BRIEF COMMUNICATION

Chloroplast DNA polymorphism in different types of cytoplasmic male sterile rice

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Abstract

The lengths of open reading frame (ORF)100 and ORF29-TrnC^{GCA}, the intronic sequence of *rps16* and the transcribed spacer of TrnT^{UGU}-TrnL^{UAA} in chloroplast from different lines of cytoplasmic male sterility (CMS) rice were studied using *indica* types, *japonica* types and common wild rice as controls. The results show that the lengths of ORF100 and ORF29-TrnC^{GCA} in CMS lines are similar to those of typical *indica*. The sequences of the *rps16* intron and the TrnT^{UGU}-TrnL^{UAA} spacer in sporophyte sterile types (wild-abortive type, Yinshui type and K type) are almost the same, and they also share a molecular marker of GTTGAG at nucleotide positions 220 - 225 in the *rps16* intron. Therefore, it is speculated that the source of these three types is the same. In contrast, a gametophyte sterile type, Yuetai A does not contain such a GTTGAG sequence in the *rps16* intron and has a unique G at position 595, which may works as a molecular marker distinguishing the sporophyte sterile type from the gametophyte sterile type. Based on the observation that CMS rice has much lower cytoplasmic polymorphism than *indica*, *japonica* and wild rice, it is concluded that CMS rice lack cytoplasm diversity. Therefore, it is important to introduce new sources of cytoplasm into hybrid rice.

Additional key words: gametophyte sterility, molecular marker, open reading frame, *Oryza sativa*, sporophyte sterility.

Rice (*Oryza sativa* L.) is one of the most important food sources. There are three important cytoplasmic male sterility (CMS) types in rice [Wild-abortive (WA), Honglian (HL) and Baro-II (BT)] which tremendously contributed to the increased yield (Pan 1982). Genetic analysis of fertility restorer (*Rf*) genes indicates that HL- or BT-CMS are controlled by single dominant *Rf* gene and WA-CMS is controlled by one or two pairs of dominant *Rf* genes, which reflects the characters of the gametophytic and sporophytic restoration CMS type (Tan *et al*. 2008). Some researchers believe that CMS is mostly related to mitochondria (Kadowaki and Harada 1989, Iwahashi *et al*. 1984, Yao *et al*. 2001, Lin *et al*. 2000). Chen (1992) found that various CMS are significantly different in terms of heterosis after examining their effect on hybrid yield. Liu *et al*. (1992) proposed that a single source of sterile cytoplasm might produce specific race and then lead to the craze of rice blast. Yan *et al*. (1998) showed that disease resistance of CMS varies greatly and suggested that expanding the source of sterile cytoplasm is one of the most effective ways to solve the problem. However, the molecular mechanism of cytoplasmic effect has not been studied in details. In particular, there is no report about the structure and function of chloroplast DNA in different types of CMS. Here we performed a comparative analysis on the chloroplast DNA sequences of different CMS, thereby advancing our understanding of polymorphism in rice cytoplasm.

The ORF100 has been reported as an efficient marker to identify whether a rice chloroplast genome belongs to

Received 5 August 2008, *accepted* 16 July 2009.
Abbreviations: CMS - cytoplasmic male sterility; HL-CMS - Honglian cytoplasmic male sterility; ORF - open reading frame; Rf - fertility restorer; rps16 - ribosomal protein s16.
Acknowledgements: This work was supported by the Key Laboratory of Hunan Province for Study and Utilization of Ethnica Medicinal Plant Resources, Huaihua University (SYSXM200905) and the key project of Hunan, China (06FJ2003). The first two authors contributed equally to the paper.
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indica or *japonica* type: the ORF100 bands of *japonica* rice lag behind those of *indica* rice because there is a 69 bp deletion in the *indica* ORF100 while a 69 bp repeat in *japonica* (Chen *et al*. 1993, Kanno *et al*. 1993). Tang *et al*. (2004) found that the *indica* type has a 32 bp insertion in the ORF29-TrnC^{GCA} (tRNA-Cys(GCA) spacer but the *japonica* type does not contain such an insertion, leading to a lag of the *indica* bands related to the *japonica* ones. Therefore, ORF29-TrnC^{GCA} can be regarded as another marker to distinguish the *indica* chloroplast genome from that of *japonica*. The intron of ribosomal protein s16 (rps16) and the spacer of TrnT^{UGU}-TrnL^{UAA} [tRNA-Thr(UGU)-tRNA-Leu(UAA)] are highly variable regions in plant chloroplast genomes (Shaw *et al*. 2005). In the present study, the polymorphism of these above-mentioned ORFs, introns and spacers in chloroplasts were examined and then the genetic diversity of CMS rice was discussed, thus providing important clues for the increasing yield of hybrid rice.

Five lines of wild-abortive type CMS rice (Jin 23A, Guangye A, V20A, Chuanxiang A and Zhenshan 97A), four lines of Yinshui type CMS rice (T98A, II-32 A, Zhong 9A and You 1A), one line of K type CMS rice (K17A) and one line of HL-CMS rice (Yuetai A) were used in this study. Meanwhile, three *indica* cultivars (9311, Guangluai sihao and Nanjing sanhao), two *japonica* cultivars (Nipponbare and Qiuguang) and two lines of common wild rice (Hainan CWR and Chaling CWR) were used as controls. DNA was extracted according to Gelvin *et al.* (1988).

PCR primers were designed by *Primer Premier* (version *5.0*) based on the chloroplast genome sequence of 9311 (GenBank Accession No. AY522329) (Table 1). The reaction system contained 2.5 mm^3 10× PCR buffer, 0.5 mm^3 forward and reverse primers, 0.3 mm^3 10 mM dNTPs, 1 U Taq, 40 ng template, and complementary ultrapure water to 25 mm^3. The reaction was set as follows: pre-denaturation at 94 °C for 4 min, then 94 °C 30 s, 50 °C 40 s, and 72 °C 50 s; the cycle was repeated for 32 times, and then 72 °C 10 min.

The PCR products were fractionated on 1 % agarose gel, and the gel images were obtained with the *GelLogic 100* image system. The target fragments were isolated from the agarose gel under UV radiation, reclaimed and purified with the reagent kit (*Tiangel* midi purification kit), and then directly sequenced. Sequence analysis on ORF100 and ORF29-TrnC^{GCA}, the rps16 intron and the TrnT^{UGU}-TrnL^{UAA} spacer was all performed by *MEGA 3.1*.

Based on the ORF100 PCR products amplified by the cp1 primer pair, we find that all the four types of CMS rice are consistent with typical *indica*. In contrast, the ORF100 bands of typical *japonica* (Nipponbare and Qiuguang) lag behind those of typical *indica* (9311, Nanjing sanhao and Guangluai sihao). The result from the ORF29-TrnC^{GCA} analysis is also compatible with the ORF100 analysis: all the bands of the four types of CMS rice are same as those of typical *indica*. Together, these results suggest that the cpDNA of CMS rice comes from *indica*.

Based on the PCR products amplified by the cp3 primer pair, we find that the size of the rps16 intron ranges from 677 to 684 bp (Table 2), and the intron contains a special *indica*/*japonica* marker at nucleotide positions 267 - 273, which is CTTTATC in *indica* but a deletion in *japonica* rice. For this segment, these lines of CMS rice are most compatible with *japonica* types.

Except for Yuetai A, all the CMS lines are nearly identical in the rps16 intron and contain a special sequence of GTTGAG at nucleotide positions 220 - 225, which might work as a molecular marker of sporophyte sterile cytoplasm. In addition, we identified two polymorphism sites: the nucleotide at position 595 in Yuetai A is G, while it is T in the other lines. The nucleotide sequence at positions 309 - 312 in Guangluai sihao is CTTT, while it is deleted in the other lines.

Based on the PCR products amplified by the cp4 primer pair, we find that the size of the TrnT^{UGU}- TrnL^{UAA} spacer ranges from 813 to 824 bp and this region contains mostly single nucleotide polymorphism (Table 3). The spacer has three distinguishable *indica*/ *japonica* nucleotide sites (positions 332 - 326, 413 and 774 - 779) and the sequences of CMS rice are almost the same as that of *indica* type. Moreover, Qiuguang has three special nucleotide sites (positions 463, 599 and 635) and Nanjing sanhao has three special nucleotide sites (positions 479, 520 and 572).

Through PCR sequencing on ORF100, Sun *et al*. (1997, 1996, 2002) showed that there was an *indica-japonica* differentiation in the chloroplast DNA of wild rice. For example, *indica-japonica* differentiation was existed in the 10 strains of Dongxiang wild rice from the same place, in which six strains were *indica* chloroplasts and 4 strains were *japonica* chloroplasts. We find that the lengths of ORF100 and ORF29-TrnC^{GCA} in Hainan CWR are consistent with typical *indica*, while that of Chaling CWR falls in line with typical *japonica*, providing some evidence for this differentiation tendency in common wild rice. However, the nucleotide sequences at positions

Table 1. Primer sequence and target fragment.

Primer	Forward sequence 5' - 3'	Reverse sequence 5' - 3'	Target fragment
cp1	GTGGACCTGACTCCTTGAA	AGCCGAGGTCGTGGTAA	ORF100
cp2	GCAGCCCAAGCGAGACT	AAGGCTCGGCGATACTG	ORF29-TrnC^{GCA}
cp3	TTTTCTCCTCATACGGCT	TAGTCTGTTCTATTCGTCCC	rps16 gene intron
cp4	AGTGGGCTTACATAACAGAAA	ACCAAGGCTCAATACAATCA	TrnT^{UGU}-TrnL^{UAA}

Table 2. Sequence divergence of rps16 gene intron and of $TrnT^{UGU}$-$TrnL^{UAA}$ spacer.

Material	rps16					$TrnT^{UGU}$-$TrnL^{UAA}$								
	52	220-225	267-273	309-312	595	322-326	413	463	479	520	572	599	635	774-779
9311	A	-	CTTTATC	-	T	-	-	G	A	A	G	T	A	-
Nanjing san hao	A	-	CTTTATC	-	T	-	-	G	C	G	A	T	A	-
Guangluai sihao	A	-	CTTTATC	CTTT	T	-	-	G	A	A	G	T	A	-
Nipponbare	T	-	-	-	T	TATAT	T	G	A	A	G	T	A	AGAAAA
Qiuguang	A	-	-	-	T	TATAT	T	A	A	A	G	C	T	AGAAAA
Jin 23A	A	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	-
Guangye A	A	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	-
V20 A	A	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	-
Chuanxiang 29A	A	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	-
Zhenshan 97A	A	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	-
T98 A	A	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	-
II-32A	A	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	-
Zhong 9 A13	A	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	-
You 1A	A	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	-
K17A	A	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	-
Yuetai A	A	-	-	-	G	-	-	G	A	A	G	T	A	-
Hainan CWR	T	-	-	-	T	TATAT	T	G	A	A	G	T	A	AGAAAA
Chaling CWR	T	-	-	-	T	-	-	G	A	A	G	T	C	AGAAAA

267 - 273 of the rps16 intron and at positions 774 - 779 of the $TrnT^{UGU}$- $TrnL^{UAA}$ spacer in Hainan CWR are consistent with typical *japonica*. Thus, although the lengths of ORF100 and ORF29-$TrnC^{GCA}$ in wild rice support the tendency of *indica-japonica* differentiation, the type of chloroplast DNA remains inconclusive. More comprehensive genetic analysis is required to determine the characteristics of wild rice chloroplast confidently.

The wild-abortive type CMS rice is the most widely planted rice in China, whose cytoplasmic donor is Hainan CWR. But this type of CMS has a special nucleotide sequence GTTGAG in rps16, suggesting that the cytoplasm is not from the common Hainan CWR but from a specific mutant. The K-type line was obtained by backcross and seed cultivation of the sterile plant, a hybrid progeny of *indica* and *japonica* rice (Yunnan *japonica* rice was female parent). With wild-abortive sterile lines as differential materials, the cytoplasm of Yinshui type was obtained by directly searching for a new sterile cytoplasm in cultivated rice (restorer line) and then through hybridization and genetic recombination between the restorer and the maintainer. However, our results show that the chloroplast DNA sequences of K type and Yinshui type are identical to those of the wild-abortive type. This is consistent with a previous study showing that there is no significant difference between the restorer and the maintainer. Therefore, the relationships among the three types of so-called sterile cytoplasms remain to be explored.

Compared with wild-abortive type, Yinshui type and K type, HL-CMS rice (Yuetai A) contains no GTTGAG sequence and has a unique G at nucleotide position 595 of the rps16 gene. This indicates that the cytoplasm of Yuetai A is different from other sterile cytoplasms at the chloroplast sequence level.

The wild-abortive type, Yinshui type and K type rice have no nucleotide polymorphism in the intronic region of rps16 and the transcribed region of $TrnT^{UGU}$-$TrnL^{UAA}$, in contrast to the homologous regions in typical *japonica* and *indica* rice that show different levels of polymorphisms. This suggests that the chloroplast genetic background of these CMS lines is the same. Constrained by the same cytoplasmic resource, the potential for increasing the yield of these CMS lines may be limited. Thus, it is becoming more and more important to search for the lines that contain novel cytoplasmic sterile source. This is a crucial step for increasing the biological diversity of rice as well as a key direction of maintaining biological safety in agriculture.

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