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Inheritance and fine mapping of fertility restoration for cytoplasmic male sterility in *Gossypium hirsutum* L.

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Abstract Genetics of CMS fertility restoration was presented through the analysis of classic genetics and molecular markers. Based on F_2 segregation of the crosses between CMS and the restoring lines, the testcrosses and $F_1 \times F_1$ populations, together with RAPD and SSR mapping, one dominant gene was identified to control the CMS fertility restoration in cotton. The strategy of genotype representation analysis (GRA) was put forward to screen the markers linked with the Rf_1 locus. Using 1,025 random decamer primers and 282 pairs of SSR primers, two RAPD and three SSR markers were identified to be closely linked to the Rf_1 gene. Among the five markers, three were co-dominantly inherited. Additionally, based on the analysis of monosomic and telesomic lines with one SSR maker, the Rf_1 locus could be located on the long arm of chromosome 4. The molecular markers available here are helpful in the development of the elite restoring lines in cotton by marker-assisted selection.

Keywords *Gossypium hirsutum* L. · Inheritance · Molecular mapping · CMS · Rf_1

Introduction

Cotton (*Gossypium* spp.) is a very important cash crop in the world. It produces the world's most important textile fiber and is the second most valuable oil and meal seed. The heterosis in cotton is highly significant, especially in improving fiber yield and quality. Cytoplasmic male sterility (CMS) is a maternally inherited trait, which is characterized by the inability to produce functional pollen but without affecting the female fertility. CMS occurs widely in higher plants and can be restored by a nuclear fertility restoring gene(s), (*Rf*). CMS and its fertility restoration (*Rf*) system have been studied intensively in many crops including rice, maize, oil rape and sunflower (Schnable and Wise 1998; Börner et al. 1998; Matsui et al. 2001).

Compared with hand-emasculation and pollination, and genetic male-sterile lines, the CMS line is much more effective and economical in the commercial production of hybrid seeds in cotton. Much effort has been made on CMS in cotton. Since 1965, several different sources of CMS have been developed, including CMS-D2-2 (Meyer 1973a, 1975), CMS-hir (Jia 1990), CMS-D8 (Stewart 1992), CMS-D4 (Meshram et al. 1994) and CMS-C1 (Zhang and Stewart 1999), and several elite CMS lines were developed in *Gossypium arboreum, Gossypium anomalum, Gossypium harknessii* and *Gossypium trilobum* cytoplasms (Zhu et al. 1998; Weaver 1982). CMS hybrid cotton cultivars have been developed and are being grown in India.

As both cotton fiber and seed are the major harvesting products, the fertility restoration of CMS is the most important target for cotton production. Meyer (1973b, 1975) developed CMS in *Gossypium hirsutum* L. by transferring the cytoplasm of the wild *G. harknessii* Brandg. diploid cotton to the tetraploid species (Sheetz and Weaver 1980). And two restorer genes, one dominant (F), and another recessive (s), responsible for fertility restoration were deduced. But later, Weaver and Weaver (1977) reported that a restorer gene (Rf_1) for fertility restoration from *G. harknessii*. Rf_1 was a partially dominant gene. Genetic modifiers were required to restore complete male fertility. But some reports (Da Silva et al. 1981; Maranhao et al. 1984) also reported that there were at least three dominant restorer genes, one of them being on chromosome (Chr.) 18, and some modifying genes on Chrs. 16, 25 and the long arm of Chr. 15, on the basis of aneuploid analyses.

Based on the observation of a 13:3 ratio, Wang et al. (1996a, b) reported that the fertility restoration of seven

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CMS lines in Upland cotton developed in China was controlled by two independent dominant genes Rf_1 and Rf_2. Rf_1 was completely dominant while Rf_2 was partial. The fertility restoration could be enhanced by a fertility enhancer gene (*E*). Zhang and Steward (2001a) found that D2R could restore CMS-D8, the Rf_1 gene from D2 as CMS-D2 functioned sporophytically. Linkage tests revealed that the *Rf* was linked with cracked root gene (*Rc*) (Weaver and Weaver 1979; Kohel et al. 1984). Another CMS-*Rf* system studied in detail was CMS-D8-D8R. The cytoplasm of CMS-D8 lines was from *G. trilobum* (DC) (Stewart 1992). The D8 restorer-gene functions at the gametophytic level. And CMS-D8 restoration was conditioned by one dominant gene (Rf_2). They also found that the Rf_1 (from D2R) and *Rf*2 (from D8R) loci were not allelic but were tightly linked with a genetic distance of 0.93 cM (Zhang and Steward 2001a, b). The genetic basis of fertility restoration to CMS has not yet been fully understood, especially for the inheritance of the CMS from *G. harknessii* (D2). Exploring the molecular mechanism of restoration will be very helpful in the utilization of heterosis and understanding of the nuclear-cytoplasm interaction. The differences in genetic research resulted most likely from the materials used in the studies, the error in fertility scoring and the size of segregating population. So, to explore the inheritance, we adopted the molecular markers and traditional genetic analysis to overcome the reported error-inducing factors.

Materials and methods

Plant genetic materials

The CMS and fertility restoring lines used in the study are listed in Table 1. Besides Zhongmiansuo (ZMS) 12A-1 in *G. barbadense* cytoplasm and ZMS 12A-2 in *G. hirsutum* cytoplasm, the fertility of the CMS lines used in the present article could be all restored by the same restoring lines with *G. harknessii* cytoplasm. This indicated that there might exist the same inheritance pattern in nuclear-cytoplasm interaction between them. The restorer lines Simian (SIM)-3R and Zheng-R were developed by recurrent backcrosses to transfer the restoring gene(s) in the fertility restoring line 0-613-2R into the cultivars SIM 3 and Zheng-4107 in our institute. The crosses for inheritance studies were made in Nanjing, China, and the F_1 plants were self-pollinated to produce the F_2.

DNA extraction and amplification

Genomic DNA of all the materials used in the study was isolated from leaves as reported in Paterson et al. (1993). DNA samples were amplified by the RAPD and SSR techniques according to Williams et al. (1990) and Zhang and Zhang (2000). The RAPD amplification procedures were as follows: DNA denaturation (94 °C for 15 s); primer annealing (35 °C for 30 s) and polymerization by *Taq* polymerase (72 °C for 1 min). After 45 cycles, the amplification products were separated by gel electrophoresis on a 1.2% (v/v) agarose gel containing ethidium bromide in 1.0 × TBE. RAPD fragments were visualized under UV light and recorded with the SX-imaging system and photos were taken. A total of 1,025 decamer oligonucleotide primers were used for RAPD from Operon Technologies and the University of British Columbia (Canada, UBC primers), and a total of 282 primers were used for SSR (Plant Genetics Inc.). The procedure for SSR analysis was reported by Zhang and Zhang (2000). DNA bands of SSR were developed with silver staining and recorded with the SX-image system.

Table 1 Plant genetic materials used in the experiment

Name	*Gossypium* cytoplasm, probably	Origin
CMS lines		
Zhongmiansuo12A-1 (ZMS 12A-1)	*G. barbadense*	Cotton Research Institute, Nanjing Agricultural University (CRI, NAU)
Zhongmiansuo12A-2 (ZMS 12A-2)	*G. hirsutum*	Handan Inst Agric Sci
Zhongmiansuo12A-3 (ZMS 12A-3)	*G. harknessii*	Cotton Research Institute, China Academy of Agricultural Sciences (CRI, CAAS)
Sumian6A-1(SM 6A-1)	*G. hirsutum*	Shanxi Cotton Research Inst; CRI, NAU
Simian3A-1 (SIM 3A-1)	*G. hirsutum*	Shanxi Cotton Research Inst; CRI, NAU
Sumian16A-2 (SM 16A-2)	*G. hirsutum*	Shanxi Cotton Research Inst; CRI, NAU
Simian3A-2 (SIM 3A-2)	*G. hirsutum*	Shanxi Cotton Research Inst; CRI, NAU
Sumian12A (SM 12A)	*G. hirsutum*	Shanxi Cotton Research Inst; CRI, NAU
Fertility restoring lines		
0-613-2R	*G. harknessii*	CRI, CAAS
501R	*G. harknessii*	CRI, CAAS
Zheng-R	*G. harknessii*	CRI, NAU
SIM3R	*G. harknessii*	CRI, NAU
Maintainer lines		
ZMS 12	*G. hirsutum*	CRI, CAAS
SIM3	*G. hirsutum*	CRI, NAU
SM6	*G. hirsutum*	CRI, NAU
SM16	*G. hirsutum*	CRI, NAU

Constructing the genotype representations

With the combination of the methods involving bulked segregation analysis (BSA) described by Michelmore et al. (1991), and NIL, the genotype-representation analysis (GRA), were used to screen the molecular markers tightly linked with the Rf_1 locus. The fertility representation with Rf_1 comprised the different restorer lines, which had the same Rf_1 in a different genetic background, the fertility representation without Rf_1 composed of some maintainer lines, and all the CMS lines used in this study consisting of the sterility representations.

Linkage analysis

A total of 1,025 random decamer primers and 282 pairs of SSR primers were used to characterize the fertility and sterility representations, as well as the F_1; the polymorphic DNAs that distinguish the representations were thought to be a probable marker tightly linked with the Rf_1 locus and further screened on the entire F_2 using the same primers.

The fertility restoring line SIM 3R was crossed with the CMS lines SIM 3A-1, Sumian (SM) 12A and SIM 3A-2, respectively, to produce F_2s. The segregation analyses of the heteromorphic marker bands and the fertility of the F_2 plants were performed using the x^2-test according to the Mendelian segregation ratio of 3:1.

The recombination ratio was determined with the MAPMAKER 3.0 Program (Lander et al. 1987) with a minimum Lod score of 3.0 using the Kosambi map-unit function.

Fertility scoring

All the cotton plants were grown in the field in Jiangpu Breeding Station, Nanjing. The performance of pollen shedding was recorded for all plants. The fertility was scored by the percentage of pollen, and flower bud size and morphology inspection; the one with large anthers, bud size and shedding pollen was scored as fertile and otherwise scored as sterile. In the fertile individuals the percentage of the pollen-shedding anther was also recorded at the same time. The observation was done at least twice during the flowering time. A minimum of two flowers per plant were classified (Sheetz and Weaver 1980; Zhang and Stewart 2001a, b). In addition, all the plants were self-pollinated.

Chromosome location

Hybrids crossed between aneuploid lines, involved monosomic lines for Chrs. 1, 2, 3, 4, 6, 7, 9, 10, 12, 16, 17, 18, 20, 23, 25 and 26, and telosomic lines for 4Lo, 4Sh, 5Lo, 11Lo, 14Lo, 15Lo, 20Sh, 22Lo, and 22Sh, of the total 26 pairs of allotetraploid cottons in the TM-1 genetic background, and *G. barbadense* cultivars 3-79, a genetic standard in *G. barbadense*, were made by USDA ARS, Crop Germplasm Research Unit in cooperation with Texas A&M University. Monosomic and telosomic TM-1 plants were cytogenetically identified and crossed with 3-79. DNA was extracted from identified F_1 aneuploid plants for chromosomal mapping of molecular markers using 3-79, TM-1, CMS and fertility restoration lines as controls. As the monosomic and mono-tel-

Table 2 Segregation of fertile and sterile plants in F_2's crossed between CMS lines and restoring lines. χ^2 (0.05, 1) = 3.84

Crosses	No. plants	No. fertile plants	No. sterile plants	χ^2 value (3:1)	χ^2 value (13:3)
ZMS 12A-1 × 0-613-2R	400	359	41	31.3705	21.8694
ZMS 12A-3 × 0-613-2R	440	366	74	15.2759	0.9548*
ZMS 12A-2 × 0-613-2R	194	253	41	23.2200	401447
SM 6A-1 × 0-613-2R	345	299	46	24.4216	6.2937
SM 16A-2 × 0-613-2R	314	284	30	39.1337	16.8313
Total	1,793	1,561	232	138.4585	39.3594
ZMS 12A-1 × Zheng-R	320	296	24	51.3375	25.8513
ZMS 12A-3 × Zheng-R	434	376	58	30.7220	7.9142
ZMS 12A-2 × Zheng-R	338	300	38	33.3885	1.0617*
SM 6A-1 × Zheng-R	300	258	42	18.7777	4.1367
SM16A-2 × Zheng-R	434	376	58	30.722	7.9142
Total	1,826	1,622	204	185.4808	68.2363
ZMS 12A-3 × 501-R	575	509	66	70.6093	19.4837
SM 16A-2 × 501R	438	405	33	53.0411	35.4340
SM 6A-1 × 501R	66	54	12	1.2929*	0.0016*
Total	1,079	968	111	123.784	50.1701
SIM3A-1 × SIM3R	196	161	35	4.9594	0.0523*
SM12A-1 × SIM3R	208	169	39	4.0064	0*
SIM3A-2 × SIM3R	231	188	43	4.6883	0.0010*
Total	635	518	117	14.2913	0.0252*
104-7A × 0-613-2R	500	408	92	11.2667	0.0205*
Xiang-A × 0-613-2R	306	261	45	16.7504	3.025*
HA-A × 0-613-2R	506	418	88	15.2200	0.5273*
ZMS12A × 0-613-2R	290	237	53	6.6391	0.0173*
Total	1602	1,324	278	49.5513	1.9607*
104-7A × 501-R	212	183	29	13.8931	3.2530*
Xiang-A × 501-R	350	319	31	47.7867	21.8400
HA-A × 501-R	272	237	35	20.7107	5.7979
Zh12A × 501-R	255	231	24	32.2209	11.3926
Total	1,089	970	119	114.2703	43.2300
Sum	8,024	6963	1,061	592.9413	160.9058

Table 3 Segregation of fertile and sterile plants in testcross and in the $F_1 \times F_1$ population

Crosses	No. fertile plants	No. sterile plants	χ^2 value (1:1) or (3:1)	Probability
(104-7A × 0-613-2R) × ZMS 12B	289	272	0.5775	0.250–0.500
(Xiang-A × 0-613-2R) × ZMS 12B	156	139	0.8678	0.250–0.500
104-7A × (104-7A × 0-613-2R)	28	23	0.3556	0.500–0.750
104-7A × (104-7A × 0-613-2R)	25	23	0.0208	0.750–0.900
HA227A × (HA227A × 0-613-2R)	57	50	0.3364	0.500–0.750
HA227A × (HA227A × HA16R)	29	17	2.6304	0.100–0.250
Total	139	113	2.4802	0.100–0.250
Heterogeneity test			0.612	0.975–0.990
(104-7A × 0-613-2R) × (HA-A × 0-613-2R)	85	30	0.02609	0.750–0.900
(HA227A × 0-613-2R) × (104-7A × 0-613-2R)	75	28	0.1586	0.500–0.750
(Xiang-A × 0-613-2R) × (HA227A × 0-613-2R)	47	7	3.6162	0.050–0.100
(SM6A-1 × 0-613-2R) × (ZMS12A-2 × 0-613-2R)	117	43	0.2083	0.500–0.750
(SM6A-1 × 0-613-2R) × (ZMS 12A-3 × 0-613-2R)	129	40	0.0965	0.750–0.900
(SM 16A-2 × 0-613-2R) × (ZMS 12A-2 × 0-613-2R)	81	13	3.6312	0.050–0.100
(SM 16A-2 × 0-613-2R) × (ZMS 12A-3 × 0-613-2R)	68	26	0.2837	0.500–0.750
Total	602	187	0.6370	0.250–0.500
Heterogeneity test			0.0001	>0.995

osomic stocks have a single chromosome or chromosome arm of *G. barbadense*, with a co-dominant molecular marker locus, if one marker genotype is similar to that of *G. barbadense* and the counterpart marker allele is not observed, the involved marker loci would be considered to be assigned on the corresponding chromosome or the chromosome arm.

Results

Inheritance of fertility restoration in CMS lines

The F_1 plants crossed between CMS lines and restorer lines were all fertile, and the flower sizes and pollen shedding were normal. That F_1 plants were complete male fertile indicated that the fertility restoration was dominant. The F_2 data seem that more than one gene was probably involved in the CMS fertility restoration (Table 2). The x^2 test suggested that two dominant genes might have certain interactions with CMS, such as (ZMS 12A-3 × 0-613-2R) F_2, (SM6A-1 × 501R) F_2, (SIM3A-1 × SIM 3R) F_2, (SM16A-2 × SIM 3R) F_2 and (SIM 3A-1 × SIM 3R) F_2. In some crosses it seems that there were three or four loci involved in the restoration. However, in the testcrosses (Table 3), a ratio of 1:1 was observed when F_1 was pollinated by the maintainer line, corresponding to a one-gene model. In the testcrosses of CMS lines, the fertile to sterile plants within the F_1 also fitted a 1:1 ratio ($x^2 = 2.4802$) although the male-sterile individuals were a little low. The segregation of the fertile and sterile plants in the $F_1 \times F_1$ was given in Table 3. In such populations, the fertile plants were 602 and the sterile 187; it also fitted the expected one-gene segregation ratio of 3:1 ($x^2_{0.05} = 0.6425$). The heterogeneity-test gave the x^2 value (3:1) of 0.0001. These results suggested that only one restorer allele was involved in the CMS fertility restoration.

Why were there so great differences in segregation for different populations? Was there any difference between the *Rf* and *rf* male and female gametes during the double-fertilization? Interaction between the cytoplasm of *G. harknessii* and the nuclei of *G. hirsutum* not only caused abortion of the pollen mother cell (PMC) but also influenced the fertility of the embryo sac (Wang et al. 1997). The phenomenon that CMS causes the impairment of female gametes was also reported in other species (Frankel et al. 1977). Zhu et al. (1998) reported that the viable pollen percentage of the (A × R) F_1 was lower than that of restorer line 0-613-2R. Our research presented here found that the mean number of normal seeds per boll (20.813 ± 9.330) in the crossing of CMS lines × homozygous individual (*RfRf*), identified in the F_2 by molecular marker selection, was higher than that (15.733 ± 12.453) in the crossing of the CMS lines × heterozygous individual (*Rfrf*)) identified in the F_2, and the mean aborted seeds per boll was 8.438 ± 6.292 to 12.537 ± 8.210, suggesting the low viability or weak competitiveness of rf pollen grains compared with the Rf pollen grains during the double fertilization. At the same time, both in the crosses of CMS lines with *RfRf* and with the *Rfrf* individual in the F_2, the mean number of normal seeds per boll (20.813 ± 9.330, 15.733 ± 12.453) was lower than that (29.375 ± 4.340, 31.400 ± 6.336) of the self-pollinated *RfRf* or *Rfrf* individual in the F_2, indicating to some extent that less seeds would be due to the influence of hand pollination, such as impairing the pistil and stigma. The number of seeds from restorer lines × maintainer lines (20.393 ± 6.540, 20.921 ± 6.266) was less than that of self-pollinated maintainer lines (26.741 ± 6.656) and restorer lines (22.245 ± 5.971), indicating partial embryo sac abortion presumably caused by the sterility cytoplasm and probably impairing the hand pollination.

As the segregation of phenotype (male fertility/sterility) in F_2s did not fit a one gene ratio of 3:1 perfectly, the two SSR primers S1231 and S1317, which could produce polymorphic DNA between parents and were not

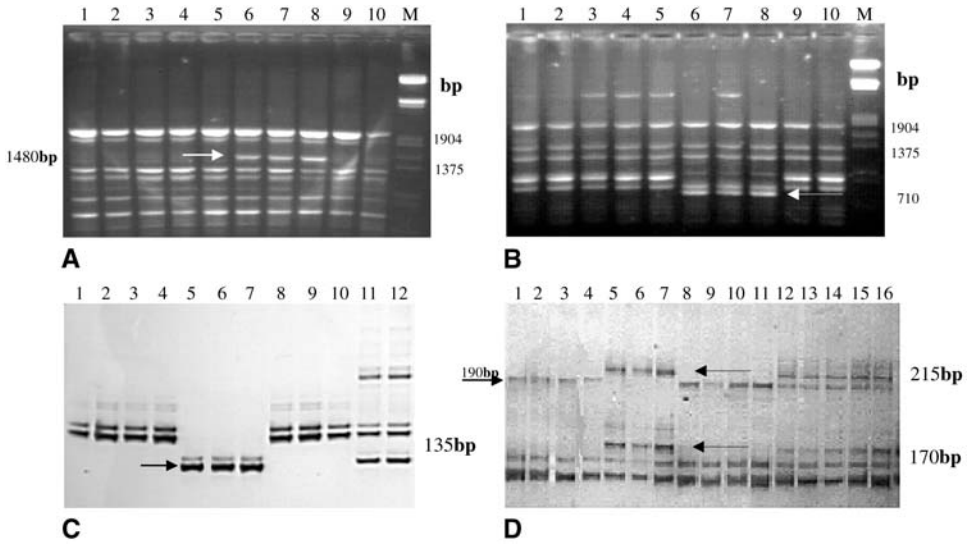

Fig. 1A–D RAPD and SSR analysis of fertility representation in GRA. **A** RAPD products with Primer NAU/RAPD/$Rf_1$3. 1–5: CMS lines (ZMS 12A-1, ZMS 12A-2, ZMS 12A-3, SM 6A-1, SIM 3A-1); 6–8: fertility restoring lines (0-613-2R, 501R, Zheng-R); 9–10: maintainer lines (ZMS 12, SIM3). **B** RAPD products with Primer NAU/RAPD/$Rf_1$5, 1–5: CMS lines (ZMS 12A-1, ZMS 12A-2, ZMS 12A-3, SM 6A-1, SIM 3A-1); 6–8: fertility restoring lines (0-613-2R, 501R, Zheng-R); 9–10: maintainer lines (ZMS 12, SIM3). **C** SSR products with Primer NAU/SSR/$Rf_1$2. 1–4: CMS lines (ZMS 12A-2, ZMS 12A-3, SM 6A-1, SIM3A-1); 5–7: fertility restoring lines (0-613-2R, 501R, Zheng-R); 8–10: maintainer lines (ZMS 12, SIM3, SM6); 11–12: F_1. (A × R). **D** SSR products with Primer NAU/SSR/$Rf_1$1. 1–4: CMS lines (ZMS 12A-2, ZMS 12A-3, SM 6A-1, SIM3A-1); 5–7: fertility restoring lines (0-613-2R, 501R, Zheng-R); 8–11: maintainer lines (ZMS 12, SIM3, SM6); 12–16: F_1. (A × R)

mapped in the same linkage group (Zhang et al. 2002) as those tightly linked with Rf_1 presented later, were used to test whether the molecular markers are segregating normally in the F_2 in which Rf_1 did not fit a 3:1 segregation. The primer S1231 generated a co-dominant maker $S1231_{210}$ and a dominant maker $S1231_{140}$. A dominant fragment $S1317_{180}$ could be amplified using S1317. The segregation ratio of the marker $S1231_{210}$ locus is 59:115:57, $S1231_{140}$ is 174 to 57, and $S1317_{180}$ is 171 to 60 in the (SM12A-1 × SIM3R) F_2. Three marker loci were segregated in a normal Mendelian manner in the F_2 and perfectly fit the expected ratio, with the x^2 value 0.01082. This result confirmed our supposition that there did exist a difference between the *Rf* and *rf* male and female gametes during the double-fertilization.

Identification of RAPD and SSR markers and linkage analysis

In present study, a genotype representation analysis (GRA) was put forward. Fertility representations were constructed at first. The sterility representation consisted of some CMS-lines including ZMS 12A-1, ZMS 12A-2, ZMS 12A-3, SIM 3A-1, SM 6A-2 and SM 16A-2, and maintainer lines (N-rfrf) without Rf_1 including ZMS 12 and SIM 3, and the fertility representation including restorer lines (*RfRf*) 0-613-2R, 501-R and Zheng-R. Though a lot of polymorphic DNAs among the CMS-lines, restorer lines and maintainer lines were observed, only two RAPD markers, NAU/RAPD/$Rf_1{3}_{1480}$ and NAU/RAPD/$Rf_1{5}_{710}$ (co-dominant markers), and three reliable SSR markers, NAU/SSR/$Rf_1{2}_{135}$, NAU/SSR/$Rf_1{1}_{170}$ and NAU/SSR/$Rf_1{4}_{215}$, amplified by two primers were identified on the fertile and sterile representations (Figs. 1, 2). It was assumed that they might be tightly linked to the Rf_1.

These two RAPD and two pairs of SSR primers were used to amplify the individual plant of three F_2s as the mapping population (Fig. 1, 2). The segregation of the markers was highly in accordance with that of the fertility restoration in these populations. There were only a few recombined individuals detected between Rf_1 and the marker loci, suggesting that the DNA markers was tightly linked to the Rf_1 (Fig. 2, Table 4). However, x^2-tests revealed that the segregation of dominant markers did not fit the expected 3:1 ratio (dominant to recessive), and that segregation of the co-dominant markers did not fit the expected ratio of 1:2:1, either (Table 4). This inheritance pattern of molecular markers was in complete accord with that of phenotype segregation. Theoretically, inheritance of a molecular marker should be in accord with the Mendelian segregation ratio for its neutral nature, just as our results were amplified by S1231 and S1317; but in this study a distorted ratio was also obtained. It was clear that due to unequal viability of *Rf* and *rf* pollen during the double fertilization, the number of sterile plants was low.

Based on the segregation of F_2 using the MAPMAKER 3.0 program, NAU/RAPD/$Rf_1{3}_{1480}$ was tightly linked to Rf_1 with a genetic distance of 0.4 cM in the (SIM3A-1 × SIM3R) F_2, 0.1 cM in the (SIM3A-2 × SIM3R) F_2, 0.6 cM in the (SM12A-1 × SIM3R) F_2; and NAU/RAPD/$Rf_1{5}_{710}$ to the Rf_1 was 1.2-cM, 0.3-cM and

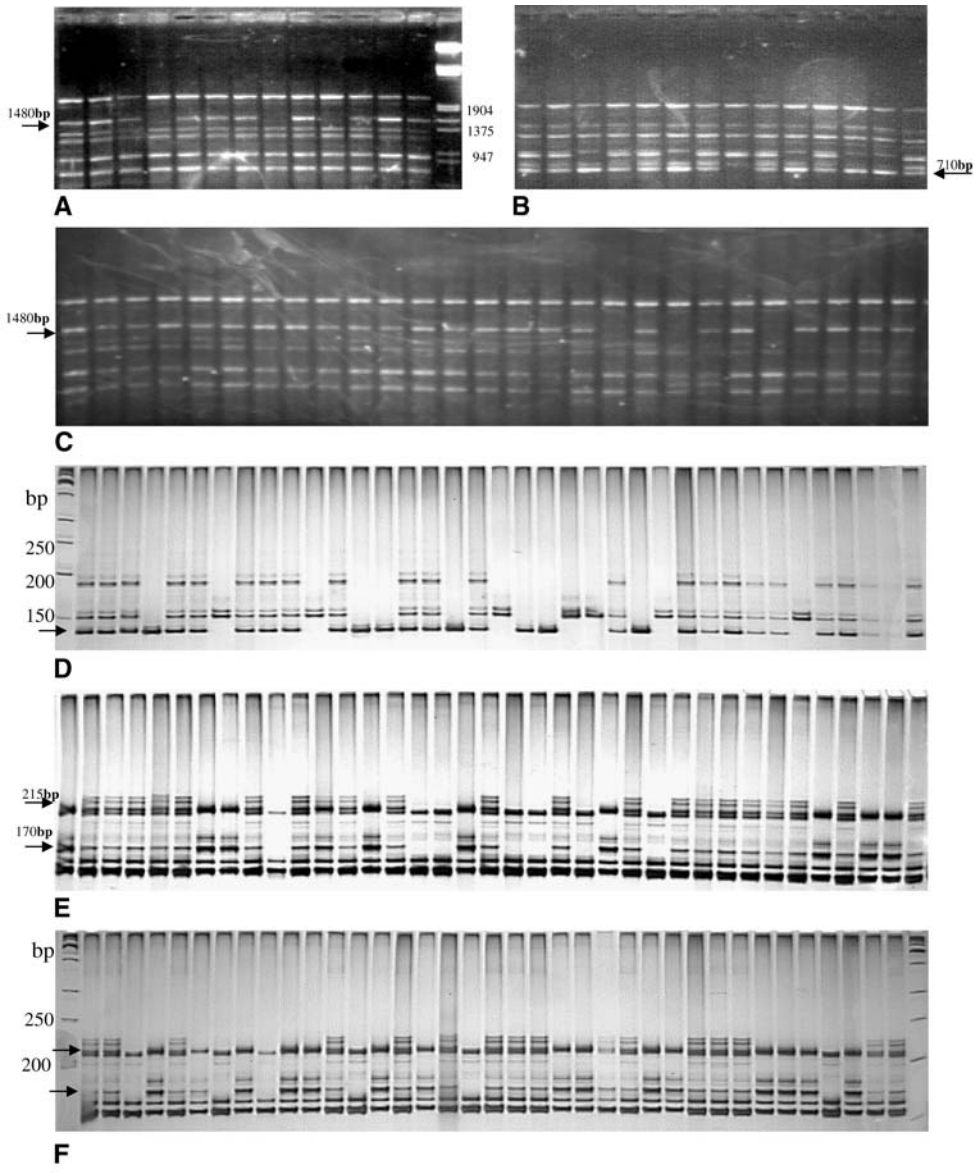

Fig. 2A–F RAPD and SSR analysis of individuals in F_2 (A × R) with Primer NAU/RAPD/Rf$_1$3 (**A, C**), NAU/RAPD/Rf$_1$5 (**B**), NAU/SSR/Rf$_1$2 (**D**), NAU/SSR/Rf$_1$1 (**E, F**)

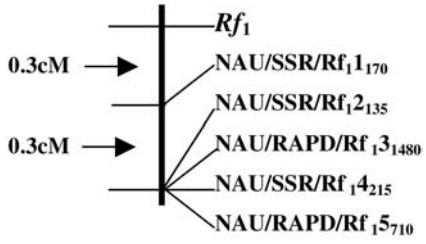

Fig. 3 Linkage map of CMS fertility restorer gene Rf_1

0.9-cM, respectively. The co-dominant marker NAU/SSR/Rf$_1$2$_{135}$ located 1.2-cM, 0.0-cM and 0.9-cM to the Rf_1 in the above three populations respectively. The co-dominant marker NAU/SSR/Rf$_1$4$_{215}$ was 1.2-cM, 0.3-cM and 0.9-cM from Rf_1. The NAU/SSR/Rf$_1$1$_{170}$, a dominant marker, was located 1.2-cM, 0.3-cM and 0.6-cM from Rf_1. Among the different populations used in this study, the genetic distances were similar, indicating that fertility restoration was probably due to the same gene. So three F_2s were pooled and used to construct a linkage test. Their linkage relationship between molecular markers and Rf_1 was presented in Fig. 3.

Chromosome mapping for Rf_1

One set of aneuploid hybrids crossed the monosomic and telodisomic lines in the TM-1 (*G. hirsutum*) genetic background as female parent, with 3-79 (*G. barbadense*) used to map the Rf_1. A SSR primer NAU/SSR/Rf$_1$4 produced a co-dominant marker, NAU/SSR/Rf$_1$4$_{215}$, in the restoring line, and an allele marker, NAU/SSR/Rf$_1$4$_{190}$, in the maintainer line was used for this purpose. In the

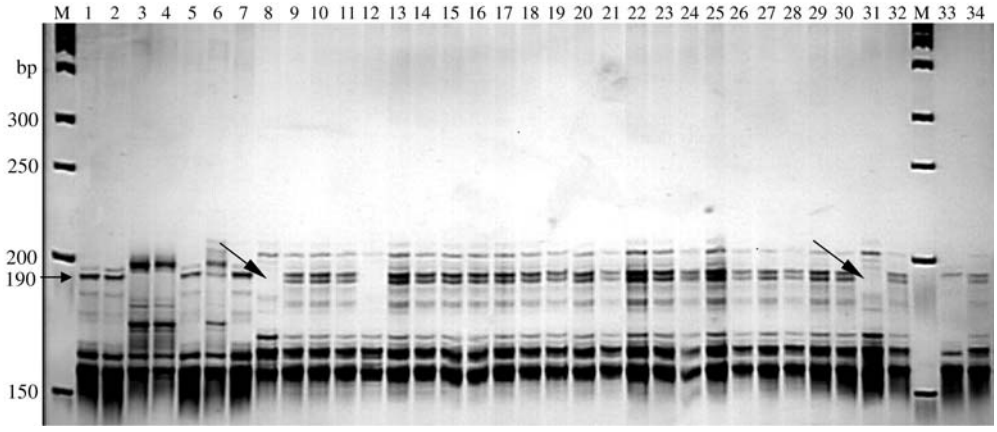

Fig. 4 SSR products using primer NAU/SSR/$Rf_1$1 in the CMS lines and the aneuploid hybrids. *Lanes 1–2* CMS lines; *Lanes 3–4* restoring lines; *Lane 5* maintainer line; *Lane 6* F_1; *Lane 7*, TM-1; *Lane 8 G. barbadense* cv Hai-7124; *Lanes 9–30* H1, H2, H3, H4, Te5Lo, H6, H7, H9, H10, Te 11Lo, 12, Te 14Lo, Te 15Lo, H16, H17, H18, H20, Te 22Lo, Te 22Sh, H23, H25, and Te 26Sh, respectively; *Lanes 31–32*, Te 4Lo,Te 4Sh; *Lanes 33–34*, H26 and Te 20Sh, respectively. *M* DNA size maker

Table 4 RAPD and SSR analysis of the F_2 individual progeny

Population[a]	No. individuals	Sterility or fertility		Marker NAU/RAPD/$Rf_1 3_{1480}$[b]		Marker NAU/RAPD/$Rf_1 5_{710}$[c]			Marker NAU/SSR/$Rf_1 2_{135}$			Marker NAU/SSR/$Rf_1 1_{170}$		Marker NAU/SSR/$Rf_1 4_{215}$		
		Sterility	Fertility	*rfrf*	*Rf-*	*rfrf*	*RfRf*	*Rfrf*	*rfrf*	*RfRf*	*Rfrf*	*rfrf*	*Rf-*	*rfrf*	*RfRf*	*Rfrf*
1	196	35	161	36	160	36	64	96	36	64	96	36	160	36	64	96
2	208	39	169	42	166	40	63	104	39	63	106	40	168	40	63	105
3	231	43	188	46	185	43	79	107	44	79	108	44	187	44	79	108
Total	635	117	518	124	511	119	206	307	119	206	310	120	515	120	206	309

[a] Population1: F_2(SIM3A-1 × SIM3R); population2: F_2(SIM3A-2 × SIM3R); population3: F_2(SM12A-1 × SIM3R)
[b] NAU/RAPD/$Rf_1$3 was not detected on one individual in population 2, two individuals in population 3
[c] NAU/RAPD/$Rf_1$5 was not detected on one individual in population 2, two individuals in population 3

chromosome mapping system, NAU/SSR/$Rf_1 4_{190}$ could not be amplified in H4 and Te 4Lo (Fig. 4). This clearly indicated that Rf_1 was located on the long arm of Chr. 4.

The gene "dose effect" in fertility restoration

By use of the co-dominant markers NAU/RAPD/$Rf_1 5_{710}$, NAU/SSR/$Rf_1 2_{135}$ and NAU/SSR/$Rf_1 4_{215}$, the genotype of the individual progeny could be identified. Combining the field-fertility scoring with molecular markers by genotype analysis, it was found that the average percent of the pollen-shedding anther is 90.749 ± 7.613% in the *RfRf* homozygous plants, and 69.64 ± 22.779% in the heterozygous plants (*Rfrf*). A *t*-test showed that the difference in pollen-shedding percent between the homozygous and heterozygous plants is very significant (t = 14.5629, $t_{0.05}$ = 1.6488), indicating that there existed a gene dosage in the CMS fertility restoration.

Discussion

The CMS-restoration system of cotton has been intensively studied, especially in the inheritance of restoring genes. The results were quite different from one another (Meyer and Meyer 1965; Meyer 1969, 1973b, 1975; Weaver and Weaver 1977; Sheetz and Weaver 1980; Wang et al. 1996a, b; Zhang et al. 2001), mainly because of the stability of the genetic materials used, the difference of fertility scoring standards, the small size of the segregating populations, and the low number of cross and environmental conditions, among other causes. Moreover, these results primarily depended upon the segregation ratio and did not monitor the gene locus. As known, the prerequisite of Mendel's normal segregation is that all the viability of female and male gametes should be equal during double fertilization; otherwise a distorted segregation ratio would be observed.

Sometimes the non-restorer allele (*rf*) is not transmitted normally through the pollen when it is in sterile cytoplasm. Because of inviability or reduced competitiveness in the non-restorer (*rf*) relative to the pollen grains with the restorer allele (*Rf*), some pollen with the non-restorer allele (*rf*) may fail in double fertilization. This was confirmed by the difference in the mean number of normal seeds per boll in the cross of the CMS lines × homozygous individual (*RfRf*) and the CMS lines × heterozygous individual (*Rfrf*) in the F_2 generation, as well as the segregation of molecular markers.

Of course, *rf* pollen grains do not have any competitiveness in the gametophytic sterility restoration system. In CMS restoration, the Rf_1 in cotton probably functions between the sporophytic and gametophytic levels, and

Rf_1 pollen gametes have partial competitiveness in fertilization. Assuming the normal behavior of female gametes in fertilization, the male *rf* gametes were unfavored to some extent in double fertilization. If the competitiveness of *rf* male gametes is 60%, like that of *Rf* gametes, we would observe a segregation ratio of 4.33 F to 1 S. If it is 50%, then the ratio is 4.988:1, corresponding with that in the (ZMS 12A-3 × 0-613-2R) F_2; if it is 35%, then the ratio is 6.692:1; if it is 30%, then the ratio is 7.696:1 and so on, which explains all the observed ratios. Although the ratio was 1:1 in test crosses ($A \times F_1$), the number of sterile plants was smaller than that of fertile plants, which also confirmed the low competitiveness of the pollen with the *rf* allele.

The phenomenon that in the presence of male-sterile cytoplasm the *Rf* male gametes were more favored in fertilization than *rf* gametes, was found in several crops. Tsunewaki (1993) found that in Mt cytoplasm, *Rf* pollen was favored three to one over *rf* pollen in fertilization. In *Aegilops crassa* Boiss. cytoplasm, the competitiveness of rfd_1 pollen is only 90% of Rfd_1 pollen (Murai and Tsunewaki 1994); besides the exotic cytoplasm, some alien chromosomes or genes also have negative effects on the transmission of certain genes (Nasuda et al. 1998). In the gametophytic CMS-S system of maize, based on the RFLP mapping information, the non-restoring allele, rf_3 was transmitted aberrantly through the male gamete (Kamps and Chase 1997).

Based on segregation analysis of the progeny and molecular markers, we know that in this CMS-Rf system DNA fragments tightly linked to the Rf_1 gene were preferentially transmitted to the next generation in cotton. Thus, one can infer that the Rf_1 gene functions as a one-dominant-gene model.

Generally, a single molecular-marker locus segregates in a Mendelian ratio. In this study, however, the segregation ratio of the DNA marker tightly linked with the *Rf* locus is distorted, and much perfectly corresponding with the ratio of sterility to fertility in the plant. Of course, interaction between the male sterility cytoplasm and the nuclei of *G. hirsutum* also influences the fertility of the embryo sac, resulting in the impairment of female gametes to some degree (Frankel and Galom 1977; Wang et al. 1997). Therefore, it can be concluded that the distorted ratio we observed may have primarily resulted from the low competitiveness of the *rf* gametes, and that the Rf_1 gene functions in a one-dominant-gene model.

As the genetic base of the *Rf* gene for CMS in cotton was not identified, few studies were done on its chromosome mapping. On the basis of monosomic analyses, Da Silva (1981) and Maranhao et al. (1984) reported that one dominant restorer gene was probably in chromosome 18D, with some modifying genes being on 16D, 25D and telesomic 15L. Linkage tests showed that *Rf* was linked with *Rc* (cracked root gene) and did not link with 13 genetic marker loci which were distributed on at least nine chromosomes, i.e. 3, 5, 6, 7, 12, 15, 16, 20, 15 (Weaver and Weaver 1979; Kohel et al. 1984). In this study, we found that the Rf_1 gene is in the long arm of Chr. 4 based on monosomic and telesomic analyses, with one co-dominant molecular marker tightly linked with it. Generally, it is assumed that the Rf_1 gene for CMS is probably from *G. harknessii*. (Meyer 1973b, 1975), hence *Rf1* is inferred to be on chromosome D. The cause for this phenomenon is probably the crossover of the subgenomes A and D in the homologous assemblage, or translocation with others, which is still to be further explored.

To increase the efficiency of screening, the molecular markers were tightly linked to the Rf_1 locus. We adopt the genotype-representation analysis (GRA), which is a combination of the methods of bulked segregation analysis (BSA), described by Michelmore et al. (1991), and NIL. Using GRA, the fertility representation with Rf_1, the fertility representation without Rf_1 and the sterility representation were constructed. Although at first more laborious work occurred during screening of the primers, possibly fewer markers were obtained compared with other strategies, once the molecular marker was obtained, and it would be surely tightly linked with Rf_1. Its high efficiency probably resulted from excluding the influence of different genetic backgrounds.

The molecular markers identified in the present work, and much closely linked to the Rf_1, are of great value in selecting and detecting the Rf_1 gene in the breeding lines, and will be very helpful for breeding new elite restorer lines efficiently. To avoid the danger of cytoplasm-specific diseases, restorer lines with normal cytoplasm are better than with sterile cytoplasm. But sometimes, breeding restorer lines with normal cytoplasm takes a much long time, since it needs testcrossing with CMS lines in each generation. By the use of these markers, especially co-dominant markers, we can easily distinguish the homozygous and heterozygous alleles, and clearly detect the existence of Rf_1. This marker-aided-selection will be a good tool for the development of restorer lines. Moreover, these tightly linked molecular markers will be a good basis for cloning the Rf_1 gene with the map-based-cloning procedures. They are also useful tools in understanding the mechanism of CMS and accelerating the process of cotton breeding.

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