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REVIEW

Two non-allelic nuclear genes restore fertility in a gametophytic pattern and enhance abiotic stress tolerance in the hybrid rice plant

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Abstract In *indica* rice, the HongLian (HL)-type combination of cytoplasmic male sterility (CMS) and fertility restoration (*Rf*) is widely used for the production of commercial hybrid seeds in China, Laos, Vietnam and other Southeast Asian countries. Generally, any member of the gametophytic fertility restoration system, 50% of the pollen in hybrid F_1 plants displays recovered sterility. In this study, however, a HL-type hybrid variety named HongLian You6 had approximately 75% normal (viable) pollen rather than the expected 50%. To resolve this discrepancy, several fertility segregation populations, including F_2 and BC_1F_1 derived from the HL-CMS line Yuetai A crossed with the restorer line 9311, were constructed and subjected to genetic analysis. A gametophytic restoration model was discovered to involve two non-allelic nuclear restorer genes, *Rf5* and *Rf6*. The *Rf5* had been previously identified using a positional clone strategy. The *Rf6* gene represents a new restorer gene locus, which was mapped to the short arm of chromosome 8. The hybrid F_1 plants containing one restorer gene, either *Rf5* or *Rf6*, displayed 50% normal pollen grains with I_2-KI solution; however, those with both *Rf5* and *Rf6* displayed 75% normal pollens. We also established that the hybrid F_1 plants including both non-allelic restorer genes exhibited an increased stable seed setting when subjected to stress versus the F_1 plants with only one restorer gene. Finally, we discuss the breeding scheme for the plant gametophytic CMS/*Rf* system.

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Introduction

In plants, cytoplasmic male sterility (CMS) is a maternally inherited trait that leads to the production of non-functional pollen. CMS is frequently caused by chimeric open reading frames in the mitochondrial genome and may be suppressed or counteracted by nuclear genes known as restorer-of-fertility (*Rf*) genes (Nair 1993; Schnabel and Wise 1998; Hanson and Bentolila 2004). This phenomenon is used widely for hybrid seed production. In addition to their commercial application, the CMS/*Rf* system also provides insight into the interactions between mitochondrial and nuclear genomes in plants (Havey 2004; Chase 2007).

In rice (*Oryza sativa* L.),three main CMS/*Rf* systems which based on pollen degeneration and genetic evidence of restoration, Wild abortive (WA), HongLian (HL) and Boro II (BT) are effectively utilized in rice cultivation (Lin and Yuan 1980; Rao 1988; Yuan 1994).The WA-CMS line is derived from wild rice and belongs to the sporophytic CMS/*Rf* system, in which more than 90% of pollen sterility can be rescued by one of two major restorer genes, *Rf3* or *Rf4*, in F_1 hybrid plants (Li et al. 2007). The HL- and BT-CMS were absorbed into the gametophytic CMS/*Rf* system, where the sterility of abortive pollen grains is

restored in a gametophytic pattern, and only those pollen grains containing the restorer gene can survive and pass it on to future generations. No more than 50% of pollen grains can be restored in F_1 hybrid plants. This phenomenon is also observed in CMS plants such as maize S, sorghum A3 and rice types CW and LD (Watanabe 1971; Ishimine and Shinjyo 1978; Bosques-Vega et al. 1987; Gabay-Laughnam et al. 2004).

HL-CMS is derived from the common red-awned wild rice *Oryza rufipogon* through backcrossing with the early-matured *indica* variety Lian Tang-Zao; the sterile pollens are spherical with no accumulated starch when stained with 1% I_2-KI solution (Zhu 1984; Rao 1988; Li et al. 2007). The pollen collapses due to expression of a mitochondrial chimeric gene region designated *atp6-orfH79* (Yi et al. 2002). Molecular dissection reveals that the expression of ORFH79 in mitochondria impairs mitochondrial function, which affects the development of male gametophytes (Peng et al. 2010). Transcription of *atp6-orfH79* can be processed by the nuclear gene designated *Rf5* (Huang et al. 2000), which is located on chromosome 10 in a region with simple sequence repeat (SSR) markers between HL01 and MRG4456 and was identified as a member of the pentatricopeptide repeat gene (PPR) family using a map-based cloning strategy (Huang et al. 2003; Hu et al. 2011). Within the last 10 years, the combination of HL-CMS line, the maintainer and the corresponding restorer has been used widely to produce F_1 hybrid seeds, which exhibit high yields, good grain quality and adaptability to environment. Although many F_1 hybrids derived from HL-CMS produce about 50% normal pollen and exhibit normal seed setting, only the hybrid varieties HongLian You 6 produce about 75% stainable pollen grains and exhibit stable seed setting, and had been widely planted in China along the Yangtze River and the South China, as well as in Laos, Vietnam, Bangladesh and other Southeast Asian countries.

Pollen fate is controlled by the interaction of the CMS and *Rf* genes of the haploid gamete in the plant gametophytic CMS/*Rf* system, in which the sterility of only approximately 50% of pollens can be restored in hybrid F_1 plants. In this study, however, approximately 75% of pollen grains were rescued the sterility in HL-CMS F_1 plants. This observation resembles neither the gametophytic (50% normal pollen) nor the sporophytic (more than 90% normal pollen) CMS restoration pattern. To understand this phenomenon, we constructed the pollen fertility segregation populations through self- and backcrossing of the HongLian You 6 hybrid, which was derived from the cross of YTA and 9311. In these fertility segregation populations, the restorer genes were subjected to both genetic and molecular analyses and a gametophytic restoration model of two non-allelic genes was discovered. When both the *Rf5* and *Rf6* restorer genes were present, the sterility of approximately 75% of pollen grains was rescued, whereas the sterility of only 50% of pollens could be rescued in the presence of only one of the two restorer genes in the hybrid F_1. Based on our discovery of two non-allelic genes in this restoration model, we propose a scheme for increased fertility that is very useful for plant breeding.

Materials and methods

Plant materials

The fertility-restorer line 9311 was crossed with Yuetai A (YTA), a CMS line derived from a cross of *indica* early-matured cultivar Lian-Tang Zao and *Oryza rufipogon* Griff, a red-awned wild rice. The resulting F_1 generation plants were backcrossed with Yuetai B (YTB) plants, which had the same nuclear background as YTA but different mitochondrial genomes, to produce BC_1F_1 seeds for coarse mapping and the F_2 plants were used for fine mapping of *Rf6* locus. Simultaneously, we performed marker-assisted selection in the BC_1F_1 population to obtain plants with a genotype of *Rf5rf5rf6rf6* or *rf5rf5Rf6rf6*, which were used to construct the segregating populations for genetic analysis of one restorer gene. The R5 and R6 were derived from the *Rf5rf5rf6rf6* and *rf5rf5Rf6rf6* self-crossed plants homozygous for the *Rf5* or *Rf6* locus, respectively. The three NILs, *Rf5*, *Rf6* and *Rf5Rf6* were developed by backcrossing with YTA. The genotypes and phenotypes of the plants used in this study are listed in Table S1.

Pollen grain observation and genetic analysis

Pollen grains from mature anther rice plants were stained with a 1% iodine-potassium iodide (I_2-KI) solution, observed under an optical microscope and classified into two groups: normal pollen with starch accumulation (stained black) and abortive pollen with a spherical shape exhibiting unstained in I_2-KI solution. Approximately 400 grains were counted per spikelet. The plants with <5% stainable pollen were categorized into the sterile class, and those with >5% were regarded as fertile. The fertile plants with 45–50, 70–75 or ≥90% stainable pollen grains were categorized as having fertility restoration abilities of 50, 75 or 100%, respectively. All plants were grown under normal condition with proper management from April to August in Wuhan. The pollen from segregating populations, including F_2 populations generated from the crosses of YTA/9311, YTA/R5 or YTA/R6 and BC_1F_1 populations derived from the crosses of YTA/9311//YTB, YTA/R5//YTB or YTA/R6//YTB, were observed. The statistical results were used for further genetic analysis.

Molecular location of *Rf6*

To mapping of the restorer gene *Rf6*, a BC_1F_1 population that included 544 plants derived from YTA/9311//YTB was screened using the SSR markers RM6469 and RM25661, both of which are tightly linked to the *Rf5* gene (Hu et al. 2011). Two DNA bulks from the sterile plants with genotype *rf5rf5rf6rf6* and the fertile plants with genotype *rf5rf5Rf6rf6* were constructed and 689 molecular markers were used to detect the recombinants. To further determination of the location of the recombinations nearest to *Rf6*, 19,355 F_2 plants derived from the crosses of YTA/9311 was used for linkage analysis, of which 3,302 plants with the genotype *rf5rf5Rf6Rf6* or *rf5rf5Rf6rf6* were detected with molecular markers. Some of the primers used in this study are listed in Table S2.

Results

A new phenotype of fertility restoration in hybrid rice

YTA is a male sterility line whose cytoplasm comes from common red-awned wild rice and fails to produce normal pollen (Fig. 1a). As a member of gametophytic CMS system, its fertility restoration was convinced in a gametophytic pattern, in which the sterility of approximately 50% of pollen in hybrid F_1 plants can be restored, as was observed in BT- or CW-CMS-type rice. The pollen grains of the hybrid variety HongLian You 6, which was derived from the cross of YTA with 9311, were observed under the microscope. In the 423 F_1 plants, approximately 75% of sterile pollen grains were rescued and stained black using a 1% I_2-KI solution (Fig. 1b). However, according to a gametophytic CMS pattern, no more than 50% of the pollen of HongLian You 6 F_1 plants was stained black. The fertility restoration phenotype in F_1 hybrid was neither similar to a gametophytic (50% normal pollen) nor a sporophytic (more than 90% normal pollen) CMS restoration pattern. To understand this phenomenon, we constructed the F_2 and BC_1F_1 genetic population to assess the cause of this phenotype.

Two non-allelic nuclear genes restore the fertility of HL-CMS rice

To analyze the pollen fertility genetically, two segregating populations, F_2 and BC_1F_1, were constructed. The pollen of individual plants was stained with 1% I_2-KI solution to count the numbers of normal and abnormal grains under optical microscopy. In the 554 BC_1F_1 plants, which were derived from the YTA/9311//YTB, there were 122 sterile plants with <5% of pollen stained black and 432 fertile plants with more than 50% stainable pollen. Of all 432 fertile plants, 156 plants approximately 75% normal pollen and 276 plants showed approximately 50% stainable pollen. A ratio of 1:2:1 among the plants that displayed 75 and 50% normal pollen and sterile ($\chi_c^2 = 4.18 < \chi_{0.05}^2 = 5.99$) (Table 1) indicates there is not one but two fertility restorer genes that function in a manner similar to two nuclear genes did in a genetic model of the gametophytic restoration pattern, in which only those pollens containing *Rf* genes develop normally (Table 2). The two *Rf* genes were located in different linkage groups and behaved according to Mendel's laws. To distinguish the two *Rf* genes, we named one *Rf5* and the other *Rf6*. Furthermore, Plants with 50% normal pollen were inferred to be the *Rf5rf5rf6rf6* or *rf5rf5Rf6rf6* genotype, and those with 75% normal pollen be *Rf5rf5Rf6rf6* (Table 2). To molecular evidence, we screened the genomes of 432 fertile plants using the tightly *Rf5*-linked markers RM6469 and RM25661, We detected 303 *Rf5*-containing plants that displayed 75% (*Rf5rf5Rf6rf6*) or 50% (*Rf5rf5rf6rf6*) stainable pollen

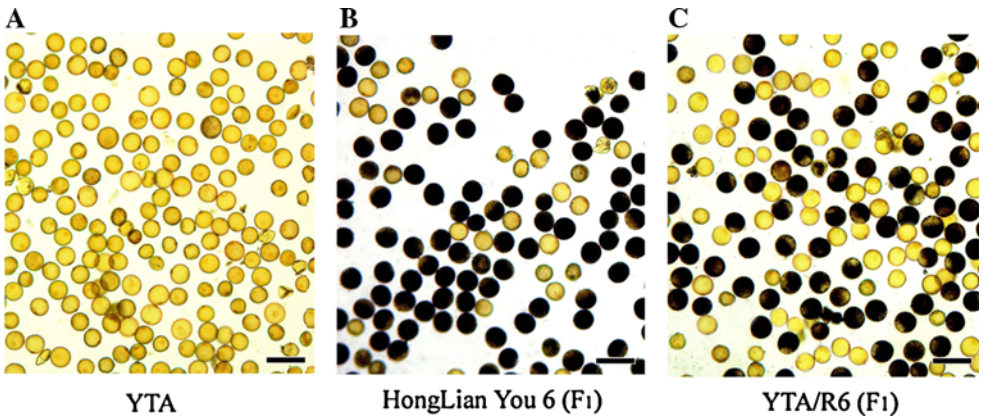


Fig. 1 Restoration of sterile pollen in the F_1 hybrids of HL-CMS plants. **a** The HL-CMS line YTA has sterile pollen grains that are spherical and display no starch accumulation when stained with a 1% I_2-IK solution. **b** The F_1 hybrid variety HongLian You 6 displays approximately 75% normal pollen grains that stain black. **c** Approximately 50% of the pollen is recovered with the restorer gene *Rf6* in YTA/R6 F_1 plants. *Bars* 50 μm

Table 1 The BC_1F_1 plants fertility genetic analysis

Plants					Chi-squared test
Numbers	Pollen genotypes	Fertility	Theory ratio	Observation numbers	$\chi^2_{0.05} = 5.99$ ($df = 2$) $\chi^2_{0.05} = 3.84$ ($df = 1$)
554	I	Fertile	1:4	156	$\chi^2_c = 4.18$
	II, III	Fertile	2:4	276	
432	IV	Sterile	1:4	122	$\chi^2_c = 2.19$
	I, II	Fertile	2:3	303	
	III	Fertile	1:3	129	

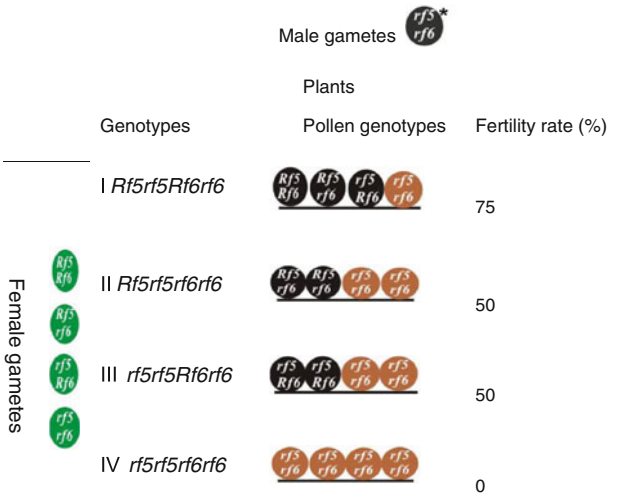
grains and another 129 plants without *Rf5*; the *Rf5*-lacking plants were likely to be of the *rf5rf5Rf6rf6* genotype (Table 2). The 303:129 ratio of plants containing versus lacking the *Rf5* gene was in accordance with the 3:1 theoretical ratio shown in the model ($\chi^2_c = 2.19 < \chi^2_{0.05} = 3.84$) (Table 1).

An F_2 population derived from the cross YTA/9311 (consisting of 19,355 plants) was subjected to the same genetic analyses as the BC_1F_1 population. We found 3,148 plants with approximately 50% stainable pollen, and 16,207 plants that showed approximately 75 or 100% stainable pollen. The ratio of plants with approximately 50% stainable pollen to other plants was 3,148:16,207, which coincided with the theoretical 2:10 ($\chi^2_c = 2.2 < \chi^2_{0.05} = 3.84$) (Table 3) ratio that was described by the two non-allelic gene restoration model in which there are 12 and not 16 genotypes are involved. That is because only the gametes containing the *Rf* gene can survive and pass the gene on to later generations (Table 4). According to this model, we speculated that those individual plants with approximately 50% stainable pollen were of the genotype *Rf5rf5rf6rf6* or *rf5rf5Rf6rf6*. In addition to analyzing the genetics behind the plant pollen phenotype, we used the *Rf5*-linked SSR markers RM6469 and RM25661 to screen the individual genomes of all 19,355 plants of the F_2 generation. There were 3,302 plants (*rf5rf5Rf6rf6* or *rf5rf5Rf6Rf6*) lacking the *Rf5* gene; the 16,053:3,302 ratio of plants containing versus lacking the *Rf5* gene is in accordance with the theoretical ratio of 10:2 ($\chi^2_c = 2.12 < \chi^2_{0.05} = 3.84$) (Table 3), as the model showed (Table 4). All of these results originated from the BC_1F_1 and F_2 genetic population analysis, thus revealing that there are two non-allelic nuclear genes, *Rf5* and *Rf6*, each individually capable of restoring the fertility of HL-CMS rice in a gametophyte restoration pattern.

The *Rf6* gene restores fertility in a gametophytic restoration pattern

Our genetic and molecular analyses showed that *Rf5* and *Rf6* were involved in HL-CMS sterility restoration, and the YTA/R5 F_1 plants showed approximately 50% normal pollen and

Table 2 The model of two non-allelic *Rf* genes genetic in BC_1F_1 population



* The ovals represent normal and abnormal pollen, respectively. Only pollen grains containing the *Rf* genes are stainable and develop normally, as shown in both Tables 4, 6 and 8. The pollen of YTB develops normally because it has the same nuclear background as YTA but different mitochondrial genomes

the *Rf5* gene functioning in the YTA/R5 F_2 and YTA/R5//YTB segregating populations in a gametophytical restoration pattern (data not shown); however, the restorer gene *Rf6* restored abortive pollens according to a gametophytic restoration pattern had not yet been validated genetically. Therefore, from the YTA/9311//YB BC_1F_2 population, we selected out the R6 (*rf5rf5Rf6Rf6*) plants exhibiting 100% stainable pollen, but lacking *Rf5*, using marker RM25661, which displayed tight linkage to the *Rf5* gene, to cross with YTA, and the hybrid F_1 plants were self-crossed to produce the F_2 population. The individual pollen grains of 106 YTA/*rf5rf5Rf6Rf6* F_1 plants were observed under the optical microscope, all individuals had approximately 50% pollen grains stained black with I_2-KI solution (Fig. 1c). In the F_2 population, which consisted of 542 plants, there were 213 individuals with approximately 50% stainable pollen and 229 plants with more than 90% fertile pollen. This ratio is consistent with the theoretical 1:1 ratio ($\chi^2_c = 0.15 < \chi^2_{0.05} =$

Table 3 The F_2 plants fertility genetic analysis

Plants					Chi-squared test
Numbers	Pollen genotypes	Fertility	Theory ratio	Observation numbers	$\chi^2_{0.05} = 3.84$
19,355	XI, XII	Fertile	2:12	3,148	$\chi^2_c = 2.2$
	I, II, III, IV V, VI, VII, VIII IX, X	Fertile	10:12	16,207	
19,355	IX, XII	Fertile	2:12	3,302	$\chi^2_c = 2.12$
	I, II, III, IV V, VI, VII, VIII XI, X	Fertile	10:12	16,503	

Table 4 The model of two non-allelic *Rf* genes genetic in F_2 population

		Male gametes			
		Rf5 Rf6	Rf5 rf6	rf5 Rf6	rf5 rf6 *
		Plants			
	Genotypes	Pollen genotypes	Fertili rate (%)	Genotypesty	Pollen genotypes	Fertility rate (%)
Female gametes: Rf5 Rf6	I *Rf5Rf5Rf6Rf6*		100	VII *Rf5rf5Rf6Rf6*		100
Rf5 rf6	II *Rf5Rf5Rf6rf6*		100	VIII *Rf5rf5Rf6rf6*		75
rf5 Rf6	III *Rf5rf5Rf6Rf6*		100	IX *rf5rf5Rf6Rf6*		100
rf5 rf6	IV *Rf5Rf5Rf6rf6*		100	X *Rf5rf5Rf6rf6*		75
	V *Rf5Rf5rf6rf6*		100	XI *Rf5rf5rf6rf6*		50
	VI *Rf5rf5Rf6rf6*		75	XII *rf5rf5Rf6rf6*		50

Table 5 The genetic analysis of *Rf6* gene in F_2 population

Plants					Chi-squared test
Numbers	Pollen genotypes	Fertility	Theory ratio	Observation numbers	
442	I	Fertile	1:2	213	$\chi^2_{0.05} = 3.84$
	II	Fertile	1:2	229	$\chi^2_c = 0.51$

3.84), as shown in (Table 5), which indicated a gametophytic restoration model (Table 6). Also, another proof had been provided by the genetic analysis of *Rf6* in the YTA/R6//YTB population (Tables 7, 8). All results demonstrated that the *Rf6* gene restores pollen sterility in a gametophytic pattern, as was observed for the *Rf5* gene.

Table 6 The model of *Rf6* genetic in F_2 population

		Male gametes		
		Rf6 rf6*		
		Plants		
		Genotypes	Pollen genotypes	Fertility rate (%)
Female gametes	Rf6	I*Rf6Rf6*	Rf6 Rf6	100
	rf6	II*Rf6rf6*	Rf6 rf6	50

Molecular mapping of the *Rf6* gene

We previously fine-mapped the restorer gene *Rf5* to chromosome 10 in the region between SSR markers RM6469 and RM25661 (Hu et al. 2011). This is a frequently observed genomic location of *Rf* genes in rice (Ahmadikhah and Karlov 2006; Kato et al. 2007), To find the molecular location of the new restorer gene *Rf6*, we selected 129 plants with the *rf5rf5Rf6rf6* genotype that were derived from the BC_1F_1 of three crosses (YTA/9311//YTB) and another group of YTA plants (*rf5rf5rf6rf6*) to constructed two pools of DNA, respectively. Three SSR markers on chromosome 8 exhibited linkage with the *Rf6* locus, and the *Rf6* gene was primarily limited to a larger genetic region between RM7037 and RM22355 (Fig. 2). To further refine the position of *Rf6*, we used additional molecular markers in the region to screen the individual genomes of recombinants. The markers RM3710, RM407, RM152 and RM3702 did not suggest a recombination event, and all four markers were linked tightly with the *Rf6* gene; however, there was still a large physical distance between RM3710 and RM3702 (Fig. 2) according to the Nipponbare genome sequence (NCBI database). To further fine-map the restorer gene *Rf6*, 3,302 plants (*rf5rf5Rf6rf6* or *rf5rf5Rf6Rf6*) were individually picked out from the F_2 population derived from the YTA/9311 cross, and these plant genomes were used to PCR detecting the *Rf6* gene and linkage analysis. A plant recombinant was detected with marker RM22242, and two plants happened to exchange marker RM3710, while no recombinant individual was acquired using marker RM407. Thus the location of the *Rf6* gene was narrowed down to a region of approximately 200 kb, according to the sequence of the Nipponbare genome, between the markers RM3710 and RM22242 on the short arm of chromosome 8 (Fig. 2). The molecular cloning of the *Rf6* gene is ongoing.

Two non-allelic restorer genes enhance low-temperature stress tolerance in hybrid F_1 plants

Previously, out of a total of 62 hybrid F_1 crosses of HL-type rice, we identified that HongLian You 6, which is a plant widely used in farming, produces approximately 75% normal pollen. We thus speculated that the plants with 75% normal pollen are more adaptable to the surrounding environment than those with only 50% normal pollen. We detected the pollen of remaining 61 hybrid F_1 crosses and found they only produced 50% fertile pollen grains. To further evaluate the ability of hybrid F_1 plants that contained either one or two *Rf* genes to adapt to the environment, we performed three crosses of hybrid F_1 populations derived from YTA/*Rf5*NIL, YTA/*Rf6*NIL and YTA/*Rf5Rf6*NIL plants and treated them with low temperature (17°C) during the young microspore stage of the pollen development process for 5 days. Compared with the untreated plant, no more than 20% of the pollen grains from the YTA/*Rf5*NIL or the YTA/*Rf6*NIL hybrid F_1 plants can be stained with I_2-KI solution, and displayed only about a 7% rate of seed setting. In contrast, approximately 42% of the pollen from YTA/*Rf5Rf6*NIL F_1 plants was normal and exhibited 74% seed setting (Fig. 3). This result shows that the cross that contained two non-allelic restorer genes in hybrid F_1 plants increased seed setting stability and retained its ability to adapt to environmental stresses such as low temperature. This is important for plant breeders interested in cultivating hybrid varieties that exhibit a good seed setting rate and environmental adaptability.

Discussion

Plant CMS provide a chance to utilize the heterosis resulting from the formation of non-functional microspores

Table 7 The genetic analysis of *Rf6* gene in BC_1F_1 population

Plants					Chi-squared test
Numbers	Pollen genotypes	Fertility	Theory ratio	Observation numbers	
258	I	Fertile	1:2	138	$\chi^2_{0.05} = 3.84$
	II	Fertile	1:2	120	$\chi^2_c = 1.12$

Table 8 The model of *Rf6* genetic in BC_1F_1 population

Male gametes		Plants	
Female gametes	Genotypes	Pollen genotypes	Fertility rate (%)
Rf6	IRf6rf6	Rf6 rf6	100
rf6	IIrf6rf6	rf6 rf6	0

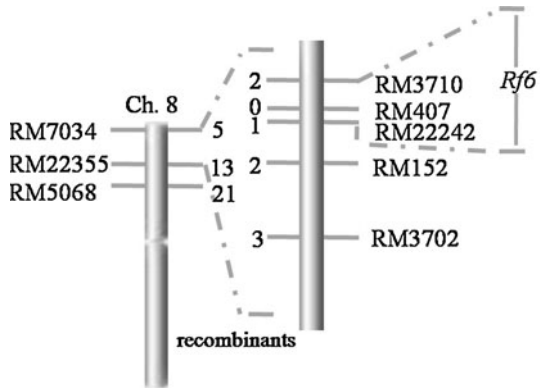


Fig. 2 Molecular mapping of the *Rf6* restorer gene to the short arm of chromosome 8

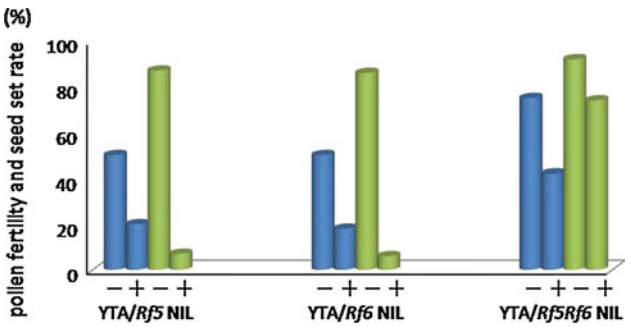


Fig. 3 Low-temperature effects on the plant pollen fertility and seed setting of the YTA/*Rf5*NIL, YTA/*Rf6* NIL or YTA/*Rf5Rf6* NIL hybrid lines. The "−" and "+" represent the control and the 17°C treatments, respectively. The *black* and *white* columns represent pollen fertility and seed setting rates, respectively

or pollen grains while female gametes develop normally, and nuclear *Rf* genes can remove the adverse impact of CMS. The system, especially sporophytic CMS/*Rf*, has been widely used in hybrid seed production, including T-maize, A1-soghum, *Ogura/Kosena radish* and WA-rice (Stephens and Holland 1954; Wych 1988; Delourme et al. 1999; Cheng et al. 2007; Jordan et al. 2010), whereas in the gametophytic CMS/*Rf* system, only a few CMS types, such as HL-type rice, especially the hybrid variety HongLian You 6 had been widely planted by famers in China. Compared with other CMS types, HL-type's normal and abnormal pollen are easily distinguished with I_2-KI solution under the optical microscope (Li et al. 2007).

Until now, there is only one restore loci had been found in same a CMS/*Rf* system such as BT-, CW-or LD- type. Here, we found that the sterility of approximately 75% of pollen from hybrid F_1 plants was rescued in HL-type CMS/*Rf* system rice. In this system, two non-allelic restorer genes, *Rf5* and *Rf6*, functioned individually in a gametophytic restoration pattern. Maybe, there are other restore loci to function together and have more pollen normally in hybrid F_1, just a program was proposed like Fig. S1, where one, two or three restorer genes which in different linkage group can rescue the observed pollen sterility by 50, 75 or 87.5%. Using the transgenic technology, the program had been proved that hybrids carrying the *Rf* gene at three loci showed 87.5% normal pollen (Komori and Imaseki 2005).

This phenomenon of multiple restorer gene loci in F_1 hybrid is very useful for hybrid breeding of gametophytic CMS plants. Here, we found the plants with approximately 75% fertility pollens can withstand environmental stresses such as low temperature and exhibit good seed setting, which is similar to the result observed in the transgenic rice hybrids (Komori and Imaseki 2005). We also screened the pollen of F_1 plants of 62 hybrid crosses derived from HL-CMS and only hybrid varieties HongLian You 6 with approximately 75% normal pollens, perhaps, that is an important factor of their adaptability to the environment.

To understand the mechanism of nuclear and cytoplasmic gene exchange, it is useful to molecularly map the *Rf* genes. In rice, many *Rf* genes have been molecularly mapped, which includes the mapping of the following genes: the two major fertility restorer genes, *Rf3* and *Rf4*, in sporophytic WA-CMS to chromosomes 1 and 10, respectively (Yao et al. 1997; Zhang et al. 1997; Ahmadikhah and Karlov 2006); the gene *Rf5* for HL- and *Rf1a*/*Rf1b* for BT- in the gametophytic CMS system to chromosome 10 (Fukuta et al. 1992; Akagi et al. 1996; Huang et al. 2000; Wang et al. 2006), the *Rf17* for CW- to chromosome 4(Fujii and Toriyama 2005) and *Rf2* for LD- to chromosome 2 (Shinjyo and Sato 1994). Here, another *Rf* gene new member, *Rf6*, for HL-CMS has been mapped to the short arm of chromosome 8. Several *Rf* genes, such as *Rf2* for T-CMS maize and *Rf17* and *Rf2* for CW-, LD-CMS rice, do not belong to the PPR family (Cui et al. 1996; Fujii and Toriyama 2009; Itabashi et al. 2011). However, most *Rf* genes, such as *Rf5* for HL-CMS (Hu et al. 2011), *Rf1a*/*Rf1b* for BT-CMS (Komori et al. 2004; Akagi et al. 2004; Wang et al. 2006), the petunia *PPR592* gene (Bentolila et al. 2002), the sorghum *Rf1* gene (Klein et al. 2005) and the radish *Rfo* or PPR-B gene (Brown et al.

2003; Koizuka et al. 2003), encode proteins containing pentatricopeptide repeats, usually target mitochondria, (Bentolila et al. 2002; Kazama and Toriyama 2003; Andres et al. 2007; Fujii et al. 2011). In this study, two PPR genes were found on the *Rf6* locus on the short arm of chromosome 8, according to the sequence available in the NCBI database. We suspect that one PPR gene is *Rf6*, and thus, molecular cloning of the restorer gene is currently underway.

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