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Research Article

Molecular markers linked to fertility restorer gene in A_2 cytoplasm male sterility of pigeon pea [*Cajanus cajan*]

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Abstract
In pigeonpea the cytoplasmic-genetic male sterility (CMS) system is considered to be a feasible approach for the development of hybrids. Identification of CMS lines and their putative restorers using molecular markers in the early stage of growth is an important and economical in long duration pigeonpea. Based on phenotyping, the 120 plants of F_2 designated as fertile and sterile. Both visual and pollen staining phenotyping unequivocally exhibited 85 fertile: 35 sterile plants in F_2 population. The segregation of F_2 for fertility restoration of A_2 cytoplasm indicated 3F:1S segregation indicating dominant monogenic control of fertility restoration. Bulk segregant analysis revealed that 10 of the 139 polymorphic decamer RAPD markers produced precisely distinct bands subjective to fertility restorer DNA bulk while, none of the SSR was found polymorphic between bulks. Two of them *viz*; OPC 7_{617} (GTC CCG ACG A) and OPK 3_{860} (CCA GCT TAG G) evinced clear and unambiguous bands for fertility restorations in F_2 population at 0.5 and 1.0 cM, respectively. They could be exploited as a precise selection tool for seminal but stubborn character like restoration of fertility in male sterile lines that has germane significance in otherwise immensely important heterosis breeding in pigeonpea.

Key Words
C. cajan, C. scarabaeoides, CGMS system, RAPD, SSR.

Introduction

Pigeonpea is an important grain legume crop of the semi-arid tropics and is widely cultivated in the Indian subcontinent, Southeast Asia, Africa and Central America. The highest production of pigeonpea occurs in India, where it is the most widely grown legume after chickpea. The production and productivity of this crop has remained static over the years and the research efforts for enhancing the productivity through pure line breeding have not been very successful. Unlike other legumes, pigeonpea is an often cross pollinated crop exhibiting 25-70% natural out crossing across locations (Saxena *et al*., 2005); efforts were made for heterosis breeding by exploiting genetic male sterility. However, the system could not be exploited due to inherent problems like labour intensiveness, huge rouging and genetic purity associated with genetic male sterility system. These problems were overcome with the development of cytoplasmic genetic male sterility system using cytoplasm of wild species (Ariyanayagam *et al.,* 1995; Tikka *et al*., 1997) and lately concerted efforts have been made to develop cytoplasmic genic male sterility (CGMS) based commercial hybrids in pigeonpea (Saxena *et al*., 2003; Acharya *et al*., 2005). The cytoplasmic genetic male sterility based hybrid programme involves three lines *viz*; male sterile (A), maintainer (B) and restorer (R).

Molecular markers are reliable diagnostic tools for various plant breeding applications and it allows analyzing thousands of genotypes during a breeding season rapidly and effectively. Molecular maker techniques provide powerful tools to identify and map the target genes. In any heterosis breeding programme selection of fertility restorer genes is a very tedious task. Molecular markers tightly linked to fertility restoration (Fr) loci have tremendous application in crop improvement. Such tightly linked molecular markers to fertility restorer genes have been identified in several crops like sorghum (Jordan *et al*.. 2010), rice (Sattari *et al*., 2008), brassica (Li *et al*., 2011), wheat (Prakash et al., 2012) and pigeonpea (Sheikh *et al*., 2011 & Bhora *et al*., 2012). However, so far similar linkage analyses have not been reported in pigeonpea because of lower level of polymorphism among the cultivated genotypes and absence of ample amount of genomic tools such as mapping populations, molecular markers and linkage maps. Marker assisted selection (MAS) can improve the breeding process by selecting molecular markers linked with gene controlling the target traits. Molecular breeding seems to be the next step for genetic improvement in pigeonpea. Pigeonpea being a long duration crop, molecular markers can be very useful for precise characterization and identification of parental lines as they can be detected at juvenile stage independent of environmental effects. The objective of present study was to identify SSRs and RAPD markers

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linked to fertility restorer gene among a male sterile line GT 304A and a fertility restorer line GTR 41 as parents that were crossed to develop F_1, F_2, BC_1 and BC_2 populations of pigeonpea.

Materials and Methods

Plant materials: The *Cajanus scarabaeoides* cytoplasmic background male sterile line GT 304A was crossed with restorer line GTR 41 to develop F_1 and subsequently derived its F_2 population under strict selfing in insect proof net house condition. BC_1 and BC_2 populations were also developed by dusting pollen of F_1 on both male sterile (GT 304A) and fertility restorer line (GTR 41). This population were planted during 2011 and 2012 at the Centre of Excellence for Research on Pulses, S.D. Agricultural University, Sardarkrushinagar, Gujarat, India.

Fertility Scoring and Sorting: Pollen fertility and the seed-setting rate were used as the main criteria for the evaluation of fertile and sterile plants. The test for pollen grains fertility and sterility was done as per Iodine + potassium Iodide stain method proposed by Singh *et al*. (2009). Fresh and opened flowers and buds were collected from each parent and F_2 plants in moist Petri-dishes and brought to the laboratory. The anther was pierced using sterilized needle on slide and one drop of 1% I_2-KI stain was added. The slides were then observed under 10X lens and photographs were captured. The darkly stain pollen depicted fertile pollens while the unstained ones evinced sterile pollens.

DNA extraction and preparation of fertile and sterile bulks: Genomic DNA was isolated from juvenile fresh leaves from parents, F_1 and each individual plant of F_2 following CTAB (Cetyl Trimethyl Ammonium Bromide) extraction method given by Sheikh *et al*. (2011). Genomic DNA from 10 fertile and 10 sterile individuals was pooled to make up the fertile and sterile DNA bulks, respectively. Bulks were also constructed from BCF_1 populations, and the resulting DNA concentration of the bulks and the parents was adjusted to 10 ng/µl.

Analysis of random amplification of polymorphic DNA (RAPDs) and microsatellite markers (SSRs): A total of 160 available decamer random oligonucleotide primers were used to ascertain polymorphism among GT 304A and GTR 41 lines, their hybrid and individual plants of F_2 as also to find out the fertility restoration gene specific marker. RAPD amplification was performed as adopted by Sheikh *et al*. (2011) and was carried out in 25 µl of reaction mix. PCR products were then subjected to electrophoresis with marker DNA of known molecular weight in 1.5 per cent agarose gel in 1X TBE buffer. PCRs for 50 microsatellite primers analysis followed the procedure described by Odeny *et al.* (2009). The amplification products were separated by electrophoresis on a 4.0% (w/v) agarose gel containing ethidium bromide in 1X TBE buffer.

Genetic linkage analysis: Segregation data obtained for polymorphic RAPD and SSR markers on the F_2 populations were used for determining the recombination and genetic distance using MAPMAKER/EXP 3.0 (Lander *et al*., 1987). Prior to linkage analysis, marker segregation data were subjected to the goodness-of-fit test (x^2) to assess deviations from the expected Mendelian segregation ratio of 3:1 at a 1% level of significance. A Kosambi map function was used to convert the recombination frequency into cM distances.

Results and Discussion

Segregation for male fertility: The study involved male sterile line (GT 304 A), fertility restorer line (GTR 41), F_1, F_2, BC_1 and BC_2 populations. Both visual and pollen staining phenotyping unequivocally exhibited 85 fertile: 35 sterile plants in F_2 population of GT 304A × GTR 41. The male sterile plants exhibited consummated sterility, while restorer line GTR 41, F_1 and BC_2 evinced 98, 86 and 99 per cent pollen fertility, respectively. Pollen fertility among 85 fertile plants in F_2 varied from 85 to 98 percent. The segregation of F_2 for fertility restoration of A_2 cytoplasm indicated 3F:1S segregation indicating dominant monogenic control of fertility restoration (x^2=1.11, P=0.28). The dominant monogenic control was corroborated by BC_1 too that segregated in the ratio of 1F:1S (x^2=2.80, P=0.09).

The segregation of F_2 population plants of cytoplasmic male sterile line and fertility restorer line segregated for pollen fertility revealed that there were 85 fertile plants in comparison to 35 sterile plants indicating dominant monogenic control of fertility restoration in A_2 cytoplasm of pigeonpea, respectively. Ours results are also supported by Kumar *et al*., (2004) and Cherian *et al*. (2006).

Identification and linkage of marker to fertility restorer gene: The fertile and sterile parents were compared to screen SSRs and RAPD markers. Of the 160 RAPD markers, 139 were polymorphic while 15 were monomorphic. They amplified 1402 bands out of which 1086 were polymorphic. This evinced on an average 7.81 polymorphic bands/primer; though the average number of amplified bands per primer were 8.76. SSR primers did show amplification but were monomorphic amplifying 50 alleles with an average of 1.66 alleles per locus. No SSR marker was found informative for fertility restoration gene. The plateau in productivity of pigeonpea has been a world concern for the last two decades and different modes to defy this mire have been

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contemplated (Acharya *et al*., 2005). Empirically microsatellite or SSR markers are ideal markers for genetic analysis and customized application for marker aided selection in crop improvement as they are highly polymorphic, reproducible, co-dominant in nature and distributed throughout the genome. However, in present study none of the 50 SSR primers was specifically informative to fertility restoration /sterility in pigeonpea. These results are not in consonance to the findings of Burns *et al*.(2001), Odeny *et al*. (2007) and Bhora *et al*. (2012) who have reported SSR markers to be more polymorphic in pigeonpea. Thus, it seems more concerted efforts are needed to develop a novel set of customized SSR markers which could be specifically linked to fertility restoration gene/genes in pigeonpea.

Further, bulk of both fertile and sterile plants of F_2, BC_1 and BC_2 population revealed that 10 of the 139 polymorphic decamer RAPD markers *viz*; OPC 7_{617}, OPK 14_{648}, OPH 5_{650}, OPA 10_{675}, OPI 16_{661}, OPK 3_{860}, OPH 4_{1062}, OPC 1_{1324}, OPP 16_{2150}, and OPA 14_{2300} produced precisely distinct bands subjective to fertility restorer DNA bulk. The number of amplified loci varied from two to twelve with a band size varying from 617 bp to 2300 bp. The polymorphism information content (PIC) value ranged from 0.46 in OPK 3 and OPA 10 to 0.89 in OPA 14 and OPC 7 (Table1).

Ten precisely distinct RAPD markers instinctively peculiar to fertility restoration gene pool were subjected to linkage analysis entailing all the 120 F_2 plants. Despite their distinctiveness, only two markers viz; OPC 7_{617} (GTC CCG ACG A) and OPK 3_{860} (CCA GCT TAG G) evinced clear and unambiguous bands for fertility restorations in F_2 population and the chi square value also shows no segregation distortion for these two markers at P = 0.01 from the expected ratios 3:1 (A: C or B: D) for the dominant markers (Figure 1 & 2). This indicated that out of 10 RAPD markers, two markers (OPC 7 and OPK 3) exhibited dominant inheritance with 3:1 segregation ratio as expected theoretically. Two informative markers OPC 7_{617} and OPK 3_{860} were located 0.5cM and 1.0 cM away from the fertility restoration locus. The result unequivocally indicated that OPC 7_{617} (0.5cM) and OPK 3_{860} (1.0 cM) were tightly linked to fertility restoration gene and could be exploited as a precise and reliable selection tool as per marker aided selection (MAS) for fertility restoration of A_2 cytoplasm based male sterility in pigeonpea. The cytoplasmic male sterile lines GT 304A and fertility restorer line GTR 41 were screened for ascertaining polymorphic markers using RAPD and SSR markers. A total of 160 RAPD and 50 SSR markers were used to detect polymorphism. The present set of RAPD primers exhibited relatively higher level of polymorphism than RAPD studies conducted earlier in pigeonpea (Choudhary *et al*., 2008a and Mishra *et al*., 2013). This could be ascribed to very diverse materials used for the present study that enabled the hence to otherwise reported non polymorphism of RAPD markers in pigeonpea as enormous polymorphic.

The marker is useful only when it is tightly linked to the gene of interest (Wang *et al*.2010). In pigeonpea, efforts have been made to distinguish male parents from the CMS lines using RFLP markers (Sivaramakrishnan *et al*., 1997). Souframanien *et al*. (2003) reported a set of RAPD markers that could distinguish each component of the CGMS system. Choudhary *et al*. (2008b) also reported a set of primers to distinguish the CMS system derived from *C. scarabaeoides* (GT 288A/B) and *C. sericus* (67A/B). In consonance to their findings, present study also indicated the pertinence of RAPD primers in discerning polymorphism up to 80 per cent in pigeonpea.

The significance of molecular marker linked to fertility restoration loci cannot be underscored for tracking the gene of interest during the course of breeding and selection for fertility restoration in a crop like pigeonpea whose productivity has been pathetically static since long (Agarwal *et al*. 2007). The present study ubiquitously revealed that OPC 7_{617} was tightly linked (0.5cM) to fertility restoration gene in F_2 segregating population and could be used as a precise and reliable selection tool as per marker aided selection (MAS) for fertility restoration of A_2 cytoplasm based male sterility in pigeonpea. These results are in consonance to the findings of Choudhary *et al*. (2008b) who have also reported two RAPD primer (OPAZ 01 1900&2100 and OPBA 1 3900&1000) linked exclusively in sterile line and six RAPD primers (OPBA 1_{9280}, OPAZ 04_{3000}, OPAZ 04_{2000}, OPA 10_{500}, OPA 2_{500}, OPH 19_{1200}, OPAQ 4_{3000}) linked to restorer lines of pigeonpea. The results are also in congruence to the findings of Laporte *et al*. (1998) in sugar beet wherein they reported RAPD markers linked to male fertility gene at 5.6cM. Wang et al., (2006) applied RAPD technique along with BSA in radish and found that a RAPD primer OPC 6_{1900} was 0.8cM away from *Rf* locus. Chandrashekar *et al*. (2013) has used RAPD analysis successfully to validate marker linked to fertility restorer gene in CGMS lines of *Brassica juncea*. Kumar *et al*. (2004) reported two RAPD primers *viz;* OPP 13_{1397} and OPW 19_{800} associated with fertility restoration genes (*Rf*) in pepper while Zhang et al., (2004) ascertained two RAPD makers (UBC 1113000 and UBC 188500) associated with fertility restoration genes in D8 CMS lines of cotton.

Significant progress has been made in cereals in constructing genetic maps besides ascertaining character specific molecular markers (Varshney *et al*., 2005). Such molecular and genomic inputs are

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prerequisites for planning and executing trait mapping and molecular breeding in any crop species. However, consequent upon narrow genetic base as also paucity of molecular markers and mapping populations (Varshney *et al*., 2010), they have cited limited availability of molecular markers and non-availability of the linkage map as the major reasons for slow progress in the molecular breeding of the otherwise important legume crop pigeonpea. Therefore, the present study has humungous practical utility in initiating marker aided selection for otherwise stubborn characters like restoration of fertility in male sterile lines having A_2 cytoplasm background.

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Table 1. RAPDs markers that putatively amplified the fertility restoration locus in the F_2 population of a cross between a male sterile line GT 304A and fertility restorer line GTR 41 in pigeonpea.

Sl. No.	Sequence (5'-3')	Size of informative band (bp)	PIC value
1.	GTC CCG ACG A	617	0.89
2.	CCC GCT ACA C	648	0.85
3.	AGT CGT CCC C	650	0.45
4.	TCT CCG CCC T	661	0.71
5.	GTG ATC GCA G	675	0.46
6.	CCA GCT TAG G	860	0.46
7.	GGA AGT CGC C	1062	0.60
8.	TTC GAG CCA G	1324	0.59
9.	CCA AGC TGC C	2150	0.76
10.	TCT GTG CTG G	2300	0.89

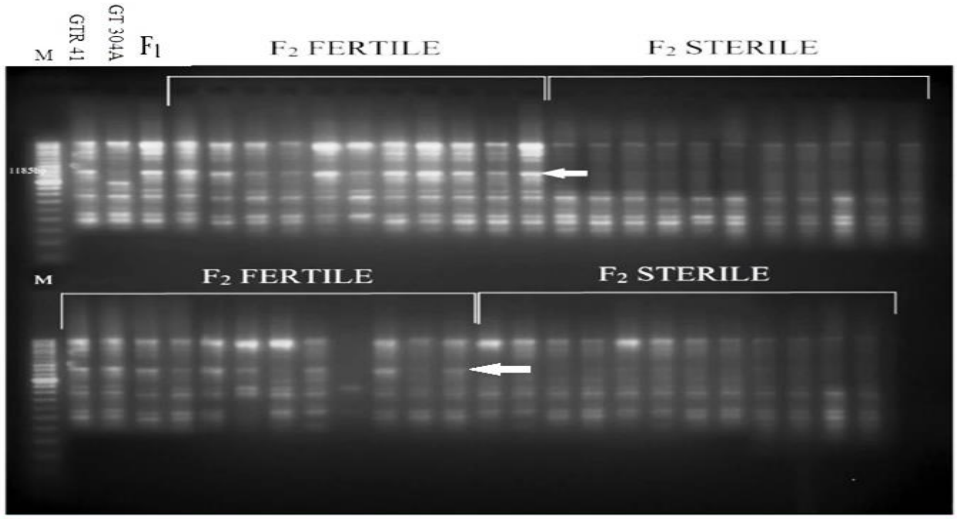

Fig.1 : Fertility restoration locus specific RAPD profile captured with primer $OPC7_{617}$.

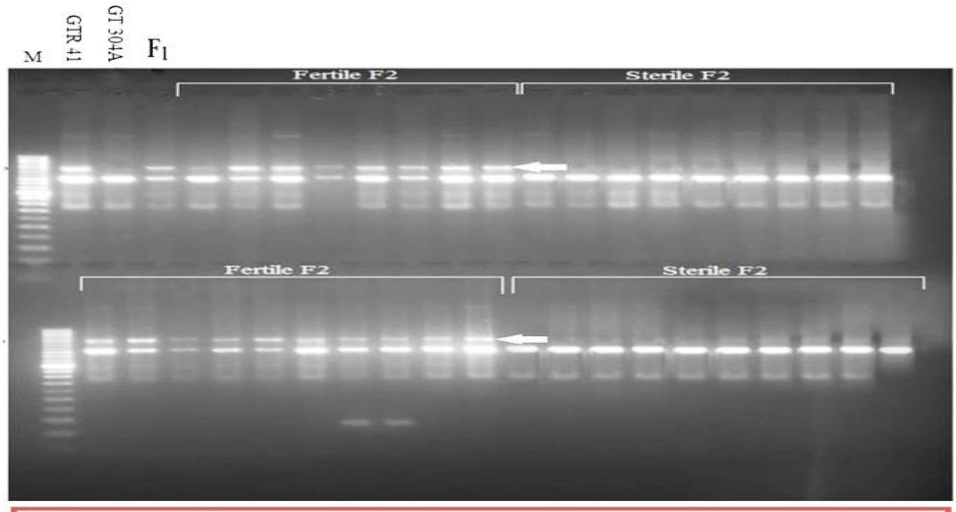

Fig.2 : Fertility restoration Locus Specific RAPD profile captured with primer OPK_{860}.