Bot. Bull. Acad. Sin. (2003) **44:** 285-289

Fine mapping of the nuclear fertility restorer gene for HL cytoplasmic male sterility in rice

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(Received December 2, 2002; Accepted May 16, 2003)

Abstract. Bulked segregant analysis (BSA) of a BC_1 population derived from congguang 41A/MiYang 23//congguang 41B was used to map the nuclear fertility restorer gene *Rf5* for HongLian (HL) cytoplasmic male sterility. The parents and two bulks representing extremely fertile and sterile plants, respectively, were screened for polymorphism with 20 microsatellite primer pairs on chromosome 10, chosen on the basis of previous research. MRG4456 is linked to the fertility restorer gene *Rf5* at a distance of 1.57 cM, and another newly developed microsatellite primer, HL01, was locked at a distance of 0.63 cM to *Rf5*. Closely linked DNA markers will facilitate not only breeding but also the purity management of hybrid seeds. Concrete steps for developing new microsatellite markers using the rice whole genomic sequence database are also described.

Keywords: Fertility restorer gene; Marker-assisted selection; Microsatellite marker; *Oryza sativa* L.

Introduction

The phenomenon of cytoplasmic male sterility (CMS), described as the inability of a plant to produce functional pollens, has been observed in over 150 plant species (Wise et al., 2002). Fertility restorer (*Rf*) genes in the nuclear genome can counteract this inability (Newton, 1988) and restore fertility to cytoplasmic male-sterile plants. Hybrid breedings based on CMS/*Rf* systems have achieved great success all over the world. Aside from its commercial exploitation, CMS offers a rare opportunity to examine the regulation of mitochondrial genes by nuclear genes in multicellular organisms.

Three primary types of CMS in rice are now known, and their heritance habits and physiological characteristics have been extensively investigated. They are Wild-rice abortive (WA), BaoTai (BT) and HongLian (HL). WA type CMS belongs to sporophytic abortion, which fails to produce normal pollen and finally forms typical abortive pollen. In contrast, BT (*japonica*.) and HL type CMS (*indica*.) belong to gametic abortion, but they are also greatly different in terms of abortive phenotype, relationship of restoration, and maintenance.

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CMS systems are usually attributed to chimeric ORFs in the mitochondrial genome (Kempken and Ping, 1998; Schnable et al., 1998; Szklarczyk et al., 2000). These ORFs encode novel proteins, which often interfere with mitochondrial function and pollen development. In many instances, the restorer gene suppression of CMS is directly associated with *Rf*-gene-dependent mitochondrial RNA modification and concurrent reduction of CMS associated protein (Schnabel et al., 1998). Although many mitochondrial genes associated with CMS have been cloned, only two *Rf* genes have been isolated until now. One is the maize *Rf* gene named *Rf2*, encoding aldehyde dehydrogenase (ALDH) (Cui et al., 1996; Liu et al., 2001); The other is Petunia Rf gene, encoding a mitochondrially targeted protein comprised of pentatricopeptide repeat (PPR) motif (Bentolila et al., 2002).

The inheritance of fertility restoration in WA type CMS has been extensively investigated. Most investigators tended to agree that restoration of WA type CMS is controlled by two nuclear genes (*Rf3*, *Rf4*) and that their chromosomal loci have been resolved (Zhang et al., 1997; Yao et al., 1997; Tan and Trangoonrang, 1998; Zhang et al., 2002). BT type CMS is restored by nuclear fertility restorer gene *Rf1*, which was mapped on chromosome 10 (Fukuta et al., 1992; Akagi et al., 1996;Yokozeki et al., 1996). HL type fertility restoration gene *Rf5* was also mapped on chromosome 10, 7.8 cM from RM258 (Huang et al., 2000). The studies reported here were undertaken to finely locate the nuclear fertility restoration gene *Rf5* for HL type CMS.

Materials and Methods

Mapping Population Development and Fertility Evaluation

A male sterile line of HL type: Congguang 41A and male sterile restorer line responding to HL type: Miyang 23 were used as parents to generate F1. The F1 was then back-crossed to Congguang 41B to generate a BC_1. The BC_1 population, consisting of 1023 individuals, was used as a mapping population for *Rf5* gene. Pollen fertility in BC_1 population and seed setting were evaluated at the flowering and maturity stages, respectively. For seed setting evaluation, the panicles were covered before flowering by a paper bag to prevent them from pollinating each other. Sterile plants contained less than 5% stainable pollen and produced no fertile seed. All others were treated as fertile. The segregation ratio of fertile to sterile plants was evaluated in the BC_1 generation based on the standard mentioned above. If individual lines contained less than 1% stainable pollen and their seed setting rates of bagged panicles were zero at maturity, they were considered as excessive male sterility lines.

Construction of the Bulks

Genomic DNAs were prepared as described by Huang (Huang et al., 2000) from the parents and from individual plants in the BC_1 population. Equal amounts of DNA from male fertile and male sterile individuals were pooled to generate a male fertile and a male sterile bulk, respectively.

Microsatellite Marker Development

Microsatellite DNA markers were produced by searching the rice whole genomic sequence database for simple repeated di-to tetra-nucleotide motifs with software. Polymerase chain reaction (PCR) primer pair consists of 17-22 nucleotides, each with a GC content around 50% (Tm Ca. 60°C), which meets the criteria of a low frequency primer-dimer, and it is preferably G-or C-rich at the 3'end. Generally, PCR product ranges between 100-250 bp.

Genetic Mapping by Microsatellite Markers

We used 20 microsatellite primer pairs (Table 1) near microsatellite marker RM258 in this study. PCR followed the procedure described by Huang (Huang et al., 2000). The PCR reaction was carried out on a PTC-100 DNA Thermal Cycler. Amplification products were analyzed with regular 4.0% agarose gel. For polymorphic microsatellite markers, the PCR products were analyzed by running a 4.0% denaturing polyacrylamide gel.

RFLP Analysis

Southern-blotting analysis followed the standard procedure described by Sambrook et al. (1989). RFLP marker probes were kindly provided by Dr. Li Zhikang (International Rice Research Institute). The genetic data was analyzed with Mapmaker software (3.0) following the instructions provided.

Results

Identification of Microsatellite Markers Linked to Rf5

DNA from Congguang 41A, Miyang 23, a DNA bulk of 15 male fertile plants, and a DNA bulk of 15 male sterile plants from the BC_1 population were used as the template for PCR analysis. 4 out of 20 primer pairs generated the

Table 1. All 20 microsatellite markers used in the experiment. MRG2510, MRG4456, RM171 and HL01 are polymorphic in BSA analysis.

NO.	SSR marker	Profile length (bp)	Fwd_primer_sequence	Reverse_primer_sequence
1	MRG0315	123	TCCTAACCACCTGCCTAATGC	TCATCCCACAGTTTCCTACCC
2	MRG2510	160	TAAGATCGTAAGATCGCGGC	AGGCAGGAAGAGGTGGAGG
3	MRG4456	160	GTCCGCTGGTTGGTATGATT	CAACAGCTACAGACACACACAGC
4	MRG4629	117	AGCTCAACTCGACAACTCCC	CCATCTCCTCTTTCACCTCG
5	MRG5100	144	TCCTCTACCAGTACCGCACC	GCTGGATCACAGATCATTGC
6	MRG5150	180	CTCGACGGAGCTCTCTTCAC	CAAGAAGCAGAGGAAAAGCG
7	MRG5455	189	TCCACATAAACCCTCGCTTC	AATTTCCTCCCCGAAGGC
8	MRG5704	240	CACACATTGCATTACGAGGG	CAGGGGCAGCTTGAATACTG
9	MRG5737	187	CATTGGGGGTGGATAAAGAG	TATCCTCTACTCCCTCGGCC
10	RM171	328	AACGCGAGGACACGTACTTAC	ACGAGATACGTACGCCTTTG
11	RM304	160	TCAAACCGGCACATATAAGAC	GATAGGGAGCTGAAGGAGATG
12	HL01	110	GGAGATGCTATAGCAGCAGTG	ATTGCTCCTTACCACCTTGC
13	HL02	126	TCCGACGAACTGACTTTCTG	AGTAGGTCCACTCCTGCCTC
14	HL03	206	CGATAGATCAGTGGCAGATC	CCTCGCTATACTACCTGTCG
15	HL04	139	AACTCCCAAAGTGTAGCGTG	CCAAACCAAGGAACAGATTG
16	HL05	128	CTTGCAGCTGATGTCCCTTC	AGGAAGCCGCTGATGTGGC
17	HL06	156	TTCTTGGACTGCACCACCC	GCAACAGATCATGTCAGCCAC
18	HL07	158	ATGAGCGCCGTGATGGTGCC	GAGTTCCTCGGCGACGTGTC
19	HL08	130	TCATCCGACACCGACAGAAC	GACCTCTAGCTAATCTCATTG
20	HL09	111	TGTTACTACCGTACTAGCAC	GTGTCGGCAAGCCATCCAT

amplicon polymorphism between parents. The frequency polymorphism generation using bulk analysis for microsatellite markers was 20.0%.

Genetic Linkage Analysis of Rf5 Restorer Gene Via Microsatellite Markers Using the BC_1 Population

The segregation ratio of fertile to sterile plant was about 1:1 (443:580), which shows only one restorer gene exists in Miyang 23. In order to estimate the genetic distance between microsatellite marker and restorer gene *Rf5*, 158 excessive male sterility lines were used for genetic mapping analysis via microsatellite markers. Two out of 158 individual lines exhibited a genetic recombination when using a microsatellite marker HL01. For microsatellite markers MRG2510 and RM171, 3 and 7 individual lines showed the genetic recombination respectively; On the other side of *Rf5*, five individual lines showed a genetic recombination when using a microsatellite marker MRG4456 (Figure 1). The genetic linkage analysis revealed that the two closed microsatellite markers, HL01 and MRG4456, flanked the *Rf5* gene locus with a genetic distance of 0.63 cM and 1.57 cM, respectively.

Genetic Fine Mapping of Rf5 Via RFLP Markers Using the BC_1 Mapping Population

To further confirm chromosome location of the restorer gene *Rf5* in rice genome, RFLP markers from the region surrounding the restorer gene *Rf5* on chromosome 10 were selected for a parental polymorphism survey based on previous results (Huang et al., 2000). Two RFLP markers, G2155 and S10019, revealed polymorphism between the parents (data not shown). The results showed that G2155 and S10019 linked to the *Rf5* locus with a genetic distance 6.1 cM and 2.7 cM, respectively, and the two RFLP markers are located in the same direction as the *Rf5* locus on the long arm of chromosome 10. However, the microsatellite markers HL01 and MRG4456 flanked the *Rf5* locus, showing the physical order on chromosome 10 as HL01-*Rf5* -MRG4456-S10019–G2155 (Figure 2). These results will accelerate the steps of isolating *Rf5* gene by a map-based cloning strategy.

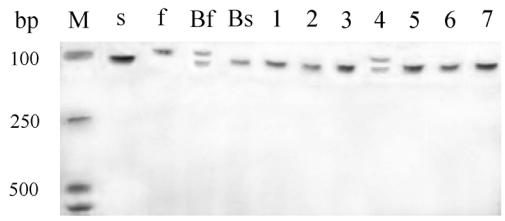

Figure 1. Amplication profiles obtained with the microsatellite primer pairs MRG4456 are revealed on 6.4% denaturing polyacylamide gel. M, DL2000 marker; s: Congguang 41A; f: Miyang 23; Bf: fertile bulks; Bs: sterile bulks; 1-7: individuals of BC_1 population; 4 is recombinant.

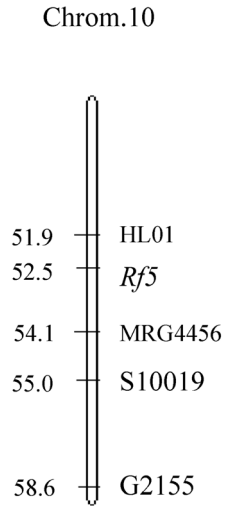

Figure 2. The location of the restorer gene *Rf5* identified by SSR and RFLP markers. Markers' names are listed to the right of chromosome, and their positions (in cM) are on the left.

Discussion

Selection of BC_1 Population

HL type CMS belongs to gametic abortion, whether pollen is fertile or not is determined by its own genotype. This is different from sporophytic abortion, in which pollen's fertility is determined by sporophytic genothype. The pollen fertilities in F_1 hybrid plants differ in their CMS, 50% have gametophytic and 100% have sporophytic cytoplesmic male sterility. Therefore, a backcross population was used here instead of an F_2 population, which is widely used in mapping the genes involved in sporophytic abortion.

Multi-Loci of Fertility Restorer Genes

This study has finely located the nuclear fertility restorer gene for the HL type CMS on Chromosome 10. Interestingly, the nuclear fertility restorer gene *Rf1* for BT type CMS has been located on chromosome 10, 3.7 cM from OSR33 (Akagi et al., 1996). Subclone Y3-8 from rice YAC clone Y4892 of RGP (Rice Genome Program) was mapped to the *Rf4* gene locus with a genetic distance of 0.9 cM, and YAC clone Y4892 was anchored to the RFLP marker S10019 (Zhang et al., 2002). Comparing various molecular linkage maps suggests that three genes are located in the adjacent region on chromosome 10. Just like the disease resistant genes (Grog et al., 1993; Mahadevappa et al., 1993; Ellis et al., 1995), the restorer genes may be clustered on chromosome 10 (Li et al., 1998).

Analysis of Microsatellite Markers

Microsatellite markers combine the rapidity, straightness, and simplicity of RAPD with the stability and reliability of RFLP. They also often detect more allelic variation than RFLP or RAPD markers (Panaud et al., 1996). Additionally, it has been discovered that simple sequence

repeat content for rice 93-11 occupys 1.7% of its whole genome (Yu et al., 2002) which proves the microsatellite marker's abundance and wide distribution in the rice genome. Therefore, microsatellite markers are the tool of choice in the genetic mapping of rice. Actually by designing new microsatellite markers in the target region, we have got the co-segrative microsatellite marker *Rf5* (unpublished data).

The results presented here indicate that microsatellite marker HL01 and MRG4456 will facilitate marker-assisted selection (MAS) of restorer lines in the CMS-HL system, which will promote the development of hybrid rice. Construction of the physical map encompassing the *Rf5* gene locus is underway. Our ultimate goal is isolating the *Rf5* gene through a map-based cloning strategy.

Acknowledgements. The author would like to thank Dr. Fu Bingyin for his excellent technical assistance. This work was supported by the National Program on Basic Research. (Grant No. 2001CB108806).

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水稻紅蓮型細胞質雄性不育恢復基因（細胞核）的精細定位

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利用群分法，以（從廣 41A X密陽 23）X從廣 41B 回交群體為基因定位群體對紅蓮型細胞質雄性不育恢復基因進行了精細定位。位於 10號染色體上的微衛星標記 MRG4456與紅蓮型細胞質雄性不育恢復基因連鎖，遺傳距離為 1.57 cM；另一個新發展的微衛星標記 HL01被定位於恢復基因的另一端，遺傳距離為0.63cM。這兩個與恢復基因緊密連鎖的微衛星標記有助於對紅蓮型細胞質雄性不育恢復系的分子標記輔助選擇，同時為用圖位克隆法分離紅蓮型細胞質雄性不育恢復基因打下了堅實的基礎。我們也詳細的敘述了利用水稻基因組測系結果發展的微衛星標記的具體方法。

關鍵詞：水稻；恢復基因；微衛星標記；標記輔助選擇。