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ORIGINAL PAPER

Genetical and molecular analysis reveals a cooperating relationship between cytoplasmic male sterility- and fertility restoration-related genes in *Oryza* species

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Abstract Although the characterization of genes associated with cytoplasmic male sterility (CMS) and fertility restoration (*Rf*) has been well documented, the evolutionary relationship between nuclear *Rf* and CMS factors in mitochondria in *Oryza* species is still less understood. Here, 41 accessions from 7 *Oryza* species with AA genome were employed for analyzing the evolutionary relationships between the CMS factors and *Rf* candidates on chromosome 10. The phylogenetic tree based on restriction fragment length polymorphism patterns of CMS-associated mitochondrial genes showed that these 41 *Oryza* accessions fell into 3 distinct groups. Another phylogenetic tree based on PCR profiles of the nuclear *Rf* candidates on chromosome 10 was also established, and three groups were distinctively grouped. The accessions in each subgroup/group of the two phylogenetic trees are well parallel to each other. Furthermore, the 41 investigated accessions were test-crossed with Honglian (gametophytic type) and Wild-abortive (sporophytic type) CMS, and 5 groups were classified according to their restoring ability. The accessions in the same subgroup of the two phylogenetic trees shared similar fertility restoring pattern. Therefore, we conclude that the CMS-associated mitotypes are compatible to the *Rf* candidate-related nucleotypes, CMS and *Rf* have a parallel evolutionary relation in the *Oryza* species.

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Introduction

Cytoplasmic male sterility (CMS), which is maternally inherited and widely found in numerous flowering plant species, causes the production of non-functional pollen (Wise and Pring 2002). A number of studies have so far found that CMS is attributable to defects in the mitochondrial genome (Schnable and Wise 1998; Stadler and Delph 2002). The majority of the CMS-associated *orf*s (open reading frames) produced by rearrangement contain pieces of the known mitochondrial genes and co-transcribe with an upstream or downstream functional gene (Hanson and Bentolila 2004). At least ten mitochondrial genes have been characterized to be involved in plant CMS, such as *nad3*, *nad5*, and *nad7* of mitochondrial complex I (Brown 1999), *cob* of mitochondrial complex III (Janska et al. 1998), *cox1* and *cox2* of mitochondrial complex IV (Song and Hedgcoth 1994; Dong and Byung 2006), and *atp1*, *atp6*, *atp8*, and *atp9* of mitochondrial complex V (Heazlewood et al. 2003; Sabar et al. 2003; Hanson and Bentolila 2004). Rice (*Oryza sativa* L.) is one of the most important crops and feeds about half of the world population. Since the discovery of the first CMS line (Shinjyo 1969), over 60 CMS lines with different origin have been developed through interspecies, intersubspecies, and even inter-varieties of *Oryza* with the

AA genome (Li and Yuan 2000). Genetical, cytological and molecular evidence has shown that the abortive characteristics among many of these CMS systems are different from each other. There are plenty of CMS systems hidden in the *Oryza* species, which would be potentially valuable resources for research on the nucleo-cytoplasmic interaction.

CMS can be restored by nuclear restorer gene (*Rf*), another important factor in the CMS system, which contains mitochondria targeting precursor and plays a critical role in conditioning the expression pattern of the abnormal CMS-associated genes at the transcriptional and/or translational levels (Budar and Pelletier 2001). Usually, one or two major restoring loci confer complete fertility restoration in a majority of CMS systems, such as *Brassica napus*, corn, and *Plantago coronopus* (Gabay-Laughnan and Laughnan 1994; Menassa et al. 1999; Van Damme et al. 2004). However, multiple *Rf* loci are usually identified in a variety of CMS systems in the natural populations within a plant species. There are over 17 *Rf* alleles for different CMS systems being reported in rice. Apart from *Rf2*, *Rf3*, *Rf17* were mapped on chromosomes 2, 1 and 4, respectively (Li et al. 2007; Fujii and Toriyama 2009), others including *Rf4* for the WA-CMS system, *Rf1* for BT-CMS, *Rf5* and *Rf6* for HL-CMS, and *qRf-10-2* for DA-CMS, etc., all lay on the long arm of chromosome 10, and are closely linked to form a gene cluster (Yao et al. 1997; Xie et al. 2002; Komori et al. 2003; Akagi et al. 2004; Liu et al. 2004; Wang et al. 2006).

Theoretically, CMS is regularly companied by the *Rf* genes in natural plant populations, CMS is the basis for the occurrence of *Rf*, and the *Rf* is the prerequisite to maintain the transmission of CMS within plant populations (Dowling et al. 2007). If a detrimental rearrangement in mitochondrial genome leads to CMS, the relevant *Rf* gene would be selected to keep the fertility restoration. The dependent evolutionary relationship between CMS and *Rf* genes in plants has been exemplified as a typical nucleo-mitochondrial interaction in early theories (Charlesworth 1981; Saumitou-Laprade et al. 1994; Budar et al. 2003). Touzet and Budar (2004) proposed that the dynamics of *Rf* gene and CMS is a coevolutionary process as an arm race in two genomes. Budar et al. (2003) also suggested that CMS may be the evolutionary force for the nuclear *Rf* generation in natural population. Similar opinions about the evolution between *Rf* and CMS are also mentioned by Li et al. (2005) and Tian et al. (2006). However, the information on the genetic diversity and evolutionary route between nuclear *Rf* and CMS factors in mitochondria of plant species is still separate and fragmentary. Seeking a better understanding of the *Rf*–CMS interaction at the genome level in *Oryza* species, we analyzed both the CMS-associated mitochondrial factors and the *Rf* candidate genes on chromosome 10, and found that there exists a highly associated relationship between CMS and fertility restoration in the *Oryza* species.

Materials and methods

Plant materials

A total of 41 *Oryza* accessions with AA genomes, including 16 *O. sativa* ssp. *indica*, 4 *O. sativa* ssp. *japonica* with wide compatibility (Zheng et al. 1994; Zhu 2000), 4 *O. glaberrima*, 8 *O. nivara*, 3 *O. rufipogon*, 1 *O. barthii*, 4 *O. glumaepatula*, and 1 *O. meridionalis*, were employed in this study (Table 1). These accessions are all fertile after self-pollination. In addition, WD08 (*O. officinalis*, CC genome, derived from China) was used as an outgroup for the phylogenetic analysis. A typical Honglian CMS (gametophytic type) line, Yuetai A (YtA), and a typical Wild-abortive CMS (sporophytic type) line, Zhenshan 97A (ZsA), were both test-crossed as female parents with the accessions (Zhu 2000). A relevant maintainer Yuetai B (YtB) for YtA and a relevant maintainer Zhenshan 97B (ZsB) for ZsA were emasculated and crossed as female parents with the accessions without fertility restoring ability for these two CMS. All the plant materials were planted in the experimental field within Wuhan University campus in the summer and Hainan Island, Hainan, China in the winter during 2004–2007.

Fertility scoring of the F_1s

Pollen stainability and seed-setting rate were used as the criteria for the evaluation of plant fertility as described by Li et al. (2005). All the values were the means of 30 assays at random.

Isolation of mitochondrial DNA and nuclear DNA

Mitochondrial DNA purification followed the method of Yi et al. (2002) and modified slightly. Briefly, about 20 g etiolated slender leaves were homogenized in 80 ml homogenizing buffer. After differential centrifugation and DNase I procedure, the pellet was resolved with lysis buffer and placed for 5 min at room temperature. After phenol–chloroform extraction, the DNA was precipitated with ethanol. Total plant genomic DNA was isolated from green leaves using the modified method of Zhang et al. (1992).

Southern hybridization

Ten CMS-associated genes, including *atp1*, *atp6*, *atp8*, *atp9*, *cox1*, *cox2*, *cob*, *nad3*, *nad5*, and *nad7*, were selected for investigating the mitochondrial DNA patterns. The

Table 1 The *Oryza* genus species used in this study

Serial	Species	Accession	Origin	Serial	Species	Accession	Origin
c1	*O. sativa* ssp. *indica*	64771	Bangladesh	c22	*O. glaberrima*	101791	Senegal
c2	*O. sativa* ssp. *indica*	66509	Sri Lanka	c23	*O. glaberrima*	101855	Burkina Faso
c3	*O. sativa* ssp. *indica*	66525	Sri Lanka	c24	*O. glaberrima*	102641	Liberia
c4	*O. sativa* ssp. *indica*	74659	Indonesia	w1	*O. nivara*	103836	Bangladesh
c5	*O. sativa* ssp. *indica*	74721	India	w2	*O. barthii*	101255	Cameroon
c6	*O. sativa* ssp. *indica*	74746	India	w3	*O. nivara*	104705	India
c7	*O. sativa* ssp. *indica*	74748	India	w4	*O. nivara*	101971	India
c8	*O. sativa* ssp. *indica*	74777	India	w5	*O. meridionalis*	104085	Australia
c9	*O. sativa* ssp. *indica*	78242	Thailand	w6	*O. nivara*	105704	Nepal
c10	*O. sativa* ssp. *indica*	78243	Thailand	w7	*O. nivara*	101978	India
c11	*O. sativa* ssp. *indica*	78263	Thailand	w8	*O. nivara*	103821	China
c12	*O. sativa* ssp. *japonica*	Moroberkan	Brazil	w9	*O. nivara*	104659	Thailand
c13	*O. sativa* ssp. *japonica*	Nangka	Indonesia	w10	*O. nivara*	106344	Myanmar
c14	*O. sativa* ssp. *japonica*	Newbonnet	USA	w11	*O. glumaepatula*	100968	Suriname
c15	*O. sativa* ssp. *indica*	Dular	India	w12	*O. glumaepatula*	105561	Colombia
c16	*O. sativa* ssp. *japonica*	Pecos	USA	w13	*O. glumaepatula*	105661	Brazil
c17	*O. sativa* ssp. *indica*	Mahsuri	India	w14	*O. glumaepatula*	105668	Brazil
c18	*O. sativa* ssp. *indica*	Nahalin	Philippine	w15	*O. rufipogon*	105696	Nepal
c19	*O. sativa* ssp. *indica*	Taichung native	China	w16	*O. rufipogon*	106321	Cambodia
c20	*O. sativa* ssp. *indica*	Mbp98	China	w17	*O. rufipogon*	100219	Thailand
c21	*O. glaberrima*	103955	Senegal				

probes were amplified with the corresponding primers following the mitochondrial genome sequences of rice (see Supplementary Table 1). Mitochondrial DNA (20 μg) was separated on 0.8% agarose gels after digestion with three double digestion combination, *Eco*RI–*Bam*HI, *Pst*I–*Sal*I and *Hin*dIII–*Xho*I (New England Biolabs, Ipswich, MA, USA) and transferred to Hybond N^+-nylon membranes, respectively. Probes were radioactively labeled by random priming with a-^{32}P-dCTP. Southern hybridization was performed in hybridization buffer at 65°C for 16 h. The membrane was washed twice at room temperature for 15 min with 2× SSC containing 0.1% sodium dodecyl sulfate (SDS) and then at 60°C for 30 min with 0.1× SSC containing 0.1% SDS. The membrane was then autoradiographed.

PCR primer design and PCR amplification for *Rf* candidates

Due to all cloned *Rf* genes contain mitochondrial precursor targeting to mitochondria and play a role in conditioning the expression pattern of the abnormal CMS-associated genes at the transcriptional or translational levels, the prediction of subcellular location for all the 81 proteins tightly linked to *Rf* genes within the 4 BAC of OSJNBa0041P03, OSJNBa0066I08, OSJNBa0017E08 and OSJNBa0078O01 on chromosome 10 was essential. So, the subcellular location for these proteins was predicted by the software of TargetP 1.1 (see http://www.cbs.dtu.dk/services/TargetP/) and the software of Mitoprot II 1.0a4 (see http://ihg.gsf.de/ihg/mitoprot.html). The genes with scored value of export to mitochondria over 0.500 by the two softwares were seen as the candidate *Rf* genes in this research.

PCR amplification was performed with Taq DNA polymerase enzyme (MBI, Fermentas, Hanover, MD, USA) using a DNA engine dyad thermal cycler (MJ Research, USA). A negative control containing all PCR components except DNA template (replaced by water) was included in every experiment to test for DNA contamination in the reagents. Amplified products were analyzed by electrophoresis on agarose gels.

Phylogenetic analysis

Each fragment produced by restriction fragment length polymorphism (RFLP) and PCR was treated as a unit character and scored as a binary code (1/0). Only distinct, reproducible fragments were scored. The 1/0 matrix was used to construct phylogenetic trees by maximum parsimony (MP) in the software package of Phylip 3.63 (Felsenstein, University of Washington, Seattle, USA). The majority rule consensus phylogenetic trees were drawn by the software of MEGA4.0 (Tamura et al. 2007). The reliability and

robustness of the phylogenetic trees were tested by bootstrapping. Bootstrap analysis was performed with 1,000 replicates.

Results

Phylogenetic analysis of CMS-associated mitochondrial genes

In order to assay the polymorphism of CMS-associated functional genes in mitochondria genome among the accessions, mitochondrial genomic DNA of all the accessions was digested with *Eco*RI–*Bam*HI, *Pst*I–*Sal*I and *Hin*dIII–*Xho*I, respectively, and hybridized with ten homologous CMS-associated mtDNA probes (see Supplementary Table 1). Then, the restriction fragments were observed and analyzed. Of these, six probes (*atp1*, *atp8*, *atp9*, *nad3*, *nad5*, and *nad7*) revealed no polymorphisms while the other probes *atp6*, *cox1*, *cox2*, and *cob* exhibited 70 scorable polymorphic bands (Fig. 1). There were 23 polymorphic bands with *atp6*, 18 bands with *cox1*, 13 bands with *cox2*, and 16 bands with *cob*.

A dendrogram (Fig. 2) was constructed based on the data matrices derived from these restriction fragments using the MP method in the Phylip 3.63 and MEGA 4.0 software packages. At a similarity coefficient value of 0.700, three monophyletic groups were revealed on the dendrogram, designated Mt-I, Mt-II, and Mt-III. Mt-I comprises four subgroups including m1, m2, m3, and m4; Mt-II comprises five subgroups including m5, m6, m7, m8, and m9, and the Mt-III comprises two subgroups including m10 and m11.

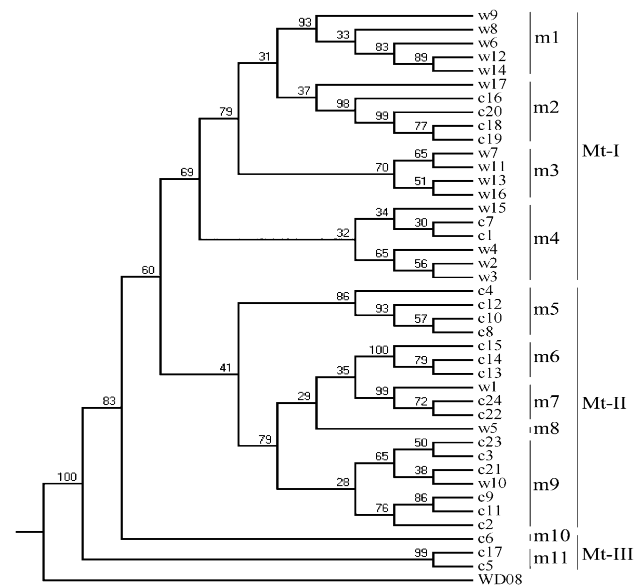

Fig. 2 Phylogenetic tree based on the RFLP pattern of mitochondrial genes. Phylogenetic tree was constructed with the MP method of the Phylip 3.63 software package based on the RFLP patterns of the CMS-associated mitochondrial genes. The names of Mt-I–Mt-III and m1–m11 on the *right* indicate the different groups and subgroups, respectively. *Numbers* at the nodes indicate the bootstrap percentages for 1,000 replicates

Phylogenetic analysis of *Rf*-related nuclear genes

20 out of the 81 putative proteins (25.92%) within the *Rf* region on chromosome 10 were predicted to be mitochondria-targeted. Among the 30 selected primer combinations covering these 20 *Rf* candidate genes, 15 of which (Table 2) produced 35 polymorphic bands. Although most protein functions among these genes were unclear, at least seven proteins containing PPR domain were putative *Rf* proteins, and others were putative functional genes according to the data of the Institute of Genomic Research (TIGR) rice genome annotation. The genome database for *O. sativa* in the NCBI (http://www.ncbi.nlm.nih.gov/) suggests that these genes span a ~440 kb segment (Fig. 3), and are tightly linked to form a gene cluster.

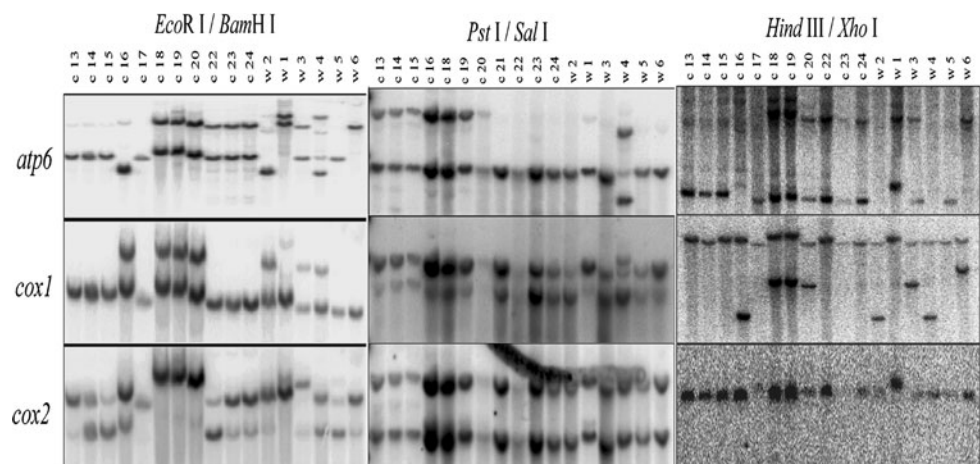

Fig. 1 Pattern of Southern hybridization of mitochondrial probes. Southern hybridization was carried out using mitochondrial gene probes *atp6*, *cox1*, and *cox2* against mitochondrial DNA completely digested with *Eco*RI–*Bam*HI, *Pst*I–*Sal*I, and *Hin*dIII–*Xho*I, respectively

Table 2 PCR primers and prediction of subcellular location of associated genes in regions of fertility restoration nuclear genes

Name	Oligonucleotides (5′–3′)	Sequence length (bp)	Locus identifier	TargetP probable value	Mitoprot probable value	Putative function[a]
41P03.1	F: GAAGAGGACGACGATACT R: CACTTCTCTCCAAATACT	1,818	LOC_Os10g35250	0.863	0.434	rf1 protein, mitochondrial precursor, putative
41P03.2	F: ATGTGGCCCGCCGCAG R: TCCTGTTGCCATGATTGG	1,612	LOC_Os10g35140	0.553	0.983	Permeases of the drug/metabolite transporter, putative, expressed
41P03.3	F: TTCTGATCCAATCATGGC R: TCCCTCCACGTGCTCGTC	1,591	LOC_Os10g35140	0.553	0.983	Permeases of the drug/metabolite transporter, putative, expressed
41P03.4	F: GCGCCGCGTCCCTACC R: CTTGATATTTGCTCCCAG	2,334	LOC_Os10g35240	0.563	0.634	rf1 protein, mitochondrial precursor, putative, expressed
41P03.5	F:ACGTGTTCGACGAATTGC R:AAATTAACTCCTCAGCCTTC	1,284	LOC_Os10g35230	0.544	0.862	rf1 protein, mitochondrial precursor, putative, expressed
17E08.1	F: CATCGTCACTGTCGTCTG R: CGTGGCTGTATTCATTCTC	1,990	LOC_Os10g35444	0.739	0.807	Expressed protein
17E08.2	F: TCAGCTATCACCTTCCTTG R: CTCTTCTCATCCTGTTTGG	2,010	LOC_Os10g35440	0.914	0.932	rf1 protein, mitochondrial precursor, putative, expressed
17E08.3	F: ATAGGCATGCACTCAACC R: ACCTTTACATGCCCTTAC	2,435	LOC_Os10g35436	0.769	0.908	rf1 protein, mitochondrial precursor, putative, expressed
17E08.4	F: TTCAGCAGCTCAAAGATTC R: TCGAGGCATCTGTCTTAG	1,902	LOC_Os10g35412	0.532	0.882	Retrotransposon protein, putative, unclassified
17E08.5	F: TGAAGAAGCCGAGGAGG R: ATGGCGGATCAGATCGAG	2,230	LOC_Os10g35390	0.805	0.563	1-Acyl-sn-glycerol-3-phosphate acyltransferase 1, chloroplast precursor, putative, expressed
78O01.1	F: AAATGATGAGTCTGCTAGG R: CATGCAGTAGAGTGTGATA	1,800	LOC_Os10g35670	0.835	0.991	RNA-binding protein, putative, expressed
78O01.2	F: ACGGGGTGATTTATTCAGC R:CAGGATGGATTTCACTTCAG	1,300	LOC_Os10g35640	0.736	0.950	rf1 protein, mitochondrial precursor, putative, expressed
78O01.3	F: ACGAGGTGAGTCATTAGC R: ACATCTCAGATCCTACCAC	2,465	LOC_Os10g35780	0.119	0.922	Hypothetical protein
78O01.4	F: GCGACCACCATGCTGCTG R: TTGGTTTGCACTTGCTTGAC	1,715	LOC_Os10g35790	0.938	0.978	rf1 protein, mitochondrial precursor, putative
78O01.5	F: CACAAATCACCAAACGAGTTC R:CTCTAACTTCAGGTGA	1,775	LOC_Os10g35810	0.770	0.996	Expressed protein

[a] Putative function derived from TIGR Rice Genome Annotation

The sequencing results of PCR fragments with the primers above were identical to the size of anticipative products, which means that these PCR products are the homologous fragments to the respective primers (data not shown). The PCR productions with 3 of 15 primers, such as 41P03.3 (LOC_Os10g35140), 17E08.4 (LOC_Os10g35412), and 78O01.1 (LOC_Os10g35670), exhibit rich polymorphisms, as shown in Fig. 4.

According to the data matrices derived from the polymorphic PCR productions with the 15 primers above, a phylogenetic tree was also constructed using MP method with the Phylip 3.63 and MEGA4.0 software packages. There were three monophyletic groups on the dendrogram at similarity coefficient value of 0.700, designated Nu-I, Nu-II, and Nu-III. Nu-I comprises three subgroups (n1, n2, and n3); Nu-II comprises two subgroups (n4 and n5); and Nu-III comprises three subgroups (n6, n7, and n8) (Fig. 5).

Fertility restoring pattern of the accessions for Honglian and Wild-abortive CMS

If the molecular polymorphic pattern represents the trends of potential restorer gene in the accessions of *Oryza*, it should be reflected with the restoring pattern for CMS in rice. In order to analysis if the status of *Rf* genes fits to the DNA pattern in the accessions, the HL-CMS line, YtA, and the WA-CMS line, ZsA, were both test-crossed as female parents with the 41 accessions, and fertility of the F_1s was

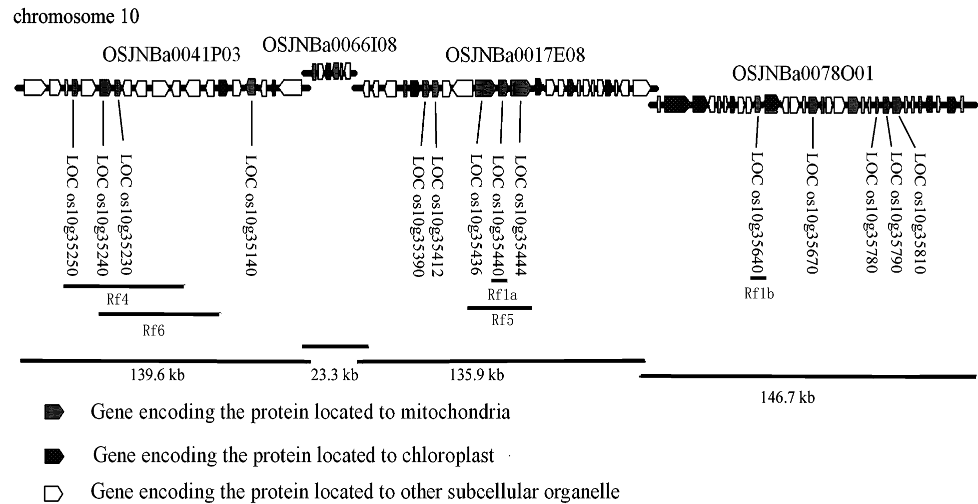

Fig. 3 A physical map of the genes in the *Rf* candidate region containing mitochondrion transit signals

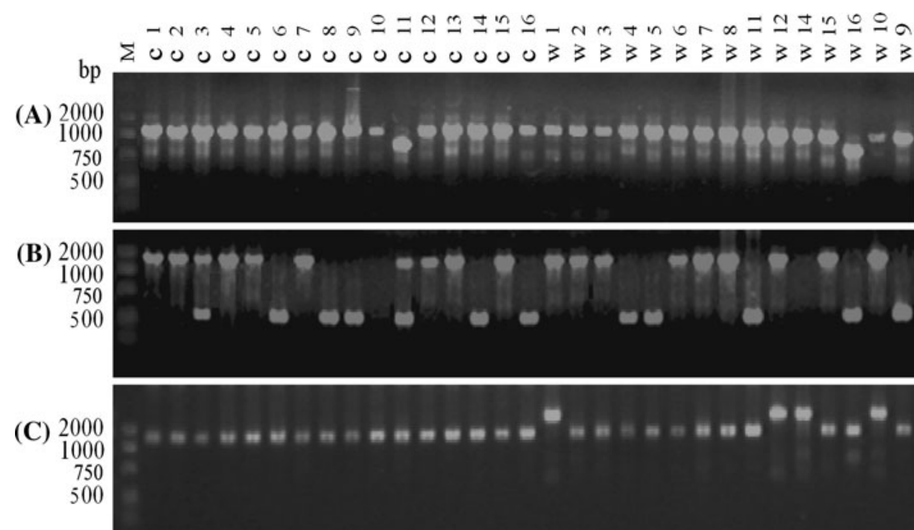

Fig. 4 PCR profiles with primers of *Rf* candidate genes, such as 41P03.3 (LOC_Os10g35140), 17E08.4 (LOC_Os10g35412), and 78O01.1 (LOC_Os10g35670). *M* presents the DNA marker DL2000, *c1–w9* presents the name of samples of *Oryza* species in the study. The *left letter* shows the code of the primers: *A* 41P03.3, *B* 17E08.4, *C* 78O01.1

carefully evaluated. The F_1s with both >10% stainable pollen and >20% seed-setting rate was regarded as fertile. We found that 29 out of 41 HL-type F_1 hybrid plants and 24 out of 41 WA-type F_1 hybrid plants were fertile (Fig. 6). The restoring spectrum of the accessions for HL-CMS (70.7%) was obviously wider than that for WA-CMS (58.5%). The fertility of the hybrid F_1s was accordant with that during 2004 and 2007 (data not shown), which suggests that the fertility of the hybrid F_1s was stable. According to the restoring features of the HL- and WA-type hybrids, the 41 accessions could be classified into 5 groups as shown in Fig. 6. Eight accessions restoring neither the HL-CMS nor the WA-CMS line belonged to group A, 4 accessions restored only the WA-CMS but not the HL-CMS line belonged to group B, 19 accessions restored both HL- and WA-CMS lines, of which, 11 accessions having a stronger restoring ability for the WA-CMS line than for the HL-CMS line belonged to group C, the other 8 accessions having a stronger restoring ability for the HL-CMS than for the WA-CMS belonged to group D, 10 accessions restoring only HL-CMS belonged to group E.

Wide hybridization is a widely used method to broaden genetic diversity in rice breeding programs, but the unpredictable reproductive isolation between species usually leads to male sterility. The accessions in this study belong to seven different species and two subspecies of *O. sativa*. The sterility of some test-crosses may be caused by reproductive barriers but not the absence of *Rf*. In order to elucidate whether the male sterility of the test-crosses comes from potential incompatibility between species and two subspecies, the maintainers YtB and ZsB were crossed as female parents with those accessions of the sterile test-crosses. The crossing compatibility of the accessions was carefully evaluated based on the natural seed-setting rate of hybrid F_1s. The results showed that the F_1 hybrids from *O. sativa* ssp. *japonica* lines (c12, c13, c14, and c16) and YtB or ZsB were all fertile, which means that the four *japonica* lines presented wide compatibility as reported by

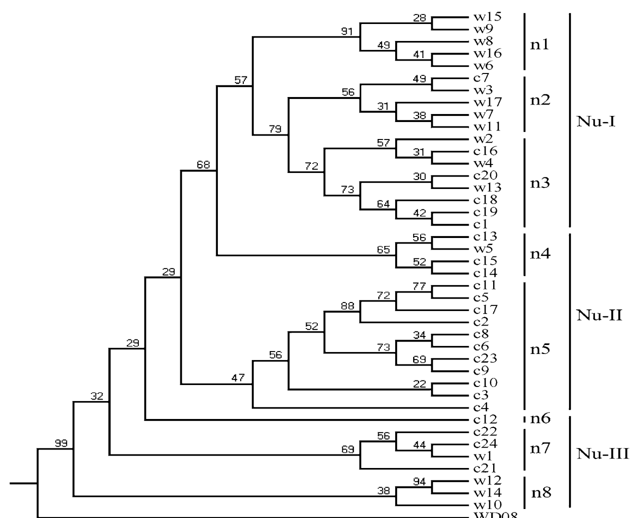

Fig. 5 Phylogenetic tree based on the PCR profiles of *Rf* candidate genes. This figure was constructed with the MP method of the Phylip 3.63 software package as that in Fig. 2. *Nu-I–Nu-III* and *n1–n8* present the name of groups and subgroups, respectively

Zheng et al. (1994). Likewise, the hybrid F_1 plants between the wild rice (w1, w3, w5, w6, w7, w8, w9, w10, w11, w12, w14, w15, w16, and w17) and *O. glaberrima* (c21, c22, and c24) and YtB or ZsB were also fertile (Fig. 7). The data indicate that the sterility of these test-crossed F_1s for these two CMS lines had nothing to do with the reproductive barriers, and the fertility of test-crosses showed the real status of fertility restorer for HL- and WA-CMS in the *Oryza* accessions.

Coherence of the clustering among the DNA patterns of *Rf*-related genes, CMS-associated genes, and the fertility restoration of accessions

When lining up sequentially all of the accessions in each group/subgroup of Figs. 2, 5 and 6, we found a well parallel relation exiting in the accessions of groups clustered from the CMS and fertility restore-associated genes. In Fig. 8, nuclear group Nu-III (n6–n8) contained accessions in mitochondrial group Mt-II (m7, 9) and subgroup m1 of group Mt-I, and also corresponded to that in group A of Fig. 6. These accessions carried no any restorer genes for Honglian and Wild-abortive CMS. Nuclear Nu-II included both subgroups n4 and n5, of which the accessions in n4 were well corresponded to that in mitochondrial subgroups m8 and m6, and fertility group B in Fig. 6, and carried restorers for WA-CMS. Nuclear subgroup n5 containing mitochondrial subgroups m11 (Mt-III), m5 and m9 (Mt-II) was completely compatible to that of fertility group C. They all were partial restorers to HL-CMS and strong restorer to WA-CMS. The accessions of nuclear group Nu-I were completely fallen in mitochondrial group Mt-I, and had relatively strong restoring ability for HL-CMS. By virtue of fertility of test-crosses F_1s for HL-CMS and WA-CMS, nuclear group Nu-I (Mt-I) was classified into two fertility groups: group D and group E. Group D was compatible to nuclear subgroup n3, and corresponded to mitochondrial subgroup m2 and part of m4 (accessions of w2, w4 and c1), the accessions were all strong restorer for HL- and WA-CMS. Group E was comprised by nuclear subgroups n1 and n2, and corresponded to m1, m3

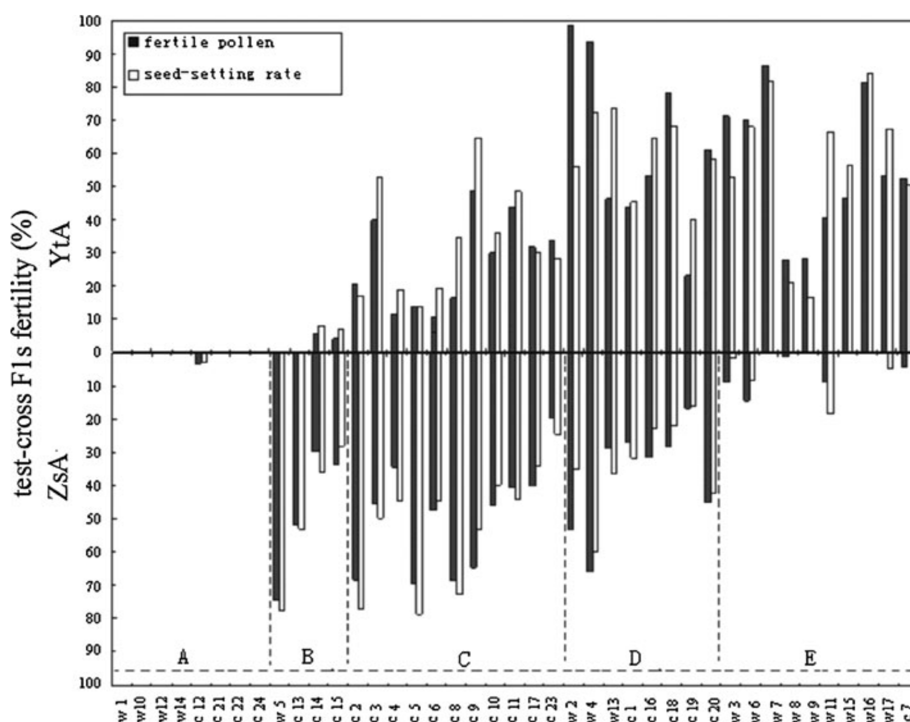

Fig. 6 Fertility of HL-type and WA-type test-crossed hybrid F_1s. The *letter below* shows the code of sharing similar fertility for two test-crossed hybrid F_1s. *A* the group restoring neither HL-CMS line nor WA-CMS line; *B* the group restoring not HL-CMS line but WA-CMS line; *C* the group restoring two CMS line, fertility for WA-CMS higher than that for HL-CMS; *D* the group restoring two CMS line, fertility for HL-CMS higher than that for WA-CMS; *E* the group restoring HL-CMS not WA-CMS line

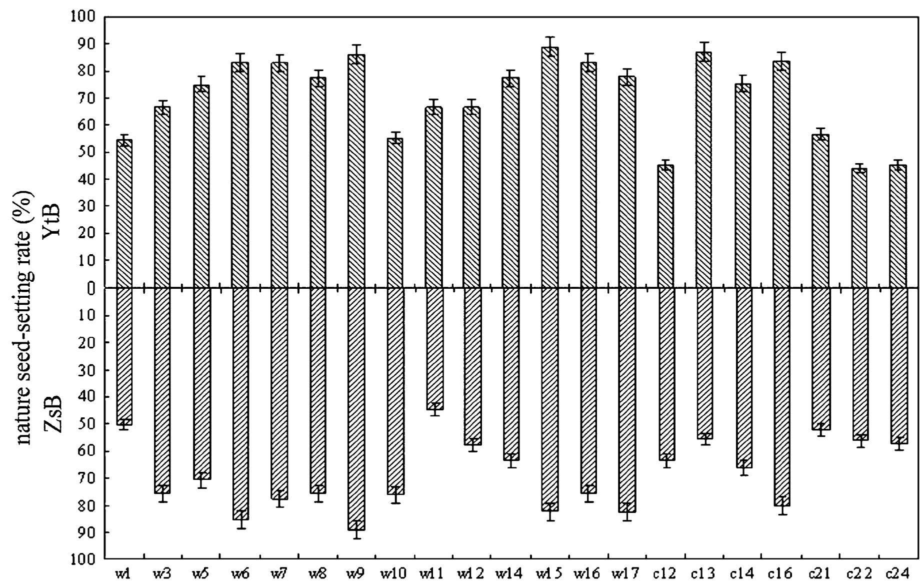

Fig. 7 Natural seed-setting rate of hybrids between YtB and ZsB as female parents and the accessions without restoring ability to HL- and WA-CMS

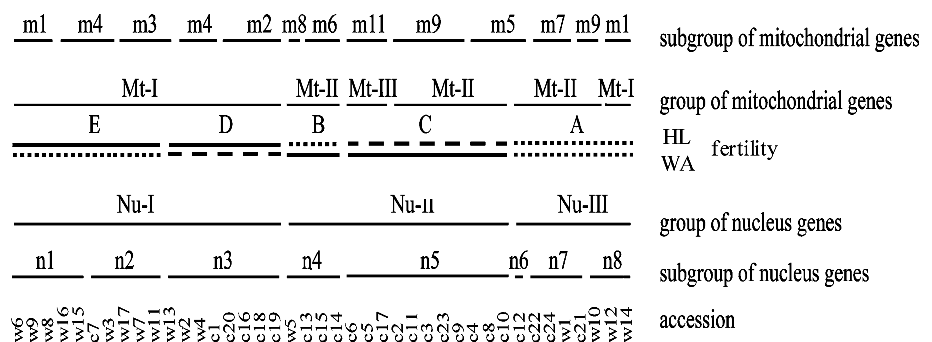

Fig. 8 Schematic comparison of the accessions in each monophyletic (sub)group and testcrosses group of Figs. 2, 5 and 6. The group and subgroup names are corresponded to that in Figs. 2, 5 and 6. *Line* represents the restoring ability of the accessions to HL- and WA-CMS: *solid line* complete restoration, *short line* partial restoration, *dotted line* no restoration

and part of m4 (w15, c7 and w3), the accessions were only stronger restorer to HL, but maintainer to WA-CMS.

From Fig. 8, it is not difficult to find that the accessions with similar fertility restoring pattern were always clustered in the same subgroup in the two phylogenetic trees. The DNA patterns of the potential *Rf* candidate genes were almost completely parallel to their fertility restoring ability to HL- and WA-CMS in the analyzed accessions, and the mitotypes based on CMS-associated genes were concordant with the distribution of *Rf* genes in *Oryza* species. So, it can be concluded that the accessions of the two phylogenetic trees are well corresponded to each other, and both of CMS and restorer genes in CMS/*Rf* systems depend on each other evolutionally in *Oryza* species.

Discussion

Diversity of CMS and *Rf* in *Oryza* species

Plant mitochondrion is featured with a great many of repeats and redundant sequences. A lot of unknown *orfs* involved in CMS are often characterized. Correspondingly, multiple CMS mitotypes carrying unique mitochondrial CMS genes have been identified in some of plant species. For example, in maize, T, S, and C-CMS have been suggested being resulted from chimeric T-*urf13*, *orf355-orf77*, and an unknown chimeric C-*atp6/atp9* region in the mitochondrial genome, respectively (Zabala et al. 1997; Wise et al. 1999). In rice, WA, BT and HL-CMS systems are three genetically different CMS systems used in commercial production. Of which, BT-CMS and HL-CMS have been confirmed being induced by chimeric *orf79* and *orfH79*, respectively, these two *orf*s share 98% identity in coding region (Yi et al. 2002). However, in WA-CMS, a polymorphic *orfB* gene was suggested responsible for male sterility (Das et al. 2010). In this research, we found that the accessions in Mt subgroups m3 and m4 held the *orfH79/orf79* fragment (PCR primer: 5′-ATGACAAATCTGCTCCGAT-3′, 5′-TTACTTAGGAAAGACTACAC-3′). Sequencing (data not shown) revealed that the accessions (w7, w11, w13, w16) carrying *orfH79* and the accessions (c1, c7, w2, w3, w4, w15) carrying *orf79* fragment fall into Mt subgroups m3 and m4, respectively, as shown in Fig. 2. This implies that each subgroup in the mitochondrial phylogenetic tree could be deemed to represent

a type of cytoplasm. If this hypothesis can be established, then, besides the cytoplasm-HL (subgroup m3) and -BT (subgroup m4), there are two other unknown cytoplasm types in group Mt-I (subgroup m1 and subgroup m2), three unknown cytoplasm types in group Mt-II (subgroup m5, subgroup m6 and subgroup m7) (Fig. 2). This means that possibly there are some CMS types genetically different to any of the known CMS systems hidden in the *Oryza* species. Although some of these need to be characterized in the future, it is consistent with the report by Pradhan and Jachuck (2000) that two shallow lowland male sterile lines, MotiA and PadminiA with Miz.4, derived from inter-variety hybridization are genetically different from the WA-CMS.

Pollen fertility of CMS lines can be recovered by *Rf* alleles via repressing the deleterious effects of CMS genes (Hanson and Bentolila 2004). The numbers and working model of *Rf* genes are specific to each of the CMS types within a plant species (Gabay-Laughnan and Laughnan 1994). Increasing documents have suggested that *Rf*s condition the working pattern of the CMS genes at different levels from DNA to protein, just as the *Fr* restorer for *Phaseolus vulgaris* does at DNA level (Mackenzie and Chase 1990), *Rf3* for CMS-S maize at the post-transcriptional level (Xiao et al. 2006), and *Rfo* restorer for CMS-Ogura rapeseed at the protein level (Bellaoui et al. 1999). In rice, molecular analysis has indicated that the WA-, HL- and BT-CMS each has at least two different alleles being detected, respectively. Of which, *Rf1a* and *Rf1b* possess different processing model to *orf79* transcript in BT-CMS (Wang et al. 2006). In this research, the investigated accessions were divided into five groups according to their restoring ability for HL- and WA-CMS. Interestingly, accessions in each of the subgroups of the nuclear phylogenetic tree were well corresponded to that of the fertility restoring pattern in Fig. 5. Which means that each of the subgroup in Fig. 5 represents a type of fertility restorer. This is highly compatible to our previous findings that there are at least three restoring loci for WA-CMS in the wild rice with AA genome (Li et al. 2005), and four alloplasmic CMS lines with variant *orfH79* haplotypes correspond to specific restorer alleles (Li et al. 2007). It reflects that *Rf* allele has multiple acting patterns and different loci in *Oryza* species.

Concert evolution between CMS and fertility restorer in *Oryza* species

During the evolutionary process, mitochondrion has transferred most of its functional genes to the nuclear genome so as to specify its role for power production in cells, and only part of the genes encoding respiratory complex subunits are retained in its genome (Boore 1999; Thorsness and Weber 1996; Hanson and Bentolila 2004). So, concerted interaction between nDNA- and mtDNA-encoded proteins becomes the precondition to keep the normal function of mitochondria. The disharmony between nuclear and mitochondrial genomes will lead to breakdown in cell growth or even cell death, which has been manifested by the survivability of hybrid cells or individuals containing alien nDNA or mtDNA from other populations or species (Schmidt et al. 2001; Budar et al. 2003). In order to keep surviving, if a natural mutation occurred in plant mitochondrial genome results in the defect of pollen development, correspondingly, a nuclear gene will also change so as to reconcile the altered mitochondrial expression pattern to recover the fertility of the mutants, which may lead to the occurrence of *Rf* genes.

A good many of references have suggested that different *Rf* alleles interact with CMS in a gene-for-gene fashion, this means that various *Rf* loci are correspondingly determined by the multiple CMS systems existed in the natural populations within a plant species (Manicacci et al. 1997; Charlesworth and Laporte 1998; Taylor et al. 2001; Van Damme et al. 2004). From our results, it is evident that the cytoplasm in *Oryza* species was distinctly divided into three groups based on the mitochondrial DNA patterns as shown in Fig. 2. While, based on PCR profiles of the *Rf* candidate, nuclear genotypes were also divided into three groups as shown in Fig. 5. The accessions between Mt subgroups and Nu subgroups were well in agreement with each other. In view of species or geographical distribution, 18 accessions in the group Nu-I include 5 species, 6 *O. nivara*, 1 *O. barthii*, 2 *O. glumaepatula*, 3 *O. rufipogon*, 6 *O. sativa* (ssp. *indica*/*japonica*), and come from Nepal, Thailand, China, Cambodia, India, Suriname, Brazil, Cameroon, Bangladesh, America and Philippine from Southeast Asia, West Africa, South America and North America. Likewise, the accessions in other groups/subgroups exhibit also the analogical characteristic. The accessions within each group/subgroup of the two phylogenetic trees have nothing to do with species or geographical origin, but are highly related to their DNA patterns of *Rf* candidates and CMS-related genes, which implies that the mitochondria CMS-related *orfs* are evolutionarily parallel to the nuclear *Rf* candidates in *Oryza* species. This finding may give us a potential way to explore new CMS cytoplasm and the corresponding *Rf* genes from wild rice resources in breeding program.

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