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ORIGINAL PAPER

Gametophytically alloplasmic CMS line of rice (*Oryza sativa* L.) with variant *orfH79* haplotype corresponds to specific fertility restorer

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Abstract For years discovery and identification of the cytoplasmic male sterility (CMS) resource in wild rice is the most intriguing events in breeding field. *orfH79*, a chimeric gene in mitochondria, has been suggested being the determinant for Honglian CMS in rice. In this report o*rfH79* gene as molecular marker to screen the wild rice, we found eight accessions with *orfH79* gene in the total 42 investigated objects. Sequence analysis revealed that there were a total of nine nucleotide substitutions resulting in the change of nine amino acids in the newly identified *orfH79* in wild rice, which further fell into seven haplotypes. In order to investigate the underlying relationship between *orfH79* haplotypes and the corresponding fertility restorers, four accessions were selected with different *orfH79* haplotype as female parents to hybridize the Honglian maintainer line, Yuetai B. After eight consecutive recurrent backcrosses, four alloplasmic CMS lines with different *orfH79* haplotype were developed. Microscopic observation exhibited that their pollen grains were spherical and clear in 1% I_2–KI solution same as that of Honglian CMS line. Moreover, these four CMS lines displayed various fertility restoring model through test cross, suggesting that each *orfH79* haplotye represents a new CMS type and corresponds to their specific *Rf* allele.

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Introduction

Cytoplasmic male sterility (CMS) in higher plants is intriguing for the utilization of heterosis to produce vigorous F_1 hybrids (Francoise and Georges 2001). The CMS lines of rice are basically divided into wild-abortive (WA), Honglian (HL) and Baotai (BT) according to their distinctive cytological and genetic character (Zhu 1984). Till now, normous CMS lines have been developed through interspecies, intersubspecies or intervariety hybridization (Virmani and Shinjyo 1988) since the first CMS line (*Oryza sativa* L.) being produced at the end of 1960s (Shinjyo 1969) in rice. In China, WA and HL hybrid rice have been broadly cultivated. In order to maintain and optimize the CMS system, however, it is necessary to develop the new alternative elites CMS line. As known that the new sterile line is derived from elite CMS donors via protoplast fusion or backcross with other maintainer lines in breeding program (Bijoya et al. 1999; Nakajima et al. 2001), broad usage of one or few of CMS cytoplasm will accumulate genetic vulnerability and latent risks in commercial production. Exploitation of new sterile cytoplasm in domesticated or wild species of rice will not only enrich the diversity of CMS for hybrid rice, but also help us in better understanding of the origin and evolution of CMS in *Oryza* species.

Since Honglian CMS is derived from common red-awn wild rice of *Oryza rufipogon*, it is possible to find the similar cytoplasm in common wild rice. Therefore, cloning of chimeric *orfH79* gene related to Honglian CMS contributes to uncover the variation and distribution of *orfH79* alleles in wild rice population (Yi et al. 2002). Furthermore, if a series of alloplasmic CMS lines could be developed with variant *orfH79* haplotypes as the accessions to cross with the Honglian maintainer line, it also contributes to gain more insight into the evolutionary relationship between

CMS alleles in mitochondria and the corresponding fertility restorers (*Rf*). For such purpose, we characterized the distribution and allele variation of *orfH79* gene in the AA-genome species, and compared the restoring–maintaining relationships among the newly developed four alloplasmic CMS lines.

Materials and methods

Plant materials

There are 42 accessions of wild rice with AA-genome from International Rice Research Institute (IRRI). They are 9 *O. rufipogon*, 4 *O. glumaepatula*, 4 *O. longistaminata*, 6 *O. barthii*, 5 *O. meridionalis*, 5 *O. glaberrima*, 9 *O. nivara* (Table 1) and 14 restorers of Honglian CMS were provided by Rice Research Institute of Hubei Province (RRIH), China (Table 5). A typical Honglian CMS line, i.e., Yuetai A (YtA), and the corresponding maintainer, i.e., Yuetai B (YtB) were used in this study. Plants were grown in the experimental fields in Wuhan University campus (summer season) and Hainan Island (winter season) during 2002–2007.

Mitochondrial DNA (mtDNA) extraction

Purification of mitochondria from young leaves was performed according to the method described by Mohammed et al. (2000) with a minor modification. All the procedures were carried out at 4°C. About 50 g of freshly harvested leaves were homogenized in 400 ml pre-cooled homogenization buffer (pH 7.2) containing 0.4 M mannitol, 40 mM MOPS (3-(*N*-morpholino)-propanesulfonic acid), 1 mM EDTA, 0.05% cysteine, 0.1% BSA and 0.03% mercaptoethonal. The homogenate was filtered through 30 μm nylon mesh and the filtrate was centrifuged at 2,000×*g* for 10 min. The supernatant was centrifuged at 10,000×*g* for 15 min and the pellet was resuspended with buffer containing 0.4 M mannitol, 10 mM MOPS and 0.1% BSA. After a second cycle of centrifugation and purification on a self-forming 32% Percoll gradient, purified mitochondria pellet was resuspended in 0.5 ml TE (pH 7.5) with 200 μg of proteinase K (Mulligan et al. 1988).

PCR amplification of *orfH79* gene related to Honglian CMS

PCR amplification was carried out according to the procedure described by Yi et al. (2002) using a pair of primers designed with the sequence of orfH79, F: 5′-ATGACAA ATCTGCTCCGATG-3′, R: 5′-CTTACTTAGGAAAGA CTAC-3′. About 60 ng mtDNA was mixed with 50 mM KCl, 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1.0 unit of *Taq* polymerase, and 1 μM of primer pair in a 25 μl reaction volume. Amplified fragments were subjected to 1.5% agarose gel electrophoresis and recovered using the DNA Extraction kit (Fermentas K0513, Canada). Then the purified and recovered DNA was cloned into the pGEM T-Vector according to manufacturer (Promega A3600, USA). This DNA fragment was sequenced by Huada Corporation (Shangshai, China).

Fertility scoring of the hybrids derived from different crosses

Hybrid plants were obtained by crossing the wild rice with Honglian maintainer YtB as male or female parent. Fertility evaluation was performed based on the following two different criteria: pollen stainability in 1% I_2–KI solution and seed-setting rate of spikelets. The plants are scored fertile if dark stainable pollen grains are more than 5% and seed-setting rate of bagged spikelets are more than 1%. Otherwise, the plants are scored sterile. All the experiments were performed with triplicates; values reported were means (±SD).

Results

orfH79 gene exists in wild rice

To determine whether there is a similar cytoplasm as Honglian's in wild rice, PCR amplification was performed to identify the wild rice accessions using the *orfH79* sequence-specific primers (H1 and H2). The same size band about 240 bp as YtA was amplified from eight out of the 42 investigated accessions, but was absent in the control of YtB (Fig. 1). This phenomenon suggests that the amplified band is specific to Honglian, and these eight accessions possess similar cytoplasm to HL-CMS. The eight accessions fell into four species, of which, w11 (101411) belonged to *Oryza meridionalis*; w15 (101971), w20 (103836), w29 (104705) and w34 (105419) belonged to *Oryza nivara*; w21 (104078) belonged to *Oryza barthii*; w42 (106158) and w46 (106321) belonged to *Oryza rufipogon*.

Single nucleotide polymorphisms (SNPs) of *orfH79* among wild rice accessions

SNP frequently occurs in alleles, which leads to the changes of the composition, structure and even the function of the allele among individuals in a certain species. In this study, we sequenced all the PCR products from the eight

Table 1 Wild rice accessions used in the study (from IRRI)

Series number	Accession number	Scientific name—species	Source (Country)
w06	100219	O.rufipogon	Thailand
w07	100968	*O.glumaepatula*	Suriname
w08	100970	*O.glumaepatula*	Brazil
w09	101213	*O.longistaminata*	Ivory coast
w10	101255	*O.barthii*	Cameroon
w11	101411	*O.meridionalis*	Australia
w12	101791	*O.glaberrima*	Senegal
w13	101855	*O.glaberrima*–Saria 480	Berkina Faso
w14	101959	O.barthii	Senegal
w15	101971	*O.nivara*	India
w16	101974	*O.rufipogon*	India
w17	102452	*O.glaberrima*	Mali
w18	102641	*O.glaberrima*	Liberia
w19	103580	*O.barthii*	Chad
w20	103836	*O.nivara*	Bangladesh
w21	104078	*O.barthii*	Nigeria
w22	104081	*O.barthii*	Nigeria
w23	104085	*O.meridionalis*	Australia
w24	104127	*O.longistaminata*	Chad
w25	104147	*O.longistaminata*	Cameroon
w26	104540	*O.glaberrima*—Ex Kano	Nigeria
w27	104599	*O.rufipogon*—Uru Wee	Sri Lanka
w28	104680	O.nivara	India
w29	104705	*O.nivara*	India
w30	105204	*O.longistaminata*—Zurha/Sukimia	Ethiopia
w31	105283	O.meridionalis	Australia
w32	105293	*O.meridionalis*	Australia
w33	105303	O.meridionalis	Australia
w34	105419	*O.nivara*–Uru Wee	Sri Lanka
w35	105561	O.glumaepatula	Colombia
w36	105661	*O.glumaepatula*–Arroz Bravo	Brazil
w37	105704	O.nivara	Nepal
w38	105736	*O.nivara*–Srange	Cambodia
w39	105887	*O.rufipogon*–Jhora	Bangladesh
w40	106036	*O.rufipogon*–Padi Hantu	Malaysia
w41	106083	O.rufipogon	India
w42	106158	*O.rufipogon*	Laos
w43	106194	*O.barthii*	Guinea
w44	106260	*O.rufipogon*	Papua New Guinea
w45	106309	*O.nivara*	Cambodia
w46	106321	*O.rufipogon*	Cambodia
w47	106344	*O.nivara*	Myanmar

wild rice accessions to compare the nucleotide mutation of *orfH79* in wild rice population. Sequencing revealed that *orfH79* in the wild relative species shared 98 to 100% similarity with that of YtA. Apart from w15 and w42 which have complete sequence to that of YtA, a total of nine nucleotide variations were identified in the other six wild relatives, of which, two nucleotides of 142T, 178T were changed to 142A and 178C, respectively, among w11, w21, w29, w34 and w46; The substitutions of 4A to 4G, 13C to 13G only shared with w34 and w46; the other five substitutions

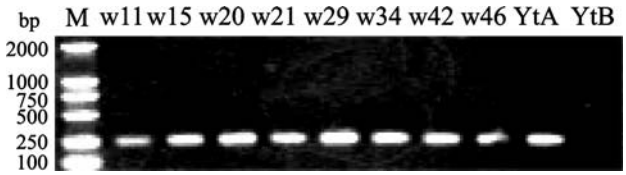

Fig. 1 PCR amplification of *orfH79* in wild rice. Only one band of 240 bp appeared in YtA and the eight wild rice accessions, indicating that the amplification was sequence specific

were dispersedly found. The frequency of nucleotide change was variant depending on accessions. There were two, one, four, four, five and four nucleotide variations being detected in w11, w20, w21, w29, w34 and w46, respectively (Fig. 2). Amino acid alignment revealed that nine amino-acid changes corresponding to the nucleotide variations occurred in the six accessions. Based on the deduced amino acids of ORFH79 peptides, the eight wild rice accessions could be classified into seven haplotypes.

Gametophytically alloplasmic CMS lines can be developed via outcross and successive backcrosses from the wild rice with *orfH79*

Mitochondrial gene of *orfH79* has been confirmed to be related to Honglian CMS. To further validate the molecular analysis, interspecies cross was conducted using four accessions carrying various *orfH79* haplotypes as maternal parents with Honglian maintainer YtB. Fertility observation showed that F_1 hybrids in w15 × YtB and w20 × YtB were partially fertile, and those in the others w34 × YtB and w46 × YtB were sterile. The stainable pollen grains of the F_1 hybrids ranged from 0 to 73.8%, and more than 50% of the abortive pollen grains were spherical. The seed-setting rate of the bagged spikelets ranged from 0 to 43.2% (Table 2). Based on the fertility of F_1 hybrids, we can deduce that w15 and w20 have their own respective restorers in the nuclear genome, but w34 and w46 nuclear genome have weak or recessive *Rf* genes for their corresponding cytoplasm.

Furthermore, the fertility of the population derived from BC_1F_1 backcrosses of w15/YtB//YtB and w20/YtB//YtB was carefully examined. We found that the segregation ratio between fertile and sterile plants closed to 1:1 (Table 3), suggesting that w15 and w20 each carries one pair of *Rf* allele. The sterile plants from BC_1F_1 or BC_2F_1 were selected to backcross as maternal parents with YtB. After eight successive backcrossing, four alloplasmic CMS lines with complete pollen sterility designed as w15A, w20A, w34A and w46A were developed, of which, more than 90% pollen grains were spherically abortive (Fig. 3), and no seed-set being observed in the bagged spikelets. This reflected that all of pollen from the newly developed CMS lines has similar cytological feature as that of HL-CMS line.

Outcrossing of YtB as maternal parent with wild rice showed no hybridization barrier

Apart from the factors in cytoplasmic genome controlling fertility of the interspecies hybrids, nuclear genome is more

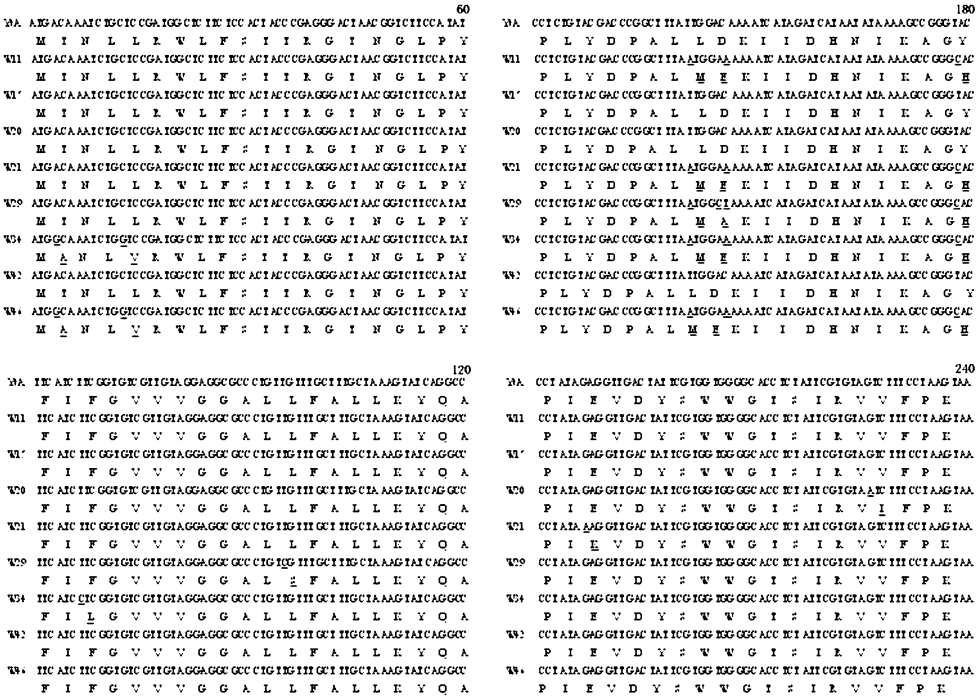

Fig. 2 Nucleotide sequences and the deduced amino acids of *orfH79* in wild rice accessions and YtA. A total of 9 nucleotide variations were found in seven wild rice accessions, which resulted in 9 amino acids change. *Letters with underline* are variant nucleotides and amino acids

Table 2 Fertility of F_1 hybrids in the crosses of wild rice with *orfH79* × YtB (%)

F_1 crosses	w15/YtB	w20/YtB	w34/YtB	w46/YtB
S-abortive microspores	20.5 ± 2.7	57.0 ± 4.1	73.1 ± 2.8	76.8 ± 4.2
T-abortive microspores	5.7 ± 1.0	34.4 ± 3.6	23.2 ± 2.7	23.2 ± 1.9
Stainable pollen grains	73.8 ± 3.6	8.6 ± 2.2	3.7 ± 0.4	0 ± 0
Natural seed-setting rate	67.0 ± 6.2	8.3 ± 1.5	0	5.7 ± 0.9
Bagged seed-setting rate	43.2 ± 3.3	1.2 ± 0.1	0	0

S-abortive spherical abortive, that the aborted pollen grains were round and stained negatively in I_2–KI solution. *T-abortive* typical abortive, that the aborted pollen grains were also stained negatively in I_2–KI solution but irregular in shape

Table 3 Fertility of sterile progenies derived from BC_1F_1 backcrosses (%)

BC_1F_1 backcrosses	w15/YtB//YtB	w20/YtB//YtB	w34/YtB//YtB	w46/YtB//YtB
S-abortive microspores	87.4 ± 3.8	82.4 ± 7.3	80.9 ± 6.0	90.6 ± 5.5
T-abortive microspores	12.6 ± 3.7	17.6 ± 2.8	19.1 ± 2.4	9.4 ± 1.6
Stainable pollen grains	0	0	0	0
Natural seed-setting rate	0	0	0	0
Bagged seed-setting rate	0	0	0	0
Number of sterile: fertile	30:38	24:29		
Expected ratio	1:1	1:1		

Complete sterile plants derived from backcrosses of BC_1F_1 were identified. The abbreviations are shown in Table 2

important to affect the fertility and seed-setting rate of the outcrossing hybrids between wild and cultivated rice (Harushima et al. 2002; Lu et al. 1998). In order to assess the possible effects imposed by the nuclear genome on the fertility of hybrids in our research, the crosses of YtB × wild rice including w20, w29 and w46 were also conducted, and the fertility of the hybrids was assayed as shown in Table 4. The results showed that if wild rice has or not of *orfH79*, all the reciprocal crosses between YtB and wild rice of w20, w29 and w46 had normal fertile pollen grains, and the natural seed-setting rate were all over 35%, and the bagged seed-setting rates all over 20%. From the fertility of the hybrid F_1s between wild rice and YtB, we found that there were great fertility difference between the reciprocal crosses from the same wild rice and YtB, but no significant difference was observed among the three crosses of YtB × wild rice (with or without *orfH79*). It means that fertility of the crosses between wild rice and YtB was

Table 4 Fertility of F_1 hybrids of YtB × wild rice (%)

F_1 crosses	YtB/w20	YtB/w29	YtB/w46
Fertile pollen grains	77.4 ± 3.05	80.3 ± 2.51	70.8 ± 7.76
Natural seed-setting rate	41.3 ± 2.07	5.7 ± 1.47	350.9 ± 2.84
Bagged seed-setting rate	26.1 ± 1.82	22.7 ± 1.53	37.4 ± 2.16

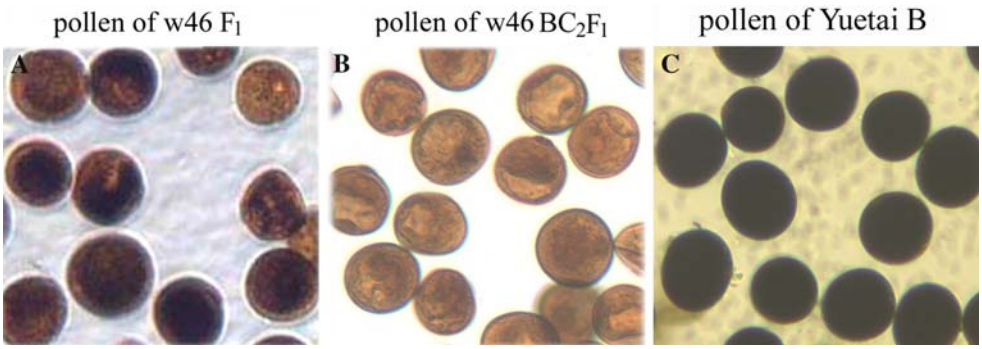

Fig. 3 Microspores of the sterile plants derived from cross of w46 × YtB. **a** The aborted microspores of w46 × YtB F_1 hybrid were slightly stained in 1% I_2–KI solution; **b** the aborted microspores of sterile plants derived from w46 × YtB BC_2F_1 were negatively stained in 1% I_2–KI solution; **c** the microspores of fertile plant YtB were darkly stained in 1% I_2–KI solution

influenced mainly by the cytoplasm in wild rice, but not by the nuclear genome.

Alloplasmic CMS lines with different *orfH79* haplotypes exhibited, respectively, specific restoring model

After eight successive recurrent backcrossing of the sterile plants in the BC_1F_1 population with YtB, four completely sterile alloplasmic CMS lines were developed from w15, w20, w34 and w46, and named temporarily as w15A, w20A, w34A and w46A. They all shared the same sterility character with that of typical Honglian CMS lines. The aborted pollen grains were all spherical and unstainable in 1% I_2–KI solution (Fig. 4a). Although these four CMS lines have the same sterility characteristic, their cytoplasm bear different *orfH79* haplotypes with various origin, and may display different restoring and maintaining character. In order to define the restoring and maintaining relationship of the newly developed alloplasmic CMS lines with different *orfH79* haplotype, w15A, w20A, w34A and w46A were tested with 14 restorer lines from Honglian CMS. Fertility analysis of the F_1 hybrids showed that the four CMS lines were all completely restored by the restorer line 3S52, and about 50% of the pollen was fertile (Fig. 4b), but the natural seed-setting rates were all over 75%, which revealed that the four newly developed CMS lines are all gametophytical restored as Honglian CMS lines. However, 95–102, Xue-xiang-zhan, Gumei2, Zhong413, Up15, MR77, BR24, etc. restorer lines displayed inconsistency with fertility restoring model to the four alloplasmic CMS lines, which can restore the fertility only one to three of the four CMS lines, respectively. This means that the restoring and maintenance relationships of the four alloplasmic CMS lines are different to each other and also inconsistent with that seen in Honglian CMS system (Table 5).

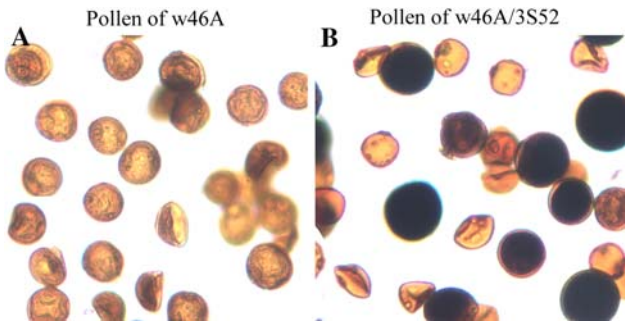

Fig. 4 Microspores of the CMS line w46A and the testcross F_1 of w46A × 3S52. **a** The microspores of the CMS line of w46A were clear in 1% I_2-KI solution; **b** the microspores from the hybrid F1 line of w46A/3S52 were 50% darkly stained in 1% I_2–KI solution

Discussion

Compatibility of outcrossing between wild relatives and cultivated rice

In the F_1 hybrids between wild rice with *orfH79* and YtB, two crosses of YtA/w34 and YtA/w46 were ranked as complete sterile, the other two crosses of YtA/w15 and YtA/w20 were scored as fertile. Further analysis revealed that the populations derived from the two fertile backcrosses segregated into sterile and fertile plants, and that the ratio between two types of plants is approaching 1:1 (Table 2). This indicates the two accessions of w15 and w20 carrying fertility restorer genes. Similar phenomenon has also been found in other wild relative populations. Charlesworth (1981), for instance, had studied the heredity of CMS system in gynodioecious species, and found that sterile cytoplasm can not be maintained except for the fixation of *Rf* gene in the populations. In the natural populations of wild radish (Yamagishi 1998), *Silene vulgaris* (Charlesworth and Valerie 1998) and *Plantago lanceolata* (Anita et al. 1997), the sterile cytoplasms coexist with their corresponding restorer genes. This is in accordance to our previous report that most of the wild rice accessions usually carry restorer alleles for HL-CMS or WA-CMS, and CMS factors coexist with *Rf* genes in wild rice populations (Li et al. 2005), which suggests that *Rf* alleles is usually coexistence with *orfH79* for keeping the sterile cytoplasm in the natural populations of wild rice as other plant species do.

The wild relative species of rice with AA genome are the primary gene pool for further rice improvement. However, reproduction isolation resulting in sterility of the inter-subspecies or interspecies hybrids within the AA genome *Oryza* species will hamper the wide utilization of valuable genes hidden in wild relatives in breeding program of rice (Naredo et al. 1997). Although we know little about the mechanism of zygote-breakdown of interspecies hybrids within AA genome species, it has been well documented that hybrid sterility is attributed to the disharmony of genotypes of the two parents (Lu et al. 1998; Matsubara et al. 2003), and there are up to 35–37 loci mapped on different chromosomes controlling hybrid sterility of inter-subspecies of *O. sativa* (Harushima et al. 2002). For example, Heuer and Miézan (2003) suggest that the sterility of hybrid of *O. glaberrima* × *O. sativa* is controlled by a locus linked to the waxy starch synthase on chromosome 6 which is independent of S-locus. Matsubara also proposes that a *Cif* allele responsible for failure of early endosperm development of the hybrid zygotes is originated from *O. rufipogon*.

It has been hypothesized that the fertility variation in the reciprocal crosses may be the result from two cases: one is the crossing incompatibility alleles on gamete hidden in the wild rice hampering the pollination of the hybrids, another is

Table 5 Fertility of the testcrosses from the alloplasmic CMS lines of rice with various *orfH79* haplotype (%)

Sterile lines	Restorer lines												
	3S52	MBP98	95-102	Huajing74	Gumei2	R644	Yue-xiang-zhan	Zhong413	C418	Feng-xiang-zhan	UP15	M401	MR77	BR24
YtA	[A]30.7 ± 2.1	48.6 ± 3.3	51.3 ± 2.4	60.6 ± 3.1	3.3 ± 0.4	43 ± 3.5	64.3 ± 2.2	10.4 ± 4.7	20.5 ± 3.4	57.3 ± 2.7	0	60.8 ± 2.6	50.1 ± 4.2	0
	[B]41.9 ± 2.7	84.7 ± 4.3	93.8 ± 0.5	88.3 ± 2.7	0	84.6 ± 2.6	85.7 ± 2.9	24.1 ± 1.3	34.3 ± 2.6	81.8 ± 1.8	0	90.5 ± 2.0	87.6 ± 1.7	0
	[C]28.8 ± 2.7	71.5 ± 4.8	73.5 ± 3.9	78.6 ± 3.6	0	59.3 ± 2.8	68.4 ± 3.7	13.7 ± 1.5	29.4 ± 1.8	73.5 ± 2.6	0	68.3 ± 1.8	63.8 ± 2.1	0
w15A	46.7 ± 5.3	23.6 ± 4.1	43.5 ± 0.4	53.3 ± 2.4	12.6 ± .08	32.5 ± 1.9	5.2 ± 0.1	0	33.5 ± 2.4	42.6 ± 3.4	0	63.4 ± 1.8	43.7 ± 1.2	32.6 ± 1.5
	78.7 ± 5.6	31.3 ± 3.1	3.4 ± 0.3	78.9 ± 2.5	23.5 ± 1.5	57.2 ± 2.5	1.2 ± 0.1	0	57.7 ± 1.7	80.1 ± 2.7	0	86.6 ± 2.9	81.5 ± 1.9	67.6 ± 2.3
	62.1 ± 4.7	23.8 ± 3.8	0	69.4 ± 2.8	26.3 ± 1.1	25.8 ± 1.3	0	0	38.2 ± 1.6	66.4 ± 3.5	0	72.4 ± 2.4	67.4 ± 1.5	54.6 ± 2.4
w20A	44.8 ± 1.8	50.3 ± 2.2	56.9 ± 3.6	15.7 ± 2.1	38.2 ± 1.3	29.6 ± 1.1	3.1 ± 0.1	0	37.4 ± 4.2	56.4 ± 3.8	43.7 ± 1.4	55.8 ± 3.1	53.8 ± 0.8	3.5 ± 0.2
	80.6 ± 1.1	79.4 ± 2.4	83.7 ± 2.7	30.6 ± 1.4	58.2 ± 2.7	61.4 ± 1.9	0	0	61.9 ± 2.9	88.2 ± 2.9	79.5 ±	87.3 ± 1.6	69.4 ± 2.6	0
	66.1 ± 1.8	60.3 ± 3.1	61.5 ± 3.0	16.4 ± 1.2	53.6 ± 2.2	35.7 ± 1.7	0	0	43.7 ± 1.6	69.3 ± 4.3	50.7 ±	67.6 ± 2.7	63.7 ± 1.7	0
w34A	60.7 ± 4.8	25.8 ± 2.6	0	44.8 ± 1.5	44.7 ± 2.6	55.3 ± 2.5	21.7 ± 1.2	40.6 ± 2.1	25.7 ± 2.1	47.3 ± 2.1	15.2 ± 1.7	63.7 ± 2.5	4.7 ± 0.1	17.3 ± 1.4
	84.9 ± 4.4	50.8 ± 3.9	0	81.5 ± 2.6	84.2 ± 2.6	83.6 ± 4.2	55.5 ±	74.7 ± 2.7	54.6 ± 1.4	84.3 ± 3.7	26.1 ±	79.2 ± 1.8	1.7 ± 0.3	25.6 ± 2.0
	66.4 ± 3.4	41.7 ± 2.4	0	63.9 ± 1.5	56.8 ± 3.1	50.7 ± 2.3	18.4 ±	57.3 ± 2.6	23.8 ± 2.1	63.6 ± 2.6	12.7 ±	64.8 ± 2.2	0	18.5 ± 1.5
w46A	54.2 ± 3.7	18.4 ± 2.2	37.7 ± 2.0	48.1 ± 0.6	8.2 ± 0.2	57.2 ± 2.1	35.7 ± 1.7	7.2 ± 1.3	37.2 ± 2.9	35.8 ± 1.3	0	45.8 ± 1.9	68.3 ± 4.9	13.8 ± 1.6
	87.5 ± 5.2	33.1 ± 1.9	87.7 ± 4.8	78.3 ± 2.3	11.5 ± 1.0	80.6 ± 2.7	61.4 ± 2.5	6.3 ± 0.6	57.2 ± 2.9	55.7 ± 2.4	0	81.2 ± 2.5	85.9 ± 2.4	21.6 ± 1.9
	71.4 ± 4.5	34.4 ± 3.3	70.8 ± 4.5	54.8 ± 1.8	2.1 ± 0.3	53.7 ± 3.4	24.9 ± 1.7	3.1 ± 0.3	20.4 ± 2.7	31.6 ± 2.6	0	43.8 ± 2.8	57.7 ± 1.7	12.7 ± 1.1

A represents the fertile microspores; *B* represents the natural seed-setting rate; *C* represents the bagged seed-setting rate of spikelet

the sterile cytoplasm disturbing the development of microspores of the hybrids (Li et al. 1997). Naredo et al. (1997) had reported that considerable degree of fertility differences was observed between the reciprocal crosses of *O. meridionalis* × *O. nivara* and *O. meridionalis* × *O. rufipogon*, whereas higher seed-set in hybrids were observed when *O. meridionalis* was used as the female parent.

Generally, the wild relatives of rice are predominantly cross-pollinated, and the crossability between *O. sativa* and wild species, especially the two species of *O. nivara* and *O. rufipogon*, seems more compatible than that of the two subspecies of *O. sativa* (Harushima et al. 2002). In 1970s, IRRI had developed a series of CMS lines including IR73382, IR73678, IR65484, etc. from interspecies crossing between *O. sativa* and its closest wild relatives with AA genome (Brar and Khush 1997). In this study, all of the hybrids between YtB as a female parent and wild rice of w20, w29 and w46 showed high fertility, which demonstrated that the wild relatives and YtB have well-crossing compatibility, while the sterility of hybrids w34/YtB and w46/YtB is caused by the cytoplasm genome but not by the nuclear factors.

orfH79 haplotypes for CMS in wild rice population correspond to their respective nuclear *Rf* alleles

To maintain the diversity of CMS system in hybrid rice, it is necessary to explore the new sterile-inducing cytoplasm resources (Horn 2002; Frank 2000). Uniformity of CMS may be fatal for the commercial production because of the high latent risk from some special pest or disease as that happened in T-CMS type of hybrid maize in American in 1970s (Ullstrup 1972). To increase CMS system in rice will be pressing for the future prospect of hybrid rice because of the WA-type hybrid rice covering over 80% of hybrid rice area planted in China (Li *et al*. 2007). Kadowaki *et al*. (1988) reported that seven different restriction patterns of mtDNA were observed in ten cytoplasmic male sterile lines of rice, of which, seven gametophytic types were all originated from *O. rufipogon* except for CMS-Boro. This reveals that variation of mtDNA exists widely in the gametophytic-type CMS rice. Similar results are also found between V-20A (typical Wild-abortive CMS line of rice) and a new CMS line from *O. perennis* Acc 104823, where about half of their mitochondrial RFLP hybridization patterns are different to each other (Dalmaciao et al. 1995). In our study, PCR and Southern blot have shown that eight out of 42 accessions carry *orfH79*, which distribute not only in *O. rufipogon*, but also in *O. nivara*, *O. meridionalis* and *O. galaberrima*. This indicates that *orfH79* exists widely in AA genomic species of wild rice and displays variant allele, which would be potentially an important CMS resource for exploitation in hybrid rice production.

Growing evidences that both of coding variations and regulatory expression differences between nuclear alleles have been observed in plant species including maize, *Arabidopsis*, rice, etc. (Thornsberry et al. 2001; Schadt et al. 2003; Fondon and Garner 2004), by comparing their sequences and relative abundance of mRNA transcripts obtained from normal individuals. Thornsberry et al. (2001) evaluated the polymorphisms of *Dwarf8* alleles in maize populations using association approaches from 92 maize inbred lines, and found that mutation and deletion of *Dwarf8* gene might affect the quantitative variation of maize flowering time and plant height. De Meaux et al. (2005) also suggested that functional variation in the Chalcone Synthase (*CHS*) *cis*-regulatory region in *Arabidopsis thaliana* can arise from a small number of nucleotide mutations. Similarly, Saitoh et al. (2004) pointed that apiculus coloration occurrence of varying degrees was caused by a series of alleles with nucleotide substitutions in coding region at the *OsC1* locus, and 17 haplotypes were found in 39 wild and cultivated rice lines. These findings above indicate that functional changes in promoters and coding regions can arise from a few mutations, pointing to promoter and regions all working as fundamental determinants of functional genetic variations in plants.

However, few reports have been involved in the relation between variation of CMS-related allele and the corresponding *Rf* genes. In our research, the four accessions with different *orfH79* haplotypes were all developed into alloplasmic CMS lines when hybridizing as maternal parents with YtB, which suggest that the gametophytic CMS determinant of *orfH79* exists multiple alleles in mitochondrial genome of rice just as that observed in *Plantago lanceolata* (Carolina et al. 1997). We propose that these *orfH79* alleles are all derived from a common ancestor, but it diverged gradually to different forms in different populations survived in various environments during the long evolutionary process. Correspondingly, *Rf* alleles evolve actively so as to fit the different mutation in mitochondria to recover the function of pollen grains.

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