Analysis of homology of small plasmid-like mitochondrial DNAs in the different cytoplasmic male sterile strains in rice

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ABSTRACT

Mitochondrial DNAs (mtDNAs) were isolated from several rice strains of Chinsurah boro II (fertile), Zhen Shan 97A (male sterile), Zhen Shan 97 (fertile), and Akihikari (normal *japonica*). Small plasmid-like DNAs, B1 and B2, which were found firstly in BT line, were cloned into plasmid vectors and mapped in terms of some of restriction enzymes. Plasmid-like DNAs were also observed in the mitochondria of Zhen Shan 97A and Zhen Shan 97 by agarose gel electrophoresis. To examine whether these plasmids were identical or not, hybridization analyses were carried out with B1 and B2 as probes. Consequently, it was suggested that two plasmids of Zhen Shan 97A were identical to B1 and B2, and one of the plasmids of Zhen Shan 97 was identical to B2. The smaller plasmid of Zhen Shan 97 had no homology to either B1 or B2. Co-existence of B1 and B2, while common, is not an obligatory feature. Because B2 was found in the fertile cytoplasm of Zhen Shan 97, an involvement of B2 with cytoplasmic male sterility (*cms*) might be denied.

1. INTRODUCTION

Cytoplasmic male sterility (*cms*) is a valuable trait for commercial production of hybrid seeds in rice. It is maternally inherited, and seems to be encoded in the mitochondrial genome (Kadowaki et al., 1986). *Cms* is a widespread phenomenon in the other higher plants, and has been studied by many investigators at the molecular levels (reviewed by Pring and Lonsdale, 1985; Newton, 1988).

Mitochondria of the male sterile cytoplasm often has small plasmid-like DNAs (Pring and Lonsdale, 1985). In maize, two linear episomal DNAs, S1 and S2, have been found in the S-type mitochondrial genome (Pring et al., 1977). The relationship between the presence of plasmids and the expression of sterility is unclear. That these plasmids were homologous to other plasmids observed in the different races of mitochondria has been reported by Weissinger et al. (1982) and Timothy et al. (1983).

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In rice, Yamaguchi and Kakiuchi (1983) reported that there were two plasmid-like molecules, B1 and B2 DNAs, in the male sterile mitochondria of BT line which was produced by Shinjo (1969), and these plasmids were absent in the fertile cytoplasm of Taichung 65. Nawa et al. (1987) found that free plasmid-like DNAs were absent in the mitochondria of fertile revertants produced from BT line. Plasmid-like DNAs were also present in the other male sterile cytoplasms (Yamaguchi et al., 1986; Mignouna et al., 1987). It is unknown yet whether the sequences of these plasmid-like DNAs are associated with *cms*, and whether there are homologies between B1 or B2 DNAs and other plasmid DNAs. In this study we isolated mitochondrial DNAs (mtDNAs) from some rice strains, and examined homology of the other plasmid-like DNAs to B1 and B2 by hybridization analyses.

2. MATERIALS AND METHODS

Plant materials

Plant materials used in this experiment are listed and characterised in Table 1. These materials except for Akihikari were propagated and maintained by calluses as described by Yamaguchi and Kakiuchi (1983), but the medium was changed to the Murashige and Skoog medium with 3% of sucrose in which 2,4-D was supplemented with 6 ppm of 2, 4-D for Chinsurah boro II, and with 4 ppm for other strains. In the case of Akihikari, seedlings which were grown at 30°C for 1 week in the dark were used as the experimental material.

Table 1. Properties of rice strains used in this experiment

Sstrain	Cytoplasm	Nucleus	Pollen fertility
Chinsurah boro II	MS (<i>bo</i>)	Rf1Rf1	fertile
BT line	MS (<i>bo</i>)	Rf1rf1	50% fertile
Zhen Shan 97A	MS (<i>wa</i>)	—	sterile
Zhen Shan 97	normal	—	fertile (maintainer)
Akihikari	normal	—	fertile (<i>japonica</i>)

MS: male sterile. *bo*: boro. *wa*: wild abortive rice from China.

Isolation of mtDNA

MtDNAs were isolated according to the method of Yamaguchi and Kakiuchi (1983). Calluses or seedlings were homogenized in buffer A (0.3 M mannitol, 50 mM Tris-HCl pH 8.0, 3 mM EDTA, 1 mM mercaptoethanol, 0.1% BSA). The homogenates were filtered with 4 layers of gauze and 2 layers of nylon mesh, and centrifuged twice at 1,500 g at 0°C for 10 min. The supernatants were centrifuged at 10,000 g at 0°C for 15 min. The pellets were suspended in buffer A

containing 10 mM MgCl₂, then DNase I was added to the final concentration of 50 µg/ml, and the mixture were incubated at 4°C for 30 min. The suspensions were centrifuged at 12,000 g at 0°C, and resuspended in buffer B (0.3 M sucrose, 50 mM Tris-HCl pH 8.0, 20 mM EDTA). This wash was repeated 3 times. The final pellets were lysed in buffer C (50 mM Tris-HCl pH 8.0, 20 mM EDTA) containing 2% sarkosyl, 50 µg/ml proteinase K at room temperature for 10 min. DNAs were precipitated with 2 volumes of ethanol after phenol-chloroform extraction. DNAs were collected by centrifugation, resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) containing 20 µg/ml RNase A, and incubated at 37°C for one hour. DNAs were repurified by phenol-chloroform extraction. After precipitation with ethanol, DNAs were resuspended in TE buffer and used in the following studies.

Cloning of B1 and B2 DNAs

B1 and B2 DNAs from BT line were separated by 0.7% agarose gel electrophoresis. Each band of these plasmids was recovered from gel and DNA was electroeluted. After purification by phenol-chloroform extraction, DNAs were digested with appropriate restriction endonucleases (purchased from Takara Shuzo Co.). B1 was cloned into the *Sma*I site of pUC13 (Pharmacia), and B2 into the *Bgl*II site of pNEO (Pharmacia). Reactions by restriction endonucleases were carried out as recommended by the supplier. Ligation, transformation of *Escherichia coli* and isolation of plasmid DNA were carried out as described in Maniatis et al. (1982). The cloned fragments were electrophoresed in agarose gel with linearized B1 and B2 molecules. Since they were the same migration as B1 and B2, and hybridized to B1 and B2 respectively, it was confirmed that the B1 and B2 molecules with full length were cloned.

Electrophoresis and Southern hybridization

Electrophoresis was carried out by horizontal type (Bio-Rad) in 0.7% or 1% agarose gel (Sigma). After electrophoresis, the bands were stained with ethidium bromide, and DNA was detected under UV illumination. Agarose gels were transferred to the nitrocellulose papers by the method of Southern (1975).

The probes were prepared from recombinant clones of B1 and B2, which were digested with restriction endonucleases and electroeluted from agarose gels. The probes were labeled with photobiotin (Bresatec), and colorimetric detection was carried out according to the manual of the supplier.

3. RESULTS AND DISCUSSION

Cloning and physical mapping of B1 and B2

B1 and B2 found in BT line were electroeluted from agarose gel, and analysed by digestion with restriction endonucleases. B1 was detected as one fragment of

2.1 kilobase pairs (kb) when digested with *Stu*I, and was cloned into the *Sma*I site of pUC13. B2 was detected as one fragment of 1.5 kb when digested with *Bgl*II, and was cloned into the *Bgl*II site of pNEO. Using these clones, physical maps were constructed by an ordinary method with respect to some restriction enzymes (Fig. 1). On the maps, no common fragment between B1 and B2 was detected.

Fig. 1. Restriction maps of B1 and B2. B1 and B2 DNAs were digested to linearize with *Stu*I and *Bgl*II, respectively. Restriction enzymes *Bam*HI, *Hind*III and *Sal*I cut neither B1 nor B2. The molecular length is indicated by kilobase pairs (kb).

Fig. 2. Agarose gel electrophoresis of mtDNA. MtDNAs (3 μ g each) from Chinsurah boro II (lane 1), Zhen Shan 97 (lane 2), Zhen Shan 97A (lane 3), and Akihikari (lane 4) were electrophoresed on 1% agarose gel, and stained with ethidium bromide. Lane M; lambda phage DNA digested with *Eco*RI and *Hind*III as molecular weight markers. Molecular weights are indicated by kb.

Comparison of homology between B1 and B2 DNAs and plasmid-like DNAs of other cytoplasm

MtDNAs were isolated from Chinsurah boro II (fertile because nucleus has the genotype of Rf1Rf1), Zhen Shan 97A (*cms*), Zhen Shan 97 (fertile), and Akihikari (fertile *japonica* rice). These mtDNAs were applied directly to electrophoresis in 1% agarose gel (Fig. 2). In the lane of Zhen Shan 97A, two plasmid-like DNAs corresponding to the migration of B1 and B2 were observed. In the lane of Zhen Shan 97, on the other hand, two plasmid-like DNAs were also observed but their migrations seemed to be different from that of Zhen Shan 97A. One migrated much the same as B2 and the another was somewhat smaller than B2. No plasmid-like DNA was found in the lane of Akihikari.

Shikanai et al. (1987) reported that in addition to B1 and B2, there were two smaller plasmid-like DNAs (B3 and B4) in mitochondria of A-58 *CMS* strain. Mignouna et al. (1987) reported there were three and four plasmid-like DNAs in mitochondria of Zhen Shan 97B and Zhen Shan 97A, respectively. In this study we could not observe all of these plasmids clearly by electrophoresis. However, the smaller plasmid of Zhen Shan 97 was similar in the migration to B4.

In order to examine whether these plasmids detected in Zhen Shan 97A and Zhen Shan 97 were homologous to B1 and B2 DNAs, hybridization analyses were carried out by using cloned B1 and B2 sequences. First, mtDNAs were digested with either *Stu*I or *Bgl*III. These enzymes restrict at one site in B1 and B2, respectively (Fig. 1). After electrophoresis in 1% agarose gel, DNAs were transferred to the nitrocellulose paper and hybridized with B1 and B2. When mtDNAs were digested with *Stu*I and probed with B1, a single band of 2.1 kb, the molecular length of which was the same as B1, was detected in the lane of Zhen Shan 97A. However, none of the hybridized band was observed in the lane of Zhen Shan 97. On the other hand, when mtDNAs were digested with *Bgl*III and hybridized with B2, a single band of 1.5 kb was detected in all of the lanes. These results suggested that the fertile mitochondria of Zhen Shan 97 had the sequence homologous to B2, but not to B1, although the male sterile mitochondria of Zhen Shan 97A had homologous sequences to both B1 and B2.

Mitochondrial main genomes of all cytoplasms which we examined in this study did not hybridize to either B1 or B2 (data not shown), suggesting that homologous sequences to B1 and B2 existed as plasmids. To confirm it, mtDNAs were digested with *Hind*III which restrict neither B1 nor B2, and hybridized to B1 or B2. Results of hybridization was shown in Fig. 4. Except for the lane of Zhen Shan 97 probed with B1, both B1 and B2 hybridized to several bands as well as their own supercoiled bands which were observed by ethidium bromide-stained gels. They were supposed to be multimers of B1 and B2 DNAs existing in various conformations (cf. Nawa et al., 1987; Mignouna et al., 1987). It was indicated that homologous sequences to B1 and B2 were present only as plasmids.

Fig. 3. Hybridization of B1 and B2 to mtDNAs. **a**, *Stu*I-digested mtDNAs from Chinsurah boro II (lane 1), Zhen Shan 97 (lane 2), and Zhen Shan 97A (lane 3), and cloned B1 (lane 4) were probed with 1 μ g of labeled B1. **b**, *Bgl*II-digested mtDNAs from Chinsurah boro II (lane 1), Zhen Shan 97 (lane 2), and Zhen Shan 97A (lane 3), and cloned B2 (lane 4) were probed with 1 μ g of labeled B2. Electrophoresis was performed in 1% agarose gel. The positions of linearized fragments of B1 (2.1 kb) and B2 (1.5 kb) are indicated by arrowheads. The fragment of 1.4 kb in lane 1 of panel **a** was supposed to be a supercoiled form of B1 not digested with enzyme.

As shown in Fig. 3, the molecular weight of each plasmid homologous to B1 or B2 was the same as B1 or B2 respectively. It suggests that two plasmids of Zhen Shan 97A were identical to B1 and B2, and in Zhen Shan 97 one of the plasmids was identical to B2. The smaller plasmid of Zhen Shan 97 was homologous to neither B1 nor B2.

B1 and B2 are the small plasmid-like DNAs found firstly in the mitochondria of BT line (Yamaguchi and Kakiuchi, 1983). In this study, we show that these plasmids also exist in the male sterile mitochondria of Zhen Shan 97A, while only B2 was present in the fertile mitochondria of Zhen Shan 97. From these results, it was supposed that there was diversity in the presense of B1 and B2, and these

Fig. 4. Hybridization of B1 and B2 to mtDNAs. *Hind*III-digested mtDNAs from Chinsurah boro II (lane 1), Zhen Shan 97 (lane 2), and Zhen Shan 97A (lane 3) were electrophoresed in 0.7% agarose gel, and probed with B1 (panel a) and B2 (panel b). The arrowheads represent the positions of the supercoiled forms of B1 and B2 from the top.

two plasmids were not necessary to exist together for stable maintainance.

In rice the presence of small plasmid-like DNAs has been observed frequently in mitochondria (Kadowaki et al., 1987). The function of these plasmid-like DNAs, however, is remained to be elucidated. As these plasmids exist independently of the presence or absence of nuclear fertility-restoring genes, *cms* may not necessarily be associated with these plasmids (Nawa et al., 1987). In this study, it was suggested that because the fertile cytoplasm of Zhen Shan 97 had a plasmid hybridizable to B2, B2 alone may not be associated with *cms*. A possibility remains, however, that the plasmid in Zhen Shan 97 has been altered to lose the putative ability of causing *cms*.

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