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RESEARCH ARTICLE

SELECTIVE GENOTYPING FOR DETERMINING THE LINKAGE BETWEEN SSR MARKERS AND A FERTILITY RESTORATION LOCUS IN *SORGHUM BICOLOR* (L.) MOENCH

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ABSTRACT

Cytoplasmic male sterility (CMS) is a genetic condition in which the male sterile plants are not able to set seeds on their own. Both cytoplasmic and nuclear genes interact to control male sterility. Restorer of fertility (*Rf*) genes restore male fertility on CMS plants. In the present study, we genetically mapped a fertility restoration locus using selective genotyping approach in a F_2 population of 296A x PVK809 cross. The *Rf* locus was identified near to the SSR marker Xtxp304 on chromosome 2. Sorghum gene, Sobic.002G057000, a member of Pentatricopeptide (PPR) protein family was putatively identified as the candidate for fertility restoration in this cross.

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INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) is an important dry-land cereal crop cultivated globally over 40 m ha. It is the fifth major cereal crop of the world after wheat, rice, maize and barley in terms of production and utilization. The current annual production of sorghum in India is 5.28 m t (FAO data -2013).
Hybrid sorghum with their significant superiority over varieties and land races for yield and adaptation has boosted sorghum production and profitability in India. Sorghum F_1 hybrid seed in India or elsewhere is mass-produced exploiting the well characterized cytoplasmic male sterility (CMS) system. Fertility restoration by dominant nuclear gene(s) is essential to mass-produce F_1 hybrid seed as it is produced on a male sterile seed parent. The identification and development of genetically diverse lines that restore fertility on CMS lines is time consuming as it involves testing for fertility restorer genes through conventional test crossing methods and progeny evaluation. Location of fertility restoration gene(s) on linkage maps and the availability of its tightly linked PCR-based DNA markers would be of considerable advantage in sorghum marker-assisted fertility restorer (R) line development.

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This would permit the classification of breeding or germplasm lines as either maintainer (B) or fertility restorer (R) without the need to go for test cross analysis. Identification of fertility restoration locus linked with DNA markers will eventually provide the foundation for map-based restorer of fertility gene isolation and its functional and comparative analysis with other cereal crops.

In this background, we undertook this study to identify the fertility restoration locus/ gene(s) involved along with its tightly linked markers in a sorghum cross.

MATERIALS AND METHODS

Plant Material

The experimental material comprised of a male sterile line 296A and a restorer line PVK809. 296A is one of the best CMS line and is the seed parent of several national and private sector rainy-season sorghum hybrids. PVK809 was a rainy-season adapted genotype. The F_1 plants of 296A x PVK809 were grown during rainy season of 2012 and were selfed using paper bags to get selfed F_2 generation seed. F_2 population was raised during post-rainy season of 2012-13 at the experimental farm of the ICAR-IIMR, Hyderabad, Telangana, India. Plants in F_2 generation were grown as a single plant per hill with a spacing

of 15 cm between plants and 60 cm between rows. Recommended agronomic practices were followed to raise good crop. Each F_2 plant was labeled and before flowering at about the boot leaf stage when the panicle emerges, panicles were covered with a paper bag to allow self-pollination and to avoid cross-pollination.

Phenotypic data collection

Around 20-25 days after flowering (around physiological maturity), each F_2 panicle was observed visually for seed set under selfing. The F_2 plants were classified as male fertile (full-seed set) or male-sterile (< 10 seeds per panicle) as suggested (Klein *et al*. 2001). The goodness of fit between the observed and expected segregation for fertility: sterility in F_2 population was tested using chi-square (χ^2) test to determine the number of genes controlling fertility restoration.

DNA extraction and PCR

Each F_2 plant was individually labeled and genomic DNA extracted from the leaf tissue using CTAB extraction method (Murray and Thompson 1980). The quality of the DNA was checked by 1% agarose gel electrophoresis and concentrations were normalized at 25-30 ng/µl. Veriti PCR system (M/s Applied Biosystem, USA) was used for amplification with following buffer composition and thermal profile: about 2 µl (i.e. 50-100 ng) of template DNA was added to the walls of well in a PCR plate. The master mix consisted of 2 µm forward primer, 2 µm reverse primer, 125 µm dNTP's (M/s Bangalore Genei Pvt. Ltd.), 1U *Taq* DNA polymerase (M/s Bangalore Genei Pvt. Ltd.), 1x PCR buffer (Tris with 1.5 mM $MgCl_2$) and the total volume was made up to 10 µl using sterile distilled water. Then, the master mix (8.0 µl) was dispensed to the PCR plate containing 2 µl template DNA. The thermal profile followed was: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation for 1 min at 94°C, primer annealing for 30 sec, primer extension for 30 sec at 72°C, and final extension at 72°C for 10 min.

Selective genotyping

Selective genotyping is a method by which linkage between the DNA marker locus and a phenotypic trait value is established. In this method, genotyping of the individuals from high and low phenotypic tails of the entire population is carried out (Darvasi *et al*., 1992). In order to locate *Rf* locus that restored fertility in F_1 hybrid of the cross 296A x PVK809, 20 Simple Sequence Repeat (SSR) markers linked with the reported sorghum *Rf* loci (*Rf1, Rf2, Rf5, Rf6*) (Jordan *et al*., 2010, 2011; Klein *et al*., 2001, 2005; Praveen *et al*., 2015) were tested for polymorphism between parental lines. Reported sorghum *Rf* loci and the details of linked markers are given in Table 1. Polymorphic makers were used for selective genotyping using the phenotypic extremes. DNA from ten sterile and ten fertile plants along with two parents (296A and PVK809) were amplified with polymorphic markers. PCR amplified products were loaded in 4% Agarose gel for high resolving products and 6% PAGE low resolving products. The data was analysed for linkage between the genetic marker with *Rf* gene locus.

RESULTS

Segregation in F_2 Population

F_1 hybrid plant was fully fertile under bagging which suggested that fertility restoration is dominant over sterility. Fertile/sterile data recorded in F_2 population based on seed set data under bagging segregated in 113 fertile and 31 sterile plants that was well fitted with 3:1 ratio ($\chi^{22}{}_{(3:1)} = 0.926$; P-value= 0.336). This clearly showed the control of single gene in restoring fertility in the population.

Markers linked to *Rf* locus

Of the several reported *Rf* loci linked markers tested for polymorphism between the 296A and PVK809 parents, SSR markers Xtxp616, Xtxp304 on chromosome 2, Xnhsbm1195 and Xnhsbm1197 on chromosome 4, Xnhsbm1083 on chromosome 5, Xtxp406 on chromosome 8 were polymorphic.

Table 1. Details of *Rf* loci linked SSR markers used in present study

Gene	CMS	LG	Marker	Poly*	Physical position (bp)	Forward primer	Reverse primer	Reference
Rf1	A_1	8	Xtxp18	M	50413700	ACTGTCTAGAACAAGCTGCG	TTGCTCTAGCTAGGCATTTC	Klein *et al*. 2001, 2005
			Xtxp400	M	50656630	CTAAGAACCGACGCGTGTATAGT	GCATCTATCTTCACTCCGATTCT	
			Xtxp406	P	50689478	GGCCTGAATCTCAGTGTTAAG	AGTTGCCTGCTTCGACACTT	
Rf2	A_1	2	Xtxp84	M	4850858	CCGATCAGCACACCAG	GTACTAGGTCCAATCCAGC	Jordan *et al*. 2010
			Xtxp211	M	4991669	TCAACGGCCAATGATTTCTAAC	AGGTTGCGAATAAAAGGTAATGTG	
			Xtxp50	M	5076938	TGATGTTGTTACCCTTCTGG	AGCCTATGTATGTGTTCGTCC	
			Xtxp616	P	5664736	GCATTTCTTTCCTGCAATGAC	GCAGACAAGATCTCACCCAAG	
			Xtxp304	P	5700017	ACATAAAAGCCCCTCTTC	CTTTCACACCCTTTATTCA	
Rf5	A_1, A_2	5	Xnhsbm1082	M	1907404	CACGTCGTCACCAACCAAC	GTTAAACGAAAGGGAAATGGC	Jordan *et al*. 2011
			Xnhsbm1083	P	2430209	TGACTGGTCAACAACGAGGA	CTCTCCCGTGCATGTACTCA	
			Xnhsbm1084	P	2659490	CATTTCACATTCAAGGTCATGG	ACATTTATGGGTGCGTGCTT	
			Xnhsbm1085	M	3313831	CGTGAATGAATGAACGAACG	GAGAGCAGAGGGGTAACTGC	
Rf6	A_1, A_2	4	Xnhsbm1142	M	351479	CATCCATATCCATGGCAACA	AATGAAGGTGGAGAGGACGA	Praveen *et al*. 2015
			SB2385	M	354575	ATCCTCTTCCTCCTCCTCCACCT	TGGTTTGGCTTGTTTACATGTTGC	
			SB2386	P	358934	GGCGGTAGGTGTAAAAAGGAAGGA	GCATGCCCTACGACTCTTGTGTCT	
			SB2387	M	375093	AAGTTTTGTCACCCGTGCAGATTT	AAGGTCAGTAGCTCGCATGATTCC	
			SB2388	M	418039	AATTATGGATGCATGGAGCAAAGC	GATGGAGGATCGAGTCACCAAAAC	
			Xnhsbm1157	M	559821	GCTCCCAAATCTCGATAACAA	ACTGGTTTAGGCGGATCCTT	
			Xnhsbm1195	P	2171353	CTAAAGGAACTCGGCGATTG	GTCGTGTCCTTCGGCATTAT	
			Xnhsbm1197	P	2195125	CTGCAGAGGTCCTAGTGACAAA	GAACGACTTATAATTTGAGCCAGA	

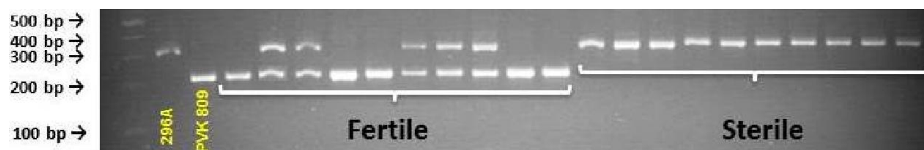

Fig1. Selective genotyping of 10 fertile and 10 sterile F_2 plants using SSR marker, Xtxp304

Hence these polymorphic markers were further used for selective genotyping. Selective genotyping analysis with extreme individuals (fully fertile and fully sterile) revealed that out of five polymorphic markers tested, markers Xtxp616, Xtxp304 co-segregated with fertility/sterility behavior of the F_2 plants. All sterile plants showed PCR amplicon band same as that of the CMS parent 296A while all the ten fertile plants exhibited amplicon band that of the parent PVK809 (Fig.1). This clearly indicated that these markers (Xtxp616 and Xtxp304) and fertility restoration locus are tightly linked i.e. they are present close-by on sorghum chromosome 2.

DISCUSSION

Understanding the genetic mechanism of male sterility/fertility restoration of CMS systems and locating its position on linkage map along with identifying linked markers is very important in sorghum breeding as it enhances the efficiency of selection for good and diversified restorer/maintainer parents to develop superior hybrids. Restoration of fertility in F_1 and pattern of segregation for fertility/sterility in F_2 generation of the present cross indicated that the restoration of fertility is a dominant trait, and is under the control of single gene. This observation is in agreement with the earlier reports of monogenic control of fertility restoration in sorghum (Murthy *et al*., 1990; Sanjana *et al*., 2010; Brown *et al*., 1996). Pentatricopeptide repeat (PPR) genes, through their RNA editing ability are known to be involved in fertility restoration in sorghum *Rf1* (Klein *et al*., 2001,2005), *Rf2* (Jordan *et al*., 2010), *Rf5* (Jordan *et al*., 2011), *Rf6* (Praveen *et al*. 2015) and in other crops (Chen *et al*., 2014). Interestingly, a PPR gene (Sobic.002G057000) was found to co-exist very near to the Xtxp616, Xtxp304 markers loci and was similar to the rice *Rf1* locus (LOC_Os10g35240.2). This locus was also identified to act as a restorer of fertility in a different sorghum genetic cross (Jordan *et al*. 2010). Therefore, the gene Sobic.002G057000 could be the candidate gene involved in fertility restoration in the cross 296A x PVK809 of the present study.

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