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Asymmetric hybridization between cytoplasmic male-sterile (CMS) and fertile rice (*Oryza sativa* L.) protoplasts

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Summary. ^{60}Co-irradiated protoplasts of the cytoplasmic male-sterile line A-58 CMS (*Oryza sativa* L.) were electrofused with iodoacetamide (IOA)-treated protoplasts of the fertile (normal) rice cultivar 'Fujiminori'. Seven of the colonies that formed were identified as cytoplasmic hybrids (cybrids): they all had the peroxidase isozymes of the fertile 'Fujiminori' parent, but contained four plasmid-like DNAs (B1, B2, B3 and B4) from the sterile A-58 CMS parent in their mitochondrial genomes. In addition, digestion of cybrid mtDNA gave a set of restriction fragments that differed from those of the parents.

Key words: Asymmetric protoplast fusion – Cytoplasmic male sterility – Electrofusion – Mitochondrial DNA – Rice

Introduction

The cytoplasmic male-sterility (CMS) phenotype is widely used in the commercial production of F_1 hybrid seed. In rice (*Oryza sativa* L.), transfer of the CMS cytoplasm requires 5–8 repeated back-crosses in order to exchange the nuclear genome of the cytoplasmic donor.

The CMS trait of irradiated protoplasts has been transmitted to various species to produce heteroplasmic fusion products (Zelcer et al. 1978; Aviv and Galun 1980; Menczel et al. 1983, 1987). In those experiments, only dicotyledonous species were used for the fusion parents. There has been no reported successful transmission of the CMS trait to a normal fertile variety of rice.

Recently, a number of hybrids have been obtained by sophisticated electrofusion methods (Bates et al. 1985; Morikawa et al. 1986; Vries et al. 1987). Somatic hybrids between rice and barnyard grass have also been obtained by electrofusion (Terada et al. 1987a).

In the present article, we report the efficient production and identification of asymmetric fused rice cells obtained by electrofusion. γ-Irradiation and iodoacetamide (IOA) inactivation were used to inhibit colony formation by the parental protoplasts, and peroxidase isozyme and mitochondrial (mt) DNA restriction patterns were used to identify the cybrids among the fusion products. Four plasmid-like DNAs, the characteristic, covalently closed circular (ccc) DNAs found in mitochondria of the male-sterile rice A-58 CMS, were found in the mitochondria of all cybrid lines. In addition, the mtDNA restriction patterns of the rice cybrids differed from those of both parents.

Material and methods

Material

Seeds of *Oryza sativa* A-58 CMS carrying the male-sterile cytoplasm of cv 'Chinsurah Boro II' were a gift from Prof. T. Kinoshita, Faculty of Agriculture, Hokkaido University. Suspension-cultured cells from the A-58 cytoplasmic male-sterile line (A-58 CMS) and the fertile cv 'Fujiminori' were produced as described elsewhere (Yamada et al. 1985, 1986).

Preparation of protoplasts

Protoplasts from suspension-cultured cells of A-58 CMS and fertile rice cv 'Fujiminori' were isolated according to the method of Yamada et al. (1985, 1986) with minor modification. A 1 g fresh weight (FW) aliquot of 3-day-old suspension-cultured cells was added to 20 ml filter-sterilized enzyme solution consisting of 1% Cellulase Onozuka RS (Yakult, Tokyo), 2% Cellulase Onozuka R-10 (Yakult), 0.1% Pectolyase Y-23 (Seishin Pharmaceutical, Tokyo), 0.8% calf serum (Nakarai Chem., Kyoto), 80 m*M* $CaCl_2$, 0.5 m*M* MES and 0.3 *M* glucose in Linsmaier and Skoog (LS) basal medium (1965). The pH of the solution was adjusted to 5.6 before it was filter sterilized. Protoplasts were

released by incubating the cells for 12 h at 26 °C without shaking. Untreated isolated protoplasts or inactivated ones were used for the fusion experiments.

Inactivation treatment

At the end of the enzyme treatment, all the A-58 CMS protoplasts in the enzyme solution were irradiated with a range of γ-rays (0.7–10.7 krad) from a ^{60}Co source (350 rad/min). The irradiated protoplasts were then washed once with the fusion chamber medium (0.5 *M* glucose and 2.5 m*M* $CaCl_2$, pH 5.6), after which they were used in the experiments on colony formation and fusion.

'Fujiminori' protoplasts were first separated from the enzyme solution, and then treated with 1.5–4.5 m*M* IOA in the chamber medium for 12 min at 4 °C in the dark. They were subsequently washed once with the chamber medium and then used in the experiments on colony formation and fusion.

Electrofusion and culture of protoplasts

Protoplasts were electrofused in a Somatic Hybridizer SSH-1 (Shimadzu Corporation, Kyoto, Japan). The electrofusion chamber used was SSH-C04 (outside dimensions, approximately 87 mm in diameter and 27 mm high; concentric ring-shaped electrodes; electrode spacing, 4 mm; electrode depth, approximately 2 mm).

Electrofusion was done on a clean bench and under microscopy. A 1.6 ml sample of protoplast mixture was combined with chamber medium containing equal amounts (2.5×10^4/ml) of A-58 CMS and 'Fujiminori' protoplasts and placed in the fusion chamber. Appropriate alternating (AC) and direct (DC) current electric field pulses were then applied to the chamber to induce protoplast fusion. Approximately 20 min after the pulses had been given, the electrically treated protoplasts were washed once with chamber medium, then cultured (2.5×10^5/ml) in RY-2 medium at 26 °C in the dark as reported previously (Yamada et al. 1985, 1986).

Isoelectric focusing

Three-gram (FW) samples of suspension-cultured cells were prepared from the fusion products, washed, and then homogenized in 6 ml buffer (0.05 *M Tris*-HCl, pH 7.5). The homogenate was centrifuged at 15,000 rpm for 30 min, after which its supernatant was collected and run through Sephadex G-25 to remove salts.

Aliquots of 100 µl extract were used for isoelectric focusing on polyacrylamide gels (pH 3.5–10). Isoelectric focusing was run for 5 h (200 v) at 4 °C. The peroxidase isozymes obtained were stained in a solution of 0.1% 3,3'-diaminobenzidine tetrahydrochloride, 0.015% H_2O_2, 0.1% NaN_3, 0.15 *M* NaCl, and 0.1 *M* phosphate buffer (pH 7.0). The gels were stained for alcohol dehydrogenase, catalase, malate dehydrogenase, glutamate dehydrogenase, acid phosphatase, aspartate aminotransferase, phosphoglucomutase, and glucosephosphate isomarase by the methods outlined by Vallejos et al. (1983).

MtDNA isolation and restriction endonuclease analysis

The procedures used to isolate and digest mtDNA were essentially those of Shikanai et al. (1987). The isolated mtDNAs from both the parents and the fusion products were digested with the appropriate enzymes: EcoRI, XhoI, SalI, BglII, PstI, and HindIII. The digests were electrophoresed at 3v/cm for 18–20 h on 0.8% agarose slab gels made with TBE buffer (89 m*M Tris*-borate, 89 m*M* Boric acid, and 2 m*M* EDTA). After electrophoresis, the gels were stained with ethidium bromide, washed briefly, and then photographed under 302 nm ultraviolet light.

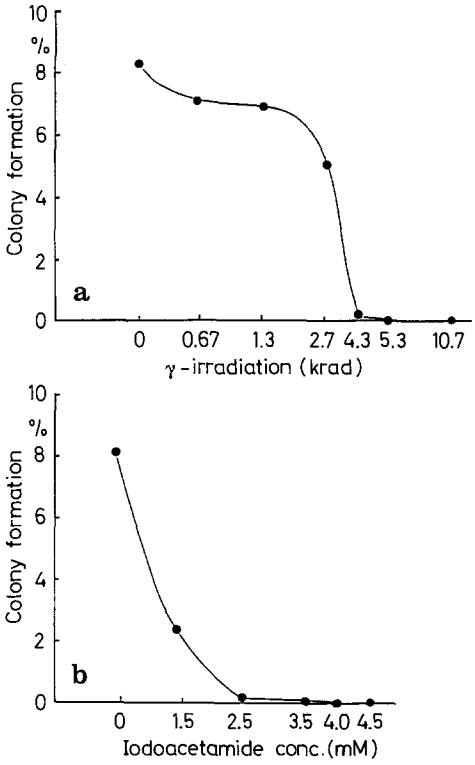

Fig. 1 a and b. Effects of inactivation treatment on colony formation by parental protoplasts. **a** γ-irradiation of A-58 CMS protoplasts. Intensity of the ^{60}Co irradiation was 20 krad/h. **b** IOA-inactivation of 'Fujiminori' protoplasts. Treatment conditions: 12 min, 4 °C. Colony formation = (no. of colonies formed)/(no. of protoplasts inoculated) × 100

Southern hybridization

The Southern hybridization method used was essentially that of Shikanai et al. (1987). Isolated mtDNAs from both the parents and the fusion products were digested with the appropriate restriction enzymes (Shikanai et al. 1987). B1 (2.1 kbp) and B4 (0.97 kbp) were digested with EcoRI and XhoI, respectively, at one site. Fragments were separated on a 0.8% agarose gel, then transferred to Gene Screen membranes (New England Nuclear) by the Southern method (Southern et al. 1975). The membranes were probed with sequences of B1 and B4 labelled with [α-^{32}p] dCTP (Amersham 3,000 Ci/mmol).

Results

Optimization of inactivation and electrofusion conditions

The effects of the inactivation treatment on colony formation by the parental protoplasts are shown in Fig. 1. After 4 weeks, only a few colonies (4×10^{-7}) were formed in the 4.3 krad γ-irradiated cultures of A-58 CMS protoplasts; no colonies appeared, however, after treatment with more than 5.3 krad γ-rays. After 4 weeks, very few colonies (1×10^{-7}) formed in cultures treated with 2.5 m*M* IOA; none were observed after treatment with more than 4.0 m*M* IOA. Therefore, two complementary

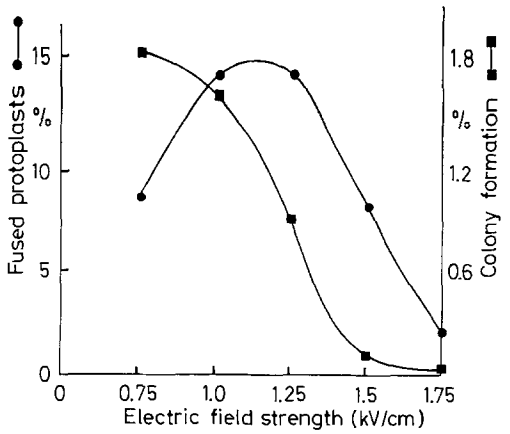

Fig. 2. Effect of DC field strength on protoplast fusion and colony formation. A single DC pulse of 40 μs was given to a 1:1 mixture of 'Fujiminori' and A-58 CMS protoplasts that had not been pretreated with γ-rays or IOA. See text for details. Protoplast fusion = (no. of fused protoplasts)/(no. of inoculated protoplasts) × 100; colony formation = (no. of colonies formed)/(no. of inoculated protoplasts) × 100

combinations of differently inactivated protoplasts were used for asymmetric hybridization: (A) A-58 CMS protoplasts were irradiated with 4.3 krad γ-rays, and 'Fujiminori' protoplasts were treated with 2.5 m*M* IOA (treatment 1); and (B) A-58 CMS protoplasts were irradiated with 5.3 krad γ-rays, and 'Fujiminori' protoplasts were treated with 4.0 m*M* IOA (treatment 2).

We also determined the effect of the voltage of the AC field on protoplast pair formation. AC fields of various strengths were applied for 60 sec at 500 kHz, the respective pair formations being 5.8%, 12.0%, 32.0%, and 21.0% for fields of 25, 50, 100, and 200 Vp-p/cm. Thus, 100 Vp-p/cm at 500 kHz for 60 sec was used as the AC field condition for pair formation.

We next examined the effect of the DC field strength on fusion frequency and colony formation for a fixed period of 40 μs. One-to-one mixtures of A-58 CMS and 'Fujiminori' protoplasts that had not been pretreated with γ-rays or IOA were electrofused at various field strengths. After the electric pulses had been applied, fused protoplasts were identifiable by their distinct morphology. The electric field strength markedly affected protoplast (homo- and hetero-plasmic) fusion frequency and colony formation – the optimal electric field strength being 1 kV/cm (Figs. 2 and 3). Fusion frequency peaked at 1 and 1.25 kV/cm, but colony formation decreased as the field strength increased. Consequently, we used a DC pulse of 1 kV/cm at 40 μs, by which we obtained a protoplast fusion of 14% and colony formation of 1.6% relative to the total protoplast population (Fig. 3).

Selection and identification of cybrids

We fused A-58 CMS and 'Fujiminori' protoplasts that had been inactivated by treatment 1 or 2 (see Inactivation

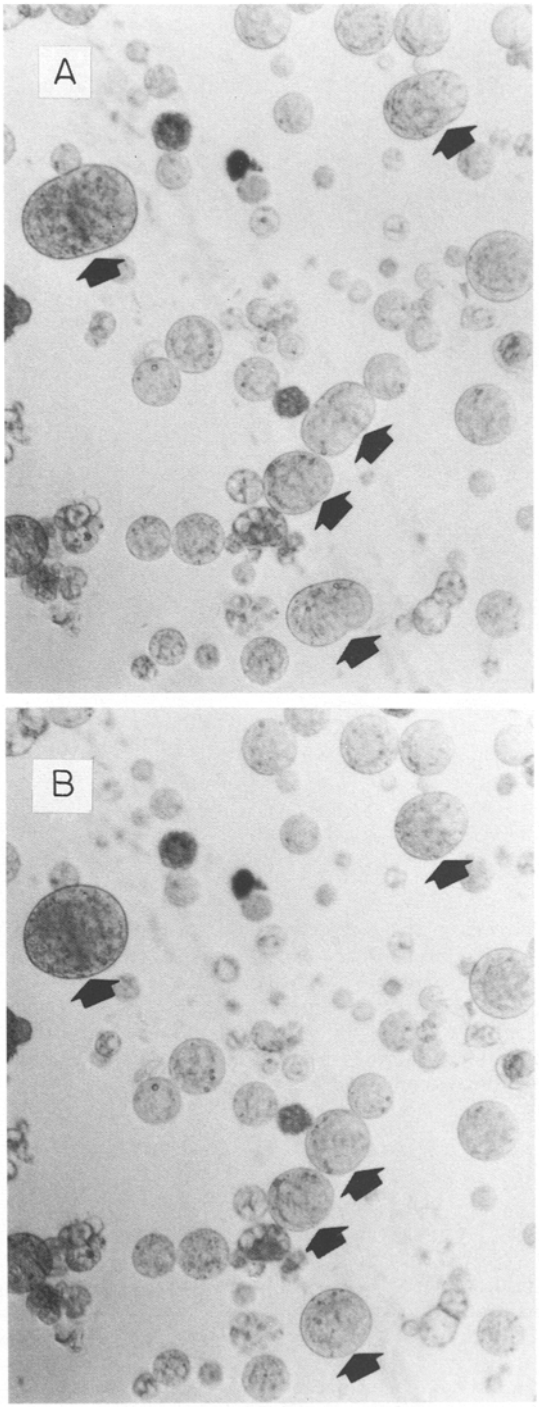

Fig. 3 A and B. Electrofusion of A-58 CMS and 'Fujiminori' protoplasts. **A** 1 min after DC pulsation. *Arrows* indicate adhering protoplasts. **B** 6 min after pulsation. *Arrows* indicate fused protoplasts

treatment) under the optimal electrical conditions described above, then cultured the protoplasts. At present (7 months after fusion), we have obtained seven colonies each from treatments 1 and 2, which we have numbered 1–1 to −7 and 2–1 to −7.

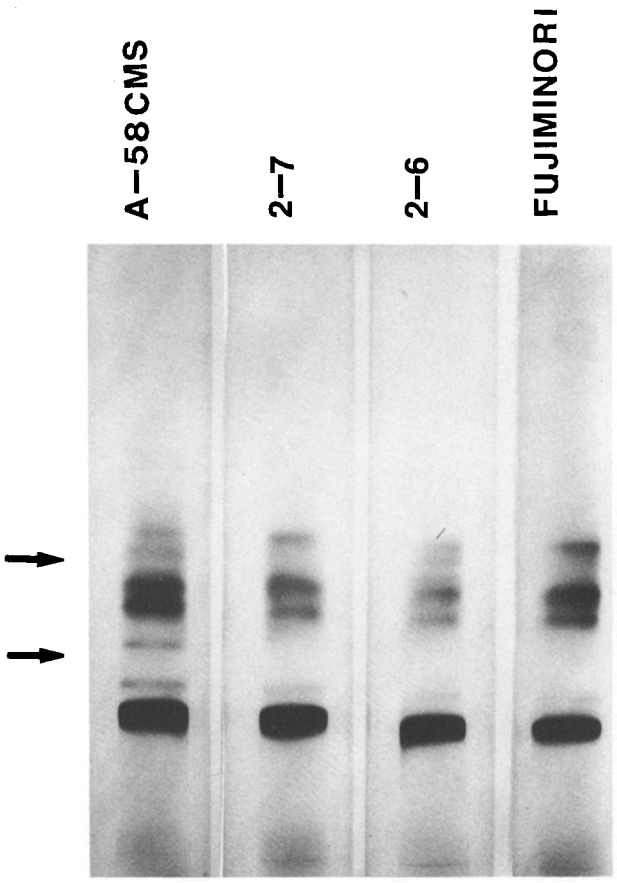

Fig. 4. Peroxidase isozyme patterns for A-58 CMS, 'Fujiminori', one cybrid line (2–7) and one hybrid line 2–6 (see text for details). The two bands in A-58 CMS indicated by the *arrows* are not present in the pattern for 'Fujiminori'. The cybrid line has a pattern identical to that of 'Fujiminori'. Line 2–6 has an isozyme pattern that differs from the patterns of both parents and has only one of the two unique bands of A-58 CMS

We next analyzed the isozyme patterns of alcohol dehydrogenase, glutamate dehydrogenase, catalase, malate dehydrogenase, acid phosphatase, aspartate aminotransferase, phosphoglucomutase, glucosephosphate isomerase and peroxidase in these cell lines. Only peroxidase showed distinct differences in its isozyme patterns in the parental A-58 CMS and 'Fujiminori' cells. The two unique bands of A-58 CMS (arrow, Fig. 4) were not present in the pattern of 'Fujiminori'. This figure also shows the peroxidase isozyme patterns of fused cell lines 2–6 and –7. Of the 14 fusion products, 10 (1–2, –3, –4, –6 and –7, and 2–1, –2, –4, –5 and –7) showed the peroxidase isozyme pattern of 'Fujiminori', and 2 (1–1 and –5) the pattern of the A-58 CMS type. Two lines (2–3 and –6) had isozyme patterns that differed from those of both parents, one of the two unique bands of A-58 CMS was present in the patterns of these two lines (Fig. 4, data for 2–3 not shown).

Parental mitochondrial genomes were identifiable by the restriction endonucleases used (BglII, SalI, HindIII, PstI, EcoRI, and XhoI). While both parents share a common set of bands, each also has its own unique bands. Figure 5 shows that the parental A-58 CMS has four unique bands, and 'Fujiminori' more than eight. After digesting the mtDNAs of the 14 fusion products with the 6 restriction endonucleases, we compared the resulting restriction fragment patterns. On the basis of these patterns, the 14 fusion products formed three groups: a heteroplasmic type (nine lines, 1–2, –4 and 2–1 to 2–7), the 'Fujiminori' type (three lines, 1–3, –6 and –7) and the A-58 CMS type (two lines, 1–1 and –5).

The heteroplasmic type had a restriction fragment pattern in which some fragments of the mixture of both parents were missing. For example, this type had two of the four A-58 CMS PstI restriction bands and six of the

Table 1. Distribution of unique parental restriction bands in fusion products

	No. of unique parental bands											
	BglII		SalI		HindIII		PstI		XhoI		EcoRI	
	A	F	A	F	A	F	A	F	A	F	A	F
Parents	6	5	1	12	1	7	4	9	2	5	1	6
Fusion products												
Heteroplasmic type	3	4	0	10	1	6	2	6	1	3	1	4
Fujiminori type	0	5	0	12	0	7	0	9	0	5	0	6
A-58 CMS type	6	0	1	0	1	0	4	0	2	0	1	0

A, A-58 CMS; F, 'Fujiminori'

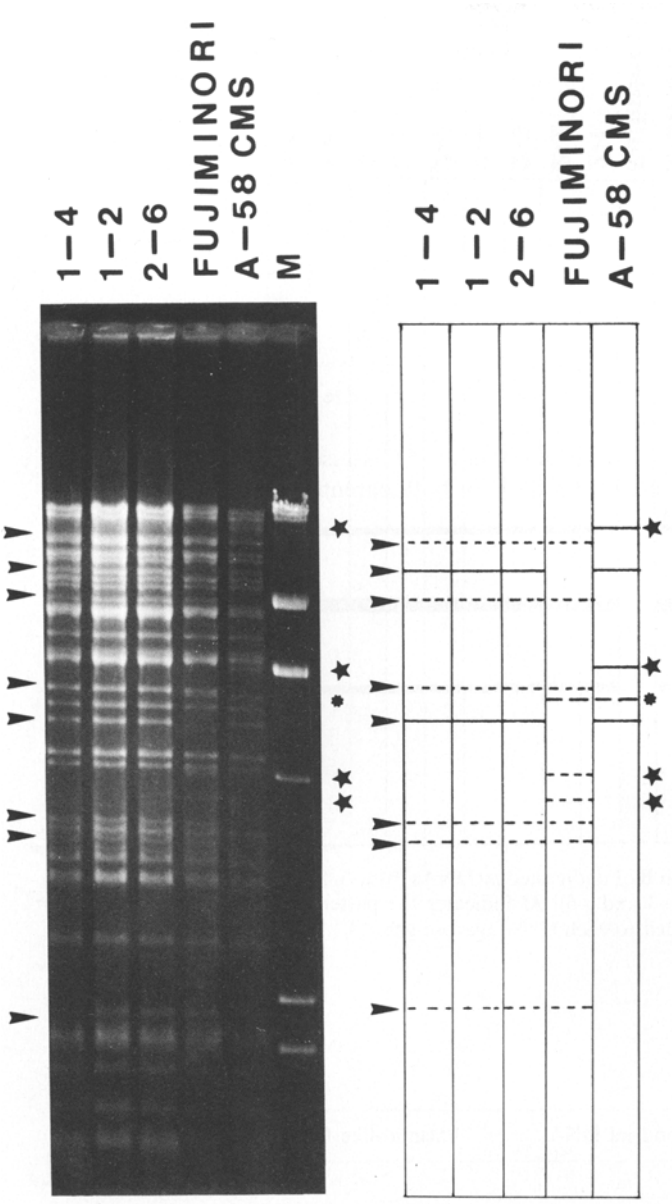

Fig. 5. Photograph of an agarose gel electrophoresis of mtDNAs that had been digested with PstI (*left*). Diagram shows unique PstI parental bands and their distribution in cybrid lines 1–2 and 1–4, and in hybrid line 2–6. The *arrows* indicate the unique parental bands present in all cybrid and hybrid lines; the *stars*, the unique parental bands that are absent from them. The *asterisk* indicates that a band common to both parents is missing in all cybrid and hybrid lines tested. A 1 μg sample of mtDNA was loaded in each well. Electrophoresis was done on a 0.8% agarose gel at 60 V for 20 h. *M* indicates the patterns of HindIII digest of bacteriophage lambda DNA

eight 'Fujiminori' PstI bands (Fig. 5, data for 2–1, −2, −3, −4, −5 and −7 not shown). A PstI band common to both parents (indicated by the asterisk in Fig. 5) was missing in the mtDNA of all the heteroplasmic type lines. The 'Fujiminori' type had an mtDNA restriction pattern that was identical to that of 'Fujiminori', and the A-58 CMS type, a pattern identical to that of A-58 CMS. The results of the restriction fragment pattern analysis are summarized in Table 1.

We earlier reported (Shikanai et al. 1987) the presence of four plasmid-like DNAs (B1, B2, B3 and B4) in the mitochondria of the cytoplasmic male sterile line A-58 CMS. We therefore decided to analyze the undigested mtDNAs of the 14 fusion products for the presence of these plasmid-like DNAs. Interestingly, both the heteroplasmic and A-58 CMS type lines had four bands attributable to these plasmid-like DNAs (Fig. 6); no plasmid-like DNAs were found in the mitochondria of the 'Fujiminori' type lines. In addition, cloned B1 and B4 were labelled with ^{32}P and used as probes in Southern blot analyses of the mtDNAs from the 14 lines. The plasmid-like DNA B1 probe hybridized to one A-58 CMS fragment of 2.1 kbp, and the B4 probe to one A-58 CMS fragment of approximately 0.97 kbp (Fig. 7).

Discussion

Metabolic complementation between irradiated and IOA-treated protoplasts, originally described for *Nicotiana* by Sidorov et al. (1981), has been successfully used to recover rice cybrids. Cybrid formation was confirmed in 7 of the 14 lines by studying their peroxidase isozyme and mtDNA restriction-fragment patterns, and their plasmid-like DNAs (Table 2). Of these 14 cell lines, 10 showed the 'Fujiminori' type zymogram of peroxidase (the 'Fujiminori' type nuclear genome). Seven of these ten lines (1–2 and 1–4, and 2–1, −2, −4, −5, and −7) were considered cybrids because they had both mtDNA restriction patterns of the heteroplasmic type and plasmid-like DNAs that originated from A-58 CMS mitochondria. Three cell lines (1–3, −6, and −7) were considered 'Fujiminori' escapes because they had peroxidase isozyme and mtDNA restriction patterns of the 'Fujiminori' type, but no plasmid-like DNA characteristic of A-58 CMS. Of the remaining four cell lines, two (1–1 and −5), which had peroxidase isozyme and mtDNA restriction patterns of the A-58 CMS type and plasmid-like DNA bands, were considered A-58 CMS escapes. The origin of the other two lines (2–3 and 2–6) is uncertain, but they could be considered hybrids since they had an isozyme pattern that differed from both parents, which consisted of one of the two unique bands of A-58 CMS, an mtDNA restriction pattern of the heteroplasmic type, and four plasmid-like DNA bands.

We successfully combined γ-ray irradiation (Sidorov et al. 1981) of the protoplasts of a sterile A-58 CMS parent with IOA treatment (Nehls 1978; Terada et al. 1987b) of a fertile 'Fujiminori' parent. The results indicate that the γ-ray dose and IOA concentration were the

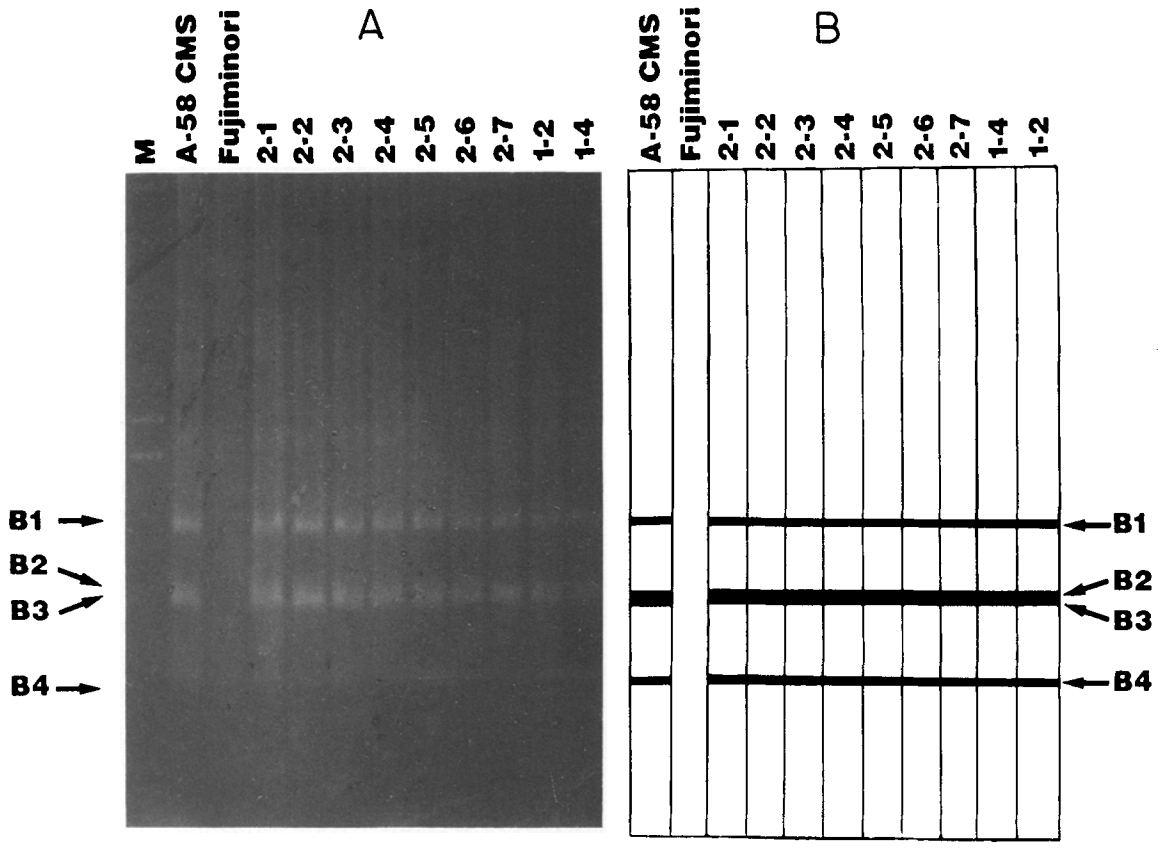

Fig. 6 A and B. Agarose gel electrophoresis patterns **A**, and a diagram **B** of undigested mtDNAs from A-58 CMS, 'Fujiminori', cybrid lines (1–1 and –4, 2–1, –2, –4, –5 and –7), and hybrid lines (2–3 and –6). *M* indicates the patterns of the HindIII digest of bacteriophage lambda DNA. A 10 μg sample of mtDNA was applied to each 0.8% agarose gel

Table 2. Properties of fusion products

Number of colonies	Peroxidase	Mitochondrial DNA	Plasmid-like DNA	Classification
1: Double-inactivation treatment 1 (4.3 krad γ-rays, 2.5 m*M* IOA)				
1–1	A[a]	A	+[b]	A escape[c]
1–2	F	A+F	+	Cybrid
1–3	F	F	–	F escape
1–4	F	A+F	+	Cybrid
1–5	A	A	+	A escape
1–6	F	F	–	F escape
1–7	F	F	–	F escape
2: Double-inactivation treatment 2 (5.3 krad γ-rays, 4.0 m*M* IOA)				
2–1	F	A+F	+	Cybrid
2–2	F	A+F	+	Cybrid
2–3	A+F	A+F	+	Hybrid
2–4	F	A+F	+	Cybrid
2–5	F	A+F	+	Cybrid
2–6	A+F	A+F	+	Hybrid
2–7	F	A+F	+	Cybrid

[a] A, A-58 CMS type; F, 'Fujiminori' type; A+F, Composed of A-58 CMS and 'Fujiminori'
[b] +, Detected; –, not detected
[c] A escpae, A-58 CMS escape; F escape, 'Fujiminori' escape

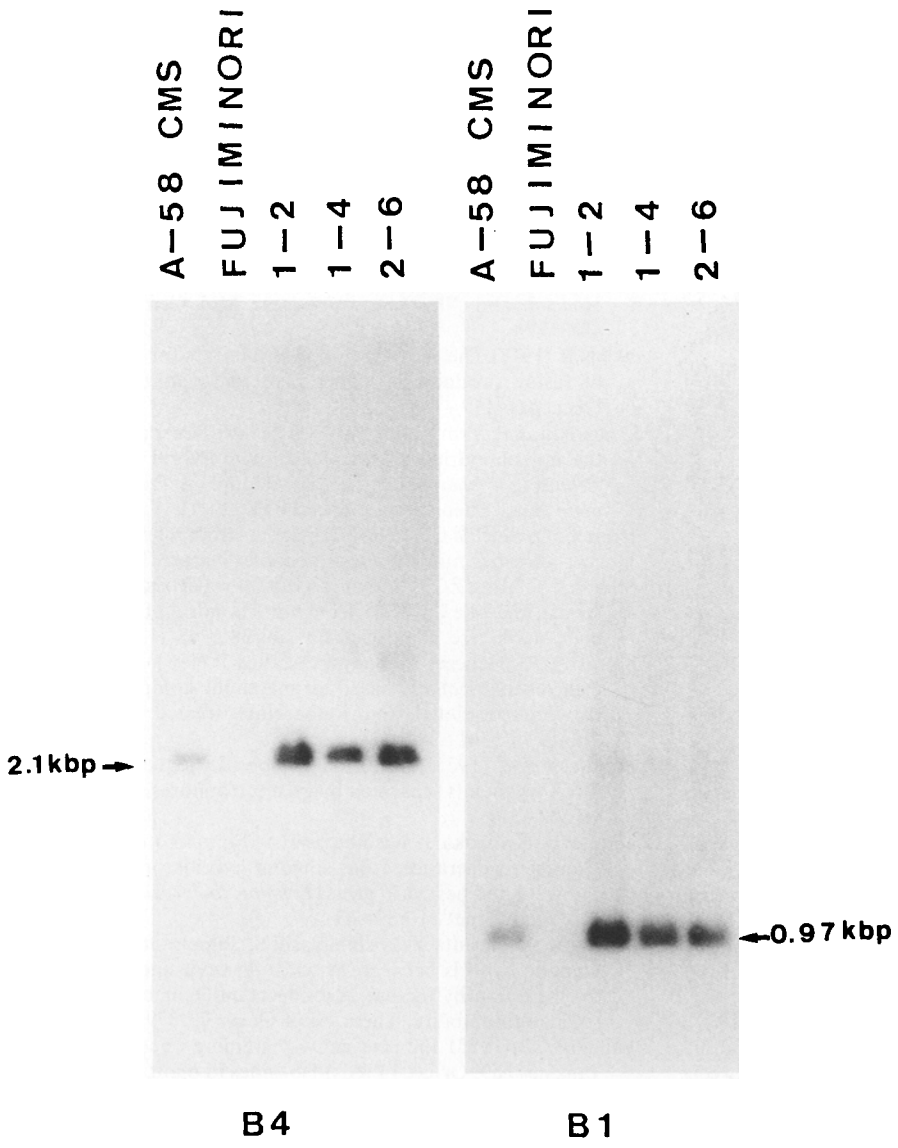

Fig. 7. Hybridization of plasmid-like DNAs (*B1* and *B4*) to mtDNAs from A-58 CMS, 'Fujiminori', cybrid lines (1–2, –4) and (2–1, –2, –4, –5 and –7; not shown); and hybrid lines (2–6) and (2–3; not shown). B1 was digested with EcoRI, and B4 with XhoI. A 1 μg sample of mtDNA was applied to an 0.8% agarose gel

most important factors for the efficient production of cybrids (Table2). After treatment 2 (5.3 krad γ-rays and 4.0 m*M* IOA), five of the seven colonies formed were cybrids (Table 2); at a lower irradiation and lower concentration (treatment 1, 4.3 krad γ-rays and 2.5 m*M* IOA), however, more than 70% of the colonies formed were A-58 CMS or 'Fujiminori' escapes.

The electrofusion program will also affect the success rate. Our results indicate that the strengths of the AC and DC fields are very important factors in obtaining high fusion frequency (see Electrofusion conditions).

The mtDNAs of the somatic hybrids reported in earlier studies differ from each other (Belliard et al. 1979; Galun et al. 1982; Nagy et al. 1981; Ozias-Akins et al. 1987), and our mtDNA digestion patterns are different from previously published patterns. Seven cybrid lines and two hybrid lines (2–3 and –6) that we analyzed showed identical restriction endonuclease patterns for each type of line (Table 1, Fig. 5). These identical patterns could be produced if recombinations were limited to specific sites on mtDNA molecules, such as repeats (Lonsdale et al. 1984; Palmer and Shields 1984; Kothari et al. 1986). It is also possible that numerous new mtDNA arrangements might be formed, but that the one found in these cybrids allows maximal mitochondrial survival (Kothari et al. 1986). The formation of identical restriction patterns for the cybrids was not a coincidence because we obtained the same type of cybrids from different culture dishes after asymmetric fusion.

The mitochondrial genomes in rice cybrids differ from that of *Daucus carota* and other species in another respect. Novel fragments not present in either parent have been found in *Daucus carota* and other species (Belliard et al. 1979; Nagy et al. 1981; Galun et al. 1982;

Matthews et al. 1985; Kothari et al. 1986; Ozias-Akins et al. 1987). Nonparental mtDNA patterns have been interpreted as being the result of genetic recombination of the parental mitochondrial genomes (Belliard et al. 1979; Galun et al. 1982). The reasons for the no novel fragments in our cybrids are not known but one possibility is that different species may have different recombination mechanisms.

The four plasmid-like DNAs present in the mitochondria of all our rice cybrids was stable even when the A-58 CMS nuclear genome was substituted for by the 'Fujiminori' nuclear genome when the mitochondria of both parents became mixed after protoplast fusion. The characteristics of four plantlets regenerated from one cybrid line (2–7) are now being investigated.

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