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A nuclear gene modifying instability of fertility restoration in cytoplasmic male sterile rice

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Summary

The present study was carried out to examine the genetic mechanism responsible for reversions to fertile phenotype detected in cytoplasmic male-sterile plants of rice. The *cms-bo* cytoplasm of Chinsurah boro II gave rise to male-sterility in plants without a gametophytic restorer gene (Rf_1). Taichung 65 (T65A) was known to be the maintainer which carries no restorer; however, Taichung 65 preserved in our laboratory (T65B) showed partial fertility (about 8% seed set) when crossed with the male-sterile plants. Unexpectedly, the seed fertility gradually increased with repeated selfings and almost fully fertile plants were obtained in the F_6 generation. The cytoplasmic substitution lines revealed that reversions to fertile phenotype resulted from mutational events at the nuclear level. The genetic experiments indicated that the partial fertility observed in the F_1 hybrid was controlled by a dominant gene, Ifr_1, which was carried by T65B. The results obtained suggested that Ifr_1 itself was associated with instability of fertility restoration in the presence of *cms-bo* cytoplasm since partially fertile plants carrying Ifr_1 always showed a tendency for gradual increase in fertility in the later generations. The results are also discussed in relation to a rapid genetic change through intensified gametic selection combined with instability.

1. Introduction

Cytoplasmic male sterility (CMS) is caused by nucleocytoplasmic interactions which lead to a failure of affected plants to produce functional pollen. Maternally inherited traits are stably transmitted to the progeny in most cases; but Laughnan and his co-workers found that spontaneous reversions to male fertility occur not infrequently in *S*-type male-sterile cytoplasm of maize (Laughnan & Gabay-Laughnan, 1983). These fertility reversions were proved to occur through changes at either the cytoplasmic or nuclear level. Interestingly, the nuclear genotype governs the frequency of reversions as well as the relative frequency of cytoplasmic and nuclear reversions (Singh & Laughnan, 1972; Laughnan & Gabay-Laughnan, 1982). Fertility reversions in the inbred line M825 of maize occurred at a high frequency (about 10%) and were predominantly cytoplasmic, while reversions in the inbred WB4 occurred at a lower frequency and were of nuclear genes. These arose through nuclear mutations giving rise to new restorers, located on different chromosomes. Recent molecular studies have revealed that cytoplasmic reversions are closely associated with reorganization of mitochondrial genomes (Escote-Carlson *et al.* 1988; Saleh *et al.* 1989). However, the genetic mechanisms inducing spontaneous reversions remains to be studied.

In rice, different types of CMS were reported on the basis of restorer genes (Shinjyo, 1969; Zhou *et al.* 1983; Virmani & Shinjyo, 1988). The *cms-bo* cytoplasm of Chinsurah boro II exhibits male sterility when combined with the nucleus of Taichung 65 (designated T65A in this paper) which has no restorer gene (Shinjyo, 1969). Fertility restoration of the *cms-bo* cytoplasm is gametophytically controlled by Rf_1. Although male sterility is stably expressed in the genetic background of T65A, Taichung 65 preserved in our laboratory (designated T65B) showed a partial restoration of fertility when crossed to completely male-sterile plants. This phenomenon was first observed by Dr Y. E. Chu (unpublished) and T65B was considered to be a weak restorer cultivar as reported for other genotypes (Shinjyo, 1975). The genetic mechanism remained to be studied since attention had been concentrated on the restoration of fertility necessary for hybrid seed production. An unexpected phenomenon was detected in the later generations of the cross between the CMS line and T65B, namely that seed fertility gradually increased with repeated selfings, giving almost fully fertile plants in the F_6 generation. The objective of this study was

to examine the genetic mechanism causing gradual reversion to the fertile phenotype. The present results give evidence of the presence of a single nuclear gene which induces reversions to fertility in CMS rice.

2. Materials and methods

The materials used were three lines, MSRf, T65A and T65B. MSRf and T65A were gifts from Dr C. Shinjyo. MSRf, derived from $BC_{12}F_4$, has the cytoplasm of *cms-bo* and its nuclear restoring gene Rf_1, which were introduced into T65A from Chinsurah boro II by successive backcrosses (Shinjyo, 1975). Both T65A and T65B (preserved in Ryukyu University and National Institute of Genetics, respectively) are Taichung 65 (Japonica type) but they are separately designated since they responded differently to *cms-bo* cytoplasm as revealed in the present study. T65A is known to carry normal cytoplasm and a non-restoring gene, rf_1. A completely male-sterile line with (*cms-bo*) $rf_1 rf_1$ (designated MSrf) was made by backcrossing MSRf × T65A F_1 with T65A. MSrf was maintained by crossing with T65A.

Fertile revertants were obtained in the F_6 generation of MSrf × T65B and one of them was designated FR37. FR37 was selfed up to F_{10} and it was further analysed in order to detect the genetic changes. Cytoplasmic substitution lines were also established among FR37, MSrf, T65A and T65B, to examine whether cytoplasmic changes were involved. The cytoplasms of FR37 and MSrf were combined with the nucleus of T65A or T65B by successive backcrossings.

Seeds were germinated in Petri dishes in early May and transplanted to a paddy field for the summer season. For the winter season, seeds were germinated in late November and were grown in a greenhouse. All the lines used were almost non-sensitive to photoperiod and they flowered in mid August (for summer season) and in late March (for winter season). Seed fertility was examined by strictly bagging before flowering to eliminate cross pollination. Two to three panicles were observed per plant.

3. Results

(i) *Instability induced by T65B*

Both T65A and T65B showed seed sets higher than 66% under bagging (Table 1), although baggings slightly reduced seed sets compared with those without bagging (higher than 80%). MSrf gave no seed under bagging and was maintained by crossing with T65A homozygous for rf_1. To examine its stability in the expression of male-sterility, 1058 MSrf plants with (*cms-bo*) $rf_1 rf_1$ were grown in an isolated field and their seed fertilities were observed without bagging (Table 2). No plant produced any seed, indicating that their expression was quite stable. In contrast, MSrf × T65B F_1 showed an average seed set of 8.3%, ranging from 6 to 9%, and the seed fertility gradually increased on repeated selfings (Table 1), suggesting that T65B responded to *cms-bo* cytoplasm differently from T65A. From the F_2 to F_4 generations, plants with different degrees of seed infertility were selected for the following generations; however, they showed similar tendencies of a gradual increase in seed fertility (Table 1). F_6 plants had a high seed set ranging from 53 to 88%. One of the fertile plants, designated FR37, was repeatedly selfed up to the F_{10} generation. Although the F_{10} plants showed a range of variation in seed sets, a high seed set in FR37 seemed to be heritable.

The occurrence of fertile segregants in the later generations of MSrf × T65B might be simply explained by the hypothesis that T65B carries recessive genes for fertility restoration and their accumulation gives rise to fertile plants in the later generations of the hybrid. This hypothesis was tested by making cytoplasmic substitution lines (Table 2). When *cms-bo* cytoplasm was combined with the nucleus of T65B (designated MSrfB), the substitution line derived from BC_7F_1 showed a seed set (6.3%) as low as that of MSrf × T65B F_1, suggesting that T65B has no recessive genes responsible for full restoration of fertility. Accordingly, the fertility restoration in FR37 must have resulted from mutational events such as found in the *S*-type cytoplasm of maize.

(ii) *Genetic changes in FR37*

Reversions from a male-sterile to a male-fertile phenotype may be due to genetic changes in either the cytoplasm or the nucleus. Firstly, the cytoplasm of the fertile revertant (FR37) was tested for cytoplasmic reversion by repeated backcrossing to T65A and T65B (Table 2). The cytoplasm of FR37 showed complete male sterility when combined with the T65A nucleus but partial fertility (7.5%) when combined with the T65B nucleus, indicating that the cytoplasm of FR37 had not changed to normal. Therefore, mutational events seemed to have occurred in the nuclear genes.

A gradual increase in seed sets observed during the F_2 to F_6 generations strongly suggested that multiple genes were responsible for the fertile revertant. FR37 produced no fully fertile F_1 hybrids in any crosses with T65A and T65B (Table 3), although the seed sets observed in the F_1 hybrids (23·6 and 26·8%) were higher than that of MSrf × T65B F_1 (8.3%). In addition, MSrf × FR37 F_2 gave fertile segregants more frequently than MSrf × T65B F_2 (Tables 1 and 3). Thus, FR37 responded to *cms-bo* cytoplasm differently from T65B. The results suggested that FR37 carried incompletely dominant genes which were responsible for the fertile phenotypes in the presence of *cms-bo*. It was difficult to detect newly arisen restorers using conventional genetic analysis because of the instability

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Table 1. *Changes in seed fertilities observed in the later generations of MSrf × T65B hybrids*

Season	Generation	Parent, fertility (%)	No. of lines	No. of plants	Seed fertility (%) 0*	5	15	25	35	45	55	65	75	> 80	Mean
Sum/82	F_1			12	—	12	—	—	—	—	—	—	—	—	8·3
Sum/83	F_2	6–8	3	36	—	12	17	5	2	—	—	—	—	—	13·4
Win/83	F_3	9–12	3	25	—	1	3	4	7	6	4	—	—	—	35·9
		31–39	2	25	—	2	1	2	10	5	3	2	—	—	37·2
	Total	9–39	5	50	—	3	4	6	17	11	7	2	—	—	36·6
Sum/84	F_4	13–23	5	29	—	—	4	7	11	5	2	—	—	—	34·9
		46–63	4	25	—	—	2	6	6	9	2	—	—	—	38·2
	Total	13–63	9	54	—	—	6	13	17	14	4	—	—	—	36·4
Win/84	F_5	15–36	4	32	—	—	—	1	3	13	11	4	—	—	47·4
		49–58	4	40	—	—	—	—	2	17	18	3	—	—	49.7
	Total	15–58	8	72	—	—	—	1	5	30	29	7	—	—	48·7
Win/85	F_6	51–61	2	12	—	—	—	—	—	—	1	6	4	1	71·5
Sum/89	F_{10}	78	1	42	—	—	—	—	—	2	7	19	11	3	68·6
	T65A			29	—	—	—	—	—	—	—	8	14	6	77·1
	T65B			33	—	—	—	—	—	—	—	7	15	9	78·9

* Shows complete sterility.

Table 2. *Seed fertilities observed when the cytoplasms of MSrf and FR37 were combined with the nucleus of T65A or T65B by successive backcrossings*

Maternal parent	Recurrent parent	Generation	No. of plants	Seed fertility (%)
MSrf	T65A	$BC_{16}F_1$*	1058	0·0
MSrf	T65B	BC_7F_1	12	6·3 ± 3·5
FR37	T65A	BC_7F_1	10	0·0
FR37	T65B	BC_7F_1	10	7·5 ± 3·1

* MSRf (*Rf*/*Rf* from BC_{14}) was crossed twice with T65A (rf_1/rf_1) eliminating Rf_1.

observed in the presence of *cms-bo*. Fortunately, no instability seemed to occur in the presence of normal cytoplasm, as mentioned later. The genetic difference was examined by pollinating MSrf or MSrfB with pollen of T65B × FR37 F_1. T65B × FR37 F_1 was fully fertile since it carried normal cytoplasm. If a major gene for fertility restoration such as Rf_1 was present in FR37, fertile and sterile plants would be expected to segregate into a 1:1 ratio in the cross. The results indicated that the genetic changes which had occurred in FR37 were heritable but were not simply explained (Table 3).

(iii) *Genetic analysis of partial fertility*

As mentioned, T65B produced partially fertile F_1 hybrids when crossed to MSrf. The genetic difference between T65A and T65B for partial fertility was examined. When MSrf was pollinated with the pollen of T65A × T65B F_1, 81 completely sterile and 77 partially fertile plants were obtained (Table 4). This observed ratio fits a 1:1 ratio, suggesting that partial fertility is controlled by a single dominant gene. As expected from this hypothesis, when T65A × T65B F_2 individuals were crossed to MSrf, three types of F_2 individuals, producing only sterile plants, only partially fertile plants, or both, could be distinguished. The observed ratio of 1:1:2 supported the above hypothesis (Table 5). The detected gene was designated

Table 3. *Segregations for seed fertility in crosses among FR37, MSrf, MSrfB and T65B*

Cross	No. of plants	Seed fertility (%) 0*	5	15	25	35	45	55	65	75	> 80	Mean
MSrf × FR37 F_1	8	—	—	4	2	2	—	—	—	—	—	23·6
MSrfB × FR37 F_1	6	—	—	2	1	3	—	—	—	—	—	26·8
MSrf × (T65B × FR37) F_1	53	—	19	28	4	2	—	—	—	—	—	14·4
MSrfB × (T65B × FR37) F_1	76	—	20	33	16	7	—	—	—	—	—	17·1
MSrf × FR37 F_2	30	—	—	8	11	6	4	1	—	—	—	28·3
FR37	20	—	—	—	—	—	3	3	7	5	2	69·3
T65B	10	—	—	—	—	—	—	—	2	5	3	74·9

FR37 was a plant derived from F_{10}.
MSrfB carries *cms-bo* cytoplasm and the nucleus of T65B (BC_7F_1).
* Shows complete sterility.

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Table 4. *Segregations for seed fertility in crosses among MSrf, MSRf, T65A and T65B*

Cross	Seed fertility (%) 0†	5	15	25	35	45	55	65	75	> 80	Total	Ratio CS:PF:F	χ^2
MSrf × (T65A × T65B) F_1	81 (81)	56	21 (77)	—	—	—	—	—	—	—	158	1:1:0	0·10 N.S.
MSrf × (T65B × MSRf) F_1	35 (35)	26	4 (30)	—	—	2	18	34 (76)	15	7	141	1:1:2	1·21 N.S.
(MSrf × T65B) × T65A F_1	34 (34)	49	11 (60)	—	—	—	—	1	1 (2)‡	—	96	1:1:0	7·19*
MSrf × (T65A × T65B) F_2§	—	18	17	13	14	6	3	—	—	—	71		

CS, PF and F show completely sterile (0 %), partially fertile (2–19 %) and fertile (46–98 %), respectively. The genotypes of MSrf and MSRf are (*ms-bo*) $rf_1 rf_1$ and (*ms-bo*) $Rf_1 Rf_1$.
N.S., non-significant.
* Significant at 1 %.
† Completely sterile.
‡ Two fertile revertants which were not included in the calculation.
§ Ten randomly selected plants which were partially fertile in the MSrf × (T65A × T65B) F_1s.

Table 5. *Segregations for seed fertility in F_2 individuals of T65A × T65B when crossed to MSrf*

No. of F_2 plants					
CS	Seg.	PF	Total	Ratio	χ^2
8	17	5	30	1:2:1	1·40 N.S.

CS and PF show plants producing only completely sterile or partially sterile segregants in crosses to MSrf. Seg. shows plants producing both completely sterile and partially sterile segregants. Mean number of plants observed was 8·1 per line.
N.S., non-significant.

Ifr_1. The *Rf_1* locus is known to carry a series of alleles which restore fertility in different ways (Virmani & Shinjyo, 1988). To examine whether *Ifr_1* is an allele at the *Rf_1* locus, MSrf was crossed with T65B × MSRf F_1. The cross gave 35 completely sterile, 30 partially fertile and 75 fully fertile plants showing a ratio of 1:1:2 (Table 4). This indicated that *Ifr_1* is independent of *Rf_1*. Thus, T65B was estimated to be homozygous for *Ifr_1* as well as *rf_1*. The observed Mendelian segregation revealed that the instability caused by T65B disappeared in the absence of *cms-bo* cytoplasm.

On the contrary, when MSrf × T65B F_1 was crossed with the pollen of T65A, the segregation ratio of completely and partially sterile plants significantly differed from the expected ratio of 1:1 (Table 4), suggesting that instability caused an altered ratio in the presence of *cms-bo* cytoplasm. Two exceptional fertile segregants were also detected in the cross although the genetic basis is unknown. A high rate of success in cross-pollinations ensures that infertility is based on male-sterility. The deviant segregation strongly suggested that in the presence of *cms-bo* cytoplasm some of the megaspores carrying *Ifr_1* also induced mutations which led to partial restoration of fertility.

(iv) *Association of* Ifr_1 *with fertility reversions*

During the present experiments, no true-breeding line showing partial fertility was obtained. Partially fertile segregants always showed a gradual increase in seed fertility in the later generations of MSrf × T65B F_1. These observations suggested that *Ifr_1* has a role in fertility reversions. If it controls only partial fertility, the homozygote for *Ifr_1* should remain low in seed set. MSrfB derived from BC_7F_1 was considered to be homozygous for *Ifr_1* as well as *rf_1* as mentioned before (Table 2). Although MSrfB had a 7·5 % seed set, the low seed set was maintained only by repeated crosses with T65B, otherwise the selfed progeny showed an

Table 6. *Changes in seed fertilities observed in the later generations of MSrfB (BC_7F_1) carrying the T65B nucleus and* cms-bo *cytoplasm*

Generation	No. of plants	Seed fertility (%) 0*	5	15	25	35	45	55	65	75	> 80	Mean
BC_7F_1	10	—	10	—	—	—	—	—	—	—	—	7·5
BC_7F_2	32	—	11	17	3	1	—	—	—	—	—	12·7
BC_7F_3	34	—	—	12	9	8	4	1	—	—	—	29·1

* Completely sterile.

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increase in seed sets (Table 6). The progeny from a cross of MSrf × (T65B × T65A) was also examined for any association of Ifr_1 with fertility reversions. If other genes were responsible for the fertility reversions, some of the partially fertile segregants ($Ifr_1/+$) would show no tendency for a gradual increase in seed fertility. In total, 10 lines were examined and they all showed a clear tendency for fertility reversion (Table 4). No completely male-sterile was found in the lines examined as noticed also in the F_2 of MSrf × T65B, suggesting preferential transmission of Ifr_1 through pollen in the presence of *cms-bo*.

4. Discussion

The present study showed that reversions to fertile phenotype occurred in CMS rice, and this led us to conclude that mutational events gave fertility reversions in the presence of *cms-bo* cytoplasm. The present results discount the possibility of cytoplasmic reversions in FR37 in terms of fertility restoring genes. FR37 cytoplasm was also tested for mtDNA modifications. *cms-bo* carries small circular plasmid-like mtDNAs which are absent in normal cytoplasm (Yamaguchi & Kakiuchi, 1982; Kadowaki *et al.* 1986; Nawa *et al.* 1987). Our unpublished data showed that FR37 carries the circular molecules, suggesting that the presence of plasmid-like mtDNAs is not necessarily associated with the male-sterile phenotype. In addition, southern blot hybridization revealed that mtDNAs of FR37 gave the same patterns as that of MSRf by using probes which specifically detect the differences between mtDNAs of *cms-bo* and normal cytoplasms (Fujimura *et al.* unpublished). These results support the conclusion that the fertility reversion observed is not at the cytoplasmic level.

Based on the results obtained, we propose here that Ifr_1 is associated not only with partial restoration of fertility but also with fertility reversions due to instability in the presence of *cms-bo* cytoplasm. The conclusion comes from the results that (1) all partially fertile plants in the F_2–F_4 of MSrf × T65B showed a tendency of fertility reversion; (2) partial fertilities in the homozygotes (MSrfB) for Ifr_1 were maintained only by crossing with T65B; (3) otherwise the seed fertility of the homozygote (MSrfB) increased on repeated selfings; and (4) no true-breeding line maintaining partial fertility could be obtained. In addition, all the partial fertility ($Ifr_1/+$) plants examined in a cross of MSrf × (T65A × T65B) always showed a tendency for a gradual increase in fertility, which strongly suggests that Ifr_1 itself is responsible for fertility reversion. Backcrossing experiments are under way to introduce Ifr_1 into MSrf and examine instability of fertility restoration due to Ifr_1. Although the backcross generation is still BC_2 at present, the resultant plants with $Ifr_1/+$ showed a gradual increase in fertility in the next generation supporting the above hypothesis.

With regard to partial restoration of fertility in *cms-bo* cytoplasm of rice, Shinjyo (1975) surveyed the distribution of restoring genes among a number of Japanese cultivars. Out of 146 cultivars examined, 19 (13%) showed partial seed-fertilities ranging from 1·7 to 11·5% while the remaining cultivars seemed to carry Rf_1 or rf_1. Although the genetic bases are unknown, there is a possibility that the 19 cultivars carry a gene similar to Ifr_1.

A question arises as to why all derivatives from MSrf × T65B F_1 showed a tendency to fertility reversion. This might result from a difference in transmission of Ifr_1 and + into the progeny in the presence of *cms-bo* cytoplasm. Without *cms-bo* cytoplasm, Ifr_1 and + were transmitted through pollen into the progeny with equal frequency, as revealed in the crosses of MSrf × (T65A × T65B) and MSrf × (T65B × MSRf). Although the cross of (MSrf × T65B) × T65A showed less completely sterile plants than expected, most of the megaspores carrying Ifr_1^+ seemed to be transmitted into the progeny, as would be expected from the fact that the plants are male-sterile but female-fertile. In contrast, MSrf × T65B F_1 gave no completely sterile plants in the later generations. This clearly shows that almost no microspore with Ifr_1^+ transmits into the progeny in the presence of *cms-bo* cytoplasm. If Ifr_1 is responsible for fertility reversions, all derivatives from the cross should carry Ifr_1 and give rise to fertility reversions in the later generations.

Why is Ifr_1 only transmitted through pollen into the progeny in the presence of *cms-bo*? This might be simply explained if Ifr_1 were responsible for instability of fertility restoration. Microspores with rf_1 become lethal in the presence of *cms-bo*, but those with Ifr_1 have a chance to change into fertile pollen because of its instability. If this is the case, Ifr_1 is expected to act gametophytically producing functional microspores, although microspores without Ifr_1 remain unchanged and inviable. Thus, intensified gametic selection operating during pollination leads to preferential transmission of Ifr_1 into the progeny. FR37 derived from the F_{10} generation still showed a range of variation in seed sets, suggesting that instability continues even in FR37. The instability of fertility observed in FR37 suggests that sterile mutations might also occur due to its instability. With regard to the occurrence of partially fertile plants in FR37, there might be an equilibrium between gametic selection and deleterious mutation. It is interesting to ask whether Ifr_1 induces alterations in other traits; however, no alteration in morphological traits has been detected so far.

In maize, the nuclear genotype also controls fertility reversions in *S*-type cytoplasm. Both cytoplasmic and nuclear reversions were detected in *S*-type cytoplasm and the relative frequencies of cytoplasmic and nuclear reversions in maize are under the control of nuclear genes. The frequency of fertile revertants in *S*-type

cytoplasm is highly dependent upon the nuclear genotype, and it gradually changes by successive backcrossings, suggesting its multigenic control (Laughnan & Gabay-Laughnan, 1982). Furthermore, nuclear revertants in maize are caused by mutant restorer genes with major effects which were mapped to several chromosomes, although most of the newly arisen restorers are deleterious compared to the naturally widespread Rf_3 (Laughnan & Gabay, 1973). Cytoplasmic reversions have not yet been found in rice. The present results suggest that nuclear reversions are caused by mutations of polygenic nature and a single nuclear gene (Ifr_1) is responsible for fertility reversions in the presence of *cms-bo* cytoplasm.

Of particular interest is intensified selection through the pollen when combined with instability. Male-sterility is of wide occurrence between and within species in plants. Numerous studies have shown that male sterility often results from nuclear-cytoplasmic interactions (Duvick, 1965; Edwardson, 1970). It should be noted that both *S* and *cms-bo* cytoplasms in maize and rice require gametophytic restorer genes for fertility restoration. Pollen gametophytic selection is apparently more effective than sporophytic selection in leading to the generation of fertile revertants. Whatever the mechanism for instability is, the present results confirm that intensified gametic selection combined with instability leads to a rapid change in the genetic content of rice.

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