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Microsatellite-Aided Screening for Fertility Restoration Genes (*Rf*) Facilitates Hybrid Improvement

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Abstract: DNA markers enabled to determine the chromosomal locations of the two *Rf* genes (*Rf3* and *Rf4*) in the wild-abortive cytoplasmic male sterility (WA-CMS) system. Four simple sequence repeats (SSRs) RM171, RM258, RM315 and RM443 were used to detect the allelic status with respect to the fertility restoration genes (*Rf3* and *Rf4*) in 300 rice cultivars or breeding lines. The results revealed that out of 300 lines, 90 lines screened had *Rf3*, 65 lines had *Rf4*, and 45 lines had *Rf3* and *Rf4* alleles. Furthermore, 45 lines selected using SSR markers were mated with a CMS line (IR58025A) to analyze their restoring ability. Offspring of all the test lines except HHZ8-SAL9DT1-Y1, HHZ5-SAL9-Y3-1 and IDSA77 exhibited higher pollen and spikelet fertility (> 80%), thus confirming they bear the *Rf* alleles. The hybrid offspring of ARH12-6-1-1-B-3-1, IR32307-10-3-2-1 and Sahel 329 had the highest pollen fertility (97.39%, 98.30% and 97.10%, respectively) and spikelet fertility (95.10%, 97.07% and 96.10%, respectively).

Key words: cytoplasmic male sterility; fertility restoration gene; heterosis; rice; simple sequence repeat

Rice is the most important staple food crop in the world. It feeds more than one-half of the world's population (Hariprasanna et al, 2006). Africa, where rice is the most rapidly growing food, will need about 3×10^7 t more rice by 2035. This amount equals to an increase upto 130% of what was rice consumption by Africans in 2010 (Seck et al, 2012).

Hybrid rice technology has contributed significantly to food security and provided rural employment in China for the last 30 years. Hybrid rice occupies about 50% of total rice field area in China (Lu and Hong, 1999). Rice hybrids increased grain yield between 15% and 20% higher than the high-yielding inbred cultivars, when farmers grew them initially in China, and thereafter in India (Mishra et al, 2003), Bangladesh (Julfiquar et al, 2003), the Philippines (Redoña et al, 2003) and Vietnam (Hoan et al, 1998). In the last decade, Côte d'Ivoire, Liberia, Madagascar, Mozambique, Nigeria, Tanzania and Uganda began evaluating and cultivating rice hybrids from China (El-Namaky and Demont, 2013). This encouraged AfricaRice to launch a breeding program for hybrid rice at its regional station in Saint-Louis, Senegal in 2010. The aims of this program are to develop and evaluate hybrids and then increase grain yield of rice through utilization of heterosis phenomenon in hybrid rice.

Cytoplasmic male sterility (CMS) is a common phenomenon in plants. It has been extensively used for preventing self-pollination in the production of hybrid seeds in various crops (Li et al, 2007). In rice, CMS systems, which consist of fertility restorers, CMS lines and maintainers, have been applied for the commercial production of hybrid seeds in China since 1975 (Yuan and Virmani, 1988; Virmani, 1996). Two major fertility restoration genes, *Rf3* and *Rf4*, are required for the production of viable pollen in wild-abortive (WA) CMS and the genes have been mapped to chromosomes 1 and 10, respectively (Yao et al, 1997; Zhang et al, 1997). Marker-assisted selection (MAS) is being explored as an important supplement to phenotypic selection in rice hybrid breeding. Bazarkar et al (2008) found that microsatellites (simple sequence repeats, SSRs) RM443 and RM315 are flanking the *Rf3* gene at genetic distances of 4.4 cM (LOD 10.29) and 20.7 cM (LOD 3.98) on chromosome

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Table 1. SSR primer pairs with their chromosomal locations and annealing temperatures.

Marker	Chromosome	Forward primer	Reverse primer	Annealing temperature (℃)
RM315	1	GAGGTACTTCCTCCGTTTCAC	AGTCAGCTCACTGTGCAGTG	55
RM443	1	GATGGTTTTCATCGGCTACG	AGTCCCAGAATGTCGTTTCG	55
RM171	10	AACGCGAGGACACGTACTTAC	ACGAGATACGTACGCCTTTG	67
RM258	10	TGCTGTATGTAGCTCGCACC	TGGCCTTTAAAGCTGTCGC	55

1, respectively. Thereafter, Nematzadeh and Kiani (2010) noted that microsatellites RM258 and RM171 are flanking to restorer gene *Rf4* at the distances of 2.9 and 3.7 cM on chromosome 10, respectively. Microsatellites RM258, RM171, RM315 and RM443 may facilitate MAS of restorer lines for a WA-CMS system in large nursery sets, thus avoiding routine testcrossing in a hybrid rice breeding program (Sheeba et al, 2009; Nematzadeh and Kiani, 2010). The objective of this research was therefore to screen for fertility restoration genes by using SSR markers.

MATERIALS AND METHODS

The screening of restoring ability by using SSR markers and testcrossing of 45 selected lines were conducted at AfricaRice Center, Saint-Louis, Senegal from 2012 to 2014.

Screening of restoring ability

SSR markers RM171, RM258, RM315 and RM443 (Table 1) were used to detect restoration genes (*Rf3* and *Rf4*) for WA-CMS in 300 rice cultivars or breeding lines (Supplemental Table 1). Three-week-old leaves of each genotype were collected for DNA extraction following the cetyl-tetramethyl ammonium bromide (CTAB) protocol for DNA isolation and purification (Murray and Thompson, 1980). After extraction, the concentrated DNA was diluted to working concentration following a dilution ratio of 1 part concentrated DNA to 3 parts TE buffer. Polymerase chain reactions were then performed in a final concentration of 10 μL on 96-well PCR plates. Typically, 10 μL PCR mixture contained 2 μL of 50 ng DNA, 0.5 μL each of 100 pmol/L forward and reverse primers, 1 μL of 10 × PCR buffer [100 mmol/L Tris (pH 8.3), 500 mmol/L KCl, 15 mmol/L $MgCl_2$ and 2 μg gelatin], 0.3 μL of 25 mmol/L $MgCl_2$, 1 μL of 40 mmol/L dNTPs, 1 U *Taq* polymerase and 4.5 μL deionized distilled H_2O. Amplification followed a profile of an initial denaturation at 94 ℃ for 4 min followed by 35 cycles of denaturing at 94 ℃ for 1 min, annealing at 55 ℃/67 ℃ for 1 min and extension at 72 ℃ for 2 min, ending with a final extension step at 72 ℃ for 5 min. A volume of 4 μL PCR product mixed with 1 μL bromophenol blue was separated on 8% polyacrylamide gel (PAGE) using 1 × TBE as buffer. Gels were stained with 10 μL ethidium bromide (0.5 mg/mL) in 200 mL of distilled water for 30 min after which gels were visualized and imaged using Syngen's G-Box gel imaging system.

Testcrossing

A total of 45 selected lines based on analysis with SSR markers linked to *Rf3* and *Rf4* were mated at AfricaRice (Saint-Louis, Senegal) with the WA-CMS line IR58025A to confirm their restoring ability in 2012. The resulting lines were evaluated along with the popular cultivar Sahel 134 (as the check) in an augmented design in 2013. Pollen and spikelet fertility were used as the main criteria for the evaluation of fertile and sterile plants. Fertility and sterility were recorded according to Virmani (1998). Mature anthers were harvested, and their pollen was stained with 1% I_2-KI solution. The numbers of dark blue (stainable) and clear pollen grains (non-stainable) in each sample were counted under an optical microscope. The seed set on a spikelet were also counted following Virmani (1998) and Li et al (2005). Agronomic traits were assessed in the fertile offspring, while complete sterile combinations were used for backcrossing with the recurrent parent to develop new CMS.

RESULTS

Screen of restoring ability using microsatellites

The SSR markers RM315, RM443, RM171 and RM258 exhibited high polymorphism among the tested lines (Fig. 1).

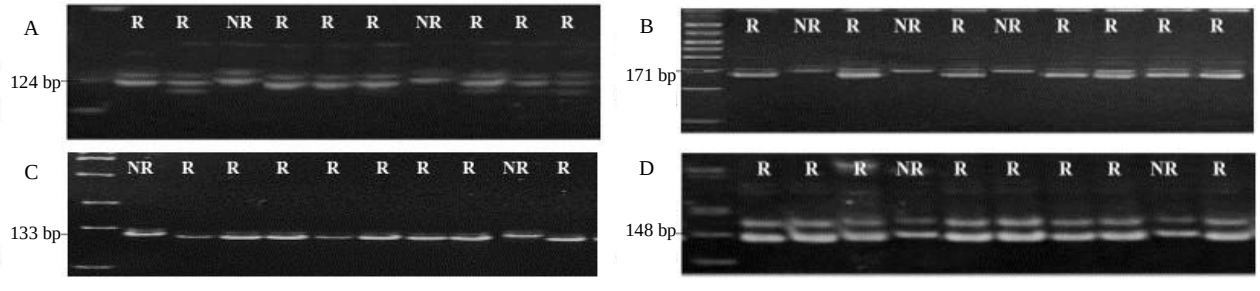

Fig. 1. Banding pattern of markers linked with *Rf3* (A and C) and *Rf4* (B and D) in cultivars and breeding lines.
A, RM443; B, RM171; C, RM315; D, RM258.
R, Restorer; NR, Non restorer.

Of the 300 lines screened by SSRs linked to *Rf3* and *Rf4*, 90 had *Rf3* allele as determined by flanking markers RM315 and RM443, whereas 65 lines had *Rf4* allele, as determined by flanking markers RM171 and RM258, and 45 lines had both *Rf3* and *Rf4* alleles (Table 2). A total of 300 lines were assayed for selection efficiency using SSR markers of fertility restoration in WA-CMS. Marker combination of RM315 and RM443 for *Rf3* and RM171 and RM258 for *Rf4* showed the maximum efficiencies of 95.5% in identification of restorers (Table 3). While the efficiencies of single allele *Rf3* and *Rf4* were 83.2% and 65.4%, respectively (Table 3).

Testcrossing

Offspring of all the test lines except HHZ8-SAL9DT1-Y1, HHZ5-SAL9-Y3-1 and IDSA77 exhibited higher pollen and spikelet fertility (> 80%), thus confirming they bear *Rf* alleles (Table 2). Pollen fertility and spikelet fertility of hybrids derived from HHZ8-SAL9DT1-Y1, HHZ5-SAL9-Y3-1, IDSA77 ranged between 60.46% and 75.62%. Moreover, the hybrid offspring of ARH12-6-1-1-B-3-1, IR32307-10-3-2-1

Table 2. Testcross performance in a Sahel location of hybrids derived from crossing the popular cytoplasmic male sterility line IR58025A with new sources of restoring ability alleles, which were previously selected using a screening with flanking microsatellites.

Cross	Pollen fertility (%)	Spikelet fertility (%)	Days to 50% flowering (d)	Plant height (cm)	Grain yield per plant (g)
IR58025A/ARH10-1-3-2-2-1	93.10	90.30	101.3	106.20	47.30
IR58025A/ARH11-1-3-3-2-1	85.70	86.40	92.4	97.30	38.40
IR58025A/ARH15-1-3-4-1-2	89.40	85.60	112.6	117.50	36.60
IR58025A/ARH13-1-3-5-1-2	83.90	87.70	90.7	95.60	48.70
IR58025A/ARH14-2-1-2-6-1	86.20	90.80	105.8	110.70	49.80
IR58025A/ARH6-2-2-3-1-3	94.50	88.90	93.9	98.80	51.90
IR58025A/ARH7-2-2-4-2-1	93.80	90.00	101.0	105.90	47.00
IR58025A/ARH8-2-2-5-2-3	82.40	82.10	94.1	99.00	40.10
IR58025A/ARH9-1-3-1-1-2	95.00	92.20	94.2	99.10	39.20
IR28025A/ARH12-13-2-B-B-1-1	90.51	94.23	92.5	97.40	32.90
IR28025A/ARH12-6-1-1-B-3-1	97.39	95.10	101.1	106.00	38.80
IR28025A/ARH15-12-1-1-B-2-1	87.70	90.93	86.6	91.50	34.30
IR28025A/ARH21-5-B-2-1	94.65	94.06	100.7	105.60	36.70
IR28025A/ARH22-2-1-B-1-1	84.01	83.07	97.6	102.50	36.30
IR28025A/ARH41-23-2-1-1-2	93.52	93.08	97.3	102.20	48.50
IR28025A/ARH42-2-2-2-1-2	95.59	93.41	90.9	95.80	35.10
IR28025A/ARH43-2-1-1-2	99.08	92.41	101.5	106.40	73.70
IR28025A/ARH46-6-6-1-1	97.00	93.37	91.6	96.50	49.90
IR28025A/HHZ8-SAL9DT1-Y1	73.21	70.95	93.0	97.90	34.00
IR28025A/HHZ5-SAL10-DT1-DT1	84.97	82.75	87.2	92.10	48.10
IR28025A/HHZ5-SAL9-Y3-1	75.62	72.36	93.1	98.00	24.30
IR28025A/IDSA77	60.46	68.90	89.9	94.80	31.00
IR28025A/NERICA-L27	92.10	91.66	96.0	100.90	45.50
IR28025A/NERICA-S-19	94.00	91.58	95.4	100.30	41.90
IR28025A/R31785	88.67	88.63	101.3	106.20	44.00
IR28025A/WITA9	94.96	94.40	110.4	115.30	52.50
IR58025A/ARH47-4-2-1-2-1	90.60	89.10	103.5	108.40	36.50
IR58025A/AD9246	91.40	87.60	98.6	103.50	64.60
IR58025A/Giza 178	90.10	88.60	88.6	93.50	54.60
IR58025A/Giza 179	89.70	87.90	97.8	102.70	50.00
IR58025A/Giza 181	98.67	94.90	99.4	104.30	50.10
IR58025A/Giza 182	95.20	91.40	101.4	106.30	58.40
IR58025A/IR32307-10-3-2-1	98.30	97.07	90.9	95.80	45.90
IR58025A/IR36	93.70	87.30	93.3	98.20	49.30
IR58025A/IR64	84.30	87.20	89.7	94.60	38.70
IR58025A/Kogoni	93.00	87.20	96.2	91.10	36.20
IR58025A/NERICA-L19	86.90	85.70	101.7	106.60	52.70
IR58025A/NERICA-L19	94.60	91.80	98.8	90.70	51.80
IR58025A/NERICA-S-44	95.62	92.33	94.9	90.80	55.20
IR58025A/Sahel 108	86.40	86.90	88.9	93.80	53.90
IR58025A/Sahel 134	89.60	89.80	82.8	87.70	54.80
IR58025A/Sahel 159	90.00	85.90	97.9	102.80	54.90
IR58025A/Sahel 328	87.80	87.00	90.0	94.90	51.00
IR58025A/Sahel 329	97.10	96.10	99.1	104.00	47.10
IR58025A/WAS127-12-1-2-1	91.60	86.16	100.5	105.40	36.00
Sahel 134 (CK)	98.50	97.33	86.5	94.51	39.90
$LSD_{0.05}$	3.2	2.9	9.4	8.6	5.6
Coefficient of variation (%)	6.8	3.4	8.8	7.7	11.1

Table 3. Efficiency of microsatellite (SSR) markers for selection of fertility restoration genes (*Rf* alleles).

Rf allele	SSR marker	Lines with positive allele	Percentage of lines with positive allele (%)	Selection accuracy (%)
Rf3	RM443	123	41.0	53.2
	RM315	136	45.3	66.2
	RM443 + RM315	90	30.0	83.2
Rf4	RM171	110	36.7	59.0
	RM258	95	31.7	65.0
	RM171 + RM258	65	21.7	65.4
Rf3+*Rf4*	RM443 + RM315 + RM171 + RM258	45	15.0	95.5

and Sahel 329 had the highest pollen fertility (97.39%, 98.30% and 97.10%, respectively) and spikelet fertility (95.10%, 97.07% and 96.10%, respectively). The testcross IR58025A/Sahel 134 was the earliest for days to 50% flowering (82.8 d) and the shortest for plant height (87.70 cm), while IR58025A/ARH43-2-1-1-2 showed the highest grain yield per plant with CMS line (73.70 g).

DISCUSSION

In this study, SSR markers RM258, RM171, RM315 and RM443 linked to fertility restoration genes (*Rf3* and *Rf4*) were successfully used for identification of potential restorer lines for WA-CMS system, thus avoiding routine testcrossing. Hybrid breeding based on CMS/*Rf* system achieved great success worldwide (Cao and Zhan, 2014). At least 90% of the rice hybrids use the wild abortive cytoplasmic source (Yao et al, 1997). In this study, combination of the two markers for *Rf3* and *Rf4* showed the maximum efficiency of 95.5% in identification of restorers. Sheeba et al (2009) reported that the selection accuracy in a set of 21 restorer lines with RM6100 from *Rf4* was 94.4%. More than 20 000 (physically mapped) SSR markers for rice are now available and being used for constructing genetic maps (http://www.gramene.org). Sattari et al (2007) and Bazarkar et al (2008) used the sequence tagged sites (STS) RG140/PvuII and S10019/BstUI for MAS for fertility restoration genes *Rf3* on chromosome 1 and *Rf4* on chromosome 10 in rice. These advances in molecular marker technology will assist screening a large number of cultivars and breeding lines with a short period and avoiding testcrossing, thus saving resources and time.

Complete sterility has not been noticed so far in testcrosses with a CMS, which indicates the difficulty to develop new CMS lines using African cultivars. Some hybrid offsprings derived from NERICA (New Rice for Africa) cultivars and CMS lines have sterility but instability of sterility in backcrossing generations (BC_1 and BC_2) leads to stopping their use in developing new CMS sources. At the same time, many released and improved rice cultivars (Sahel 108, Sahel 134, Sahel 159, Sahel 328, Sahel 328, NERICA-L19 and NERICA-S-19) showed high fertility with the CMS lines. Some breeding line (ARH43-2-1-1-2) and rice varieties (AD9246 and Giza 182) showed high grain yield per plant with CMS lines. These adapted cultivars with high yield can be used as restorer to develop hybrid with higher adaptability in West Africa.

SUPPLEMENTAL DATA

The following material is available in the online version of this article at http://www.sciencedirect.com/science/journal/16726308; http://www.ricescience.org.

Supplemental Table 1. Rice cultivars or breeding lines used in this study.

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