization of the mitochondrial genome as well as its interactions with the nuclear genome vis-a-vis male sterility in the *wild abortive* cytoplasm in rice. We had used as many as 16 mitochondrial gene probes to characterize the three rice cytoplasms, namely, male-sterile, maintainer and the restorer type (Seth et al. 1996). The RFLP analysis of the three male-sterile rice lines that contained *WA* cytoplasm revealed that, for the 16 major mtDNA probes at least, there was no detectable polymorphism, thereby suggesting an apparent homogeneity of the lines. We therefore decided to employ the powerful RAPD approach for analysing overall variability in eight lines of rice, of which two contained the *WA* cytoplasm. In this paper, we describe for the first time the application of the RAPD technique to the determination of diversity in an organelle (mitochondrial) genome, in rice. The RAPD data enabled not only the sterile and maintainer lines to be distinguished but even the lines containing the *WA* cytoplasm.

Materials and methods

Plant material

Seeds of IR58025A, IR62829A (both CMS lines containing *WA* cytoplasm), IR58025B, IR62829B, V20B (all maintainers for *WA* CMS lines) and PonniR and IR10198-66-2R (both restorers for the CMS lines) were obtained from Dr. M. Rangasamy, TNAU Coimbatore, India; Dr. N. P. Sarma, DRR Hyderabad, India and Dr. Vijaykumar, Maruteru, India. Seeds of the Japanese maintainer line RCPL1-2C were kindly provided by Dr. H. S. Gupta, ICAR Complex, Barapani, India.

Isolation of mtDNA

MtDNA was isolated from etiolated seedlings of all the eight lines as described earlier (Nath et al. 1993) but without the Proteinase K treatment and CsCl gradient purification steps. Etiolated seedlings were used to minimize plastid contamination, while mitochondrial pellets were treated with DNase to remove extra-mitochondrial (nuclear) DNA before further processing.

RAPD amplification and agarose gel electrophoresis

DNA was amplified using 40 arbitrary primers from Kit A (primer OP-A01 to OP-A20) and Kit B (primer OP-B01 to OP-B20), both from Operon Technologies, USA, according to Williams et al. (1990). All amplification reactions were performed in a final volume of 25 µl and contained 10 pmole of the 10-mer primer (Operon Technologies), 25 ng template DNA, 200 µ*M* each dNTP (Pharmacia), 1.5 m*M* Mg^{++} and 1 Unit *Taq* DNA polymerase (Bangalore Genei, Bangalore, India). Reactions were cycled 45 times at 94°C for 60 s, 35°C for 90 s and 72°C for 120 s with a final extension at 72°C for 300 s, in a DNA Robocyler (Stratagene GmbH, Germany). After completion of the amplification, 2.5 µl 10 × blue dye was added to the samples. From this, a 10-µl aliquot was loaded into each slot in a 1.2% agarose gel made in 1 × TAE buffer and electrophoresed at a constant current until the blue dye had run two-thirds of the total gel length.

RAPD data analysis

The fragment sizes of the amplification products were estimated from the gel by comparison with a standard molecular-weight marker (λDNA double digested with *Hin*dIII and *Eco*RI, Bangalore Genei, India). For each primer, a matrix of all the bands present in different DNAs was generated using '1' when the band was present and 'O' when the band was absent. Similarities of profiles were determined using the algorithm of Jaccard (1901) in the RAPDISTANCE package (ver 1.03, Armstrong et al. 1994). A UPGMA dendrogram was generated from the similarity data following the method of Sokal and Sneath (1963).

MtDNA RFLP analysis

MtDNA was first digested with *Hin*dIII as per the manufacturers' instructions, and Southern blots on nylon membranes (Hybond N, Amersham Int, UK) were prepared as described previously (Sane et al. 1994). The S-blots were probed with a $\gamma[^{32}P]$-ATP end-labelled oligonucleotide probe specific for *orf156* (kindly provided by Dr. J. M. Grienenberger, France). Hybridization with the radiolabelled probe was carried out in 5 × SSC, 5 × Denhardt's solution, 1 m*M*

EDTA and 100 μg/ml sheared calf thymus DNA at 30°C overnight. Washing was carried out in 2 × SSC for 2 × 15 min at room temperature. Blots were autoradiographed as described earlier (Sane et al. 1994).

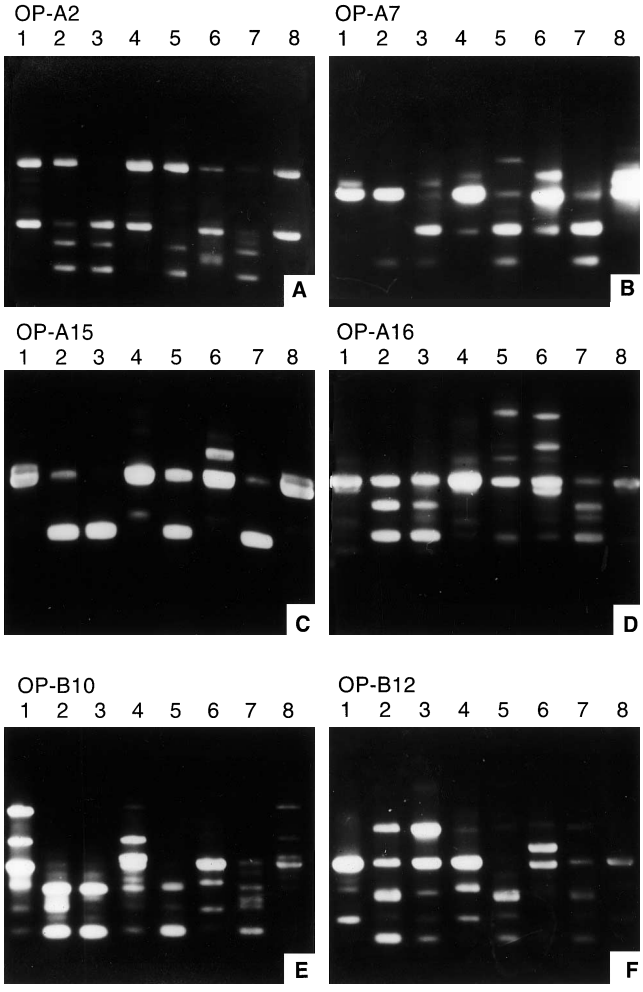

Fig. 1A–F RAPD agarose gel electrophoresis profiles obtained with eight rice mtDNAs using primers OP-A02, OP-A07, OP-A15, OP-A16, OP-B10 and OP-B12. *Lanes 1–8* are, respectively, IR58025A, IR62829A, IR58025B, IR62829B, V20B, RCPL1-2C, PonniR and IR10198-66-2R

Results

Mitochondrial DNA from two male-sterile, four maintainer and two restorer lines of rice was studied using 40 RAPD primers (20 primers each from the A and B kits, Operon Technologies, USA). Of these, 32 primers resulted in discrete amplification profiles. RAPD profiles from 25 of these 32 primers that resulted in easily scoreable and consistent profiles with all eight templates were selected for determining the similarity amongst the genotypes. These 25 primers amplified one to eight fragments ranging in size from 0.3 to 3.5 kbp in each of the eight template mtDNAs.

The amplification profiles with 6 primers, namely OP-A02, OP-A07, OP-A15, OP-A16, OP-B10 and OP-B12, are shown in Fig. 1 and are representatives of the characteristic, though mostly polymorphic, profiles that a majority of the primers generated. Some of the primers like OP-A11 and OP-B07 resulted in a highly polymorphic profile with no similarity amongst the individual lines (Fig. 2 A, B). In contrast, the profile produced by the primer OP-A12 was mostly monomorphic (Fig. 2C). A cumulative matrix of all the profiles obtained with the 25 primers was prepared as described and resulted in 254 informative bands, which were used to compute pairwise similarities amongst the eight mtDNAs. The UPGMA dendrogram was constructed from the pairwise similarities and is shown in Fig. 3. A specific clustering was observed amongst the eight mtDNAs such that the DNAs from the lines IR58025A, IR62829B, IR10198-66-2R and RCPL1-2C were grouped together, while the remaining four mtDNAs from lines IR62829A, PonniR, IR58025B and V20B grouped together. We further analysed the same DNA as used for RAPD by probing a S-blot of *Hin*dIII-digested mtDNAs with an *orf156*-specific oligonucleotide probe (Fig. 4), and our results confirmed that the distinct grouping of IR58025A and IR62829A (both containing the *WA* cytoplasm and indistinguishable by RFLP analyses) on the basis of RAPD data was not an aberration.

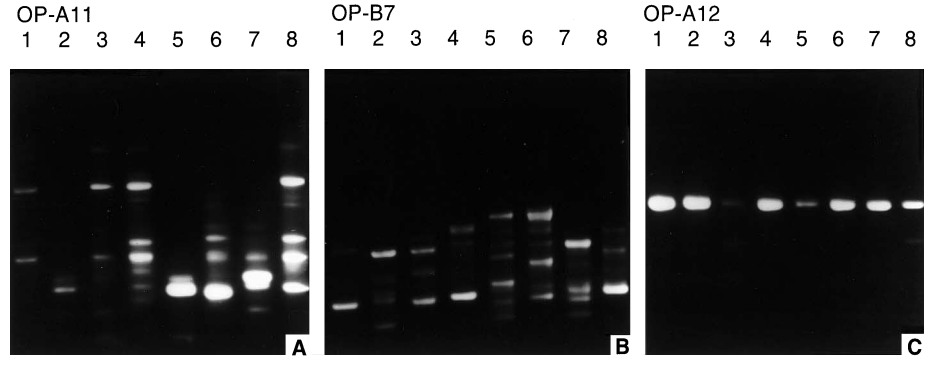

Fig. 2A–C RAPD agarose gel electrophoresis profiles of the eight rice mtDNAs obtained using the primers OP-A11 (**A**) and OP-B07 (**B**) and primer OP-A12 (**C**). *Lanes 1–8* are as in **Fig. 1**

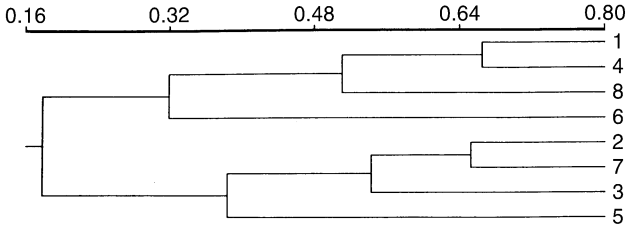

Fig. 3 UPGMA dendrogram generated from the total RAPD data of the eight rice mtDNAs. The *numbers* to the *right* of the dendrogram correspond to *lanes* denoted by the same numbers in **Fig. 1**

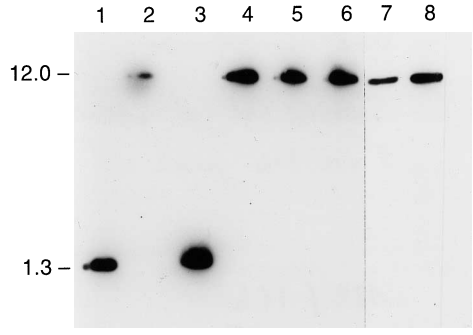

Fig. 4 MtDNA RFLP autoradiogram for S-blot of the *Hin*dIII-digested eight rice mtDNAs probed with oligo-probe specific for the *orf156*. *Lanes 1–8* are respectively, IR58025A, IR58025B, IR62829A, IR62829B, PonniR, IR10198-66-2R, RCPL1-2C and V20B. *Numbers* to the *left* of the panel indicate sizes in kilobasepairs

Discussion

A number of studies have been carried out to detect and analyse the diversity, if any, in the organelle genomes using RFLP and gene-specific PCR (Holford et al. 1991; Weihe et al. 1991; Ishii et al. 1993; Satoh et al. 1993; Lack et al. 1995; Dumolin et al. 1995). To our knowledge, no studies on the diversity of organelle genomes had ever been reported in which isolated organelle DNAs were specifically used as the templates in RAPD-PCR, until recently (Lorenz et al. 1994, 1997). This is surprising considering the RAPD technique actually enables several miniscule changes in the genome to be distinguished whereas the RFLP technique only detects changes affecting either the restriction endonuclease cleavage sites or large-scale deletions and insertions between two restriction sites. A possible reason for this is that the organelle DNAs are prepared only after elaborate purification strategies have been adopted. Consequently, the rapidity and simplicity that is essentially associated with RAPD is actually reversed when purified organelle genomes have to be used as templates. However, in a situation where it becomes essential to actually characterize diversity in the specific organelle genomes and where the RFLP approach is uninformative, as in the present studies, it is essential that purified organelle DNAs be used as templates for amplification with arbitrary sequence decamer primers. The detection of variation in cytoplasmic genomes, especially the mitochondrial genomes, is important in CMS lines. It is therefore logical that, in such situations, the diversity in cytoplasmic genomes be determined not only by the RFLP approach but also by RAPD analysis. Such studies can also perhaps delineate the evolution of the combinations of mitcohondrial, chloroplastic and nuclear genomes of the present-day species and/or varieties.

In rice, the elite cytoplasm *Wild Abortive* (*WA*) has been used for the production of several commercial hybrids using the CMS system of this cytoplasm. We have undertaken a progamme that involves analysis of the CMS phenomenon in rice and the molecular changes and interactions associated with it. The RAPD profiles in the present studies have been obtained with 25 primers selected from amongst the 40 primers that were originally tested using isolated and purified mtDNAs. Although attempts were made to minimize nuclear and plastid DNA contamination (as described in the Materials and methods), the contribution by these DNAs, when present as contaminants, cannot be entirely ruled out. However, it is unlikely that they would significantly affect the outcome of the results. In a majority of the cases, the mtDNAs from the different lines resulted in different products. In a few instances the profiles were composed of bands of different intensities, but our experimental design did not allow testing of quantitative differences amongst the template DNAs. The dendrogram in Fig. 3 indicates that the different lines are distinguishable from each other. The two sterile (A) lines could almost always be clearly distinguished from their respective maintainer (B) lines. Since the respective A and B lines are isonuclear, the clear distinction between these lines indicates that the differences are indeed cytoplasmic in origin and do not arise from bands amplified by contaminating nuclear DNA. The four B lines and the two R lines could also be distinguished from each other on the basis of the RAPD data. Surprisingly, the two A lines, which are both believed to contain the *WA* cytoplasm, also resulted in distinct RAPD patterns with most of the primers. This is an important result. Previous studies had revealed that the two male-sterile lines of the 'IR58025' and 'IR62829' varieties having the same *WA* cytoplasm were indistinguishable with as many as 16 mtDNA-specific RFLP probes (e.g. Fig. 4), while their respective maintainers could be distinguished from each other with *rps14* and *cox3* (Seth et al. 1996). The observations made by the breeders as well as our RFLP data (Seth et al. 1996) thus indicated that the *WA* cytoplasm is apparently homogeneous. If this is indeed so, there are potential pitfalls associated with the use of a single homogeneous cytoplasm source in breeding since the deleterious characters associated with this cytoplasm can automatically be introgressed into a number of hybrid lines at the same time. As

is well known, the T-cytoplasm in maize conferred susceptibility to fungal pathogen toxins and, consequently, the use of T-cytoplasm in hybird maize production had to be discontinued (Levings and Brown 1989).

The ability to distinguish between individual organelle genomes and especially within a given CMS cytoplasmic source is of considerable significance. While just such a distinction of cytoplasms within a supposedly identical source for CMS (the Owen's type of cytoplasm) was successfully achieved in sugar beet with mitochondrial RFLPs (Weihe et al. 1991), a similar approach in rice was uninformative. However, the RAPD approach used in the present studies enabled a clear distinction to be made between the two CMS lines having *WA* cytoplasm. This approach now allows us to screen all supposedly identical CMS lines for the presence of divergent types among them. Thus, the technique provides possibilities for assessing and using cytoplasms, which, though similar with respect to traits like CMS, still have considerable variations, to overcome, the problems that may be associated with identical cytoplasms in hybrid seed production.

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