Theor Appl Genet (1995) 91:563–567

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Stable inheritance and expression of the CMS traits introduced by asymmetric protoplast fusion

Received: 12 October 1994 / Accepted: 27 January 1995

Abstract The donor-recipient protoplast fusion method was used to produce cybrid plants and to transfer cytoplasmic male sterility (CMS) from two cytoplasmic male-sterile lines MTC-5A and MTC-9A into a fertile japonica cultivar, Sasanishiki. The CMS was expressed in the cybrid plants and was stably transmitted to their progenies. Only cytoplasmic traits of the male-sterile lines, especially the mitochondrial DNAs, were introduced into the cells of the fertile rice cultivar. More than 80% of the cybrid plants did not set any seeds upon selfing. Sterile cybrid plants set seeds only when they were fertilized with normal pollen by hand and yielded only sterile progenies. This maternally inherited sterility of the cybrid plants showed that they were characterized by CMS. The CMS of cybrid plants could be restored completely by crossing with MTC-10R which had the single dominant gene *Rf-1* for restoring fertility. These results indicated that CMS was caused by the mitochondrial genome introduced through protoplast fusion. The introduced CMS was stably transmitted to their progenies during at least eight backcross generations. These results demonstrate that cybrids generated by the donor-recipient protoplast fusion technique can be used in hybrid rice breeding for the creation of new cytoplasmic male-sterile rice lines.

Key words *Oryza sativa* L. · Cytoplasmic male sterility (CMS) · *atp6* · Cybrid · Protoplast fusion

Introduction

Cytoplasmic male sterility (CMS) is widely known in higher plants. It inhibits the development of mature pollen and is due to incompatibility between nuclear and cytoplasmic gene products (Newton 1988; Levings and Brown 1989). In rice, CMS was initially observed by Katsuo and Mizushima (1958) who reported that the CMS trait was expressed when the nuclear genome of *Oryza perennis* M was replaced with that of the japonica cultivar Fujisaka 5. A number of cases of nuclear-cytoplasm combinations causing CMS in rice have been reported (Li and Zhu 1986; Virmani and Shinjyo 1988).

When the nucleus of Chinsurah Boro II (indica rice) was replaced with that of Taichung 65 (japonica rice), the resulting combination was cytoplasmic male-sterile (Shinjyo 1969). This CMS system is known as the ms-bo type or BT type. BT-type CMS is restored by the nuclear gene *Rf-1* which was initially identified in Chinsurah Boro II (Shinjyo 1975).

It takes about 6–9 years to convert fertile cultivars into cytoplasmic male steriles by the recurrent backcrossing method. Recently, a new system that enables the CMS trait to be transferred into the fertile line was established using donor-recipient protoplast fusion in *Nicotiana* (Zelcer et al. 1978), *Brassica* (Barsby et al. 1987; Menzel et al.), and *Daucus* (Tanno-Suenaga et al. 1988). After the nuclei of the donor parent have been inactivated by X-or γ-irradiation, the cytoplasm of the donor is selectively introduced into the recipient by cell fusion. In rice, cybrid plants were created by such a donor-recipient protoplast fusion technique between fertile cultivars and cytoplasmic male-sterile lines. In these studies, mitochondrial genomes of the CMS lines were introduced into recipient plants (Akagi et al. 1989; Kyouzuka et al.; Yang et al. 1989).

The cytoplasmic male-sterile *Brassica* cybrids obtained through protoplast fusion became fertile through dynamic changes in the mitochondrial genomes (Bonhomme et al. 1991). Although most of the rice cybrid plants studied earlier were sterile (Akagi et al. 1989), we wished to study; (1) the stability of inheritance of newly created CMS, and (2) the complete restoration of fertility in progenies of cybrid plants with the *Rf-1* gene.

Thus, in this study we have evaluated the sterility and the restoration of fertility of rice cybrids and their

Communicated by G. S. Kush

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progenies and we report on the genetic nature of sterility in these rice cybrid plants and discuss the advantage of this method for producing new cytoplasmic male-sterile lines.

Materials and methods

Plant materials

MTC-5A is a cytoplasmic male-sterile line with the nuclear genome derived from a japonica rice, Reimei, and the cytoplasm from an indica rice, Chinsurah Boro II. MTC-9A is another male-sterile line with the nuclear genome from a japonica rice, Syurei, and cytoplasm from IR-24 (Li and Zhu 1986). These two lines (MTC-5A and MTC-9A) are classified into two different types, BT (ms-bo or BT-type) and Liao (LI-type), respectively (Li and Zhu 1986; Virmani and Shinjyo 1988). The restorer strain used here for both the BT- and the LI-type of CMS has the nuclear genome from Taichung 65 with *Rf-1*, a nuclear restorer gene which had been introduced from Chinsurah Boro II (Shinjyo 1975). This strain, designated here as MTC-10R, was referred to as BT-A in the original report (Shinjyo 1975). Sasanishiki is a fertile Japonica cultivar.

Production of cybrids

Cybrid plants were produced as described previously (Akagi et al. 1989). The cytoplasm-donor protoplasts of CMS lines were X-irradiated at 125 krad (1.25×10^3 Gy) (2 krad/min), and the recipient protoplasts of the fertile cultivar were treated with 30 mM of iodoacetamide (IOA) for 10 min at 27 °C. The population density of protoplasts was adjusted to 2×10^7/ml. The suspension of X-irradiated protoplasts and that of the IOA-treated protoplasts were mixed at a 2:1 ratio, and then electrofused. Plants were regenerated from the electrofused protoplasts.

Analysis of mitochondrial DNA

Calli were initiated from the shoot apex of regenerated cybrid plants. Suspension cultures were established by transferring these calli to liquid medium (Fujimura et al. 1985). Mitochondrial DNAs were prepared from suspension-cultured cells as described previously (Akagi et al. 1989). The mitochondrial DNAs were digested with the restriction endonucleases *Bgl*II and *Hin*dIII. The resultant fragments were separated by electrophoresis on an 0.8% agarose gel.

Chromosome counting

The chromosomes counts were made according to Nishibayashi and Kaeriyama (1986), but with the modification of statining the chromosomes with 4',6-diamidino-2-phenylindole (DAPI) (Akagi et al. 1989). Chromosomes counts were made from more than four metaphase plates in each sample.

Examination of the character and restoration of sterility of cybrids

The regenerated plants were transplanted to pots. The fertility of selfed progenies was investigated by enclosing panicles with bags. The seed fertility was estimated from the ratio of fertile spikelets in a panicle.

Results

Production of cybrid plants

The protoplasts of the cytoplasmic male-sterile donor lines were X-irradiated to inactivate their nuclei before cell fusion. We also inactivated the protoplasts of recipient Sasanishiki by IOA treatment. Colonies regenerated only after the X-irradiated protoplasts of MTC-9A had been fused with the IOA-treated prtoplasts of Sasanishiki, but no colonies were formed without cell fusion. Metabolic complementation between nuclear and cytoplasmic compartments restores the ability of cell division in fused protoplasts (Zelcer et al. 1978). The plants regenerated from these colonies after transferring onto regneration medium were putative cybrids. Similarly, putative cybrid plants were also observed when another cytoplasmic male-sterile line, MTC-5A, was used as a cytoplasmic donor.

Characterization of cybrid plants

We selected two and four putative sterile cybrid plants with the cytoplasm of MTC-5A and MTC-9A, respectively, from amongst 72 plants which had normal morphological features. The nuclear characters were determined by analyzing their chromosome numbers and their morphology. All of them had 24 chromosomes and were morphologically quite similar to the recipient, Sasanishiki. These results indicated that no chromosomes had been transferred from the CMS parents.

The origin of the mitochondrial genomes of these cybrids was determined from the restriction patterns of their mitochondrial DNAs. All plants had the fragments specific to the each CMS parent (Figs. 1 and 2, arrow A) in addition to the fragments specific to the fertile parent (Figs. 1 and 2, arrow B). The restriction patterns of mitochondrial DNAs from some of the regenerated plants were different from that of either parent (Figs. 1 and 2, arrows C and D).

These findings showed that only the cytoplasmic traits, and not the nuclei, of male-sterile lines were introduced into the fertile cultivar. The regenerated plants were cytoplasmic hybrids (cybrids) which had the nucleus of the fertile cultivar and the cytoplasm of both parental strains.

Sterility of the cybrid plants

Table 1 shows the fertility of the cybrid plants. Among 142 regenerated cybrid plants, we selected 72 which had normal morphology, and then studied their seed fertility. More than 80% of these cybrid plants did not set any selfed seed. On the other hand, only one plant was sterile among 35 control plants regenerated from the protoplast of Sasanishiki without cell fusion. These findings suggested that the sterility of the cybrids had been caused by the cytoplasmic traits introduced from the parents.

We crossed ten sterile cybrid plants with Sasanishiki in order to examine the female fertility of sterile cybrids. All cybrid plants set many seeds (Table 2). These results showed that male-sterile cybrid plants were female fertile.

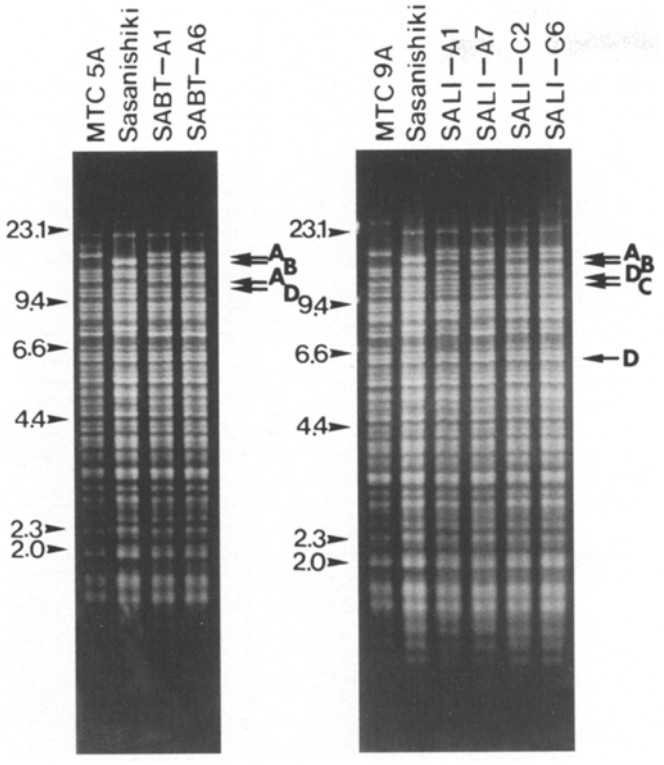

Fig. 1 *Bgl*II restriction patterns of mitochondrial DNA from both parental and cybrid plants. *Arrows* indicate both MTC-5A and MTC-9A CMS (*A*)-, Sasanishiki (*B*)- and cybrid (*C*)-specific bands. *Arrows D* indicates the band absent from cybrids

Fig. 2 *Hin*dIII restriction patterns of mitochondrial DNA from both parental and cybrid plants. *Arrows* indicate both MTC-5A and MTC-9A CMS (A)-, Sasanishiki (B)- and cybrid (C)-specific bands

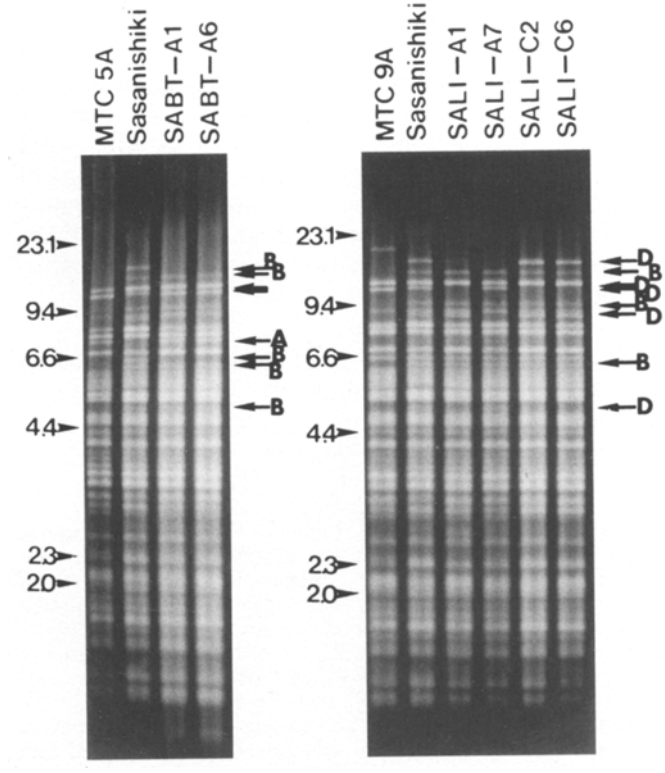

Table 1 Fertility of cybrid plants

Source of cytoplasm	Number of diploid plants		Ratio of sterile plants (%)
	Sterile	Fertile	
MTC-9A	41	8	83.7
MTC-5A	21	2	91.3
Control[a]	1	34	2.9

[a] These plants were regenerated from protoplasts of Sasanishiki without cell fusion

The cyrbid plants were male sterile

In order to determine whether the male sterility of cybrid plants was caused by the cytoplasmic or the nuclear genome, the fertility of the progenies from the crosses between sterile cybrids and Sasanishiki was analyzed. Seven and three cybrid plants with the cytoplasm of MTC-5A and MTC-9A, respectively, yielded only sterile progeny (Table 2). All BC_2 progenies also failed to set any selfed seeds (Table 2). These findings demonstrated that the cytoplasmic traits caused the male sterility of the cybrid plants. The cybrid plants were therefore CMS. The CMS trait of the cybrid plants was stable during at least seven generations of backcrossing with Sasanishiki.

Restoration of cytoplasmic male sterility of the cybrid plants

To further determine whether or not the CMS of cybrid plants was caused by the introduced cytoplasmic traits we examined the fertility restoration of CMS cybrids by the *Rf-1* gene. Fifteen BC_1 plants of eight lines of sterile cybrids were crossed with MTC-10R, which has the single dominant gene *Rf-1* for fertility restoration. A high proportion of fully fertile plants was observed in all F_1 progenies. All except one progeny had panicles containing 75–95% seed set. The range of seed fertility of the F_1 progenies was similar to that of 'Sasanishiki A', which had been bred by the recurrent-backcrossing method (Table 3). One of the F_1 progeny SALI-A1 also had fertile panicles but seed set was only 45% (Table 3). This suggested that somaclonal mutation had occurred in the SALI-A1 strain. The restoration of fertility implied that the cytoplasmic traits introduced from both MTC-5A and MTC-9A resulted in CMS.

Discussion

Donor-recipient protoplast fusion can efficiently introduce cytoplasmic traits into another line in a single step in rice (Akagi et al. 1989). The examination of morphological features, the chromosome number, and the mitochondrial genomes of the cybrid plants indicated that only the mitochondrial genome of the donor was

Table 2 Fertility of the progenies of sterile cybrid plants backcrossed with Sasanishiki

Source of cytoplasm	Strain of sterile cybrid plants	BC_1 progenies[a]		BC_2 progenies[b]	
		Number of plants	Seed fertility (%)	Number of plants	Seed fertility (%)
MTC-9A	SALI-A1	8	0	6	0
	A5	5	0	23	0
	A7	8	0	6	0
	A9	8	0	10	0
	SALI-C2	6	0	–	–
	C4	8	0	11	0
	C6	8	0	7	0
MTC-5A	SABT-A1	7	0	24	0
	A5	8	0	12	0
	A6	7		–	–

[a] Progenies from the cross of sterile cybrid plants and Sasanishiki
[b] Progenies from the cross of BC_1 progenies and Sasanishiki

Table 3 Restriction of fertility in BC_1 progenies of sterile cybrid plants with MTC-10R, the restorer line for BT and the LI-type of CMS

Source of cytoplasm	Strain of sterile[a] cybrid plants	Number of F_1 plants tested	Seed set per panicle					
			0%	<20%	<40%	<60%	<80%	<100%
MTC-9A	SALI-A1-1	3	0	0	0	1	0	2
	A5-1	24	0	0	0	0	2	22
	-2	16	0	0	0	0	0	16
	-3	15	0	0	0	0	0	15
	-4	2	0	0	0	0	1	1
	A7-1	5	0	0	0	0	1	4
	A9-1	10	0	0	0	0	0	10
	SALI-C2-1	24	0	0	0	0	1	23
	-2	19	0	0	0	0	1	18
	C4-1	23	0	0	0	0	0	23
	-2	49	0	0	0	0	0	49
MTC-5A	SABT-A1-1	41	0	0	0	0	3	41
	-2	17	0	0	0	0	0	17
	-3	36	0	0	0	0	0	36
	A5-1	30	0	0	0	0	1	29
MTC-5A	Sasanishiki A[b]	5	0	0	0	0	1	4

[a] Eight strains of CMS cybrid plants were used for the test of fertility restoration. One to three BC1 plants of each strain were crossed with the restorer line MTC-10R
[b] The cytoplasm of Sasanishiki A was introduced by conventional recurent backcrossing

inherited. Thus, the cybrid-production technique for transferring only the CMS trait to fertile rice is reproducible.

The occasional modification of the mitochondrial DNA of the cybrid plants may be the result of a rearrangement of the parental mitochondrial genome. In rice cybrids, frequent inter-parental recombination of mitochondrial genomes occurred, and the recombinant genomes, as well as the parental ones, segregated during protoplast culture (Akagi et al., in preparation). The appearance of about 20% of fertile cybrid plants (Table 1) might be the result of the elimination of the CMS trait by segregation (Akagi et al. 1994).

Chimeric genes, created by the recombination of mitochondrial genomes, may also be responsible for CMS (Newton 1988; Levings and Brown 1989). Because inter-molecular recombination between parental mitochondrial genomes occurs frequently in rice cybrids (Akagi et al., unpublished data), new CMS genes might be created in the rice cybrids in addition to the CMS trait introduced by cell fusion. The fertility restoration of the CMS of cybrid plants by the nuclear gene *Rf-1* suggests that CMS is caused by the traits introduced from the parental lines, and not by mutations in the mitochondrial genome of these cybrid plants.

The stable transmission to their progenies and the complete restoration of the CMS by *Rf-1* demonstrated that the cybrids produced by donor-recipient protoplast fusion are useful for creating new CMS rice cultivars for hybrid rice production. It takes about 8 months (from callus induction to seed set by crossing with recipient cultivars) to produce new CMS lines by the donor-recipient protoplast fusion method, whereas it takes about 6 years by the conventional recurrent-backcrossing method.

Acknowledgements We thank to Dr. H. Shimada for critical reading of our manuscript and valuable discussion.

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