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Genetic diagnosis of cytoplasmic male sterile cybrid plants of rice

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Abstract Twelve Japanese rice cultivars were converted to CMS by asymmetric protoplast fusion with MTC-5A, the cytoplasm of which was derived from an indica rice, Chinsurah Boro II. With the exception of the cybrids that had a nucleus from Hoshiyutaka, most of these cybrid plants were sterile. The unique sequence downstream from the mitochondrial *atp6* of MTC-5A was specifically amplified in the sterile cybrid plants by PCR. All progenies of the cybrid plants carrying this unique sequence were sterile. On the other hand, in some of the sterile cybrid plants in which the unique sequence was not amplified by PCR, fertility was recovered in their progenies. Somaclonal mutation may have caused sterility in these cybrids. Only the cybrid plants that had the unique sequence detected by PCR were CMS. Thus, the CMS plants can be selected rapidly and easily by PCR, at an early stage of plant regeneration. Soon after transplanting the regenerated plants to a green house, fertile cybrids and sterile cybrids produced by somaclonal mutation can be removed. These findings also show that the unique region downstream from *atp6* is tightly linked with the CMS phenotype.

Key words *Oryza sativa* L. · Cytoplasmic male sterility (CMS) · Cybrids · PCR · *atp6*

Introduction

Cytoplasmic male sterility (CMS) occurs in rice, when the cytoplasm of a japonica rice is replaced with that of an indica rice or wild rice (Katsuo and Mizushima 1958; Li and Zho 1988). CMS is due to incompatibility between nuclear and cytoplasmic gene products, which results in a failure to develop mature pollen grains. As CMS excludes the possibility of self-pollination, it is commercially applicable to hybrid seed production (Newton 1988).

Reorganization of mitochondrial genomes has been reported in CMS plants, and in many cases results in the creation of chimeric genes whose products may be responsible for CMS (Dewey et al. 1986; Young and Hanson 1987; Singh and Brown 1991; Handa and Nakajima 1992). In rice, recombination 3′ to *atp6* in the Chinsurah Boro II cytoplasm results in a chimeric sequence configuration (Iwabuchi et al. 1993; Akagi et al. 1994) which includes a novel open reading frame, *orf79* (Akagi et al. 1994), and may inhibit the expression of *atp6*.

We created cybrids which had cytoplasmic traits from an indica rice transferred to a japonica rice by asymmetric protoplast fusion (Akagi et al. 1989). There were two types of cybrids; fertile and CMS. The fertility of cybrid plants may be recovered by the segregation of the CMS trait during protoplast culture (Akagi et al. 1994). Some of the cybrid plants may have become sterile by somaclonal mutation. We cannot distinguish between fertile and sterile plants before flowering. Even after flowering, we cannot distinguish whether the sterility of cybrid plants is caused by the CMS trait or by somaclonal mutation.

In the present study, we have converted several Japanese cultivars by asymmetric protoplast fusion, and have developed a selection method for the CMS cybrids in regenerated plants. Because it was suggested that the region downstream from mitochondrial *atp6* may be involved in the rice CMS (Iwabuchi et al. 1993; Akagi et al. 1994), we analyzed this region by PCR, and found it to be completely linked to the CMS phenotype. We describe here a method for the identification of the CMS cybrid plants at an early stage of plant regeneration.

Material and methods

Plant materials

MTC-5A was used for a cytoplasmic donor. Its cytoplasm had been derived from an indica rice, Chinsurah Boro II. Twelve cultivars

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(Nipponbare, Nipponbare-246, Nipponbare-249, Notohikari, Norin 22, Toyosato, Houyoku, Mutuhomare, Tsugaruotome, Hokuriku 122, Chiyohonami and Hoshiyutaka) were used as recipients. Nipponbare-246 and Nipponbare-249 were selected from plants regenerated from Nipponbare protoplasts. The cybrid cells were created by asymmetric fusion between X-irradiated protoplasts of MTC-5A and iodoacetamide-treated protoplasts of 12 Japanese cultivars according to a previously described method (Akagi et al.1989). Calli were regenerated from protoplasts and plants were regenerated from the callus according to the method of Fujimura et al. (1985).

Preparation of total and mitochondrial DNA

Crude DNA was extracted from leaf tissue using the technique of Edwards et al. (1991). Mitochondrial DNA was isolated from suspension-cultured cells of Nipponbare and MTC-5A by a previously described method (Akagi et al. 1989)

PCR and primers

PCR was carried out using a Thermal Cycler 480 (Perkin-Elmer). Thirty PCR cycles were performed, with 1-min denaturation at 94°C, 1-min annealing at 60°C, and 2-min polymerization at 72°C with Ampli*Taq* DNA polymerase (Perkin-Elmer). Primers p1 (5′-ATGGGTTTGAATCAGAGAGA-3′), p2 (5′-ATTCAATTATGAAATTACTC-3′), p3 (5′-ATGGCAAATCTGGTCCGATG-3′) and p4 (5′-ACTTACTTAGGAAAGACTAC-3′) were used. Figure 1A shows the location of the primers. Primers p1 and p2 were located in the coding sequence of the *atp6* gene. Therefore, these sequences were present in all strains. On the other hand, primers p3 and p4 were located in the unique *orf79* sequence downstream from the *atp6* of MTC-5A (Akagi et al. 1994).

Results and discussion

Conversion of several fertile cultivars to CMS

To convert fertile cultivars to CMS, the cytoplasmic trait of MTC-5A, which were derived from Chinsurah Boro II, was transferred to 12 Japanese cultivars by protoplast fusion. About 100 plants were regenerated from each cybrid callus. However, 30–70% of them showed a tetraploid phenotype and these plants were sterile with only a few exceptions setting a few selfed seeds (data not shown). All of the analyzed 11 plants with this phenotype had 48 chromosomes (data not shown). Most of the diploid cybrid plants were sterile and did not set selfed seeds, except for the cybrids that had a nucleus from Hoshiyutaka (Table 1). The restorer genes for Chinsurah Boro II are widely distributed in the tropics where indica varieties are grown (Shinjyo 1972). Because Hoshiyutaka was bred by crossing japonica and indica rice, it might carry a restorer gene. The remaining 11 cultivars were assumed to have no such restorer genes.

Recombination between two parental mitochondrial genomes occurs in rice cybrids, and recombinant genomes, as well as parental ones, segregated during protoplast culture (unpublished data). The male-fertile cybrid plants may have lost the CMS trait by somatic segregation.

Detection of the region downstream from *atp6*

The downstream region from *atp6* in the mitochondrial genome of MTC-5A was closely associated with the appearance of CMS. In this region *orf79* is coded, which is a chimera of another mitochondrial genome region (Akagi et al. 1994). We examined whether or not this chimeric region was specifically amplified in MTC-5A by PCR.

The primers p1 and p2 were located in the coding sequence of the *atp6* gene. Because this gene is essential for mitochondrial function, these primer sequences are presented in the mitochondrial genomes of both MTC-5A and Nipponbare. The primers p3 and p4 are located in a unique sequence downstream from the *atp6* of MTC-5A (Fig. 1A). When PCR was carried out with primers p1 and p2, the predicted size of the DNA fragment was amplified in both fertile and CMS strains. On the other hand, a specific amplification in MTC-5A was observed with primers p3 and p4 (Fig. 1B), but was not detected in fertile Japanese cultivars by PCR.

Table 1 Conversion of 12 cultivars to CMS by asymmetric protoplast fusion with MTC-5A

Cultivar	Number of diploid cybrids regenerated		Ratio of sterile cybrids (%)	Number of lines maintained
	Fertile	Sterile		
Nipponbare	5	27	81.3	1
Nipponbare-246[a]	1	26	96.3	2
Nipponbare-249[a]	1	24	96.0	12
Notohikari	11	53	82.8	11
Norin 22	2	46	95.8	13
Toyosato	1	19	95.0	6
Houyoku	0	10	100	4
Mutuhomare	2	23	92.0	12
Tugaruotome	6	35	85.3	7
Hokuriku 122	6	10	62.5	2
Chiyohonami	1	26	96.3	0
Hoshiyutaka	19	2	9.5	0

[a] These two lines were selected from plants regenerated from Nipponbare protoplasts

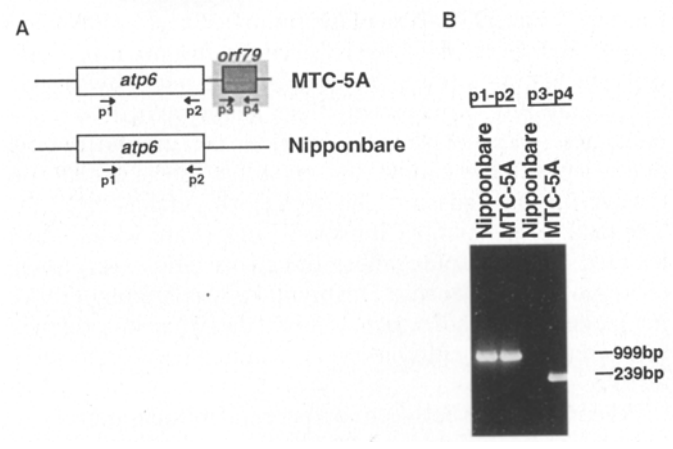

Fig. 1A, B PCR-amplification of the region downstream from mitochondrial *atp6*. **A** shows the structure around the *atp6* of MTC-5A and Nipponbare. The locations of the primers (*P*) are indicated. Primers p1 and p2 are located in the coding sequence of the *atp6* gene of both mitochondrial genomes (Akagi et al. 1994). Primers p3 and p4 are located in the unique *orf79* sequence downstream from the *atp6* of MTC-5A. **B** shows the results of amplification with primers p1 and p2, and p3 and p4. The size of the fragments amplified by PCR is indicated

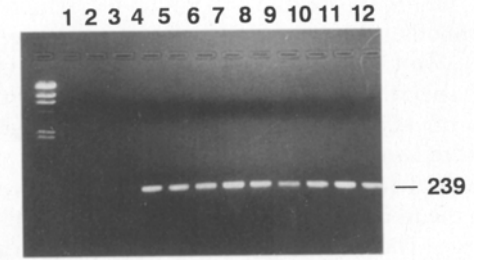

Fig. 2 Detection of the unique sequence of MTC-5A by PCR in cybrid plants between MTC-5A and Tsugaruotome. *Lanes 1–3* indicate the fertile cybrid plants. *Lanes 4–12* indicate the CMS cybrid plants, the progenies of which were sterile. The size of the amplified fragment is indicated

The amplification of the fragment completely linked to CMS

PCR determination of the downstream region from *atp6* was carried out using both the fertile and the sterile regenerated cybrid plants fused between MTC-5A and Tsugaruotome. All cybrid plants in which the DNA fragment was amplified with the primers p3 and p4 were sterile (Fig. 2, lanes 4–12). On the other hand, the cybrid plants in which no amplification with p3 and p4 was observed set selfed seeds (Fig. 2, lanes 1–3). Non of the progeny between these sterile cybrids and Tsugaruotome ever set selfed seeds (data not shown). Therefore, sterile cybrid plants of MTC-5A and Tsugaruotome were CMS. This indicates that fertile cybrid plants can be selected from CMS cybrid plants by PCR.

In the case of the eight cybrids between MTC-5A and Hokuriku 122, six sterile cybrids recovered fertility in their

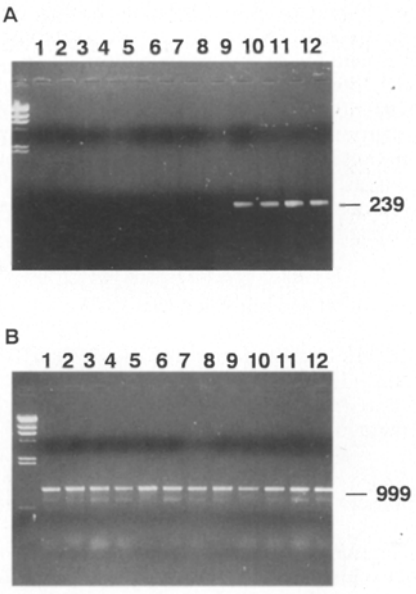

Fig. 3A, B PCR amplification of the sequence around the *atp6* gene in the cybrid plants between MTC-5A and Hokuriku 122. **A** shows the amplification of the unique sequence of MTC-5A with primers p3 and p4. **B** shows the amplification of the sequence corresponding to the *atp6* gene with primers p1 and p2. *Lanes 1 and 2* are Hokuriku 122, *lanes 3–8* are the fertile recovered progenies of the sterile cybrids, *lanes 9–10* indicate the CMS progenies of the sterile cybrid line 1A, and *lanes 11–12* indicate those of 2A. The size of the amplified fragments is indicated

progenies, while two were CMS. The progenies of these two CMS lines (Fig. 3A, lanes 9–10, 11–12) had the unique sequence downstream from *atp6*, whereas this sequence was not detected by PCR in fertile progenies (Fig. 3A). The sequence corresponding to the *atp6* gene was amplified in both fertile and CMS plants (Fig. 3B). Similarly, among the cybrids between MTC-5A and Chiyohonami, all progenies of four independent sterile cybrids recovered male fertility. The downstream region from *atp6* was not detected in these progenies (data not shown). These findings suggest that somaclonal mutation causes sterility in some cybrid plants. They also indicate that the method described here can distinguish the difference between two types of sterility, caused either by the cytoplasmic traits or by somaclonal mutation at an early stage of the regeneration of cybrid plants.

We have maintained a total of 70 independent CMS lines of ten cultivars, excepting Hoshiyutaka and Chiyohonami. We determined the progenies of these maintained CMS lines by this method. All carried the unique sequence. No exception was observed among these lines (data not shown). Therefore, this method is reproducible.

Thus, the region downstream from *atp6* was completely linked with CMS. These findings support previous observations (Iwabuchi et al. 1993; Akagi et al. 1994) that the downstream region from *atp6* is tightly related with CMS and that it may inhibit the expression of *atp6*.

We conclude that the selection method described here is very useful for the breeding of new CMS cultivars by asymmetric protoplast fusion because the fertile plants and the sterile plants produced by somaclonal mutation can be discarded at an early stage of plant regeneration so that only the CMS cybrid plants can be selected.

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References

Akagi H, Sakamoto M, Negishi T, Fujimura T (1989) Construction of rice cybrid plants. Mol Gen Genet 21:501–506

Akagi H, Sakamoto M, Shinjyo C, Shimada H, Fujimura T (1994) A unique sequence located downstream from the rice mitochondrial *atp6* may cause male sterility. Curr Genet 25:52–58

Dewey RE, Levings III CS, Timothy DH (1986) Novel recombinations in the maize mitochondrial genome produce a unique transcriptional unit in the Texas male-sterile cytoplasm. Cell 44:439–449

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genome DNA for PCR analysis. Nucleic Acids Res 19:1349

Fujimura T, Sakurai M, Akagi H, Negishi T, Hirose A (1985) Regeneration of rice plants from protoplasts. Plant Tissue Cult Lett 2:74–75

Handa H, Nakajima K (1992) Different organization and altered transcription of the mitochondrial *atp6* gene in the male-sterile cytoplasm of rapeseed (*Brassica napus* L.). Curr Genet 21:153–159

Iwabuchi M, Kyouzuka J, Shimamoto K (1993) Processing followed by complete editing of an altered mitochondrial *atp6* RNA restores fertility of cytoplasmic male-sterile rice. EMBO J 12:1437–1446

Katsuo K, Mizushima U (1958) Studies on the cytoplasmic difference among rice varieties, *Oryza sativa* L. I. On the fertility of hybrids obtained reciprocally between cultivated and wild varieties. Japan J Breed 8:1–5

Li Z, Zho Y (1988) Rice male-sterile cytoplasm and fertitliy restoration. In: Hybrid rice. Int Rice Res Inst, Manila, Philippines, pp 85-102

Newton KJ (1988) Plant mitochondrial genomes: organization, expression and variation. Annu Rev Plant Physiol Plant Mol Biol 39:503–532

Shinjyo C (1972) Distributions of male sterility inducing cytoplasms and fertility restoring genes in rice. II. Varieties introduced from sixteen countries. Japan J Breed 22:329–333

Singh M, Brown GG (1991) Suppression of cytoplasmic male sterility by nuclear genes alters expression of a novel mitochondrial gene region. Plant Cell 3:1349–1362

Young EG, Hanson MR (1987) A fused mitochondrial gene associated with cytoplasmic male sterility is developmentally regulated. Cell 50:41–49