イネミトコンドリアに存在するプラスミド様DNAの核内相同部位に関する連鎖分析

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Linkage analysis of the nuclear homologues of mitochondrial plasmid-like DNAs in rice

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ABSTRACT

A genetical study on the nucleotide sequences of the nuclear DNAs which share homology with rice mitochondrial plasmid-like DNAs, B1, B2, B3 and B4 was carried out. Restriction fragments of the nuclear DNAs hybridized with these plasmid-like DNAs showed polymorphisms in their length between Indica and Japonica rice cultivars. The hybridized signals found specifically in Indica or Japonica cultivars segregated in the F_2 population derived from a cross between these two subspecies. The observed ratio of the nuclear homologues in the F_2 population demonstrated that they were transmitted according to the Mendelian inheritance. The co-segregation of homologues was examined and the linkage was detected between the B1-nuclear homologue of Japonica and the B4-nuclear homologue of Indica, and also between the nuclear homologues of B2 and B3 of Indica. The linkage between the B1-nuclear homologue of Japonica and the B4-nuclear homologue of Indica was conserved in the different rice cultivars.

1. INTRODUCTION

Small circular and linear DNAs called plasmid-like DNAs are sometimes found in plant mitochondria (Pring and Lonsdale, 1985). Intensive study about two linear plasmid-like DNAs (S1 and S2) present in the maize cms-s cytoplasm (Pring et al., 1977) reveals their virus-like features and their involvement in the rearrangement of the main mitochondrial genome (Levings and Brown, 1989). Very little is known, however, about the origin and the function of mitochondrial plasmid-like DNAs in plants.

In rice (*Oryza sativa* L.), four small circular plasmid-like DNAs designated B1, B2, B3 and B4 have been observed (Yamaguchi and Kakiuchi, 1983; Mignouna et

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al., 1987; Nawa et al., 1987; Shikanai et al., 1987). From the sequence data, Shikanai et al. (1989) detected conserved sequences between four kinds of rice plasmid-like DNAs and maize 1.9 kb or 1.4 kb plasmid-like DNAs, suggesting that they might have evolved from a common ancestral sequence. Interestingly, these molecules share homology with neither the main mitochondrial genome nor the chloroplast genome, but do with the nuclear genome (Sakamoto et al., 1990; Shikanai et al., 1987). Sakamoto et al. (1991) examined the B1- and the B2-nuclear homologues and found that they showed restriction fragment length polymorphisms (RFLPs) among rice cultivars and that they inherited consistently.

In this study, we performed the segregation and the linkage analysis about the nuclear homologues of B1, B2, B3 and B4 to examine their relationship, using the RFLPs found between Indica and Japonica rice cultivars.

2. MATERIALS AND METHODS

Plant materials

For RFLP analysis, 144 plants of the F_2 individuals generated from a cross between Kasalath (Indica, maternal parent) and FL134 (a mutant marker line of Japonica, paternal parent), 70 plants of the F_2 individuals from crosses between Dao Ren Qiao (Indica) and FL102 (Japonica), between Hong Xie Nuo (Indica) and FL27 (Japonica), and between Liuzhou Baoya Zao (Indica) and '74 F4-9 (Japonica), respectively, were used. For the isolation of B3 and B4 molecules, the calluses induced from the seeds of Zhen Shan 97A, a cms-line which had a WA cytoplasm, were used.

DNA preparation

Total DNAs were isolated from leaves of each of the F_2 individuals and their parents essentially according to the method of Murray and Thompson (1980). Mitochondrial DNAs were isolated from the calluses of Zhen Shan 97A as described by Sakamoto et al. (1989).

Molecular cloning of rice mitochondrial plasmid-like DNAs

B3 and B4 from the mitochondria of Zhen Shan 97A were separated by agarose gel electrophoresis. Both B3 and B4, recovered from the gel, were cloned into the *Xho*I site of the pBluescript II phagemid vector (Stratagene), and used to transform *E. coli* JM109 cells. The identity of these molecules to those from Bo cytoplasms was confirmed by the hybridization experiment and their restriction maps. B1 and B2 were cloned previously (Sakamoto et al., 1989).

Southern hybridization analysis

DNAs were electrophoresed in 0.8% agarose gel and blotted to nylon filters

according to the protocols of Maniatis et al. (1982). DNA probes of B1, B2, B3 and B4 were recovered from recombinant plasmid and labelled with [α-^{32}P] dCTP using the random prime DNA labelling kit (Amersham, 3000 Ci/mmol). Pre-hybridization of the filters was carried out in a solution containing 50% (v/v) Formamide, 4×SSPE, 0.5% Blotto (5 mg/ml Non fat powdered milk, 0.1 mg/ml Sodium Azide), 1% SDS and 0.5 mg/ml Salmon sperm DNA at 42°C over night. Hybridization was done in the pre-hybridization solution containing 25 ng/filter labelled DNA at 42°C over night. The filters were washed twice in 6×SSC, 0.2% SDS at 42°C for 30 min, and in 2×SSC, 0.2% SDS at 42°C for 1 hr. Autoradiographs were obtained from the exposure of X-ray film (Kodak) to the filters at −80°C for 1 to 4 days with the aid of intensifying screen.

3. RESULTS

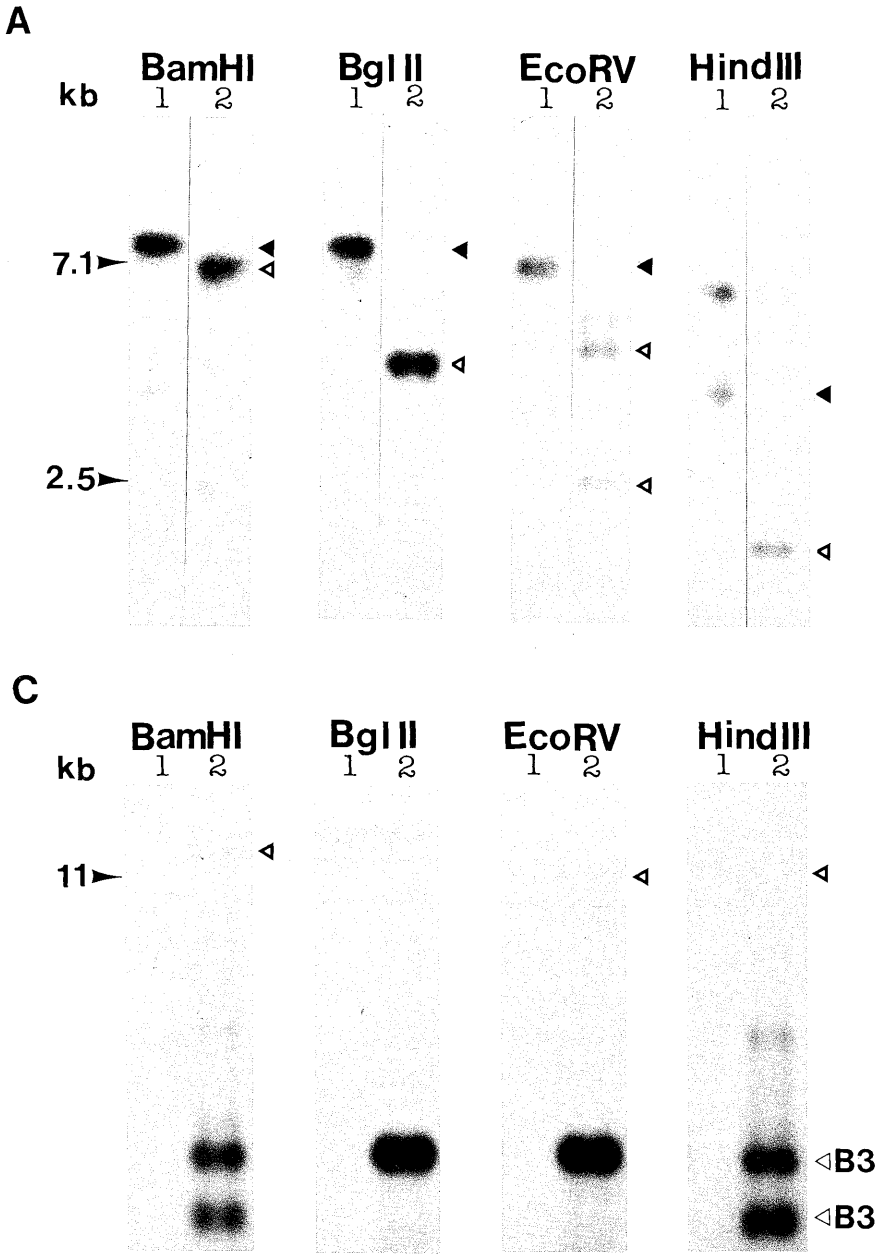
Detection of RFLPs between Indica and Japonica rice cultivars probed with mitochondrial plasmid-like DNAs

Total DNAs isolated from Kasalath (Indica) and FL134 (Japonica) were digested with restriction endonucleases, *Bam*HI, *Bgl*II, *Eco*RV or *Hin*dIII and hybridized with cloned B1 (Fig. 1A), B2 (Fig. 1B), B3 (Fig. 1C) or B4 (Fig. 1D). In every panel, hybridization signals considered to indicate the nuclear DNA fragments which share homology with mitochondrial plasmid-like DNAs showed RFLPs between Indica (lane 2) and Japonica (lane 1). The strongly hybridized signals in the low molecular weight region of the filters (panel B, C, D lane 2) indicated the plasmid-like DNAs in mitochondria because their restriction pattern agreed well with the physical maps of them.

As plasmid-like DNAs shared homology neither with the main mitochondrial genome nor with the chloroplast genome (Sakamoto et al., 1991), clearly hybridized signals in the high molecular weight region were considered to indicate the homologues of these molecules in the nuclear genome. The possibility of misidentifying the signals corresponding to the multimeric forms of plasmid-like DNAs as these nuclear homologues was excluded by observing the segregation of the signals in the F_2 population (as described later).

Segregation of the nuclear homologues of plasmid-like DNAs in the F_2 population

The RFLPs of the nuclear homologues detected between Indica and Japonica rice cultivars made it possible to analyze their segregation. To examine the segregation of these nuclear homologues in the F_2 population derived from a cross between Kasalath (Indica) and FL134 (Japonica) rice cultivars, the total DNAs isolated from 143 or 144 plants of the population were digested with restriction endonucleases and hybridized with cloned B1, B2, B3 and B4 (Fig. 2). We used restriction endonuclease *Eco*RV for the analysis probed with B1, B2 and B3, and *Bgl*II for B4 for the clearness of polymorphic signals. In consequence, among


A
BamHI
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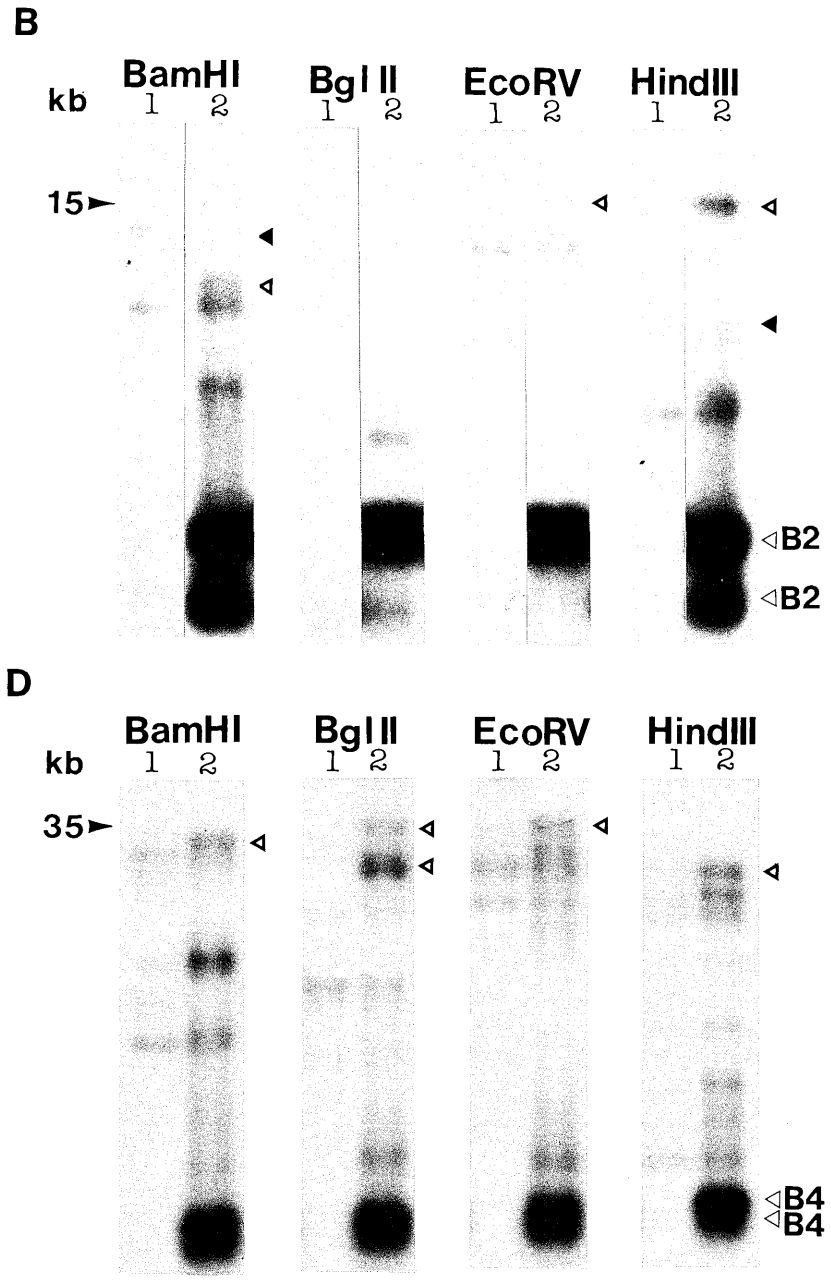


Fig. 1. A, B, C and D. Hybridization of mitochondrial plasmid-like DNAs to total DNAs from Japonica and Indica rice cultivars. Total DNAs from FL134 (lane 1) and Kasalath (lane 2) were digested with *Bam*HI, *Bgl* II, *Eco*RV or *Hin*dIII, electrophoresed in agarose gel, transferred to nylon filters and hybridized with B1 (panel A), B2 (panel B), B3 (panel C) or B4 (panel D). The signals of the nuclear homologues found specifically in Japonica or Indica were indicated by solid or open triangles, respectively, together with one of the plasmid-like DNAs in mitochondria.

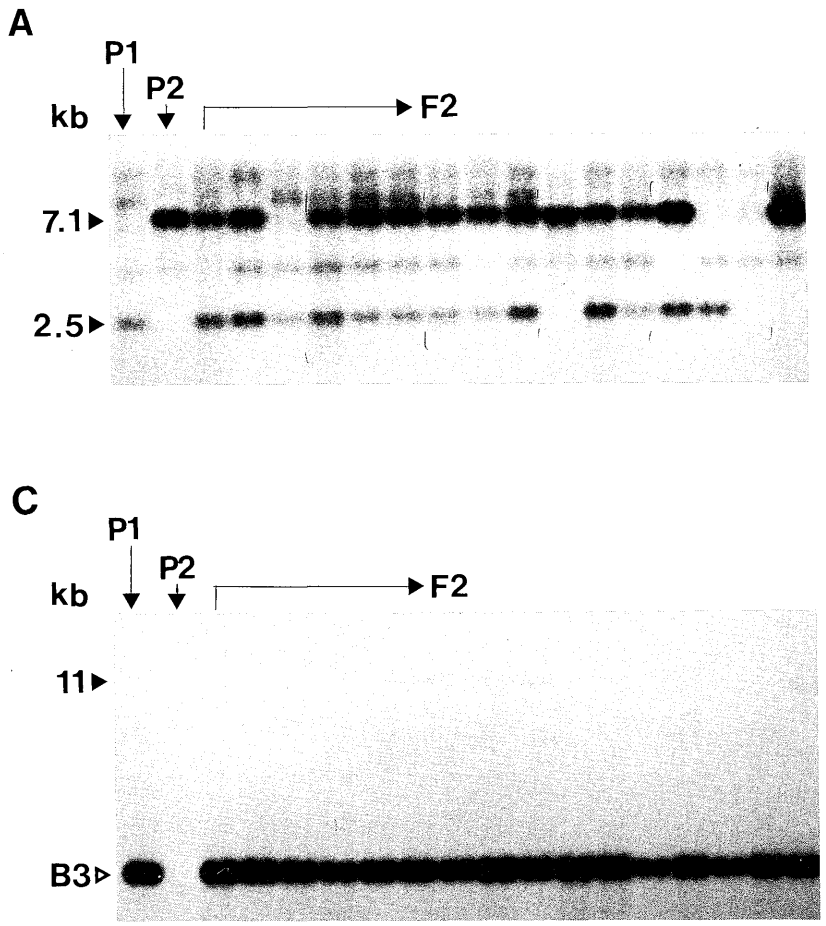
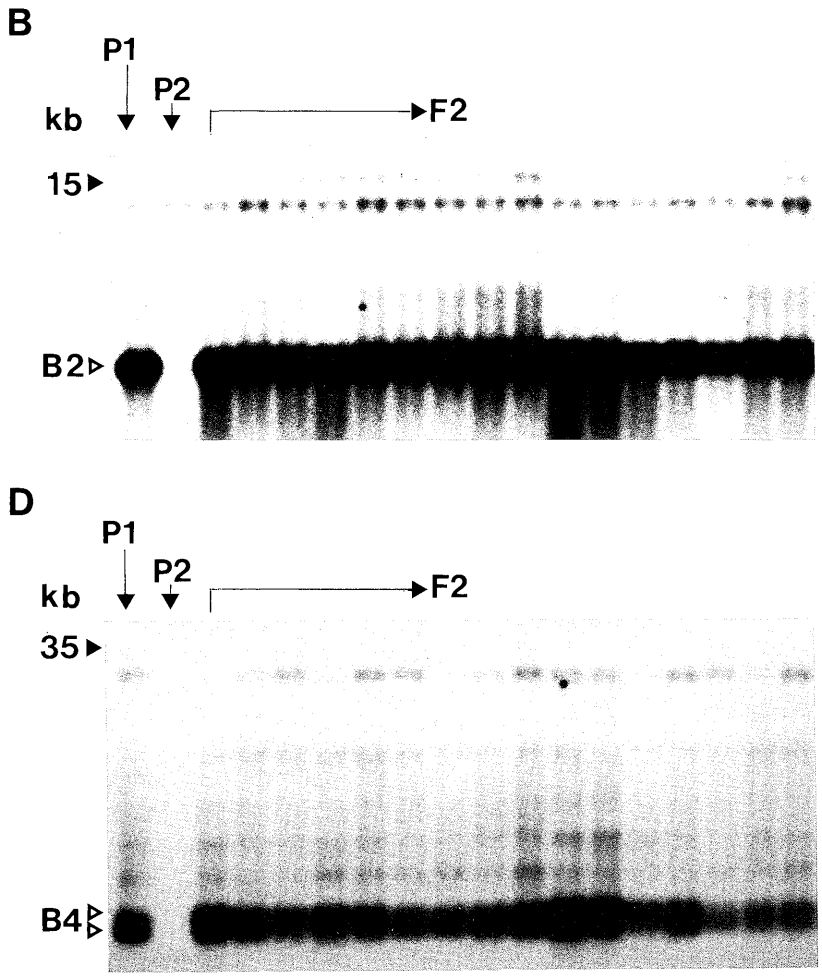


Fig. 2. A, B, C and D. Hybridization of mitochondrial plasmid-like DNAs to total DNAs from Indica and Japonica rice cultivars and the F_2 individuals derived from a cross between these two subspecies. Total DNAs from Kasalath (P_1), FL134 (P_2) and from F_2 individuals were digested with *Eco*RV (panel A, B and C) or *Bgl*II (panel D), electrophoresed in agarose gel, transferred to nylon filters and hybridized with B1 (panel A), B2 (panel B), B3 (panel C) or B4 (panel D).

several signals indicating nuclear homologues, we could detect the signals segregated in the F_2 population. Concerning the B1- and the B2-nuclear homologues, the B1-homologous 2.5 kb fragment transmitted from P_1 (Indica), the B1-homologous 7.1 kb fragment from P_2 (Japonica) and the B2-homologous 15 kb fragment from P_1 segregated clearly in the F_2 population (Sakamoto et al., 1991). Eleven kb fragment from P_1 segregated when probed with B3, and 35 kb fragment from P_1 also segregated when probed with B4.

The data of segregation and the calculation of χ^2s testing for the deviation from the expected ratio 3:1 were shown in Table 1. These fragments, with the



exception of B1-homologous 2.5 kb fragment, segregated approximately in the ratio of 3:1 in the F_2 population. This indicates that these homologues of mitochondrial plasmid-like DNAs stably located on chromosomes in the nuclear genome and transmitted according to the Mendelian inheritance. The deviation from the 3:1 ratio observed in the B1-homologous 2.5 kb fragment might be accounted for the linkage to the gametophyte gene which affects pollen fertility (Sakamoto et al., 1991). The strongly hybridized signals found in the low molecular weight region of the blots probed with B2, B3 and B4 were thought to indicate the mitochondrial plasmid-like DNAs inherited maternally (Fig. 2B, C, D). Any F_2 individuals did not have the signal of B1 (Fig. 2A), because Kasalath did not have B1 in mitochondria.

Table 1. Segregation ratio of homologous fragments to plasmid-like DNAs in the F_2 population and the χ^2s testing for the deviation from the ratio 3:1

Fragment		Segregation ratio	χ^2 (d.f.=1)
B1	2.5 kb (Indica)	129:15	16.33**
B1	7.1 kb (Japonica)	108:35	0.02
B2	15 kb (Indica)	103:40	0.67
B3	11 kb (Indica)	109:34	0.11
B4	35 kb (Indica)	105:38	0.19

** Significant at 1% level.

Linkage between the nuclear homologues of plasmid-like DNAs

Autoradiographs from F_2 filters probed with B1, B2, B3 and B4 were scored for segregating genotype and the linkages between the homologues were analyzed. We tried to detect the linkage between the homologues according to the χ^2 method of analysis (Mather, 1951; Kochert et al., 1989). χ^2s testing for the co-segregation of two homologues were shown in Table 2. χ^2s were calculated from the observed frequencies of the four classes of genotype regarding the presence or absence of the homologue. Judging from the significant value of χ^2, we could detect the linkages between the B1-homologue of Japonica and the B4-homologue of Indica (i.e., in the repulsion phase), also between the B2-homologue of Indica and the B3-homologue of Indica (i.e., in the coupling phase). The recombination value between the B2- and the B3-homologues of Indica calculated by Maximum Likelihood method was 0.18 ± 0.04. The one between the B1-homologue of Japonica and the B4-homologue of Indica, being in repulsion phase, did not exceed 0.17, which was estimated from the distance between the rice RFLP markers mapped outside these homologues (will be published elsewhere). The recombination values between other nuclear homologues varied from 0.39 to 0.49, confirming the absence of the close linkage.

Table 2. χ^2s for one degree of freedom testing for the co-segregation of the two homologues in the F_2 population

Fragment		B1 2.5 kb	B1 7.1 kb	B2 15 kb	B3 11 kb	B4 35 kb
B1	2.5 kb (Indica)		1.44	0.06	0.25	0.22
B1	7.1 kb (Japonica)			0.01	0.02	17.3**
B2	15 kb (Indica)				47.4**	1.31
B3	11 kb (Indica)					0.85
B4	35 kb (Indica)					

** Significant at 1% level.

Conservation of the linkage between nuclear homologues of plasmid-like DNAs among different rice cultivars

To examine whether the linkage detected above was conservative in other rice cultivars, we investigated the linkage between the B1-homologue of Japonica and the B4-homologue of Indica in three different F_2 populations. Indica cultivars used here are different from the Kasalath regarding their origin. These cultivars are from China (i.e., Sinica), whereas Kasalath is from India. We used three groups of 70 plants of F_2 individuals derived from crosses between Indica and Japonica rice cultivars.

Table 3 shows the results of segregation and linkage analysis about these populations scored from the autoradiographs probed with B1 and B4. In all these three populations, both the B1-homologue of Japonica and the B4-homologue of Indica segregated in the ratio of 3:1. χ^2s for their co-segregation showed significance at 5% level, indicating that the linkage between the homologues was conserved in these rice cultivars.

Table 3. Segregation and linkage analysis of the nuclear homologues of B1 and B4 in the different F_2 populations

Cross	Segregation ratio				χ^2s for linkage (d.f.=1)
	B1B4	B1b4	b1B4	b1b4	
Dao Ren Qiao×FL102	41	19	10	0	4.34*
Hong Xie Nuo×FL27	32	16	20	2	4.65*
Liuzhou Baoya Zao×'74 F4-9	34	16	19	1	5.67*

B1B4, B1b4, b1B4 and b1b4 indicate genotypes regarding the presence or absence of the B1-homologous 7.1 kb fragment and the B4-homologous 35 kb fragment. The capital letter and the small letter stand for the presence and the absence of the homologues, respectively.
* Significant at 5% level.

4. DISCUSSION

The existence of rice mitochondrial plasmid-like DNAs and their nuclear homologues may indicate the transfer of genetic information has occurred between these two organelles. However, it remains unclear whether the origin of these sequence is nuclear genome or mitochondrial genome. We cannot still exclude the possibility that these sequences were introduced into these two organelles independently (e.g., incorporation of viral DNA). In this study, we found the two linkages between the B2- and the B3-nuclear homologues of Indica (coupling phase), and between the B1-nuclear homologue of Japonica and the B4-nuclear homologue of Indica (repulsion phase).

But there seems to be no linkage of plasmid-like DNA sequences in mitochon-

dria as follows. These plasmid-like molecules share extensive sequence-homology neither with the main mitochondrial genome (Sakamoto et al., 1990; Shikanai et al., 1987), nor with the plasmid-like molecules each other (Shikanai et al., 1989). Therefore, it is unlikely that intra- or inter-molecular recombination via homologous sequences had occurred to generate the linkage of plasmid-like DNA sequences in mitochondria.

The mechanism(s) to generate the linkage detected in nuclear genome could be explained in some ways. One explanation is that these DNA sequences integrated into the nuclear genome randomly and that some of them located in the neighborhood each other on the same chromosome by chance. In fact, there are apparently some other copies of the nuclear homologues because several signals were found when total DNAs were hybridized with each of the plasmid-like DNAs. These signals were unsegregated in the F_2 population and could not be subjected to the linkage analysis. The other explanation is that the chromosomal region around these homologues had higher capacity for the DNA integration than other part of the chromosomes.

The existence of plasmid-like DNAs in mitochondria shows much diversity among other rice cultivars (Kadowaki et al., 1988) as well as the varieties used in this study (Table 4). We analyzed the distribution of B1, B2, B3 and B4 in the mitochondria among the cultivated rice from all over the world and found the co-existence of B2 and B3 frequently (Kanazawa et al., in preparation). There might be some preference to maintain the plasmid-like molecules in mitochondria. This preference could be caused by unequal distribution of mitochondrial DNA molecules during the mitochondrial division, or by different replication efficiency of each molecules.

Table 4. Existence of plasmid-like DNAs in mitochondria of the rice cultivars used in this study

Cultivar	B1	B2	B3	B4
Kasalath (Indica)	−	+	+	+
Dao Ren Qiao (Sinica)	−	+	+	−
Hong Xie Nuo (Sinica)	−	+	+	−
Liuzhou Baoya Zao (Sinica)	−	+	+	−
FL27, FL102, FL134, '74 F4-9 (Japonica)	−	−	−	−

+: Plasmid-like DNAs exist in mitochondria. −: does not exist.

Contrary to this diversity, the nuclear homologues of mitochondrial plasmid-like DNAs were found universally in cultivated rice including *Oryza glaberrima* (Sakamoto et al., 1991). The conservativeness of the linkage we detected in the nuclear homologues also indicate their stability on the chromosome. These results suggest that the nuclear homologues of the plasmid-like DNAs are transmitted consistently through the varietal differentiation in rice cultivars.

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