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Molecular analysis of organelle DNA of different subspecies of rice and the genomic stability of mtDNA in tissue cultured cells of rice *

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Summary. Chloroplast (ct) and mitochondrial (mt) DNAs were isolated from two subspecies of rice (*Oryza sativa*), *japonica* (Calrose 76) and *indica* (PI353705) and compared by restriction endonuclease fragment pattern analysis. Similarly, PI353705 (A5) mtDNA was also compared with the mtDNA of its long term tissue cultured line, BL2. Variation in the ctDNA of the 2 subspecies was detected with two (*Ava*I and *Bgl*I) of the 11 restriction endonucleases tested, whereas their mtDNAs showed considerable variation when restricted by *Pst*I, *Bam*HI, *Hin*dIII and *Xho*I endonucleases. Thus, the chloroplast DNA was more highly conserved than the mtDNA in the subspecies comparisons. Only minor variation was observed between the restriction endonuclease patterns of the mtDNAs of BL2 and A5. Southern blots of mtDNA were hybridized with heterologous probes from maize and spinach organelle genes. Differences were found in the hybridization patterns of the two subspecies for six of the eight (mitochondrial and chloroplast) probes tested. Two of the seven (mitochondrial) probes (*cox*II and 26S rRNA) detected tissue culture generated variation in mtDNA. The relative values of restriction endonuclease and hybridization patterns for studying phylogenetic and genetic relationships in rice are discussed.

Key words: Rice (*Oryza sativa*) – Mitochondrial DNA – Chloroplast DNA – Restriction pattern – Tissue culture

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Introduction

Rice (*Oryza sativa*) is the staple food for more than one-third of the world's population. The species consists of three major subspecies, *indica, japonica* and *javonica*. Hybrid formation between subspecies is often difficult and progenies are often only partially fertile. Additionally, these subspecies respond differently to anther and tissue culture techniques requiring different media and hormone formulations.

Restriction endonuclease fragment patterns of organelle DNA have been used as a measure of diversity and relatedness between species and subspecies in a large number of economically important plant genera such as *Hedysarum* (Baatout et al. 1985), *Daucus* (DeBonte et al. 1984), *Teosinte* (Timothy et al. 1979) and *Cucumis* (Ward et al. 1981), to mention a few.

Southern blot hybridization patterns of total rice nuclear DNA hybridized to probes of repeated rice nuclear sequences (Pental and Barnes 1985) and probes of total rice chloroplast DNA (ctDNA) (Ichikawa et al. 1986) were used to study the interrelationships among different species of rice. Restriction endonuclease and hybridization patterns have been used to monitor genetic changes in the mitochondrial and chloroplast genomes induced by protoplast fusion, tissue or cell culture. These reports show varying degrees of stability of those genomes during in vitro culture. No ct- or mitochondrial DNA (mtDNA) changes were observed either in carrot cells cultured for 10 years (Matthews and DeBonte 1985) or in wheat after one cycle of anther culture followed by chromosome doubling (Rode et al. 1985). McNay et al. (1984) observed minor changes in the mtDNA of maize when hybridization patterns of plant and 4-year-old suspension culture mtDNAs were compared using the plasmid-like mtDNA probes, S1

and S2, from cytoplasmic male sterile maize. Ozias-Akins et al. (1987) reported that quantitative differences in the restriction patterns of pearl millet mtDNA occurred during 5 months of cell suspension culture.

The rice tissue culture line, BL2, was derived from PI353705, which is similar to Assam 5 and belongs to the *indica* subspecies (Schaeffer and Sharpe 1981). That culture line was initiated as a haploid callus culture from anthers that spontaneously doubled its chromosome number and was subsequently regenerated into whole plants. The cell line has been maintained in suspension culture for 7 years and it was of interest to measure organelle DNA changes that occurred during that period.

The objectives of this research were twofold: (1) study the rice organelle DNA variability at the subspecies level and evaluate the effectiveness of restriction endonuclease restriction patterns and hybridization patterns using heterologous gene probes as parameters in phylogenetic studies; and (2) evaluate the rice mitochondrial genomic stability during long-term cell culture by measuring the mtDNA alterations that occurred during long-term cell culture of BL2.

Materials and methods

Materials

The rice genotypes used in this investigation were Calrose 76, a *japonica* subspecies designated as C76, and PI353705, an *indica* subspecies similar to Assam 5 (designated as A5). The A5 seed used in this study was of the same lot used to originate the cell line. The cell suspension line of A5 (Schaeffer and Sharpe 1981) was maintained in cell suspension for 7 years.

DNA isolation

Mitochondrial DNAs were isolated from 10-day-old etiolated seedlings of C76 and A5 using the method of Smith et al. (1987). Etiolated seedling tissues were ground in a Waring blender using a saline extraction buffer followed by differential centrifugation to isolate mitochondria, and then treated with DNAse to remove extramitochondrial DNA. Mitochondrial DNAs were purified by precipitating SDS-protein-carbohydrate complexes with potassium acetate, precipitating mtDNA with isopropanol followed by phenol, phenol-chloroform and chloroform extraction, then a second isopropanol precipitation.

MtDNA was isolated from cell suspension cultures of BL2 using the method of DeBonte and Matthews (1984). Briefly, cell suspensions were ground in liquid N_2, suspended in buffer containing sorbitol as an osmoticum, then mitochondria were separated by differential centrifugation. After lysis, mtDNA was purified by centrifugation in a CsCl-bisbenzamide gradient.

Chloroplast DNAs were isolated from light-grown 12-day-old seedlings using the method of Hirai et al. (1985). In this method, leaf tissue was ground in liquid N_2, suspended in a buffer containing sucrose as an osmoticum followed by purification of chloroplasts by centrifugation through a sucrose step gradient. After lysis, chloroplast DNA was purified using phenol, phenol-chloroform and chloroform extraction.

Restriction endonuclease analysis

Restriction endonuclease digestions of the mt- and ctDNAs were made with *Bam*HI, *Hin*dIII, *Pst*I and *Xho*I, and additionally, ctDNAs were digested with *Ava*I, *Bgl*I, *Eco*RI, *Sal*I, *Sac*I, *Pvu*I and *Xba*I. DNA fragment separation was done by gel electrophoresis in 0.8% agarose in TPE or TBE buffer (pH 8.0). Gels were run at room temperature either for 5 h at 5 volts/cm or 16 h at 2 volts/cm. Gels were stained with 0.5 μg/ml ethidium bromide for 45 min, destained in deionized water for 10 min and photographed over short wavelength UV light using the appropriate filter and film. *Hin*dIII-digested lambda DNA alone or with *Hae*III-digested 0X174 DNA were used as molecular size markers.

Isolation and preparation of probes

Maize clones containing mitochondrial genes cytochrome *c* oxidase subunit I (*cox*I) (Isaac et al. 1985), subunit II (*cox*II) (Fox and Leaver 1981); F_1–F_0 ATPase subunit 6 (*atp*6) (Dewey et al. 1985a), subunit 9 (*atp*9) (Dewey et al. 1985b), and subunit alpha (*atp*A) (Braun and Levings 1985); ribosomal genes (26S) (Dale et al. 1984) and (18S-5S) (Chao et al. 1984) were provided by C.S. Levings III of North Carolina State University, Raleigh/NC, USA. A clone containing the chloroplast ribulose 1,5-bisphosphate carboxylase large subunit gene (*rbc*L) from spinach (Erion et al. 1981) was provided by N. Brot, Roche Institute of Molecular Biology, Nutley/NJ, USA.

The maize gene clones were restricted and the maize inserts separated from the vectors by electrophoresis, then those inserts were recovered using the low melting temperature agarose or NA45 membrane (Schleicher and Schuell, Ine) method. In the first method, the fragment of interest was cut from the gel and DNAs were recovered from the agarose by melting the agarose at 65 °C, followed by phenol, phenol-chloroform and chloroform extraction, and ethanol precipitation. Alternatively, regular agarose was used to separate fragments, the gels were sliced directly below the fragment of interest, and a strip of NA45 membrane was inserted. The gel was then electrophoresed until the fragment of interest was bound to the membrane. The membrane was removed and DNAs were eluted and recovered according to supplier's recommendations. DNA probes were labeled by nick translation as described by Rigby et al. (1977). Unincorporated nucleotides were removed by chromatography through Sephadex G-50, and the labeled probes were denatured at 100 °C for 5 min.

Southern blotting and hybridization of DNA

Unidirectional transfers were carried out using either nytran or nitrocellulose membranes according to Southern (1975). Prehybridizations and hybridizations were carried out in sealed bags either at 42 °C using 50% formamide or at 65 °C without formamide. For the 65 °C method, prehybridization was carried out for 1 h using Denhardt's solution, SSC, and denatured salmon sperm DNA. The labeled, denatured probe was injected into the hybridization bag. Following hybridization for 16 h, membranes were washed twice in 3× SSC (1× SSC = 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0) and once in 0.3% SSC at 65 °C.

For the 42 °C method, prehybridization and hybridization methods were carried out for 2 h and 16 h, respectively, according to Maniatis et al. (1982). Post hybridization washes were carried out at 45 °C using 2× SSPE (1× SSPE = 0.15 *M* NaCl, 10 m*M* NaH_2PO_4, 1 m*M* EDTA, Ph 7.4) and 0.2% SDS with agitation three times for 5 min each, followed by two

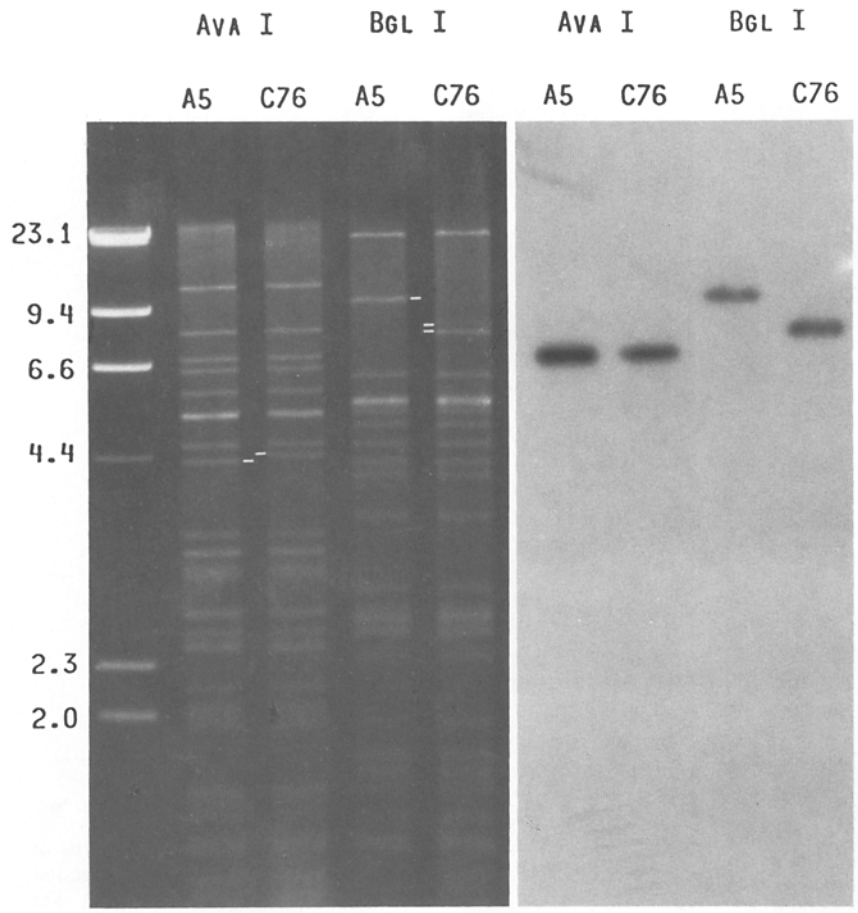

Fig. 1. (*Left*) Restriction fragment patterns of ctDNAs from A5 and C76 generated by *Ava*I and *Bgl*I are shown. *Dashes* (–) indicate the fragment differences between the rice genotypes. *Left lane* contains the *Hin*dIII-digested lambda and *Hae*III-digested 0X174 marker DNA and the sizes of those marker fragments are shown in kb. (*Right*) Autoradiographs of the above mentioned ctDNAs hybridized by the ^{32}P labeled *rbcL* gene probe shows the hybridization patterns

washes each for 20 min using 1× SSPE and 0.1% SDS. After washing, membranes were air dried and autoradiographs were made using standard procedures.

Results

Chloroplast DNA restriction patterns

Chloroplast DNA from C76 and A5 produced different restriction fragment patterns when digested with *Ava*I and *Bgl*I (Fig. 1, *left*); no differences were observed when the DNAs were digested with *Eco*RI, *Bam*HI, *Hin*dIII, *Pst*I, *Sal*I, *Sac*I, *Pvu*I, *Xba*I and *Xho*I. *Ava*I produced 29 fragments of which 28 were identical in both subspecies and variant fragments were a 4.28 kb fragment in A5 and a 4.33 kb fragment in C76. The *Bgl*I endonuclease cleaved at 23 sites in A5 and 24 sites in C76 from which 22 resulting fragments were identical in both subspecies. A5 had an 11.4 kb fragment not found in C76, and C76 had 8.5 (weak) and 8.0 kb fragments not found in A5. *Eco*RI, *Bam*HI, *Hin*dIII, *Pst*I, *Sal*I, *Sac*I, *Pvu*I, *Xba*I and *Xho*I produced 24, 22, 20, 14, 8, 12, 8, 11 and 12 fragments, respectively, in both subspecies (not shown).

Chloroplast DNA hybridization patterns

Blots containing the ctDNAs of C76 and A5 digested with *Ava*I, *Bgl*I, *Eco*RI, *Bam*HI, *Hin*dIII, *Pst*I, *Sal*I, *Sac*I, *Pvu*I, *Xba*I and *Xho*I were hybridized with the *rbc*L probe. Only *Bgl*I showed differences between the two subspecies; this probe hybridized to a 11.4 kb fragment in A5 and an 8.0 kb fragment in C76 (Fig. 1).

Mitochondrial DNA restriction patterns

Restriction patterns of C76, A5 and BL2 mtDNA digested with *Pst*I, *Bam*HI, *Hin*dIII are shown in Fig. 2. These enzymes cleaved A5 mtDNA into 37, 52 and 52 fragments, respectively. The estimated size of the mitochondrial genome in these subspecies ranged from 325–375 kb based on the assumption that there was only one fragment in each band. Considerable variation in restriction patterns was observed between C76 and A5 (Fig. 2). Due to the many fragment differences produced by each enzyme, a detailed description of that variation is omitted. Restriction with *Pst*I produced maximum pattern variation between the mtDNAs of

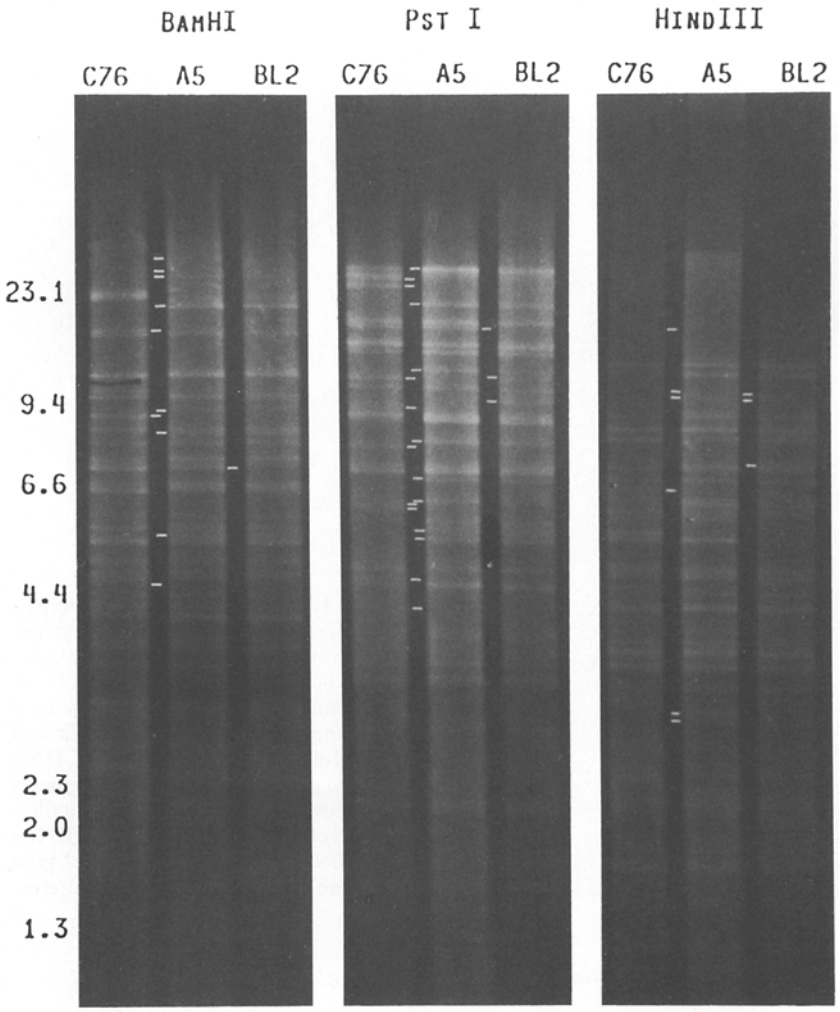

Fig. 2. Restriction fragment patterns of mtDNAs from C76, A5 and BL2 digested with *Bam*HI, *Pst*I and *Hin*dIII are shown. *Dash* (–) indicates fragment differences between the two adjacent lanes. Sizes (in kb) of the markers described in Fig. 1 are listed on the *left*

Table 1. Restriction fragment summary of C76, A5 and BL2 mtDNAs restricted with *Pst*I, *Bam*HI and *Hin*dIII

Summary item	Restriction endonuclease		
	*Pst*I	*Bam*HI	*Hin*dIII
Total no. of C76 fragments	38	48	50
Total no. of A5 fragments	37	52	52
No. of fragments common to both C76 and A5	29	45	48
No. of fragments differing between C76 and A5	17	10	6
Total no. of BL2 fragments	38	51	50
No. of fragments common to both A5 and BL2	36	51	49
No. of fragments differing between A5 and BL2	3	1	3

the two subspecies followed by restriction patterns of *Bam*HI and *Hin*dIII, respectively (Table 1).

Variation in restriction pattern was also observed between A5 and its tissue culture line, BL2 (Fig. 2). When *Pst*I-digested mtDNAs of A5 and BL2 were compared, a 13.4 kb fragment of A5 was not present in BL2, and two BL2 fragments of 11.2 and 9.2 kb were not present in A5 (Fig. 2). When digested with *Bam*HI the only difference was an 8.0 kb fragment in A5 not present in BL2; when cut with *Hin*dIII two A5 fragments of sizes 10.5 and 9.8 kb were absent in BL2, and a 7.7 kb fragment present in BL2 was absent in A5.

Mitochondrial DNA hybridization patterns

The blots of *Pst*I-digested mtDNAs of the three genotypes were hybridized with *atp*6 and 18S-5S ribosomal probes. Hybridization patterns of both probes showed differences between C76 and A5, but no differences were noted between A5 and BL2 (Fig. 3). The *atp*6 gene probe hybridized to four C76 fragments, five A5 fragments and five BL2 fragments. Three fragments (27.1, 9.2 and 3.9 kb) were common across the 3 genotypes, two A5 and BL2 fragments [16.7 (weak) and 6.3 kb] were not present in C76, and a 22.9 kb (weak) C76 fragment was absent in A5 and BL2. The 18S-5S probe hybridized to two fragments of 27.1 and 3.4 kb across

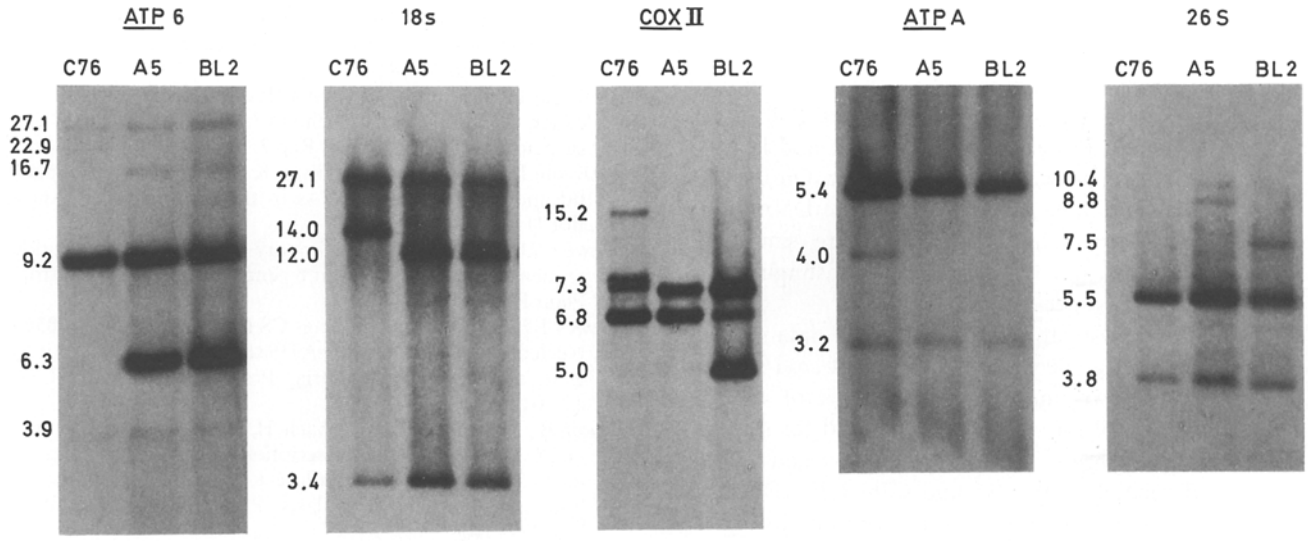

Fig. 3. Hybridization patterns of C76, A5 and BL2 mtDNAs are shown. *Top left* and *center* shows the patterns of *Pst*I-digested mtDNA hybridized by the maize *atp*6 and 18S-5S rRNA gene probes, respectively. *Top right* and *bottom left* show the patterns of *Bam*HI-digested mtDNA hybridized by the maize coxII and *atp*A gene probes, respectively. *Bottom right* shows the patterns of *Hin*dIII-digested mtDNA hybridized to the maize 26S rRNA gene probe. Numbers to the left of each autoradiograph indicate the sizes (in kb) of the hybridized fragments

the three genotypes and to a fragment of 14.0 kb in C76 and 12.0 kb in A5 and BL2 (Fig. 3).

Blots of *Bam*HI-digested mtDNA of the three genotypes were hybridized to the *cox*II and *atp*A maize genes. The *cox*II probe hybridized to four C76 fragments, two A5 fragments and three BL2 fragments (Fig. 3). Two fragments (7.3 and 6.8 kb) were common to all three genotypes, two fragments of 15.2 (weak) and 8.7 kb were found only in C76, and a 5.0 kb fragment was found only in BL2.

Hybridization patterns of the *atp*A probe showed variation between C76 and A5 but not between A5 and BL2 (Fig. 3). This probe hybridized to two fragments [5,4 and 3.2 (weak) kb] in all genotypes and a 4.0 kb (weak) fragment found only in C76.

Hybridization patterns of the 26S ribosomal gene probe to *Hin*dIII-digested mtDNAs also showed variation in all three genotypes (Fig. 3). That probe hybridized to two fragments of 5.5 and 3.8 kb that were common to all genotypes, two additional A5 fragments of 10.4 and 8.8 kb (both weak) that were absent in BL2 and C76, and to a 7.5 kb BL2 fragment that was not present in A5 or C76.

Hybridization patterns of the *cox*I gene probe to *Hin*dIII-digested mtDNAs and the *atp*9 gene probe to *Xho*I-digested mtDNAs showed no differences among the three genotypes (not shown). The *cox*I probe showed homology with four fragments (9.2, 8.2, 3.0 and 1.5 kb) and *atp*9 probe showed homology with only one fragment (10.4 kb).

Discussion

Both C76 and A5 had unique ct- and mtDNAs as distinguished by restriction endonuclease digestion. Substantial restriction endonuclease differences were observed between the mtDNAs of representatives of the two subspecies with the four restriction endonucleases used, whereas only two of the 11 endonucleases used detected minor variation in ctDNAs. Similar to many other species, e.g., carrot (DeBonte et al. 1984), *Hedysarum* (Baatout et al. 1985) and teosinte (Timothy et al. 1979), the ctDNA of rice is more highly conserved than the mtDNA in the two subspecies. That higher conservation is expected because the chloroplast genome is simple and consists of only one circle with little repetitive DNA; whereas, the plant mitochondrial genome is much larger and more complex with repetitive segments that promote recombination to give an array of genomic subcircles derived from a master circle and thus, altered restriction patterns (Lonsdale 1984).

The size of the rice mitochondrial genome was estimated to be approximately 325–375 kb. This is an underestimation of the genome size because the stoichiometry of repeated DNA fragments was not taken into account.

Our aim was to investigate the complexity of the organelle DNA and determine whether the variation present was sufficient to distinguish both divergent and closely related plant genotypes. At the subspecies level in rice, mtDNA restriction endonuclease fragment pat-

terns are complex and highly variable, and can be very useful for phylogenetic study. However, those patterns may have reduced value when genetically more divergent materials such as different species are studied. CtDNA at the subspecies level is simple and lacks the variability needed for phylogenetic study. On the other hand, hybridization patterns of the mtDNAs using heterologous organelle gene probes are simple, yet highly variable, and may be ideal to distinguish subspecies as well as species.

In this investigation, hybridization data revealed that all the organelle gene clones except *cox*I and *atp*9 distinguished two important subspecies of rice and should be useful for genetic analysis and for screening recombinant cybrids. The *rbc*L probe revealed a significant difference between A5 and C76 *Bgl*I ctDNA profiles and, therefore, should also be useful in distinguishing these two subspecies of rice.

The differences observed in restriction and hybridization patterns between A5 and BL2 demonstrate that mtDNA changes have taken place during cell culture. This result is not consistent with the findings of Matthews and DeBonte (1985) for carrot and Rode et al. (1985) for wheat. In carrot, no organelle restriction pattern alterations were noted over time in cultured tissue. In wheat, no organelle DNA alterations were observed when dihaploids were compared to their parental strains. Our results agree with the findings of McNay et al. (1984) in maize and Ozias-Akins et al. (1987) in pearl millet. Both groups of workers reported quantitative changes in the mtDNA restriction fragments due to tissue culture.

Our results indicate that use of organelle gene probes will be very useful in monitoring protoplast fusion and plant regeneration from protoplasts as well as for the phylogenetic study of rice. Markers identified by the organelle gene probes used in this investigation may serve as molecular markers for such studies.

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