MOLECULAR BIOLOGICAL STUDIES ON MITOCHONDRIA
OF
RICE WITH MALE-STERILE CYTOPLASM

KOH-ICHI KADOWAKI

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To my family and to Yukari

CONTENTS

ABBREVIATIONS

ATP	adenosine triphosphate
atpA	F_1-ATPase α subunit gene
atp6	ATPase subunit 6 gene
atp8	ATPase subunit 8 gene
atp9	ATPase subunit 9 gene
cms	cytoplasmic male sterility or cytoplasmically male-sterile
cob	apocytochrome b gene
coxI	cytochrome oxidase subunit I gene
coxII	cytochrome oxidase subunit II gene
DNA	deoxyribonucleic acid
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
kb	kilobase pairs
kDa	kilodaltons
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
O. barthii	Oryza barthii A. Chev.
O. breviligulata	Oryza breviligulata A. Chev.
O. glaberrima	Oryza glaberrima Steud.
O. rufipogon	Oryza rufipogon Gsiff.
O. sativa	Oryza sativa L.
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS	sodium laurylsulfate
TE buffer	10 mM Tris-HCl, 1 mM EDTA (pH 8.0)
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
urfRM	unidentified open reading frame of rice mitochondria

INTRODUCTION

When plants do not produce viable pollen, they are termed as "male-steriles". Male-sterility can be affected by the environment, nuclear genes, and cytoplasmically inherited genes. When the male-sterile trait is inherited in a non-Mendelian fashion, it is designated cytoplasmic male-sterility (cms). Cms occurs in many plants and observed at least 140 plant species (Laser and Lersten, 1972). Since the cms trait normally does not affect female fertility, male-sterile plants are able to set seed when provide viable pollen. This seed is the product of cross-pollination. In hybrid seed production, the male parent often carries a nuclear restorer gene(s) that suppress the cms trait and restores fertility to the hybrid.

Rice plants (O. sativa and O. glaberrima) are very important crop and cultivated in 145 x 10^6 ha, 475 x 10^6 MT in the world (FAO production year book, 1986). In spite of the great importance to both human nutrition and the economy in many nations in the world, our knowledge of rice genetics is a little and lags far behind that of other important food crops such as maize, wheat, barley, and tomato. We are/will be continuously taking many approaches to increase yield of rice. One of the approaches is to use heterosis breeding and plant breeders have taken advantage of the cms trait in the production of hybrid seed, since rice is a monoecious plant and self-pollination is usually observed (Fig. 1).

The aim of this study

Cms is an important character but the mechanism of cms has not been elucidated in rice as any other plants yet. Furthermore, informations of mitochondrial nucleic acids which was assumed to be

related with cms are almost nothing in rice. In this study, the author has intended to reveal the mechanisms of cms of rice in respects to molecular biological and genetical aspects as follows. Mitochondrial DNA was different between rice strains with normal and male-sterile cytoplasms (Chapter I). Based on the mitochondrial DNA analyses of ten strains of cms rice, alterations of mitochondrial DNA are strongly suggested to be involved in the occurrence of cms rice (Chapter II). Divergence of mitochondrial plasmid-like DNA of cultivated rice was shown and geographical distribution of characteristic cytoplasms was examined (Chapter III). Mitochondrial polypeptide synthesis and transcription were changed by introduction of fertility-restorer gene and a possible DNA fragment associating cms was determined and discussed (Chapter IV). These studies will give us the basic information for both actual breeding and genetic system of plant mitochondria.
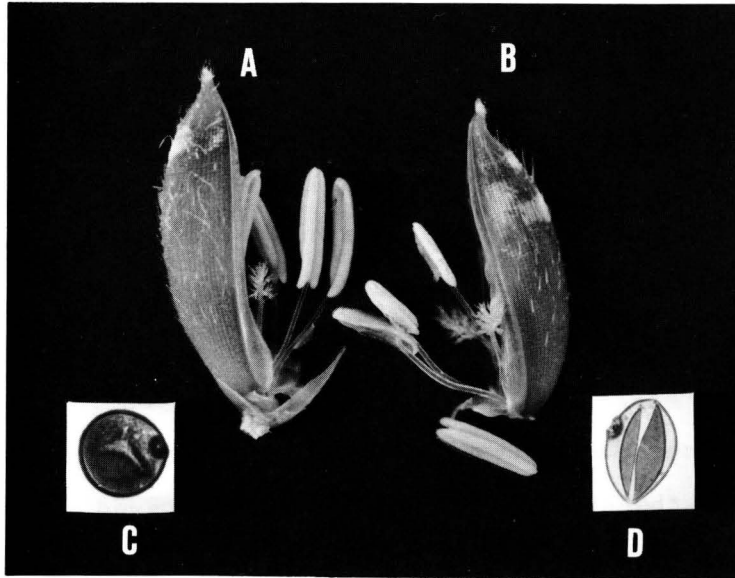
Fig. 1. Flower morphology of Japonica rice (<u>O. sativa</u>, cultivar "Reimei") with normal (A) or cms-R male-sterile cytoplasm (B). Pollen grains of each flower (C and D) were stained with 1% acetoorcein.

CHAPTER I   Differences in the mitochondrial DNA between normal and cytoplasmically male-sterile rice

INTRODUCTION

Cms which interferes with pollen maturation is a maternally inherited trait and widely distributed among many higher plants. To produce a F_1 hybrid plant, cms is a very useful character as it enables to prevent the occurrence of self-fertilization. Molecular analyses have been actively pursued in many plants to understand cms, and differences in the characteristics of mitochondrial DNA between cms and fertile lines of a number of species have been described (Pring and Levings, 1978; Galun et al., 1982; Boeshore et al., 1983; Palmer et al., 1983; Mikami et al., 1985; Ricard et al., 1986; Boutin et al., 1987; Smith et al., 1987). On the other hand, in a few species, the chloroplast genome or viral basis has been implicated with the sterility trait (Grill and Garger, 1981; Jigeng and Yi-nong, 1983).

In rice, several types of male-sterile cytoplasm have been identified in Japonica rice (Katsuo and Mizushima, 1958; Kitamura, 1958; Shinjyo, 1972). However, little is known about the molecular mechanism of cms rice. In this chapter, the author has used rice strains with male-sterile or normal cytoplasm and compared organelle DNAs by restriction endonuclease analysis to identify genetic difference between the two cytoplasms. Organelle associated with cms in rice is speculated.

MATERIALS AND METHODS

Plant materials

Japonica rice cultivar "Taichung 65" with cms-Bo male-sterile cytoplasm was originally developed by Shinjyo (1969). Restorer of

fertility gene was characterized by Shinjyo (1969, 1975). Indica rice cultivars with cms-Gam, WA, and MS-577 male-sterile cytoplasms, respectively, were originally supplied by Dr. Virmani, International Rice Research Institute (Philippines).

Isolation of chloroplast DNA

Chloroplast DNA was isolated by the same method as that applied by Bookjans et al.,(1984).

Preparation of mitochondrial DNA

Mitochondrial DNA was prepared by a modification of the procedure of Umbeck and Gengenbach (1983) as follows. Rice seeds were germinated in the dark on two layers of cheesecloth in a container at 28° C for 14 days. The etiolated leaves (ca. 10 cm long) were harvested and homogenized with a mortar and pestle in 0.4 M sucrose, 50 mM Tris-HCl, 5 mM Na_2EDTA, 0.1% bovine serum albumin, and 5 mM 2-mercaptoethanol (pH 7.5) at 5 ml/g initial weight (i.w.). The homogenate was passed through four layers of cheesecloth and two layers of miracloth (Calbiochem) and centrifuged for 10 min at 1,000 x g. The supernatant obtained was centrifuged for 10 min at 12,000 x g and the pellets were suspended in 0.3 M sucrose, 50 mM Tris-HCl, 0.1 mM Na_2EDTA, 0.05% bovine serum albumin, and 1 mM 2-mercaptoethanol (pH 7.5) at 0.5 ml/g i.w. After centrifugation for 10 min at 1,000 x g, the supernatant was put into a new tube. $MgCl_2$ (final concentration 10 mM) and DNase I (10 µg/g i.w., Sigma) were added to the supernatant to remove extra mitochondrial DNA. After this was left for 1 h at room temperature, DNase I was removed by underlaying the reaction mixture with two volumes of 0.6 M sucrose, 10 mM Tris-HCl, and 20 mM Na_2EDTA (pH 7.2) and then centrifuging it for 20 min at 12,000 x

g. Pellets were suspended again in the same buffer and centrifuged twice. The final pellet was suspended in (0.1 ml/g i.w.) 50 mM Tris-HCl and 10 mM Na_2EDTA (pH 8.0), (25 µl/g i.w.) filtered 10% SDS, and (1.5 µl/g i.w.) 20 mg/ml proteinase K (Sigma), and incubated for 120 min at 37°C. The lysis solution was deproteinized by the addition of (0.02 ml/g i.w.) filtered 7.5 M ammonium acetate and an equal volume of chloroform and TE buffer-saturated phenol, and gentle mixing. After centrifugation for 15 min at 7,000 x g, the upper aqueous phase was treated again with chloroform and TE buffer-saturated phenol, and centrifuged under the same conditions. Absolute ethanol and sodium acetate were added to the last upper phase (final conc. 70%, v/v; 0.1 M) and the mixture was left overnight at -20°C. Mitochondrial DNA was collected by centrifugation at 8,000 x g for 15 min, washed in 70% ethanol twice, and dried under reduced pressure. The precipitate was dissolved in H_2O just before use.

Restriction enzyme reaction and agarose gel electrophoresis

In restriction endonuclease analysis, mitochondrial DNAs were completely digested according to the instruction manual except that the reaction time was 3 h. In detection of plasmid-like DNA, mitochondrial DNAs were treated in 100 mM NaCl and 10 mM Tris-HCl (pH 7.5) with RNase A (final conc. 0.3 µg/µl, Boehringer Mannheim) for 3 h at 37°C. DNA molecules reacted were separated on a 0.7% agarose gel (Sigma, type II) with a running buffer containing 0.089 M Tris, 0.089 M boric acid, and 0.002 M Na_2EDTA, (pH 8.3) at 1.5 volts per cm for 15 h in a submarine type of apparatus. The gel was stained for 20 min in 0.5 µg/ml ethidium bromide, illuminated with short-wave UV light, and photographed through Kodak wratten

gelatin filters (No. 2E and No. 23A) on Polaroid Type 665 or 667 film.

Film scanning

Polaroid negative film was scanned by using a densitometer (Toyo, DMU-33C) with a slit 0.2 mm wide.

RESULTS AND DISCUSSION

Comparison of chloroplast DNA isolated from normal and male-sterile cytoplasms

Comparisons of organelle DNAs from normal and male-sterile cytoplasms were done so as to identify the organelle associating with cms rice, since cms is a maternally inherited trait. Firstly, chloroplast DNAs isolated from normal and cms-Bo male-sterile cytoplasms were digested with XhoI (Fig. I-1) or PstI (data not shown) and separated by a 0.7% agarose gel electrophoresis. Fragment patterns produced were the same as reported by Ishii et al., (1988). No distinguishable difference was detected in the restriction fragment patterns between the two types of cytoplasms. These results suggest that both cytoplasms have similar genetic information on chloroplast.

Differences in the mitochondrial DNA between normal and male-sterile cytoplasms

Mitochondrial DNAs were purified from normal and cms-Bo cytoplasms, and electrophoresed in a 0.7% agarose gel after incubation with PstI. While most restricted fragments were common to both cytoplasms, several bands were specifically identified in the fertile or the sterile cytoplasm, suggesting mutations and/or rearrangements of the mitochondrial DNA (Fig. I-2, A and B).
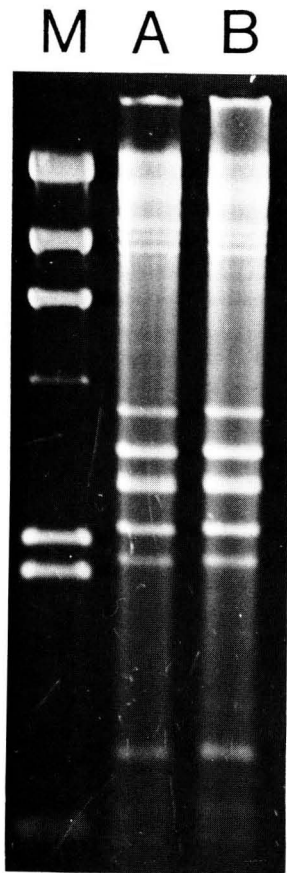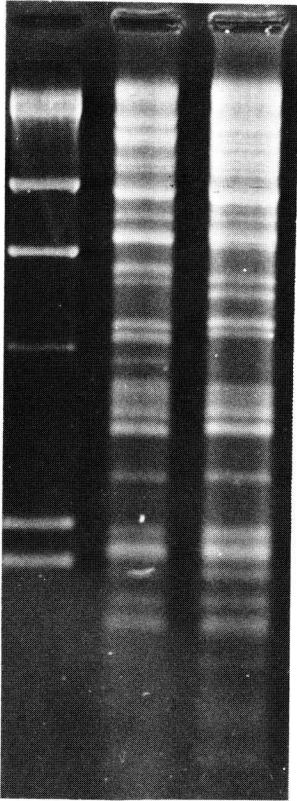
Fig. I-1. Restriction fragment patterns of rice chloroplast DNAs isolated from normal (A) and cms-Bo cytoplasms (B). Chloroplast DNA was digested with <u>Xho</u>I and fragments produced were separated on a 0.7% agarose gel. Lane M shows phage lambda DNA digested with <u>Hin</u>dIII as size marker.
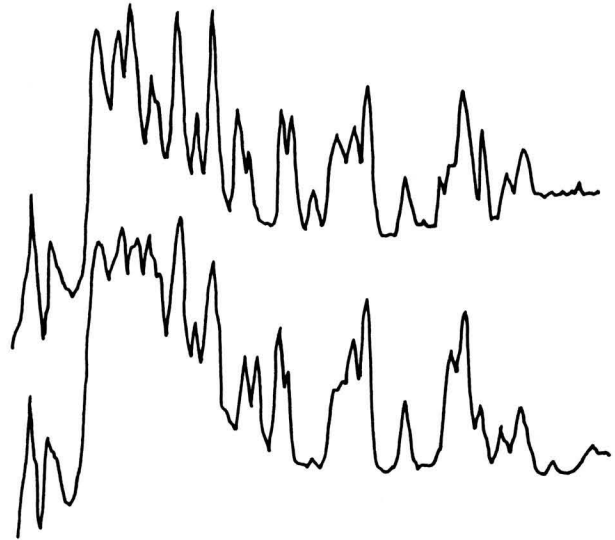
Fig. I-2. Electrophoresis on a 0.7% agarose gel of rice mitochondrial DNA digested with <u>Pst</u>I. Mitochondrial DNAs were isolated from normal (A) and cms-Bo cytoplasms (B). Lane M shows molecular weight marker; lambda DNA digested with <u>Hind</u>III. Negative film obtained in this experiment was scanned with a densitometer. Panels C and D represent profiles of the pattern of DNA restriction fragments from lanes A, B respectively. The top of the gel is on the left hand side. One peak of this figure represents one DNA fragment. The concentration of the DNA fragment reflects the area of peak.

Based on the film scanning investigations on the restriction fragment patterns, significant differences were shown in the pattern and the concentration of the DNA fragments (Fig. I-2, C and D). These results showed variation in the mitochondrial genome organization between normal and cms-Bo cytoplasms. When the mitochondrial DNAs were separated after incubation with RNase, four plasmid-like DNAs with 1.60, 1.25, 1.09, and 0.96 kb were identified in cms-Bo cytoplasm, while no plasmid-like DNA was observed in normal cytoplasm (Fig. I-3). Yamaguchi and Kakiuchi (1983) find two plasmid-like DNAs with 1.5 and 1.2 kb, in cms-Bo cytoplasm. Two additional DNA molecules with 1.09 and 0.96 kb were found in the same cytoplasm in this study. This difference might be derived from the difference of plant materials, since Yamaguchi and Kakiuchi used tissue cultured cells, and young seedlings were used in this study.

To confirm the different organization of mitochondrial DNA between male-sterile and normal cytoplasms, another three male-sterile cytoplasms; WA, cms-Gam, and MS-577 were investigated. Comparison of restricted fragment patterns showed differences between the male-sterile and its maintainer (normal) cytoplasms (Fig. I-4). Above results strongly suggest that the occurrence of cms of rice is associated with difference(s) of mitochondrial DNA.
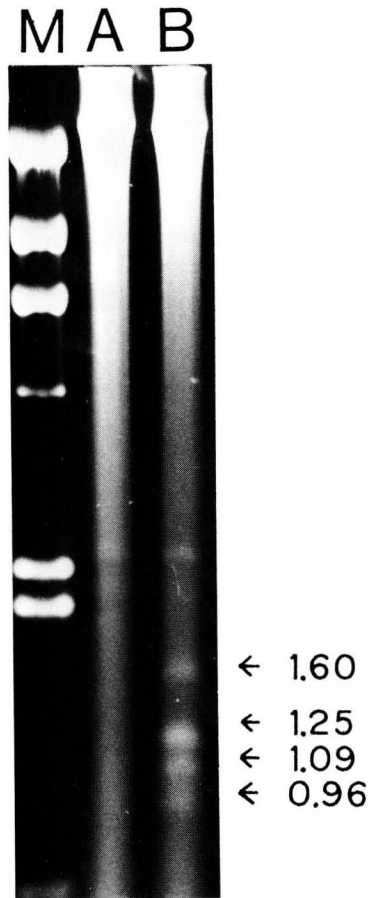
Fig. I-3. Agarose gel electrophoresis of rice mitochondrial DNAs isolated from normal (A) and cms-Bo cytoplasms (B). Lane M indicates <u>Hin</u>dIII digestion fragments of phage lambda DNA. Mitochondrial DNAs obtained were incubated with RNase and electrophoresed on a 0.7% agarose gel. Arrows show plasmid-like DNA and their molecular weights are given in kb.

Fig. I-4. Differences in the PstI-digested mitochondrial DNA between male-sterile cytoplasm (A) and cytoplasm of its maintainer line (B). Lanes A-1, A-2, and A-3 show male-sterile cytoplasms WA, cms-Gam, and MS-577, respectively. Lanes B-1, B-2, and B-3 show maintainer cytoplasms (normal cytoplasms) of lanes A-1, A-2, and A-3, respectively. Electrophoresis was performed on a 0.7% agarose gel.

CHAPTER II   Property of mitochondrial DNA in cytoplasmically male-sterile rice

II-1   Mitochondrial DNA polymorphism in male-sterile cytoplasm of rice

INTRODUCTION

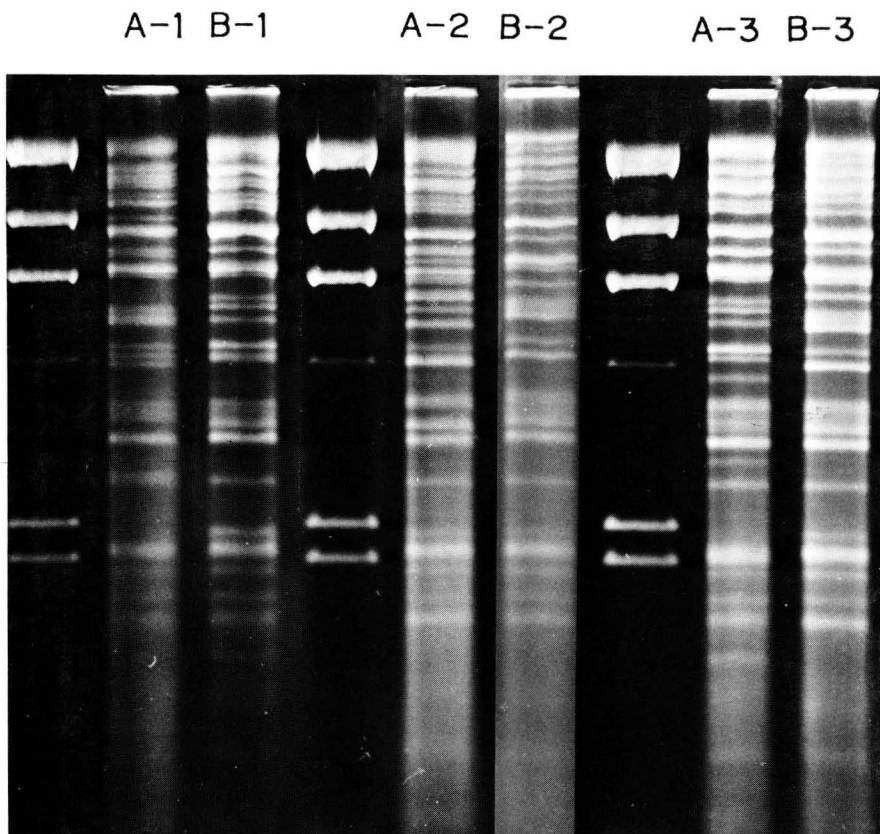Cms is a maternally inherited trait, common in higher plant, that causes a plant to fail to produce functional pollen grains. The male-sterile phenotype is caused by interactions between nuclear and cytoplasmic factors (Clayton, 1950). Extensive surveys of mitochondrial DNA variation have been done for many kinds of plants to examine the extent of polymorphism and relatedness of cytoplasms (Pring and Lonsdale, 1985). Differences in mitochondrial nucleic acids are correlated with variation in the restoration of fertility in cytoplasms of the cms-S subgroup of maize (Sisco et al., 1985). In rice, however, analysis of rice mitochondrial DNAs of high molecular weight has not been done. Male-sterile cytoplasms of rice were found in about 75 strains of O. sativa, O. rufipogon, and O. glaberrima, but in no strains of O. barthii or O. breviligulata (Shinjyo, 1984) and trying to introduce into Japonica rice by recurrent back-crossing.

In this study, we used ten strains of rice with male-sterile cytoplasm that are important in the production of hybrid seeds and are being characterized genetically now. The purpose of the work reported in chapter II-1 is to analyze the mitochondrial DNA with high molecular weight so as to examine the characteristics of the mitochondrial DNAs and to identify relationships among the strains.

MATERIALS AND METHODS

Plant materials

The ten strains of rice with male-sterile cytoplasm of different origins were used and listed in Table II-1.

Table II-1. Male-sterile cytoplasms of rice used as sources of mitochondrial DNA

Name of cytoplasm	Species of cytoplasmic donor	Type of male sterility	Source[a]	Reference
(cms-Bo)	O. sativa	gametophytic	R	Shinjyo, 1975
(cms-R)	O. rufipogon	gametophytic	N	Maruyama (unpubl.)
(cms-UR89)	O. rufipogon	gametophytic	R	Shinjyo (unpubl.)
(cms-UR93)	O. rufipogon	gametophytic	R	Shinjyo (unpubl.)
(cms-UR102)	O. rufipogon	gametophytic	R	Shinjyo (unpubl.)
(cms-UR104)	O. rufipogon	gametophytic	R	Shinjyo (unpubl.)
(cms-UR106)	O. rufipogon	sporophytic	R	Shinjyo (unpubl.)
(WA)	O. rufipogon	sporophytic	N^I	Lin and Yuan, 1980
(cms-Gam)	O. sativa	sporophytic	N^I	Lin and Yuan, 1980
(MS-577)	O. nivara		N^I	Virmani et al., 1981

[a] R: University of the Ryukyus, Japan
   N: National Agriculture Research Center, Japan
   N^I: Supplied by Dr. Virmani, International Rice Research
       Institute, Philippines

Mitochondrial DNA analysis

Rice seeds were germinated in the dark on two layers of cheesecloth in a container at 28°C for two weeks. Mitochondrial DNA isolation and purification, · restriction endonuclease analysis, and agarose gel electrophoresis were done as described previously (Kadowaki et al., 1986).

Oligonucleotides

Oligonucleotides were synthesized by a DNA Synthesizer (Model 380B, Applied Biosystems). The synthesized oligonucleotides had a part of the 5' coding region of the following genes; maize cob, 5'-

AACAACCTATATACTCCACACT-3' (Dawson et al., 1984); maize atp6, 5'-
CCTGGAGGCGGCGGACCAGTAACA-3' (Dewey et al., 1985a).

Southern blot analysis

Restricted mitochondrial DNAs separated by agarose gel
electrophoresis were blotted to nitrocellulose membrane filters
(Bio-Rad Laboratories) by the method of Southern (1975).
Prehybridization in 6 x SSC, 1 x Denhardt's solution, 0.5% SDS,
denatured salmon sperm DNA (100 μg/ml), and 50 mM sodium phosphate
(pH 6.5) lasted 20 h at 37°C with gentle agitation. As a
hybridization probe, the oligonucleotide was 5' end-labeled with [
γ-^{32}P]ATP (Amersham, >5000 Ci/mmol) with T4 polynucleotide kinase
(Takara Shuzo Co., Ltd.) according to the procedure described in
Maniatis et al., (1982). Hybridization in 6 x SSC, 1 x Denhardt's
solution, 20 μg/ml tRNA, 50 mM sodium phosphate (pH 6.5), and the
labeled-probe DNA lasted 20 h at 30°C with gentle agitation. The
membrane was washed twice in 6 x SSC and 50 mM sodium phosphate (pH
6.5) at 30°C for 15 min, twice at 37°C for 15 min, and twice at 45
°C for 15 min. The membrane was exposed to Fuji RX X-ray film
covered with an intensifying screen (Du pont) at -80°C for 15 h.

RESULTS AND DISCUSSION

The mitochondrial DNAs of ten strains of cms rice were
prepared and examined by restriction endonuclease analysis. As
shown in Fig. II-1, the patterns of PstI-digested fragments were
the same for cms-Bo and cms-UR104 and also for cms-UR89, cms-UR93,
and cms-UR102 cytoplasms. Other cytoplasms had fragment patterns
that did not resemble that of any other. Thus, there were seven
different patterns for the ten examined. We did not detect any
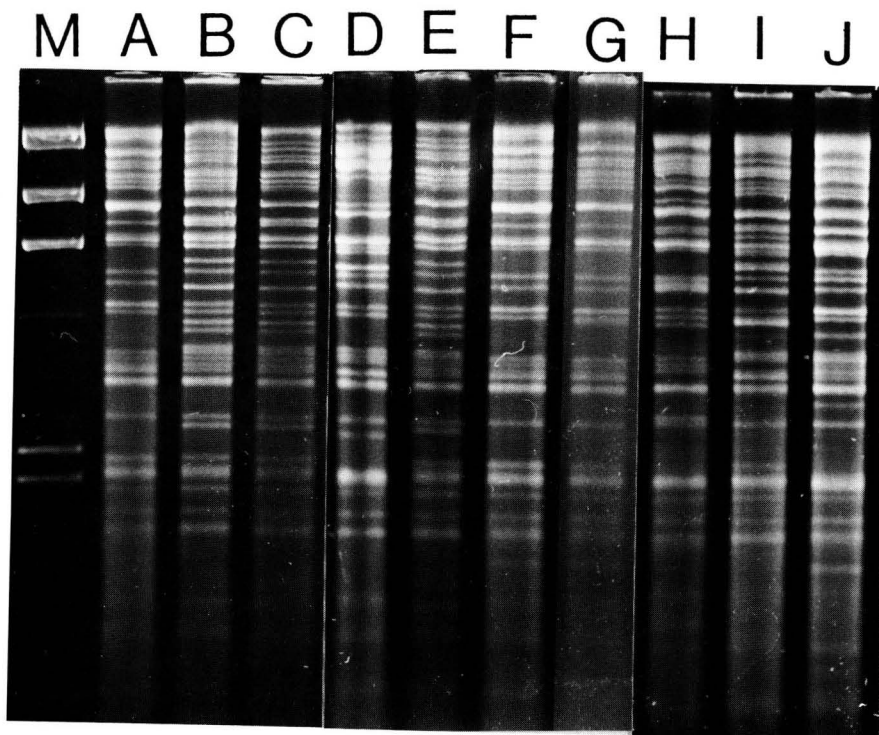difference in the fragment pattern by BamHI or XbaI analysis for

Fig. II-1. Electrophoretic patterns of PstI-digested mitochondrial DNAs from cytoplasmically male-sterile rice on 0.7% agarose gel. Lanes: M, HindIII-digested lambda DNA; A, (cms-Bo); B, (cms-UR89); C, (cms-UR102); D, (cms-R); E, (cms-UR93); F, (cms-UR104); G, (cms-UR106); H, (WA); I, (cms-Gam); J,(MS-577).

cms-Bo and cms-UR104 or for cms-UR89, cms-UR93, and cms-UR102 cytoplasms (data not shown). It is too early to draw conclusions about the evolutionary relationships among the ten strains of cms rice from these results alone. However, we speculate that the smaller the mitochondrial DNA diversity, the closer the phylogenetic relationship. It is noteworthy that several cytoplasms derived from different origins had the same restriction profile for mitochondrial DNA. These cytoplasms will provide useful information for the study of the evolution of cytoplasm of rice.

Summation of the sizes of the rice mitochondrial DNA restriction fragments gave genome sizes of about 174 kb of a unique sequence, and more than 280 kb when multiple bands were included in the calculation. The number of fragments of rice mitochondrial DNA produced by PstI or BamHI digestion (data not shown) was much smaller than that of maize mitochondrial DNA (Borck and Walbot, 1982). These results suggested that the molecular weight of rice mitochondrial DNA is smaller than that of maize mitochondrial DNA, and that the rice mitochondrial DNA is less complex (Lonsdale et al., 1984).

Six strains of rice, with either cms-Bo, cms-UR89, cms-UR93, cms-UR102, cms-UR104, or cms-UR106 cytoplasms, were used to determine the organization of the cob and atp6 genes in male-sterile cytoplasms. EcoRI-digested fragment patterns stained with ethidium bromide were the same for cms-Bo and cms-UR104, and also for cms-UR89, cms-UR93, and cms-UR102 cytoplasms. The other restriction profiles could be distinguished from one another (Fig. II-2A). Oligonucleotide probes for the cob and atp6 genes were
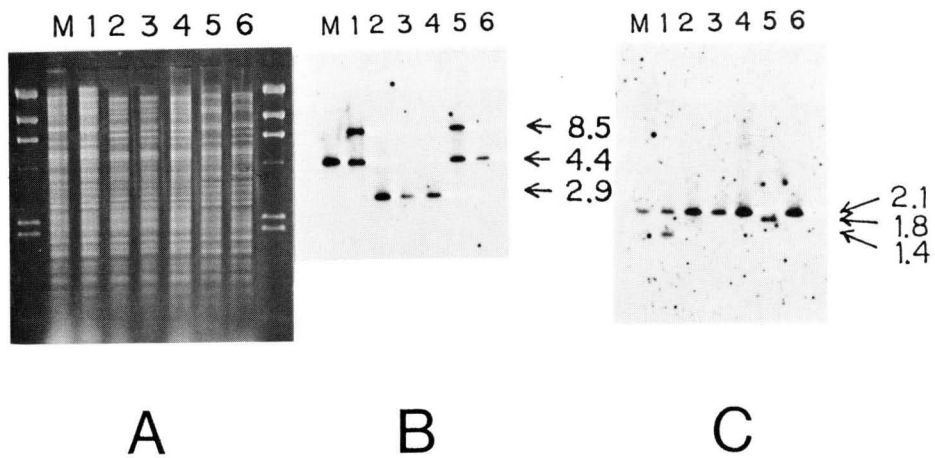
Fig. II-2. Agarose gel electrophoretic patterns of EcoRI-digested mitochondrial DNA from the following male-sterile cytoplasms [A]; cms-Bo (lane 1), cms-UR89 (lane 2), cms-UR93 (lane 3), cms-UR102 (lane 4), cms-UR104 (lane 5), and cms-UR106 (lane 6). Lane M shows the EcoRI digested mitochondrial DNA from normal cytoplasm of the maintainer line. Southern blot analysis of panel A with the oligonucleotide probes for cob gene [B] and atp6 gene [C].

prepared, and Southern blot analyses were performed on the EcoRI-digested fragments. When the probe for the cob gene was used, two hybridization signals of 6.6 and 4.2 kb were observed in cms-Bo and cms-UR104 cytoplasms, a single hybridization signal of 2.9 kb in cms-UR89, cms-UR93 and cms-UR102 cytoplasms, and a 4.2 kb signal in cms-UR106 and normal cytoplasms (Fig. II-2B). When the probe for the atp6 gene was used, two hybridization signals of 2.1 and 1.4 kb were observed in cms-Bo cytoplasm, one signal of 1.8 kb in cms-UR104 cytoplasm, and a 2.1 kb signal in the other cytoplasms (Fig. II-2C). It was noteworthy that the cms-Bo cytoplasm could be further differentiated from cms-UR104 in atp6 gene organization even though the restriction fragment profiles analyzed by agarose gel electrophoresis, of both cytoplasms, have not been distinguishable. This result indicates that not all restricted mitochondrial DNA fragments having same electrophoretic mobility are necessarily composed of the same sequences. The above results showed the heterogeneity of mitochondrial DNA in six male-sterile cytoplasms, and suggested a differential gene organization around the cob and atp6 genes in seven mitochondrial DNAs from male-sterile and normal cytoplasms. The presence of reiterated sequences, active in recombination, are thought to result in a complex multipartite organization of the mitochondrial genome of maize (Lonsdale et al., 1984). Isaac et al. (1985a) suggest a point of divergence near the atpA gene of maize, through which the gene is rearranged, and thus differentiating cms lines of maize. The intra- or intermolecular DNA recombinational events in rice mitochondria, such as in the above reports, may have generated duplication of the cob gene in cms-Bo and cms-UR104 cytoplasms, and of the atp6 gene in cms-Bo cytoplasm during the evolution of rice.

The cytoplasms were finally divided into the following eight groups; cms-Bo; cms-UR89, cms-UR93, and cms-UR102; cms-UR104; cms-UR106; WA; cms-Gam; and MS577 according to the restriction fragment patterns and Southern blot analyses of the mitochondrial DNA (Kadowaki and Harada, 1989a). The results of this present study do not contradict the biological data of fertility restoration patterns of the cytoplasms to different fertility restorer genes (Shinjyo, data unpubl.). However, classification of cytoplasms by Shinjyo was more sensitive because three cytoplasms, cms-UR89, cms-UR93, and cms-UR102, could be further differentiated to cms-UR102; cms-UR89 and cms-UR93. The above findings strongly suggest that the different expression of the cms trait in rice is associated with mitochondrial DNA variation. Similar results are obtained by Sisco et al. (1985) who observe that fertility differences are correlated with mitochondrial nucleic acid variation in at least three subgroups of cms-S cytoplasms of maize.

II-2 Heterogeneity of mitochondrial plasmid-like DNAs in cytoplasmically male-sterile rice

INTRODUCTION

The mitochondrial DNA of higher plants consists of complex and heterogeneous molecules (Leaver and Gray, 1982). This is unlike animal mitochondrial DNAs, which are usually organized in a single large circular molecule. In addition to the main genome of high molecular weight, many discrete smaller DNA or RNA molecules are observed in plant mitochondria. These molecules represent amplified or autonomous portions of the mitochondrial genome created by recombination or replication (Sederoff, 1984). Plasmid-like DNAs can be either linear or circular in form and are found in plants that are fertile or cms (Pring and Lonsdale, 1985). Sequence homology of plasmid-like DNAs within species or between species has been analysed (Pring et al., 1982; Palmer et al., 1983) to study the origin of plasmid-like DNA or the relationships among cytoplasms. Several reports show that reversion of male steriles to the fertile condition may be accompanied by the disappearance of unique plasmid-like DNAs and changes in the mitochondrial chromosomal DNA in maize and rice. (Levings et al., 1980; Nawa et al., 1987). In maize, several DNAs of low molecular weight are present in both cms and normal lines (Kemble and Bedbrook, 1980). Relationship between the plasmid-like DNA and fertility restoration patterns or mitochondrial translation products have been investigated (Kemble et al., 1980; Forde et al., 1980). On the other hand, plasmid-like DNAs are found in only two rice male-sterile cytoplasms; cms-Bo and WA cytoplasms (Yamaguchi and Kakiuchi, 1983; Mignouna et al., 1987)

In chapter II-2, ten strains of cms rice were characterized

for plasmid-like DNAs; the molecular form of the plasmid-like DNAs, and their sequence diversity among cytoplasms. In addition, sequence homology between mitochondrial plasmid-like DNAs of rice and maize was examined.

MATERIALS AND METHODS

Plant materials

Ten strains of cms rice (O. sativa) were studied; the cytoplasms were cms-Bo, cms-R, cms-UR89, cms-UR93, cms-UR102, cms-UR104, cms-UR106, WA, cms-Gam, and MS-577. The restoration of male-sterility and the species of donors of the above cytoplasms are described by Kadowaki et al., (1988b). Four strains of maize (Zea mays L.) were used: TC4 x A188, TC6 x A188, TC5 x A188, and A188. Maize cytoplasms were cms-T, cms-C, cms-S, and N, respectively.

Preparation and fractionation of mitochondrial DNA

Rice seeds or corn seeds were germinated in the dark on two layers of cheesecloth or on vermiculite in a container at 28°C for 14 and 5 days, respectively. Mitochondrial DNA isolation and purification, incubation with RNase A, and agarose gel electrophoresis were done as described previously (Kadowaki et al., 1986, 1988c).

Hybridization of plasmid-like DNA on nitrocellulose filters

After gel electrophoresis, DNAs were transferred to nitro-cellulose membrane filters (Bio-Rad Laboratories) by the method of Southern (1975). Prehybridization in 50% formamide, 5 x Denhardt's solution, 5 x SSC, 50 mM sodium phosphate (pH 6.5), 1% glycine, and denatured salmon sperm DNA (250 µg/ml) lasted 20 h at 42°C with gentle agitation. Hybridization probes were prepared by plasmid-

like DNA eluted from low-melting-point agarose gel (LGT, Nakarai Chemicals, Co., Ltd.) and labeled with $[\alpha-^{32}P]dCTP$ (Amersham,>3000 Ci/mmol) by the multiprime DNA labelling system (Amersham). Hybridizations were performed in 50% formamide, 1 x Denhardt's solution, 5 x SSC, 20 mM sodium phosphate (pH 6.5), denatured salmon sperm DNA (100 µg/ml), and denatured labeled-probe DNA for 20 h at 42°C with gentle agitation. Hybridized membranes were washed twice in 2 x SSC with 0.1% SDS at 42°C for 15 min and twice in 1 x SSC with 0.1% SDS at 42°C for 15 min. Membranes were finally washed with 1 x SSC to remove SDS, air-dried, and exposed to Fuji RX X-ray film covered with an intensifying screen (Du Pont) at -80°C for 1 to 3 days.

RESULTS AND DISCUSSION
Plasmid-like DNAs in cms rice mitochondria

Mitochondrial DNAs from ten cms strains of rice were fractionated on 0.7% agarose gels without restriction endonuclease digestion. These strains were cms-Bo, cms-R, cms-UR89, cms-UR93, cms-UR102, cms-UR104, cms-UR106, WA, cms-Gam, and MS-577. Plasmid-like DNAs were observed in all of the male-sterile cytoplasms along with high-molecular-weight DNAs (Figs. II-3 and 4). Two plasmid-like DNAs of 1.09 and 0.96 kb were observed in cms-R, four of 2.30, 1.60, 1.09 and 0.96 kb were observed in cms-UR106, five of 2.30, 1.60, 1.25, 1.09, and 0.96 kb were observed in cms-Bo, cms-UR93, cms-UR102, cms-UR104, WA, cms-Gam, and MS-577, and six of 2.30, 1.70, 1.60, 1.25, 1.09 and 0.96 kb were observed in cms-UR89 cytoplasm. There were many more copies of these molecules relative to the high-molecular-weight DNA like mitochondrial plasmid-like DNAs of other plants (Pring and Lonsdale, 1985). This was determined from densitometric measurements corrected for
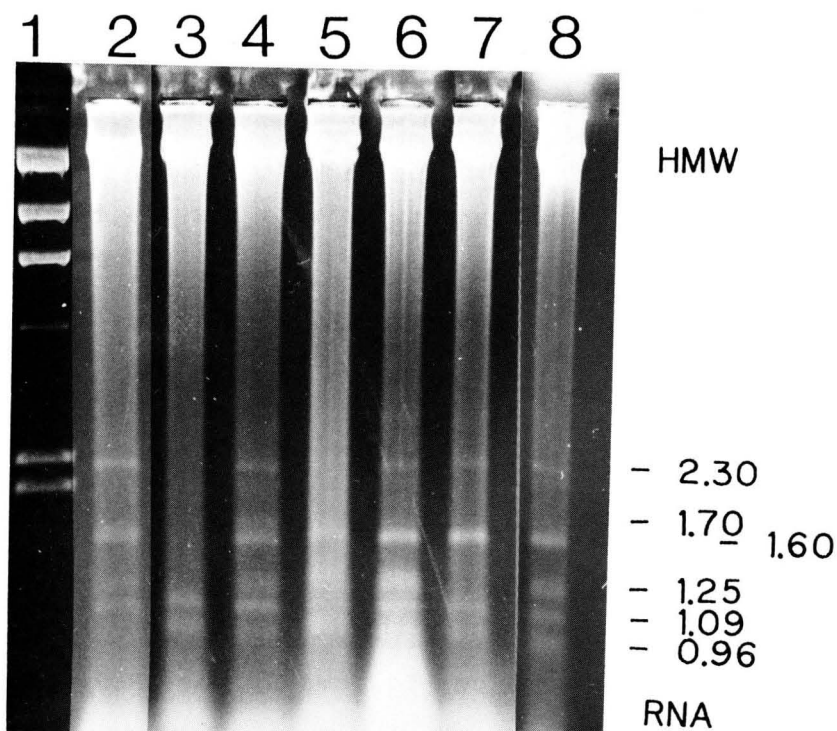
Fig. II-3. Electrophoresis of mitochondrial DNAs from male-sterile cytoplasms of rice on 0.7% agarose gel. Mitochondrial DNAs were loaded on the gel after incubation with RNase A. Lane 1, lambda DNA digested with <u>Hind</u>III; lane 2, cms-Bo; lane 3, cms-R; lane 4, cms-UR89; lane 5, cms-UR93; lane 6, cms-UR102; lane 7, cms-UR106; lane 8, cms-UR104. Molecular weights of plasmid-like DNAs are given in kb. HMW and RNA refer to the position of the high-molecular-weight mitochondrial DNA and digested RNA molecules, respectively.

Fig. II-4. Electrophoresis on 0.7% agarose gel of mitochondrial DNA after incubation with RNase A. Lanes 1-3 indicate WA, cms-Gam, and MS-577 male-sterile cytoplasms, respectively. Lanes B show maintainer (normal) cytoplasms of correspondent lines.

molecular weight (data not shown). Apparent heterogeneity was observed in the plasmid-like DNA between the male-sterile cytoplasms. The fact that some of these bands represent different conformations of the same plasmid-like DNA is discussed later.

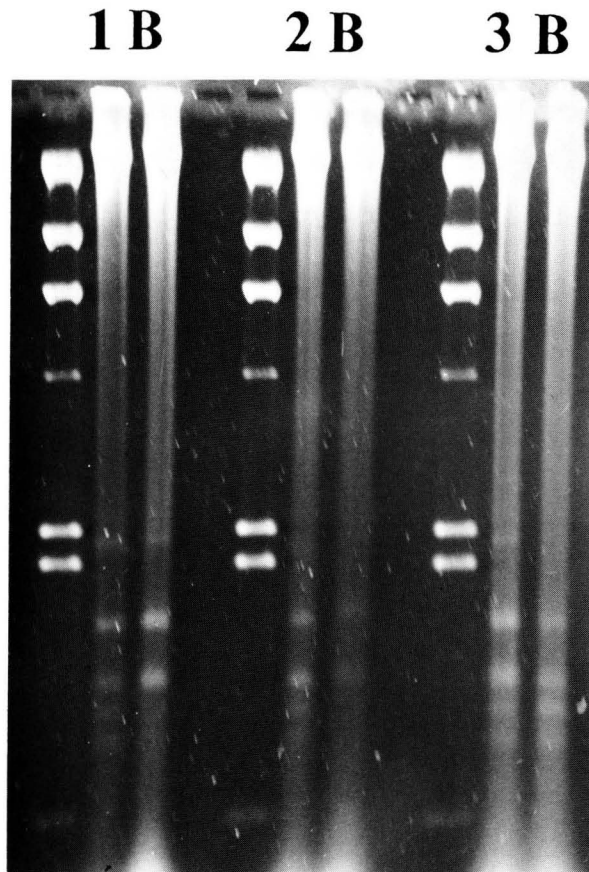Plasmid-like DNAs were also observed in the maintainers (Indica rice) as well as cms lines in Fig. II-4. Kadowaki et al. (1988c) find plasmid-like DNAs in most of rice cultivars (O. sativa L.) and show the diversity of cytoplasm of rice cultivars by the pattern of plasmid-like DNAs. Maintainer lines of Japonica rice have not plasmid-like DNA (Kadowaki et al., 1986, 1988c). Characters of Japonica rice cytoplasms are different from male-sterile cytoplasms of which donor species are O. nivara, O. rufipogon, and Indica rice with respect to the presence of plasmid-like DNA. Above results suggest that properties of mitochondrial DNA from O. nivara and O. rufipogon are closer to Indica rice than Japonica rice. This may present a good clue to study the process of rice cultivation.

Linear plasmid-like DNA in rice mitochondria

Comparison of the electrophoretic patterns of plasmid-like DNAs from cms-Bo that were prepared with or without proteinase K showed a difference in the 1.09 kb DNA molecule (Fig. II-5), similar to the linear plasmid-like DNAs S1, S2, and n of cms-S that only appeared as discrete bands in the gel after proteinase K treatment (Fig. II-5), as reported by Kemble and Thompson (1982). The autoradiograph of Fig. II-5 indicated that there was no 1.09 kb DNA without proteinase K treatment. This suggests that plasmid-like DNAs, except for the 1.09 kb DNA molecule, did not contain protein with their DNAs, while the 1.09 kb DNA molecule may
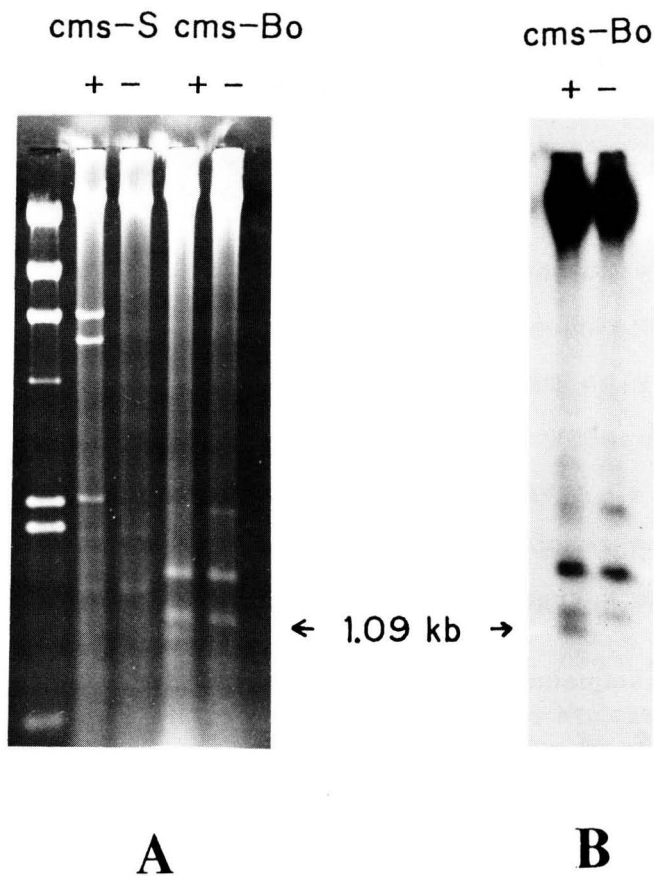
Fig. II-5. Electrophoresis on 0.7% agarose gel of mitochondrial DNAs. A: ethidium bromide staining of the gel. B: autoradiograph of the same gel after hybridization with the ^{32}P-labelled 1.60, 1.25, and 1.09 kb DNAs of cms-Bo. m, lambda DNA digested with <u>Hind</u>III; cms-S, mitochondrial DNA from cms-S maize; cms-Bo, mitochondrial DNA from cms-Bo rice; +, treated with proteinase K (240 µg/ml, 120 min, 37°C); -, not treated with proteinase K before phenol extraction. Arrowheads indicate the missing 1.09 kb DNA of cms-Bo without proteinase K treatment. S1, S2, and n indicate the linear plasmid-like DNAs of maize.

be in the linear form with protein attached like the S1, S2, and n
of maize or N1 and N2 of sorghum.   This 1.09 kb DNA may be the
smallest linear plasmid-like DNA that has been found in plant
mitochondrial DNA thus far; a unique strain of Brassica has 11.3
kb linear DNA (Palmer et al., 1983), maize has linear DNAs of 7.5,
6.4, 5.4, and 2.35 kb (Pring et al., 1977; Weissinger et al.,
1982), and Sorghum has 5.8 and 5.4 kb linear DNAs (Pring et al.,
1982). It is not clear whether this 1.09 kb linear DNA can
integrate into mitochondrial chromosomes like S1 and S2 (Schardl et
al., 1984). The actual sizes of plasmid-like DNAs except for the
1.09 kb DNA may be much larger, due to circular form, than the
sizes estimated above. The 1.60 and 1.25 kb DNAs observed may be
the 2.2 and 1.5 kb respectively, according to the report of Nawa et
al., (1987). The reason why the 2.30 kb band of cms-Bo was diffuse
after proteinase K treatment is unknown.

**Differences in sequence homology among mitochondrial plasmid-like
DNAs in cms rice**

The 1.25 kb DNA is present in eight of the cytoplasms, all
but cms-R and cms-UR106 (Figs. II-3 and 4). Sequence homology
among plasmid-like DNAs was examined to investigate sequence
diversity and conservation. The 1.25 kb DNA of cms-Bo was eluted
from an agarose gel, radioactively labelled and used as a probe.
Under low stringency conditions (1 x SSC, 0.1% SDS, 42° C), the
1.25 kb probe hybridized strongly to itself and to 1.70 kb DNAs;
suggesting conservation of sequence homology in the same size
plasmid-like DNA. With a prolonged exposure it hybridized to 2.30
kb in cms-Bo, cms-UR89, cms-UR93, cms-UR102, and cms-UR104 (data
not shown). The 1.70 kb band observed by ethidium bromide

staining (Fig. II-3, lane 4) was probably the open circular form of the 1.25 kb DNA. A similar result was obtained in the experiment with the 1.60 kb DNA from cms-Bo used as a probe. That is, the 1.60 kb DNA had high homology to the 1.60 kb DNA itself and to 2.30 kb DNA in all cytoplasms except for cms-R (data not shown). The plasmid-like DNA of 2.30 kb was probably the open circular form of the 1.60 kb DNA. It was noteworthy that the 1.25 kb DNA lacked homology to the plasmid-like DNAs of cms-UR106 and cms-R, and a homologous-signal was not detected even after prolonged exposure. This finding indicated that the 1.25 kb plasmid-like DNA was not present in cms-UR106 and cms-R; and that the 1.60 kb plasmid-like DNA was not present in cms-R. Figure II-6 showed the presence of DNAs homologous to the 1.09 kb plasmid-like DNA of cms-R in all of the cms strains as observed by ethidium bromide staining (Fig. II-3), while the 1.09 kb DNA apparently lacked sequence homology with the other plasmid-like DNAs with different sizes. However, this DNA common to the seven strains of cms rice was not detected in their maintainer Reimei (Japonica rice) even by Southern hybridization (data not shown). Above results suggest that plasmid-like DNAs of the same size between different cytoplasms are the same molecules. During the preparation of this chapter, Sakamoto et al. (1989) show high sequence homology of plasmid-like DNAs between cms-Bo and WA. Our data also agree with theirs. Hybridization signals observed in high molecular weight mitochondrial DNA (Figs. II-, 5-7) may be due to the presence of sequences in the principle mitochondrial genome homologous to the probe DNAs as reported (Nawa et al., 1987) or contamination in the gel-purified probe DNA is a possibility. However, the cytoplasmic
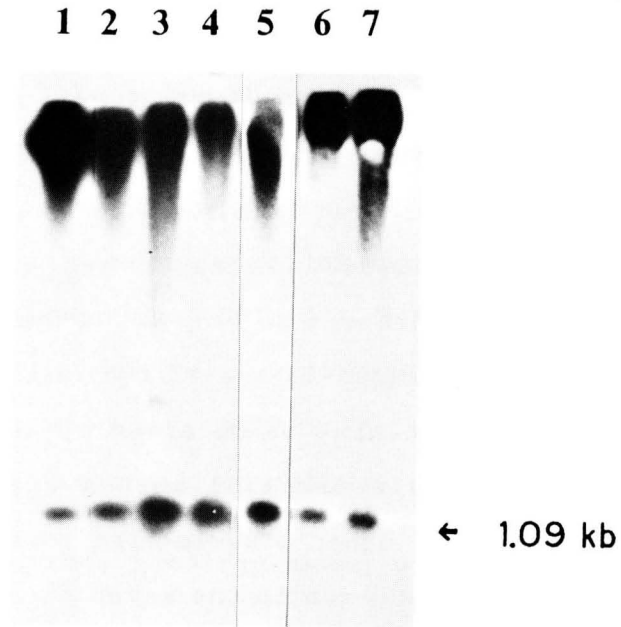
Fig. II-6. Autoradiograph of the gel after hybridization of the ^{32}P-labelled 1.09 kb plasmid-like DNA of cms-R to undigested mitochondrial DNA from seven strains of rice with cms.
Lane 1, cms-Bo; lane 2, cms-R; lane 3, cms-UR89; lane 4, cms-UR93; lane 5, cms-UR102; lane 6, cms-UR104; lane 7, cms-UR106.

differences in plasmid-like DNAs were apparent in these experiments by agarose gel electrophoretic analysis and Southern blot analysis.

Comparison of mitochondrial plasmid-like DNAs between rice and maize in sequence homology

To find the extent of sequence homology of plasmid-like DNAs and the etiological relationships of such DNAs of rice and maize, four cytoplasms of maize were examined: male-sterile cytoplasms cms-S, cms-T, and cms-C, and normal cytoplasm N. Plasmid-like DNAs of 1.60 kb, 1.25 kb, and 1.09 kb of cms-Bo were radioactively labelled and used as hybridization probes. Under low stringency conditions (1 x SSC, 0.1% SDS, 42°C), no homology was detected between pairs of the three plasmid-like DNAs of cms-Bo and those of maize even after long exposure (Fig. II-7). Smeary extra-bands observed after long exposure (Fig. II-7, lane 6) was probably due to multimeric forms of the plasmid-like DNAs or the contamination of DNA molecules having the same electrophoretic mobility, though no hybridization signal with the probes was detected in plasmid-like DNAs of maize. The terminal sequences of linear plasmid-like DNAs N1 and N2 of Sorghum have some sequence homology with the S1 and S2 DNAs of maize (Pring et al., 1982; Baszczynski and Kemble, 1987), while the linear plasmid-like DNA of the 1.09 kb DNA of cms-Bo, like the 11.3 kb linear plasmid-like DNA of Brassica (Palmer et al., 1983), had no detectable homology with the S1 and S2 DNAs. Above result suggested that the plasmid-like DNAs in rice and maize cytoplasms are independent DNA molecules, though Shikanai et al. (1987) find some short sequences (conservativeness is up to 80 nucleotides) in plasmid-like DNA between 2.1 kb of rice and 1.9 kb of maize after DNA sequence comparison with computer. However, the
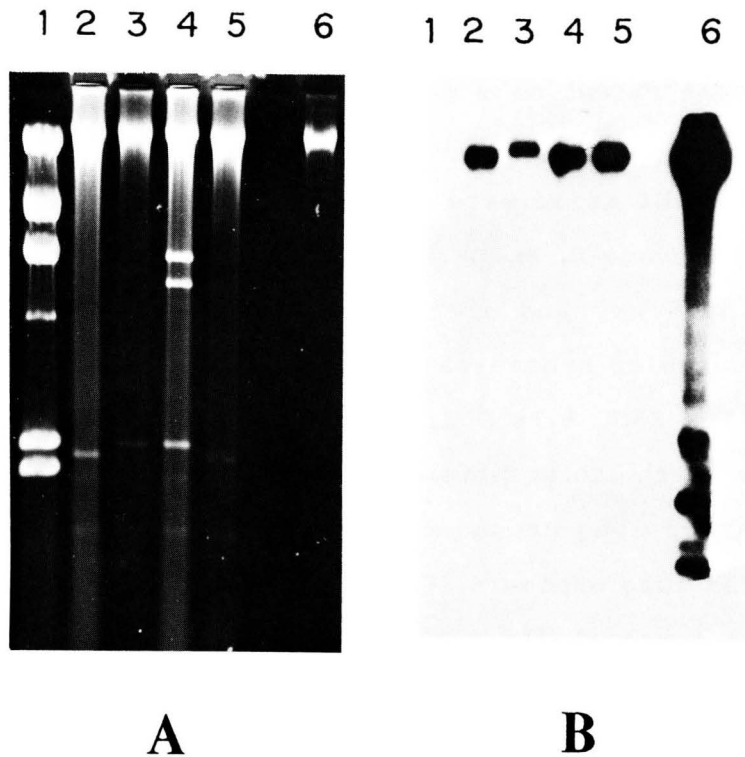
Fig. II-7. Southern blot analysis of plasmid-like DNAs of maize with plasmid-like DNAs of cms-Bo.
A: 0.7% agarose gel electrophoretic pattern of mitochondrial DNAs after incubation with RNase A.
B: prolonged exposure of the autoradiograph of panel A after hybridization.
Lane 1, lambda DNA digested with HindIII; lane 2, cms-T; lane 3, cms-C; lane 4, cms-S; lane 5, N; lane 6, cms-Bo. Hybridization probes used are the ^{32}P-labelled 1.60, 1.25, and 1.09 kb plasmid-like DNAs from cms-Bo.

molecules are of interest in the study of DNA replication in plant mitochondria because of their smallness, and their different forms. The plasmid-like DNAs had also homology with high molecular weight mitochondrial DNA of maize and rice, although the extent of sequence homology was different. The hybridization signals of the rice plasmid-like DNAs to the maize principle genome might be due to the contamination of the probe with sequences of the rice principle mitochondrial genome or the plasmid-homologous sequences in the maize principle mitochondrial genome.

The evolutional relationship of the plasmid-like DNAs between maize and rice and amplification mechanism of plasmid-like DNA are still unknown.

CHAPTER III Distribution of mitochondrial plasmid-like DNA in cultivated rice (O. sativa) and its relationship with varietal groups

INTRODUCTION

The analyses of mitochondrial DNA of different plants by agarose gel electrophoresis and by electron microscopy have shown plasmid-like DNA molecules of low molecular weight that differ in size, stoichiometry, and molecular form; e. g., Brassica (Palmer et al., 1983); Sorghum (Pring et al., 1982; Chase and Pring, 1985); sugarbeet (Powling, 1981); Oenothera (Brennicke and Blanz, 1982); tobacco (Dale et al., 1983); Zea mays (Kemble and Bedbrook, 1980; Weissinger et al., 1982); Vicia faba (Boutry and Briquet, 1982; Negruk et al., 1982); Linum usitatissimum, Datura inoxia, Glycine max, Petunia hybrida, and Phaseolus aureus (Bailey-Serres et al., 1987); rice (Yamaguchi and Kakiuchi, 1983); and wheat (Handa et al., 1984).

In maize, the plasmid-like DNAs fall into specific size classes when analysed by agarose gel electrophoresis, and the relationship between the plasmid-like DNA and cytoplasmic characteristics conferring male-sterility has been investigated (cf. Kemble et al., 1980). The assay of plasmid-like DNAs is a simple way to investigate the cytoplasms of maize (cf. Weissinger et al., 1982). The presence of the mitochondrial plasmid-like DNAs is regardless of nuclear background (Kemble and Bedbrook, 1980; Pring et al., 1982), but in some cases, the plasmid-like DNA copy number is affected by the nuclear genotype (Carlson et al., 1982; Erickson et al., 1986; Kemble et al., 1986). An association between mitochondrial plasmid-like DNA and the occurrence of cms has also been suggested in some plants (Levings

et al., 1980; Nawa et al., 1987). In rice, differences in mitochondrial DNA between the maintainer and cms strains (Kadowaki et al., 1986) and among the ten strains with cms (Kadowaki et al., 1988b) have been analyzed by restriction endonuclease analysis. Plasmid-like DNAs have been identified by agarose gel electrophoresis in mitochondria from cms-Bo (Yamaguchi and Kakiuchi, 1983) and WA cytoplasm (Mignouna et al., 1987). In ten strains with cms, the plasmid-like DNAs were heterogeneous in molecular weight, sequence homology, and molecular form (Kadowaki and Harada, 1989b). These results suggest an extensive divergence in plasmid-like DNA, as well as in the high molecular weight DNA, the so called 'master chromosome', of cms rice mitochondria. However, the mitochondrial DNAs of rice cultivars have not yet been investigated, and there is therefore no information on the homogeneity of their cytoplasms or on the presence of plasmid-like DNAs. The importance of the cytoplasm in specification of vital plant functions such as photosynthesis, sugar and fatty acid metabolism, ATP production, cms, and disease susceptibility is well established. Plant mitochondria are generally considered to be maternally inherited (Levings and Pring, 1976), so analyses of rice mitochondrial DNA will, by allowing the classification of rice cultivars from a cytoplasmic viewpoint, provide useful information for both breeding programs and for the study of the phylogenetic and evolutionary relationships in cultivated rice.

In this chapter, the author describes the results of study by agarose gel electrophoresis of plasmid-like DNA in 102 lines of rice cultivars. This method was used for its simplicity in detecting the molecular basis of mitochondrial DNA divergence, rather than restriction endonuclease analysis which gives complex

fragment patterns. Plasmid-like DNA was found in most of cultivated rice mitochondria, and the relationship between banding patterns of plasmid-like DNA and the varietal groups of cultivated rice was discussed.

MATERIALS AND METHODS

Plant materials

  For electrophoretic analysis of mitochondrial DNA, 102 lines of rice cultivars from collections of genetic stocks provided by the Laboratory of Plant Germplasm Introduction, National Institute of Agrobiological Resources, Japan, were used. They were either indigenous or pure lines. Classification of rice cultivars into varietal groups, such as Indica rice, etc., was based on isozyme analysis, the distribution of gametophyte genes, the degree of hybrid seed sterility, and the morphological and physiological characters (Nakagahra, 1978, 1986). For electron microscopic analysis of mitochondrial DNA molecules, cultivars "Taichung 65" (Japonica) and "Auslaljira" (Indica) were used.

Preparation of mitochondrial DNA and agarose gel electrophoretic analysis

  Rice mitochondrial DNA was extracted, purified, and analyzed by 0.7% agarose gel electrophoresis by the methods of Kadowaki et al., (1988b) without restriction enzyme treatment. About 15 g of etiolated leaves was used as the starting material and the nucleic acids (6-7 µg) obtained were incubated with 0.3 µg/µl RNase A (Sigma) for 120 min at 37°C and then immediately put on the gel. The presence or absence of the plasmid-like DNAs was assessed from photographs, so only samples with an adequate quality of DNA and

with highly resolved electrophoretic patterns were used.

Electron microscopy of small circular mitochondrial DNAs

The mitochondrial DNA molecules isolated were dissolved in 20 mM Tris-HCl (pH 8.0) and 2 mM EDTA and spread by the protein monolayer method of Kleinschmidt (1968).   Samples   were mounted on films of carbon-coated parlodion (Mallinckrodt, St. Louis, MO, U.S.A.) and examined under a JEOL 100C electron microscope at 80 kV.

RESULTS

Presence and distribution of plasmid-like DNAs in cultivated rice mitochondria

The mitochondrial DNAs were isolated from   etiolated seedlings of rice cultivars, incubated with RNase A, and separated by electrophoresis on 0.7% agarose gels. The 102 rice cultivars examined consisted of 69 Indica and 33   Japonica rice lines. The mitochondrial plasmid-like DNAs were identified in gels stained with ethidium bromide. Six plasmid-like DNAs with molecular masses of   2.40, 2.30, 1.60, 1.25, 1.09, and 0.96 kb were found in lanes 4 and 9 of Fig. III-1. Three plasmid-like DNAs with molecular masses of 2.40, 1.60, and 1.25 kb were found in lanes 5, 6, and 8. One plasmid-like DNA of 2.40 kb was found in lanes 3, 7, and 10. Of the 102 cultivars examined, 65 cultivars had plasmid-like DNAs with differences in their banding patterns, indicating the heterogeneity of mitochondrial DNAs in rice cultivars.

We classified the cytoplasms into the following three types, based on the electrophoretic banding patterns of their plasmid-like DNA. However, because of the small quantities of the 2.40 and
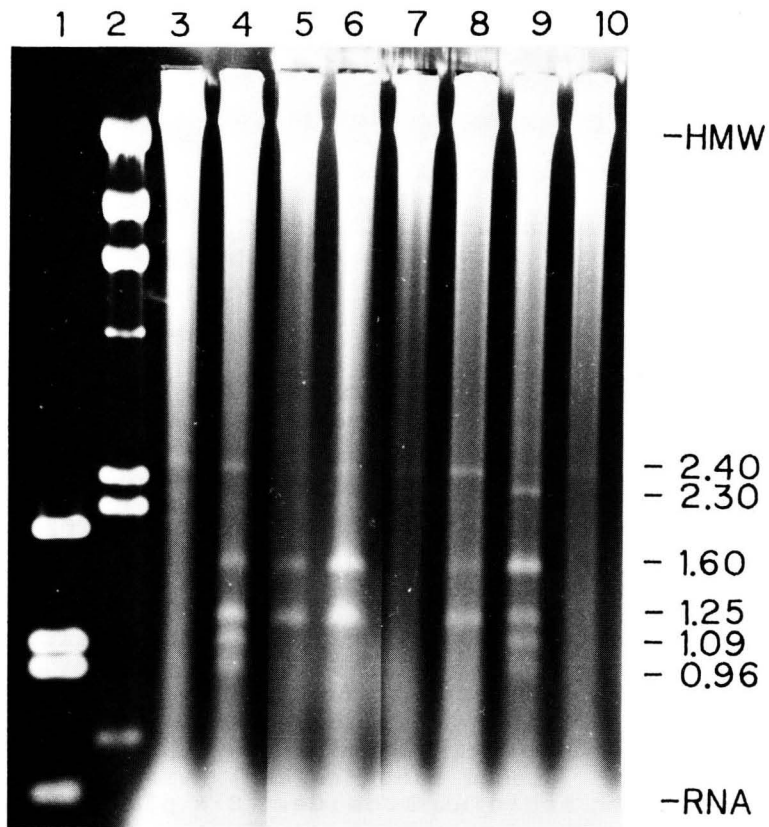
Fig. III-1. Distribution and variation of the mitochondrial plasmid-like DNAs in rice cultivars. Mitochondrial DNAs isolated from etiolated rice seedlings were incubated with RNase A and separated by electrophoresis on 0.7% agarose gel without restriction enzyme treatment.

Lane 1, pBR322 digested with BstNI, as size marker; lane 2, lambda DNA digested with HindIII, as size marker; lane 3, "Nan jing xiang dao"; lane 4, "JENA Col. No. 015"; lane 5, "Xuan chang quang"; lane 6, "Dali zaoxian"; lane 7, "Yun 83-132"; lane 8, "Dao ren qiao"; lane 9, "Leulikelash"; lane 10, "China 830".

Varietal groups of the cultivars are as follows: Indica rice, lanes 4-6 and 8-10; Japonica rice, lanes 3 and 7. The molecular weights (kb) of plasmid-like DNAs are shown in the right margin of the figure. HMW and RNA refer to high molecular weight mitochondrial DNA molecules and digested RNA molecules, respectively.

Table III-1. Polymorphism of mitochondrial plasmid-like DNA in Indica and Japonica rice

Banding patterns of plasmid-like DNA[a]	Indica	Japonica
Type I	46 (66.7)[b]	1 (3.0)
Type II	13 (18.8)	0 (0)
Type III	10 (14.5)	32 (97.0)
	69	33

[a] Type I cytoplasm has four plasmid-like DNAs of 1.60, 1.25, 1.09, and 0.96 kb.
  Type II cytoplasm has two plasmid-like DNAs of 1.60 and 1.25 kb, but dose not have 1.09 or 0.96 kb DNAs.
  Type III cytoplasm has no plasmid-like DNA or different plasmid-like DNAs of Type I or II.

[b] The numbers in parentheses show percentages in each varietal group.

2.30 kb plasmid-like DNAs, the decision on their presence or absence was sometimes difficult. Type I cytoplasm had four plasmid-like DNAs of 1.60, 1.25, 1.09, and 0.96 kb. Type II cytoplasm had the plasmid-like DNAs of 1.60 and 1.25 kb, but not of 1.09 or 0.96 kb. Type III cytoplasm had no plasmid-like DNA, or different plasmid-like DNA of 1.60, 1.25, 1.09, or 0.96 kb.

The relationship between the banding patterns of mitochondrial plasmid-like DNA and the varietal groups of the rice cultivars was investigated (Table III-1). Cytoplasms of Japonica rice were mainly Type III, but the cytoplasms of Indica rice were Types I, II, or III. These results were, in part, in agreement with the findings of Ishii et al., (1986), who showed that the restriction fragment pattern of chloroplast DNA of the ecospecies Indica is different from those of ecospecies Japonica and Javanica (included in Japonica rice in a broad sense), and that the restriction patterns of the latter two ecospecies were identical. Our results

show even more heterogeneity within the Indica rice cytoplasms, indicating that mitochondrial DNA analysis may be more sensitive than chloroplast DNA analysis for investigations of the divergence of rice cytoplasms.

Size distribution of small circular DNA under the electron microscope

Mitochondrial DNA molecules were prepared from the cultivars "Taichung 65" (Japonica), and "Auslaljira" (Indica), which had mitochondrial plasmid-like DNA banding patterns of Types III and I, respectively. Electron microscopic observations of rice mitochondrial DNA molecules not only showed small circular DNA molecules, but also huge heterogeneous DNA molecules that were mainly linear, which may be preparation artifacts (data not shown). Supercoiled small DNA molecules were identified in addition to open circular DNA molecules. These small circular molecules were observed in both cultivars regardless of the detection of plasmid-like DNAs by agarose gel electrophoresis. Figure III-2A, B, E, and F showed partially loosened supercoiled DNA molecules, and C, D, G, and H showed open circular DNA molecules. The contour lengths of the open circular DNA molecules were between 210 and 900 nm in "Auslaljira" and between 180 and 1100 nm in "Taichung 65" (Fig. III-3). In both cultivars, the modal size of the molecules ranged between 300 and 350 nm, and their histogram profiles looked similar.

We also observed circular molecules that might have been the expected replicative intermediates (Fig. III-4). The lengths of solid and dotted lines in each molecule were equal. Contour lengths of the solid lines in Fig. III-4, A and B were 690 and 380
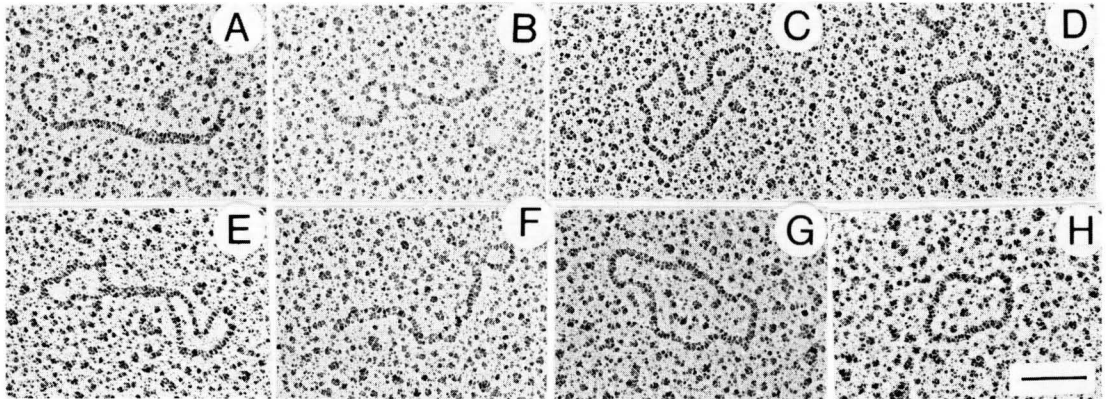
Fig. III-2. Small supercoiled and open circular DNA molecules in rice mitochondria observed by electron microscopy. A-D, DNA molecules from the cultivar "Taichung 65" (Type III*; banding pattern of plasmid-like DNA). E-H, DNA molecules from the cultivar "Auslaljira" (Type I*; banding pattern of plasmid-like DNA). A, B, E, and F show partially loosened supercoiled DNA molecules, and C, D, G, and H show open circular DNA molecules. The length of the bar is 83 nm.

* see Table III-1.
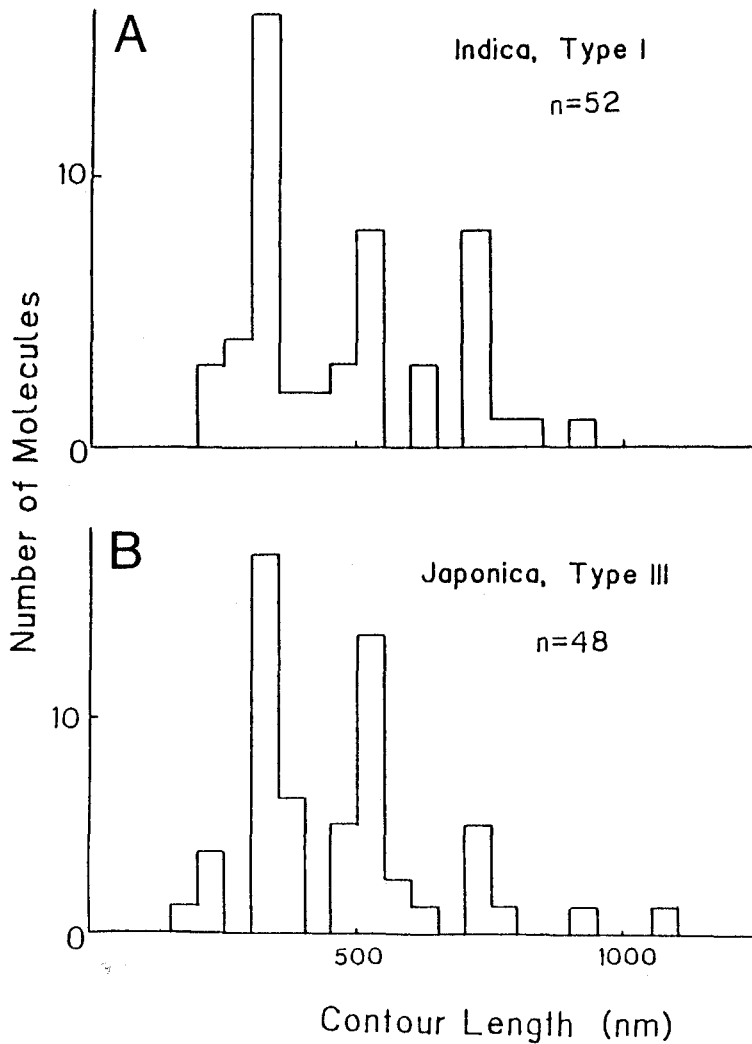
Fig. III-3. Histograms of the contour lengths of small circular DNA molecules observed in electron microscope preparations of mitochondrial DNAs. A, DNA molecules from the cultivar "Auslaljira"; B, DNA molecules from the cultivar "Taichung 65". n: Number of molecules in each sample.

nm, respectively; indicating 2.0 and 1.1 kb molecules.

DISCUSSION

The small circular DNA molecules observed by agarose gel electrophoresis and electron microscopy in this study may have been the product of intramolecular or intermolecular recombinations in direct or inverted repeat sequences of mitochondrial DNA, as proposed for Brassica campestris (Palmer and Shields, 1984) and maize (Lonsdale et al., 1984). Plasmid-like DNAs like those in Fig. III-1 should be highly amplified, small DNA molecules, because of their high molar ratio to the master chromosomal DNA. Regardless of the identification of plasmid-like DNAs by agarose gel electrophoresis, we found small circular DNAs, with similar size distribution profiles, in both cultivars (Figs. III-2 and 3). The actual sizes of the 1.60 and 1.25 kb molecules in Fig. III-1 are estimated as 2.2 and 1.5 kb, respectively, by Nawa et al., (1987), because those molecules are circular, and the size of the 1.09 kb molecule is thought to be 1.09 kb because of its linear conformation (Kadowaki and Harada, 1989b). Each of plasmid-like DNAs observed in Fig. III-1 seemed to correspond with a peak in Fig. III-3 when the length of 1 kb was taken to be 340 nm, and replicative intermediates of small circular molecules were found in Fig. III-4. Therefore, the detection of plasmid-like DNA by agarose gel electrophoresis was concluded to be not due to the existence of small DNA, but to the stoichiometry of that.

Neither the mechanism nor the factors involved in the high amplification of mitochondrial plasmid-like DNA is known. There may be three possible mechanisms involved in the regulation of specific DNA amplification. First, the plasmid-like DNA may be autonomously
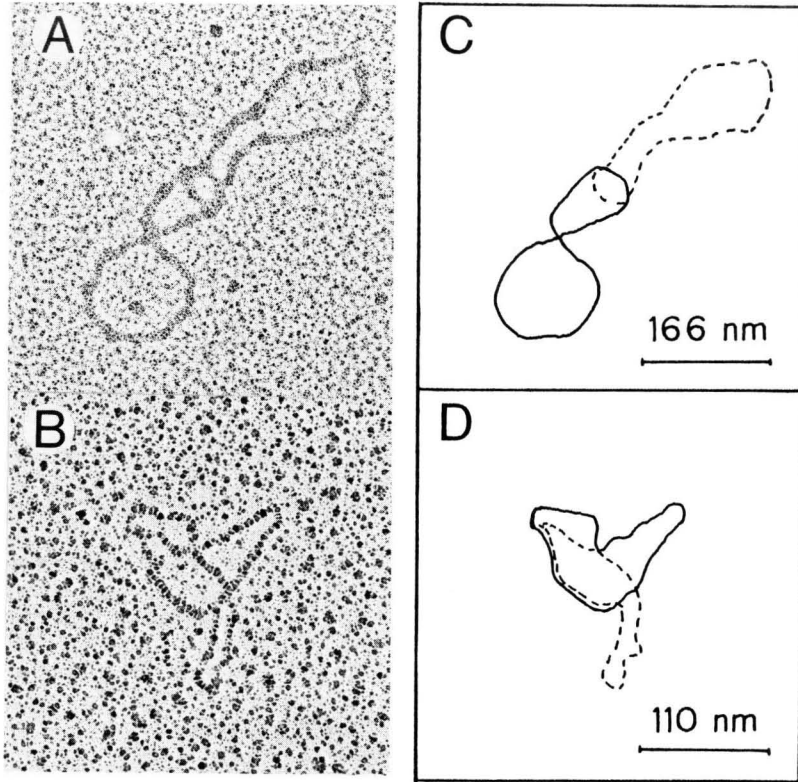
Fig. III-4. Electron micrographs of replicative intermediates of low molecular weight molecules from rice mitochondrial DNA preparations. Panels C and D show schematic representations of A and B, respectively. Solid and dotted lines show DNA molecules. Sizes of bars in each frame is given under the figures.

replicated by its own product. However, this seems unlikely, because the plasmid-like DNAs of rice are very small; not enough of an open reading frame has been identified to allow coding for a functional protein in the plasmid-like DNA B1 and B4 of rice mitochondria (Shikanai et al., 1987; Shikanai and Yamada, 1988), which has a similar electrophoretic mobility to the 1.60 and 0.96 kb DNAs shown in Fig. III-1. Second, amplification of specific DNA may be regulated not by nuclear DNA but by other mitochondrial DNA of high molecular weight, as in the reports of Kemble et al., (1980) and Pring et al., (1982) who obtained characteristic banding patterns of mitochondrial DNA regardless of the nuclear background. In this case, the plasmid-like DNAs would be good molecular markers for distinguishing between cytoplasms, because the mitochondria are maternally inherited. The third alternative for the amplification of plasmid-like DNA is regulation by nuclear DNA, as in the reports of Erickson et al., (1986) and Kemble et al., (1986) in which the plasmid-like DNA copy number is affected by the nuclear genotype. In this case, the gene(s) that regulate amplification might be included among the genes that specify the rice varietal groups or be closely linked to them. However, our preliminary results do not all agree with this possibility. The plasmid-like DNAs found in Indica rice are present even after recurrent back-crossing to the Japonica rice (Kadowaki and Harada, 1989b), but the effects of specific nucleo-cytoplasmic interactions on the presence of plasmid-like DNA are still unknown.

Cultivated rice consists of two independent species: O. sativa and O. glaberrima. Many different approaches have been taken to

Table III-2. Relationships between banding patterns of mitochondrial plasmid-like DNA and varietal groups of rice[a]

Banding patterns of plasmid-like DNA	Indica		Japonica		
	A	B	C	D	
Type I	38 (84.4)	8 (33.3)	0	1 (5.9)	47
Type II	2 (4.4)	11 (45.8)	0	0	13
Type III	5 (11.1)	5 (20.8)	16 (100)	16 (94.1)	42
	45	24	16	17	102

[a] Varietal groups were categorized according to isozyme analysis, distribution of gametophyte genes, degree of hybrid sterility, morphological characteristics, etc., by Nakagahra (1978, 1986).

A: Indica rice collected from India, Sri Lanka, and Bangladesh.
B: Sinica (Hsien) rice collected from South China and Vietnam.
C: Javanica rice collected from hilly areas of Southeastern asia and tropical islands.
D: Japonica rice collected from Japan and Northern china.

characterize the cultivars of O. sativa into varietal groups. Morinaga (1954) and Chang (1976) classified rice cultivars into the three varietal groups of Indica, Japonica, and Javanica rice on the basis of the degree of hybrid seed fertility after intervarietal crossing experiments. This classification is almost the same as that proposed by Oka (1958), which was based on differences in the physiological and morphological characteristics, and that by Matsuo (1952), based on morphological characteristics. Nakagahra (1978, 1986) further classifies the Indicas into Indica and Sinica (Hsien) rice, according to the conventional methods mentioned above, as well as by analyses of gametophyte genes and isozymes. Glaszmann (1986) classifies the varieties into six groups by analyses of several isozymes. In our experiments, rice cultivars were classified into three groups by the electrophoretic banding patterns of mitochondrial plasmid-like DNA. Cytoplasms of Japonica rice were uniform, while those of Indica rice were heterogeneous.

The relationship between the banding patterns of mitochondrial plasmid-like DNA and the four varietal groups, Indica, Sinica, Javanica, and Japonica, of Nakagahra was examined (Table III-2). Genetic differences were found in the rice cytoplasms. The great majority of Indica cytoplasms were Type I, the majority of Javanica and Japonica cytoplasms were Type III, and the Sinica cytoplasms were mixtures of Types I, II, and III. Plasmid-like DNAs were identified in all of the cytoplasmically male-sterile rice strain, the cytoplasms of which are from O. rufipogon (Kadowaki and Harada, 1989b), generally considered to be the ancestor of O. sativa. The plasmid-like DNA identified in this study has high sequence homology with that of O. rufipogon (Kadowaki and Harada, 1989b); indicating that cytoplasms of Indica rice have etiologically closer relationship with those of O. rufipogon than those of Japonica rice.

CHAPTER I V   Alterations of mitochondrial gene expression by introduction of fertility-restorer gene

INTRODUCTION

Genome size of plant mitochondrial DNA, reported to range from 218 kb of <u>Brassica</u> <u>campestris</u> (Palmer and Shields, 1984) to 2400 kb of muskmelon (Ward <u>et</u> <u>al</u>., 1981), is much larger and more complex in arrangement than that of other organisms. In spite of its large and variable size of the plant mitochondrial genome, the number of gene in mitochondrial DNA is, with one or two exceptions, similar to those described in other organisms (Hawkesford and Leaver, 1987). Plant mitochondrial DNA genes from normal cytoplasms, whose nucleotide sequences have been determined, are as follows; <u>coxI</u>, <u>coxII</u>, <u>atpA</u>, <u>cob</u>, <u>atp6</u>, <u>atp9</u>, 26S rRNA, 18S rRNA, 5S rRNA, several tRNAs, and <u>atp8</u> (cf., Isaac <u>et</u> <u>al</u>., 1985b; Kao <u>et</u> <u>al</u>., 1984; Braun and Levings, 1985; Dawson <u>et</u> <u>al</u>., 1984; Dewey <u>et</u> <u>al</u>., 1985a; Dewey <u>et</u> <u>al</u>., 1985b; Dale <u>et</u> <u>al</u>., 1984; Spencer <u>et</u> <u>al</u>., 1984; Spencer <u>et</u> <u>al</u>., 1981; Gray and Spencer, 1983; Bland <u>et</u> <u>al</u>., 1986; Hiesel and Brennicke, 1985). In addition, unique genes supposed to be associated with cms have been determined in some species of plant (Dewey <u>et</u> <u>al</u>., 1986; Young and Hanson, 1987).

Differences in the mitochondrial DNA of rice with normal and male-sterile cytoplasms have been detected by the restriction fragment analysis, and the survey of plasmid-like DNA (Yamaguchi and Kakiuchi, 1983; Kadowaki <u>et</u> <u>al</u>., 1986; Shikanai <u>et</u> <u>al</u>., 1987; Nawa <u>et</u> <u>al</u>., 1987; Mignouna <u>et</u> <u>al</u>., 1987). In this study, we tried to localize those differences, to investigate the genome organization associated with cms, by Southern blot analysis using synthesized oligonucleotides as probe DNAs which would be sensitive to determine the gene arrangement. A DNA fragment

uniquely observed in cms-Bo cytoplasm was cloned and its transcription was affected by introduction of restorer of fertility gene coded on nuclear. Nucleotide sequence of the DNA fragment was determined and the data obtained were discussed. In vitro mitochondrial polypeptide synthesis was carried out and a polypeptide presumed to be related with cms was found.

MATERIALS AND METHODS

Plant materials

Three sets of rice plant materials were used. The cytoplasmically male-sterile and restored lines had been produced from sexual crossing. In all set, the nuclear background for the male-sterile, restored, and normal cultivars is cultivar "Taichung 65". Restorer allele from cultivar "Chinsurah BoroII" had been transferred into the nuclear background "Taichung 65" of cms-Bo male-sterile cytoplasm by recurrent backcrossing (Shinjyo, 1975).

Mitochondrial DNA isolation, restriction enzyme digestion, and agarose gel electrophoresis

The procedures have been previously described by Kadowaki et al., (1988b).

Oligonucleotides

Oligonucleotides (21-26 mer) were synthesized by a DNA Synthesizer (Model 380B, Applied Biosystems). The synthesized oligonucleotides had a part of the 5' coding region of the following genes (sequences are shown in Table IV-1); rice coxII, maize coxI, maize cob, maize atp6, maize atp9, and maize atpA.

Table IV-1. Nucleotide sequences of synthesized oligonucleotides

Gene	Sequence	Reference
coxI	5'-CCAGCAGGAGGGGGAGACCCAA-3'	[Isaac et al., 1985b]
coxII	5'-GTGATGCTGCGGAACCATGGC-3'	[Kao et al., 1984]
atp6	5'-CCTGGAGGCGGCGGACCAGTAACA-3'	[Dewey et al., 1985a]
cob	5'-AACAACCTATATACTCCACACT-3'	[Dawson et al., 1984]
atpA	5'-CCTGCGGGAAAGGCCATGTTAGGCCG-3'	[Braun et al., 1985]
atp9	5'-GCTGGAGCTGCTACAATTGCTTTA-3'	[Dewey et al., 1985b]

Southern blot analysis

     Restricted mitochondrial DNAs separated by agarose gel electrophoresis were blotted to nitrocellulose membrane filters (Bio-Rad Laboratories) by the method of Southern (1975). Prehybridization in 6 x SSC, 1 x Denhardt's solution, 0.5% SDS, denatured salmon sperm DNA (100 μg/ml), and 50 mM sodium phosphate (pH 6.5) lasted 20 h at 37°C with gentle agitation. As a hybridization probe, the oligonucleotide was 5' end-labeled with [γ-^{32}P]ATP (Amersham, >5000 Ci/mmol) with T4 polynucleotide kinase (Takara Shuzo Co., Ltd.) according to the procedure described in Maniatis et al., (1982). Hybridization in 6 x SSC, 1 x Denhardt's solution, 20 μg/ml tRNA, 50 mM sodium phosphate (pH 6.5), and the labeled-probe DNA lasted 20 h at 30°C with gentle agitation. The membrane was washed twice in 6 x SSC and 50 mM sodium phosphate (pH 6.5) at 30°C for 15 min, twice at 37°C for 15 min, and twice at 45 °C for 15 min.   The membrane was exposed to Fuji RX X-ray film covered with an intensifying screen (Du pont) at -80°C for 15 h.

Protein synthesis by isolated mitochondria and analysis of in vitro translation products

Mitochondria were isolated from 14-days old etiolated shoots by the procedures described by Forde and Leaver (1980). The conditions used for labeling mitochondrial translation products with [^{35}S] methionine and the procedures used for their analysis by SDS polyacrylamide slab gel electrophoresis have been described by Forde et al., (1980). Fluorography of the gel has been described by Kadowaki et al., (1988a).

Cloning and characterization of mitochondrial DNA fragments

Mitochondrial DNA was partially digested by EcoRI restriction endonuclease. Restricted mitochondrial DNA fragments were ligated into EcoRI site of phagemid Bluescript M13 (Stratagene Co., Ltd.). The ligated DNAs were transformed into competent cells of E. coli strain JM109 (recAI, Δlacpro, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, F'traD36, proAB, lacI^{q}ZΔM15). Transformed cells were selected on agar plates containing 25 μg/ml of ampicillin, 0.03% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 1.3 mM isopropyl-β-D(-)-thiogalactopyranoside. Colony hybridization was carried out by the procedures described by Maniatis et al., (1982).

Mitochondrial RNA isolation and Northern blot-hybridization analysis

Mitochondrial RNA was isolated from 14-days old etiolated shoots by the procedure of Siculella and Palmer (1988). RNA was electrophoresed in 1.2% agarose gels containing 37% formaldehyde, 40 mM MOPS (pH 7.0), 10 mM sodium acetate, 1 mM EDTA for 3 h at 60 V. RNA was transferred to nitrocellulose papers in 20 x SSC and the papers were baked at 80°C for 3 h under vacuum. Probe DNAs were electrophoretically separated and recovered from low-melting

point agarose gel, and radiolabeled to a specific activity of 1 x 10^9 cpm/µg of DNA by using a random primer labeling kit (Amersham) and [α-^{32}P] dCTP (Amersham, 3000 Ci/mmol). Prehybridizations were carried out at 42°C for 12 h in 50% formamide, 5 x SSC, 50 mM sodium phosphate (pH 6.5), 250 µg/ml of denatured salmon sperm DNA, 0.1% Ficoll, 0.1% polyvinylpyrolidone, and 0.1% bovine serum albumin. Hybridizations with individual ^{32}P-labelled probes were done at 42°C for 12 h in 4/5 volumes of prehybridization solution and 1/5 volumes of 50% (w/v) dextran. The membrane filters were washed with 2 x SSC buffer containing 0.1% SDS at 42°C and finally with 0.1 x SSC buffer containing 0.1% SDS at 50°C for 1 h. Autoradiography was carried out for 1 day by exposures of X-ray film (RX type, Fuji Photo Film Co., Ltd.).

Nucleotide sequencing analysis

Nucleotide sequences were determined for mitochondrial DNA fragment containing cob gene. Single-stranded DNA from deletion mutants of Bluescript phagemids, precipitated by the polyethyleneglycol method, was used for sequencing of nucleotides by the dideoxy-chain termination reaction (Sanger et al., 1977) using universal primer M13 (Takara Shuzo Co., Ltd.). The DNA sequence data obtained from autoradiograms were handled by personal computer PC-9801 using the software DNASIS (Hitachi Seiki Co., Ltd.).

RESULTS

**In organelle protein synthesis and effect of nuclear restorer gene
on the 31 kDa protein**

Mitochondria were isolated from etiolated rice shoots and
their translation products were labeled in vitro with [^{35}S]
methionine. Two lines used have same cms-Bo cytoplasm and have
same nuclear genotype except only in having restorer of fertility
genes. Electrophoresis of the translation products of mitochondria
from the two lines revealed almost same sets of approximately 20
polypeptide species, ranging in molecular weight from 10,000 to
54,000 (Fig. IV-1). However, the abundance of a 31 kDa
mitochondrial polypeptide was significantly reduced in rice with
cms-Bo cytoplasm that have been restored to fertility by the
nuclear restorer gene Rf-1[a]. This observation is similar to the
results of Forde and Leaver (1980) in which a 13 kDa polypeptide
unique to the cms-T cytoplasm is dramatically reduced in T-restored
mitochondria when in organelle protein synthesis products of
isolated mitochondria are compared. The 13 kDa polypeptide
synthesized in E. coli confers sensitivity to the fungal pathogen
Bipolaris maydis toxin (Dewey et al., 1988) and the male-sterile
corn plants of which cytoplasm is cms-T are particularly
susceptible to Bipolaris maydis toxin (Hooker et al., 1970).
Therefore, the 13 kDa protein is believed to be associated with the
cms trait. It is not be confirmed yet that the 31 kDa protein is
involved in the occurrence of cms of rice like the 13 kDa maize
protein. However, the 31 kDa protein is a strong candidate of
protein associated with cms.
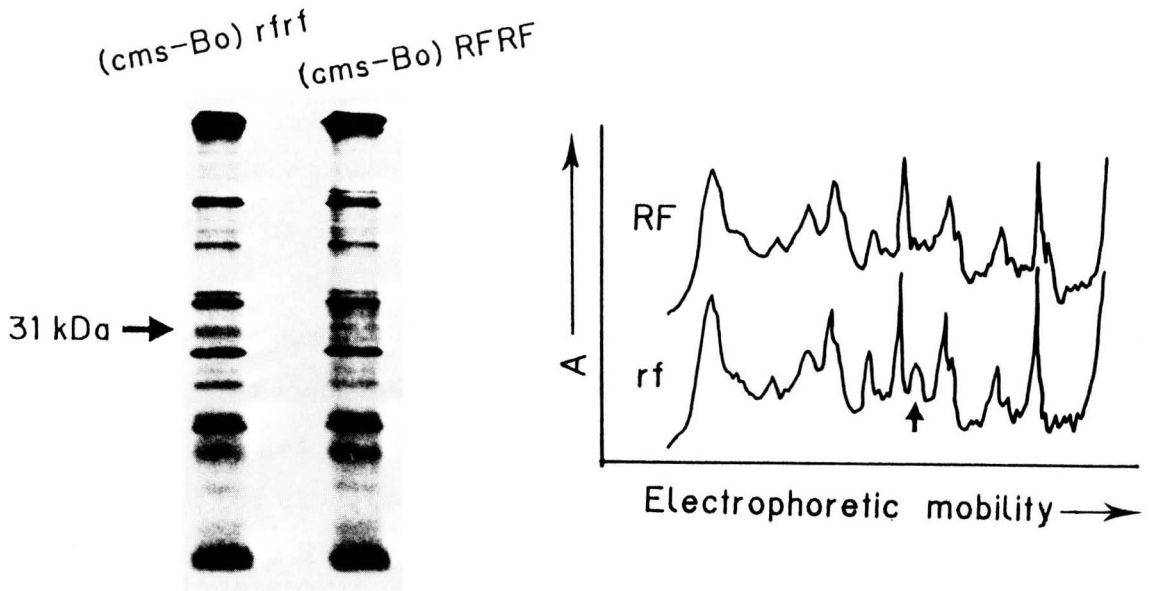
Fig. IV-1. Fluorogram of SDS polyacrylamide gel electrophoresis of mitochondrial polypeptides from male-sterile [(cms-Bo) rfrf] and· fertility-restored [(cms-Bo) RFRF] lines of rice. Mitochondria were isolated and incubated for 90 min in a medium containing [^{35}S] methionine and an energy-generating system. The mitochondrial polypeptides synthesized were separated by SDS slab gel electrophoresis and the gel was treated with EN^{3}HANCE before being dried. Arrow indicates polypeptide not common to both lines. Fluorogram of labeled mitochondrial translation products was scanned with a densitometer.

Southern blot analysis of restricted mitochondrial DNA with oligonucleotide probes

Mitochondrial DNAs prepared from cms-Bo and normal cytoplasms were digested with PstI, and separated by 0.7% agarose gel electrophoresis (Fig. IV-2, A). Mitochondrial DNAs from the above cytoplasms showed different fragment patterns as reported previously by Kadowaki et al., (1986). Southern blot analysis was used to determine the organization of mitochondrial DNA from normal and male-sterile cytoplasms. Oligonucleotides having the 5' coding region of either a coxI, coxII, atp6, atpA, atp9, or a cob gene (sequences shown in Table IV-1) were chemically synthesized by the DNA synthesizer and used as hybridization probes. All six probes hybridized specifically with digested DNA fragment(s) (Fig. IV-2, B-G). Southern blot analysis showed that normal and cms-Bo cytoplasms had the same hybridization signal patterns when probes for the coxI, coxII, atp9, and atpA genes were used (Fig. IV-2, B-E). However, different mobilities were observed between the two cytoplasms when probes for the cob and atp6 genes were used (Fig. IV-2, F and G). In addition, when using the cob gene, two hybridization signals of 25 and 7.8 kb were identified in cms-Bo cytoplasm, and only one signal of 25 kb in the normal cytoplasm (Fig. IV-2, F). These results may indicate a differential gene organization around the PstI-digested fragments, which include the cob and atp6 genes, in the two cytoplasms.

Differential gene organization of the cob and atp6 genes in mitochondrial DNA between normal and cms–Bo cytoplasms

Fig. IV-2F suggested that the copy number of the cob gene was
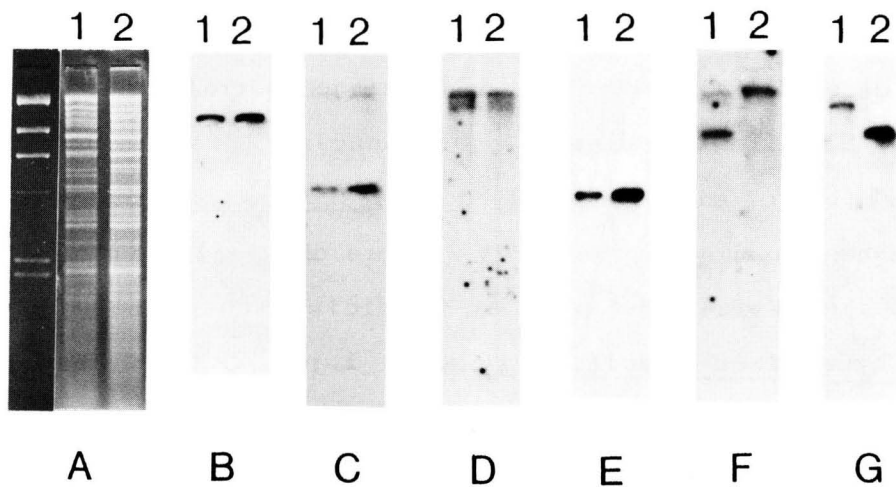
Fig. IV-2. Agarose gel electrophoretic patterns of PstI-digested mitochondrial DNA from cms-Bo (lane 1) and normal (lane 2) cytoplasms [A]. Hybridization of panel A with the synthesized oligonucleotide probes for the following genes; coxII [B], coxI [C], atp9 [D], atpA [E], cob [F], and atp6 [G].

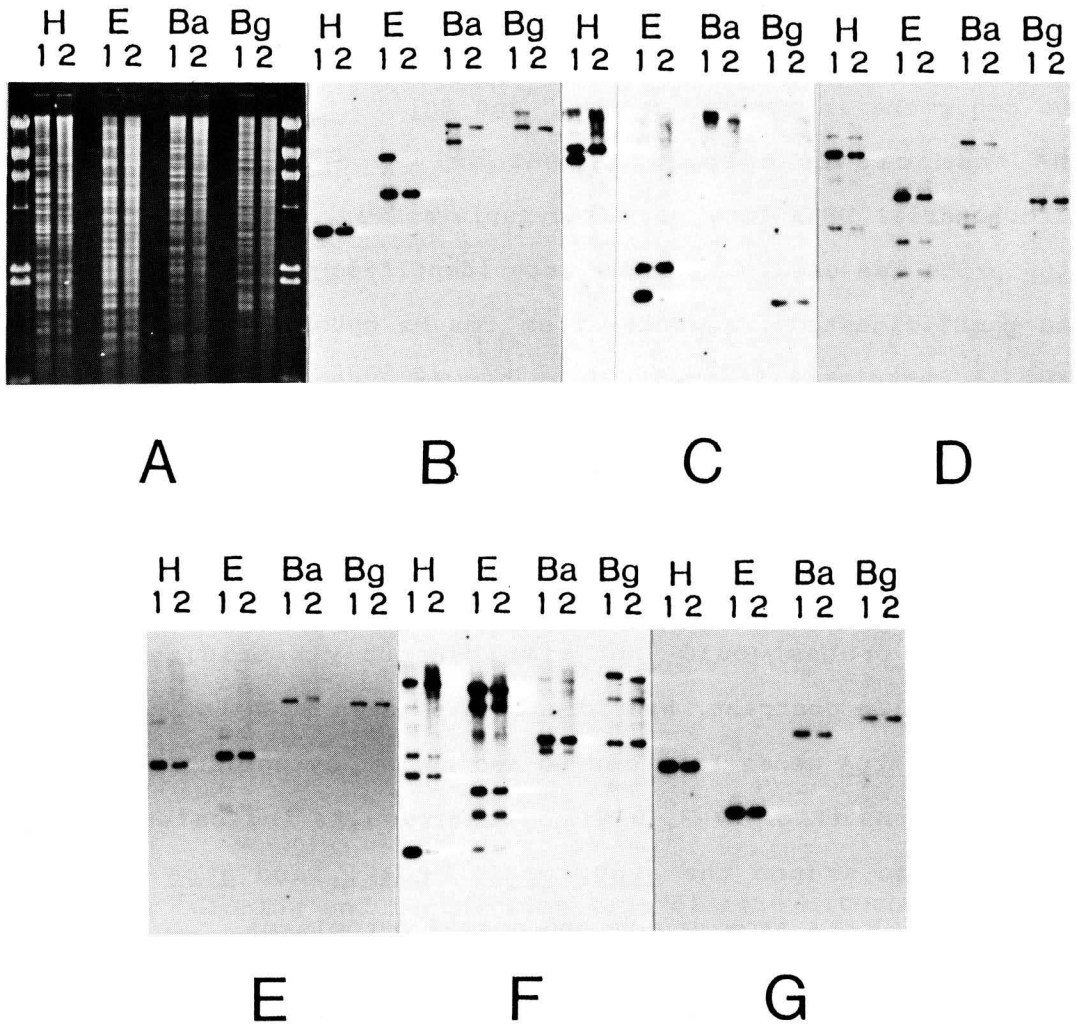**Fig. IV-3.** Agarose gel electrophoretic patterns of restricted mitochondrial DNA from cms-Bo (lane 1) and normal (lane 2) cytoplasms [A]. Southern blot analysis of panel A with synthesized oligonucleotide probes for the following genes; cob [B], atp6 [C], coxI [D], coxII [E], atp9 [F], and atpA [G]. Restriction endonucleases reacted are indicated on the panels as abbreviations: H, HindIII; E, EcoRI; Ba, BamHI; Bg, BglII.

different in mitochondrial DNA between cms-Bo and normal cytoplasms. To determine the copy number and to investigate the organization of the six genes, another four restriction enzymes, HindIII, EcoRI, BamHI, and BglII, were used for Southern hybridization analyses. There were two hybridization signals with the cob probe in EcoRI-, BamHI-, and BglII-digested mitochondrial DNA from cms-Bo cytoplasm, but only one in the restricted mitochondrial DNAs from normal cytoplasm (Fig. IV-3, B). When the atp6 probe was used, two bands were identified in EcoRI, HindIII-, and BamHI-digested fragments from cms-Bo cytoplasm, and only one band in restricted fragments from normal cytoplasm (Fig. IV-3, C). Above results suggested that mitochondrial DNA from cms-Bo cytoplasm had twice the copy numbers of the cob and atp6 genes than mitochondrial DNA from normal cytoplasm, since mitochondrial DNAs were completely digested with the restriction enzymes and the short nucleotide probes would not simultaneously hybridize over two fragments. In contrast, hybridization signals with the atp9, coxI, coxII, and atpA genes from cms-Bo and normal cytoplasms showed the same patterns (Fig. IV-3, D-G). These results indicated the same organization around the atp9, coxI, coxII, and atpA genes of mitochondrial DNA from cms-Bo and normal cytoplasms.

Cloning and characterization of DNA fragments homologous to cob gene

To know the gene expression of cob gene which is uniquely observed in cms-Bo cytoplasm, cloning of mitochondrial DNA fragments was carried out. Isolated mitochondrial DNAs from normal and cms-Bo cytoplasms were partially digested with EcoRI and cloned into Bluescript phagemid vector. Recombinant plasmids were
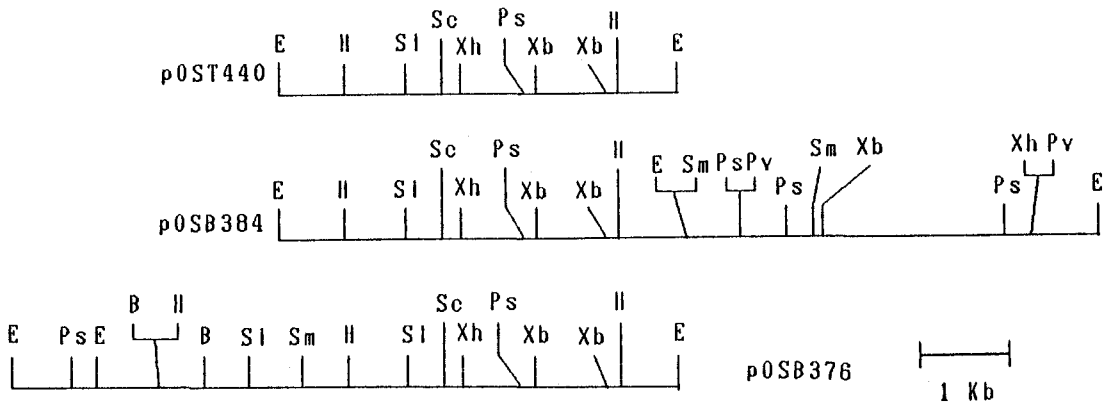
Fig. IV-4. Cloning and restriction maps of rice mitochondrial DNA fragments containing the cob gene and franking sequences. A pOST440 clone contained DNA fragment from normal cytoplasm. pOSB376 and pOSB384 clones possessed DNA fragments from cms-Bo cytoplasm. The oligonucleotide probe for cob gene had homology to 3 kb HindIII fragments of the three clones. Abbreviations: B, BamHI; E, EcoRI; H, HindIII; Ps, PstI; Pv, PvuII; Sc, SacI; Sl, SalI; Sm, SmaI; Xb, XbaI; Xh, XhoI.

obtained were screened for the cob gene by colony hybridization analysis. Five positive clones were selected and those clones were further characterized by their physical maps. Judging from their physical maps, two clones were finally isolated from mitochondrial DNA of cms-Bo cytoplasm, designating pOSB376 and pOSB384; and one was isolated from normal cytoplasm, designating pOST440; respectively (Fig. IV-4). Above results completely agree with the result of Southern blot-hybridization analysis (Fig. IV-3) that shows 6.6 and 4.2 kb EcoRI bands in cms-Bo cytoplasm, a 4.2 kb EcoRI band in normal cytoplasm, and a 3.0 kb HindIII band in both cytoplasms. Comparison of physical maps of the clones suggested that the cob gene is duplicated in the mitochondrial DNA of cms-Bo cytoplasm, while all of the cultivated Japonica rice plants tested has one copy of cob gene (data not shown).

Transcriptional alteration associated with cms

To confirm that mitochondrial DNA fragment cloned in pOSB376 was expressed in vivo and to investigate whether the gene transcription was affected by fertility-restorer gene, specific gene probes were hybridized to purified total mitochondrial RNA. Mitochondria were isolated and total mitochondrial RNA was purified. The RNA was fractionated by electrophoresis on formaldehyde-agarose gel and transferred to nitrocellulose filter. When a 6.6 kb EcoRI fragment of pOSB376 was used as a probe, one major transcript of >2.3 kb was detected (Fig. IV-5A). This is considerably larger than the 1164 bases which specify the coding region of the cob gene but this observation is also reported in maize cob gene by Dawson et al., (1984). Interestingly, fertility-restorer gene coded by nuclear affected the transcription of the 6.6 kb EcoRI fragment of
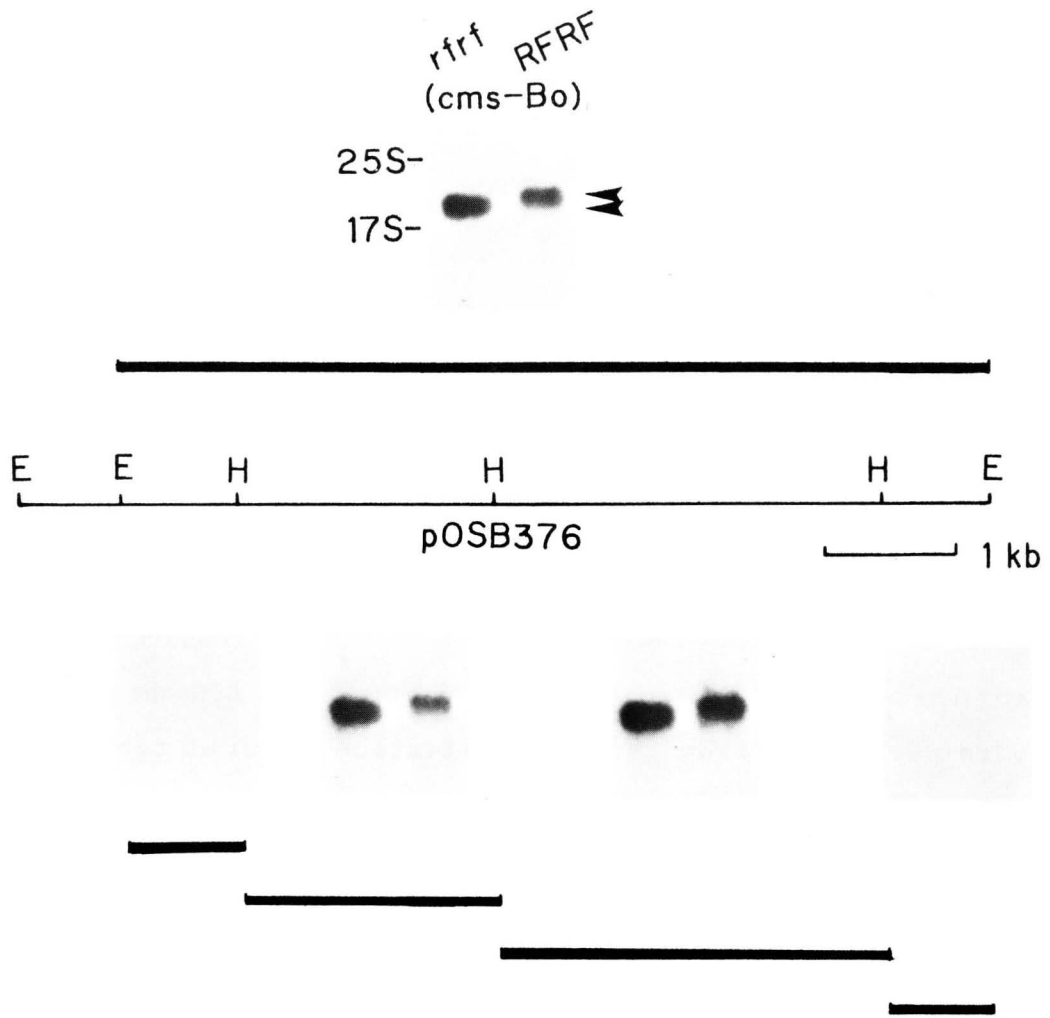
Fig. IV-5. Northern blot analysis of pOSB376 in male-sterile (nuclear genotype, rfrf) and fertility restored (RFRF) lines of the rice with cms-Bo cytoplasm. Mitochondrial RNAs were isolated from each line and separated by 1.2% agarose gel containing formaldehyde. Probe DNAs used are shown as bold lines.

pOSB376 and resulted in about 200 nucleotides longer transcript.
Northern-blot hybridizations by different restriction fragments as
probes showed that transcription was occurred in a HindIII-HindIII
DNA fragment of 5 kb. Above results suggested that the DNA fragment
of pOSB376 was involved in the occurrence of cms.

Nucleotide sequence analysis of cms-associated DNA fragment

The DNA sequences of pOSB376, pOSB384 and pOST440 were
partially determined by the chain-termination method. Nucleotide
sequence data obtained were analysed using software DNASIS.
pOSB384 and pOST440 were revealed to contain intact cob genes. On
the other hand, one unidentified open reading frame was observed
in pOSB376. The unidentified open reading frame had high sequence
homology to maize cob gene at 5' coding region of 1st-1002nd but
had almost no homology with the cob gene between the following
sequences at 3' coding region (Fig. IV-6). These results suggested
that intra- or intermolecular recombination was brought about at
1003rd nucleotide from the A of initiation codon of cob gene and
the fused new gene was constructed. Stop codon was found 1089th
from A of the initiation codon and this unidentified open reading
frame was designated as urfRM (unidentified reading frame of rice
mitochondria) which capable of coding for 41 kDa polypeptide. No
significant homology to the DNA sequence of 1003rd-1089th from the
initiation codon of urfRM gene could be found in any of the Genbank
database sequences, even though the entire mammalian mitochondrial
genome sequences is available. Hydropathy analysis of carboxyl
terminal regions of urfRM and rice cob genes show that both have
comparatively hydrophobic profiles (data not shown), although the

```
         A  L  P  F  F  K  E  M  Y  V  R  S  S  S  F  R  P  I  H  Q
urfRM    GCTTTACCTTTTTTTAAAGAAATGTATGTGCGTAGTTCAAGTTTTCGACCGATTCACCAA
         ****************************************************************
cob      GCTTTACCTTTTTTTAAAGAAATGTATGTGCGTAGTTCAAGTTTTCGACCGATTCACCAA
         A  L  P  F  F  K  E  M  Y  V  R  S  S  S  F  R  P  I  H  Q


         G  I  F  W  L  K  I  L  L  P  W  V  P  L  G  F  I  G  F  F
         GGAATATTTTGGTTGAAAATCCTCCTTCCCCGGGTTCCACTAGGATTTATAGGTTTTTTT
         **************   *     *   *    * * *    *   *   * **       *
         GGAATATTTTGGTTGCTTTTGGCGGATTGCTTACTACTAGGTTGGATCGGATGTCAACCT
          G  I  F  W  L  L  L  A  D  C  L  L  L  G  W  I  G  C  Q  P


         C  P  D  I  P  E  R  A  K  P  F  F  S  Y ---
         TGCCCGGATATCCCCGAGCGTGCTAAGCCTTTTTTTTTCATACTAAGTATTTTTTGTGTG
           **        *    *   *    *     ***  ***  *  **    *  **  ** *    *
         GTGGAGGCACCATTTGTTACTATTGGACAAATTTCTTCTTTCTTTTTCTTCTTGTTCTTT
          V  E  A  P  F  V  T  I  G  Q  I  S  S  F  F  F  F  L  F  F



         TGTGGATCCCGGCTGCTGCGACAGGGGGACA
           *  *  *     *  *  * ***   *   *   *
         GCCATAACGCCCATTCCGGGACGAGTTGGAAGAGGAATTCCAAAATATTACACGGAATAG
          A  I  T  P  I  P  G  R  V  G  R  G  I  P  K  Y  Y  T  E ---
```

Fig. IV-6. DNA sequence of 3' part of urfRM gene. The predicted amino acid sequence was translated according to the universal genetic code except CGG was translated as tryptophan (W) as proposed by Fox and Leaver (1981). Upper sequences indicate urfRM gene and lower ones maize cob gene reported by Dawson et al., (1984). Identical residues are shown by *.

number of amino acid residues is different. Relationship between the urfRM gene and the 31 kDa polypeptide is still unknown.

DISCUSSION

Heterogeneity of mitochondrial DNA of cultivated rice and wild rice is identified and geographical characters of the cytoplasms are observed (chapter II and III). Above results may imply that co-operated evolution between mitochondria and nuclear is generated, accompanied with geographical differentiation. Cms may be produced when cytoplasms are exchanged with such sexually isolated plants in specific combinations. Differential organization of mitochondrial DNA is strongly suggested to be involved in the occurrence of cms (chapter I and II). To localize the mitochondrial DNA region controlling the cms phenotype in rice, the DNA fragment uniquely observed in mitochondrial DNA from cms-Bo cytoplasm was obtained and its transcription was affected by the restorer-fertility gene. Nucleotide sequence analyses show that the fragment carries sequences homologous to 5' part of the coding region of rice cob gene but is flanked by non-homologous sequences to any genes; indicating generation of urfRM gene from intra- or intermolecular recombinational events of mitochondrial DNA (Fig. IV-6). Similar observations are found as followed. The TURE 2H3 DNA fragment containing the T-urf13 gene, which presumably arose through a series of novel recombinations within and between mitochondrial and chloroplast genomes, is suggested to be associated with the T type male sterility of maize (Dewey et al., 1986; Dewey et al., 1988). Rearrangements located within or near atpA gene influence the pattern of its transcripts in cms sunflower (Siculella and Palmer, 1988), The above results show that gene alterations are involved

in differential gene expression and association with cms. Product of urfRM could localize as a membrane-bound protein since hydrophobic profiles are similar to that of rice cob gene, although enzymatic activity is not examined. The structural analysis by computer of translational product of urfRM gene suggests one possibility for molecular mechanism of cms rice. If product of urfRM gene behaves like that of rice cob gene, as a competitor, respiratory activity might be decreased and energy generating system could not catch up the speed of pollen maturation. Such perturbation in respiratory activity may be critical to the anthers during meiosis, while aberrant efficiency in respiration may be tolerated the remainder of the plant.

Many factors are thought to be associated with pollen maturation and its degradation. The author believes the urfRM gene and/or the 31 kDa polypeptide is one of the strong candidates involving in the interference of pollen maturation and resulting in its degradation. In addition, the author thinks the restorer of fertility gene codes a mitochondrial RNA processing enzyme which processes the prematured mitochondrial RNA and changes it to translatable form.

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SUMMARY

CHAPTER I   Differences in the mitochondrial DNA between normal
              and cytoplasmically male-sterile rice

Chloroplast DNAs and mitochondrial DNAs were isolated from
normal and male-sterile cytoplasms of rice.   Restriction
endonuclease analysis of chloroplast DNA showed no detectable
difference in the two cytoplasms. On the other hand, mitochondrial
DNAs from the two cytoplasms showed different fragment patterns.
Mitochondrial plasmid-like DNAs were observed in the mitochondrial
DNA from cms-Bo male-sterile cytoplasm, while those were not
detected in normal cytoplasm.   Above results suggested that
mitochondria were involved in the occurrence of cytoplasmic male-
sterility.

CHAPTER II   Property of mitochondrial DNA in cytoplasmically
              male-sterile rice

   **II-1 Mitochondrial DNA polymorphism in male-sterile
       cytoplasm of rice**

Mitochondrial DNAs were isolated and purified from ten
strains of rice plants with male-sterile cytoplasm. The
mitochondrial DNAs were analyzed by the restriction endonucleases
and Southern blot hybridizations. Restriction fragments length
polymorphism was observed among the mitochondrial DNAs analysed,
that is, there were eight different patterns for the ten examined.
Above results are well correspond to the results of fertility-
restoration patterns of cytoplasm by restorer genes. Our results
show that there are a variety of mitochondrial DNAs in
cytoplasmically male-sterile rice and suggest that the differences
of mitochondrial DNA is involved in the different expression of

rice male-sterility.

II-2   Heterogeneity of mitochondrial plasmid-like DNAs
in cytoplasmically male-sterile rice

Mitochondrial DNAs were prepared from ten strains of rice with

male-sterile cytoplasms and fractionated by agarose gel elect-

rophoresis to investigate plasmid-like DNA in different male-

sterile cytoplasms. Cms-R cytoplasm had two plasmid-like DNAs of

1.09 and 0.96 kb, cms-UR106 cytoplasm had three plasmid-like DNAs

of 1.60, 1.09, and 0.96 kb, and the others had four plasmid-like

DNAs of 1.60, 1.25, 1.09, and 0.96 kb. Cytoplasmic properties of $\underline{O}$.

$\underline{rufipogon}$ and $\underline{O}$. $\underline{nivara}$ used as source of male-sterile cytoplasms

are suggested to be closer to Indica rice than Japonica rice.   The

DNA molecule of 1.09 kb from cms-Bo cytoplasm was suggested to be

intimately associated with protein(s) in linear form and the other

three DNA molecules seemed to be circular forms by the experiment

of extraction with or without proteinase K.   Southern hybridization

analysis showed the different sequence homology between the four

plasmid-like DNAs of different size classes in the cytoplasm, and

high sequence homology between the plasmid-like DNA of the same

size. Some of the plasmid-like DNAs from cms-Bo cytoplasm showed no

sequence homology between those in cms-T, cms-C, cms-S, and N

cytoplasms of maize.

CHAPTER III   Distribution of mitochondrial plasmid-like DNA in
cultivated rice ($\underline{O}$. $\underline{sativa}$) and its relationship
with varietal groups

Mitochondrial plasmid-like DNA was found in most of more than

100 rice cultivars ($\underline{O}$. $\underline{sativa}$) by the use of 0.7% agarose gel

electrophoresis.   The DNA was diverse in molecular weight and

number, and by electron microscopy, small circular DNAs of

different sizes were seen, in addition to the DNAs of high
molecular weight, even in the cultivars in which the plasmid-like
DNA was not detected by agarose gel electrophoresis. The detection
of the plasmid-like DNAs by agarose gel electrophoresis did not
depend on their presence or absence, but on their high
stoichiometry. The relationship between cytoplasms with the
plasmid-like DNAs and the varietal (for example, Indica rice)
groups was close. The geographical distribution of characteristic
cytoplasms was found.

**CHAPTER IV   Alterations of mitochondrial gene expression by
                introduction of fertility-restorer genes**

Southern blot analysis was used to identify differences in
the characteristics of mitochondrial DNAs of rice with normal and
male-sterile cytoplasms. Signals homologous to a probe for a <u>cob</u>
or an <u>atp6</u> gene were different in electrophoretic mobility between
the two cytoplasms. Furthermore, the copy number of the <u>cob</u> gene
was considered to be twice as many in the mitochondrial DNAs of
cms-Bo cytoplasm than that of normal cytoplasm. In four other DNA
probes for a <u>coxI</u>, a <u>coxII</u>, an <u>atpA</u>, and an <u>atp9</u> gene, no
differential gene organization was observed between the two
cytoplasms. A DNA fragment uniquely observed in cms-Bo cytoplasm
was cloned, designating pOSB376, and its transcription was altered
by introduction of fertility restorer gene. Nucleotide sequence
analysis showed default <u>cob</u> gene in pOSB376. In addition, <u>in vitro</u>
mitochondrial polypeptide synthesis was carried out and a 31 kDa
polypeptide was found in only male-sterile plant. Fertility
restorer gene was suggested to be mitochondrial RNA processing
enzyme.

LIST OF PUBLICATIONS

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Chapter I is described in reference (A).

Chapter II-1 is described in reference (C).

Chapter II-2 is described in reference (H).

Chapter III is described in reference (E).

Chapter IV is described in references (F).

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Koh-ichi Kadowaki