Identification of AFLP markers linked to sterility mosaic disease in pigeonpea *Cajanus cajan* (L.) Millsp.

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**Abstract**
Sterility mosaic is an important disease of pigeonpea causing substantial loss in yield. In the present study bulk segregant analysis (BSA) was employed to identify simple sequence repeats (SSR) and amplified fragment length polymorphism (AFLP) markers associated with sterility mosaic disease (SMD) resistance in the F2 population of the cross involving TTB 7 (susceptible) and BRG 3 (resistant) parents. A total of 156 SSRs and 16 AFLP primer pairs were surveyed for identification of polymorphic markers between the parents and DNA bulks of resistant and susceptible F2 individuals. Out of 10 polymorphic SSRs between the parents, none of them were polymorphic between the DNA bulks. From 13 polymorphic AFLP primer combinations between the parents, two AFLP primer pairs generated 4 markers (E-CAA/M-GTG150, E-CAA/M-GTG60, E-CAG/M-GCC120 and E-CAG/M-GCC150) which were polymorphic between the resistant and susceptible bulks indicating that these markers are linked to SMD and located at a map distance of 5.7, 4.8, 5.2 and 20.7 cM respectively. The markers E-CAA/M-GTG150 and E-CAA/M-GTG60 were linked in coupling phase to the susceptible dominant allele amplifying only in susceptible individuals which, can be effectively used for marker assisted selection (MAS).

**Keywords:** pigeonpea - sterility mosaic disease - AFLP - linkage - marker assisted selection.

**INTRODUCTION**
Pigeonpea is one of the major grain legume crops of the tropics and subtropics. The Indian sub continent accounts for about 90% of the global production and the diseases of pigeonpea of economic importance are fusarium wilt, sterility mosaic disease and phytophthora blight. Among these diseases, sterility mosaic disease (SMD) is considered to be the most important disease (Kannaiyan *et al.*, 1984) and at times can cause yield loss upto 95 per cent. The disease is caused by pigeonpea sterility mosaic virus (PPSMV) and transmitted by eriophyid mite (*Aceria cajani* Channabasavanna) (Kumar *et al.*, 2003). Control of the disease by chemical method though effective but economically not feasible and non eco-friendly (Nene *et al.*, 1989). Breeding for resistant varieties is considered to be one of the most effective and economic methods of reducing crop losses and has received top priority. Despite the great advances in genomic technology observed in several crop species, the availability of molecular tools such as microsatellite markers is limited in pigeonpea and till date no molecular map is available. Very few reports pertaining to identification of trait specific markers are available in pigeonpea. Kotresh identified two RAPD markers (704 bp and 500 bp linked with fusarium wilt susceptibility) using F2 populations derived from contrasting parents GS l (susceptible) (Kotresh *et al.*, 2006), ICPL 87119 (resistant) and ICP 8863 (resistant). Choudhury screened 108 RAPD markers to identify cytoplasmic male sterile (CMS) lines derived from crosses between wild (*Cajanus scarabaeoides* and *C. sericeus*) and cultivated pigeonpea (Choudhury *et al.*, 2008). Identification of molecular markers linked to sterility mosaic disease allows screening of cultivars and segregating generations at seedling stage and reduce the need for maintaining virulent isolates of the pathogen and subsequently use in MAS. Realizing the importance of such an investigation, the present study was carried out to identify SSR and AFLP markers linked to SMD in pigeonpea.
MATERIALS AND METHODS

Plant materials

The material for the study comprised of two diverse genotypes selected based on previous reports for their resistance levels. The resistant parent BRG 3 (AICRP on pigeonpea, Bangalore, India) differed from the susceptible parent TTB 7 (Karnataka, India) with respect to high level of intrinsic resistance to SMD. The genotypes were screened against SMD following “Leaf stapling technique” during rainy season, 2006 to confirm their resistance levels. The resistant parent BRG 3 was crossed with the susceptible parent TTB 7 to generate F2 population consisting of 121 plants for phenotyping against SMD and genotyping using linked AFLP markers.

Phenotyping for SMD resistance

F2 seeds of TTB 7 x BRG 3 along with their parents were raised in polybags for SMD screening following “Leaf stapling technique” during rainy season, 2007. SMD infected leaves containing mites were stapled to leaves of test plants at 3-4 leaf stage. As the stapled leaflets from the infected plants gets dried, mites from the infected leaves migrate to healthy leaf and inoculates the virus. Test plants were scored for incidence of SMD at 15 days interval up to 75 days. Any plant will be considered as ‘susceptible’ if it shows mosaic symptoms while, the other will be considered as ‘resistant’. The goodness of fit to mendelian segregation of resistant and susceptible plants in the segregating population was tested by chi-square test. The significance of Chi-square was tested against the table value with (n-1) degrees of freedom, where ‘n’ is the total number of segregating classes (Stansfield, 1986).

Collection of plant tissue and DNA extraction

Before stapling the infected leaves to test plants, all the plants were labelled and leaf samples were collected from all the F2 plants and their parents and stored at -20°C for DNA extraction. Two to three young leaves collected from 15 - 20 day old seedlings of parents and 121 F2 plants were used for DNA extraction. The CTAB method of genomic DNA extraction was followed with minor modifications. The quality and quantity of DNA was determined through agarose gel electrophoresis using 0.8 % agarose gel.

SSR analysis

A total of 156 pigeonpea specific SSR primers obtained from Dr. Rajeev K. Varshney, ICRISAT, Hyderabad, India were used to identify polymorphism between the resistant parent BRG 3 and susceptible parent TTB 7. Polymerase chain reaction was performed in a final volume of 10.0 µl containing 2.0 µl template DNA (5 ng / µl), 0.3 µl of 15 mM MgCl2, 3.0 µl of 1mM dNTPs, 0.2 µl of 10 pmol primer mix and 0.2 unit of Taq polymerase and 1.0 µl of 10X Taq buffer. For the majority of SSR markers studied the reaction was processed as followed.

The annealing temperature varied with different primers. Three different touch-down annealing temperatures viz., 55-45, 60-55 and 65-60 were used for PCR amplification. All the SSR primers were initially tested for amplification in agarose (2.0%) and then polyacrylamide gel electrophoresis (6.0 %) was used for detection of polymorphism between the parental genotypes.

AFLP analysis and bulk segregant analysis

The AFLP method was generated using the protocol of Keygene Inc. (Vos et al., 1995). A total of 16 primer combinations selected were used for AFLP analysis. Genomic DNA (250 ng) was digested with 4 units each of Mse I and Eco RI restriction endonucleases at 37°C for 3 hrs, and then 70°C for 15 min. The digested DNA were ligated with Eco RI and Mse I adapters with T4 DNA ligase for 16-18 hrs at 37°C. The ligated DNA was diluted to 1:5 in TE buffer (10mM Tris-HCl (pH 8.0), 0.1 mM EDTA) and stored at -20°C. Polymerase chain reaction (PCR) was performed in two consecutive reactions: a pre-selective and selective PCR. In the pre-selective reaction, genomic DNA was amplified using an AFLP primer pair, each having one selective nucleotide. Accordingly, a 3.0 µl diluted ligation product, 2 µl each of Mse I (7.5 ng / µl) and Eco RI (7.5 ng /µl) pre-selective primer, 2 µl dNTPs (1 mM), 1 µl 10X PCR buffer were mixed in 1.0 unit of Taq polymerase and used for pre-selective reaction in total volume of 10.0 µl. The pre-selective amplification was performed with an amplification profile of 94°C for 30 s, annealing at 56°C for 1 min, extension at 72°C for 1 min, repeated for 20 cycles, and then at 10°C for 30 min. Pre-selective PCR amplification was confirmed by gel electrophoresis and the amplified product was diluted to 1:5 in TE buffer and used as template for the selective amplification using AFLP primers, each containing three selective nucleotides. Selective PCR amplification was performed in 10.0 µl reactions consisting of 3.0 µl pre-selective template DNA, 2.0 µl each of Eco RI (7.5 ng / µl) and Mse I (7.5 ng / µl) selective nucleotide primer, 2.0 µl dNTPs (1 mM), 1.0 µl 10X PCR buffer and 1.0 unit of Taq polymerase. Selective amplification was performed with a cycling profile of 94°C for 30 s, 65°C for 30 s reducing by 0.7°C /cycle to 56°C, 72°C for 1 min for 11 cycles, followed by 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min for 24 cycles followed by 10°C for 30 min. PCR were carried out separately for each primer pair and the products were denatured immediately by adding 8.0 µl.
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of stop loading dye to each sample. The samples were heated to 94°C for 5 min to cause denaturation and then cooled to 10°C for 5 min. Finally, the product was stored at -20°C. Electrophoresis of the samples was carried out on polyacrylamide gels, by loading 3.0 μl of each DNA sample. Electrophoresis was carried out at 1200V for 3.0 hrs until the dark blue dye ran off. The gels were then separated and developed by silver staining technique.

Bulk segregant analysis

Bulked segregant analysis was carried out as described (Michelmore et al., 1991) with the polymorphic SSR and AFLP markers. Resistant and susceptible DNA bulks were prepared from F2 individuals of cross TTB 7 x BRG 3 by pooling aliquots containing equal amount of DNA (0.5µg) from each of the eight resistant and eight susceptible F2 individuals based on their reaction to SMD.

Marker segregation and linkage analysis

The putatively linked AFLP markers from bulk segregant analysis were used for linkage analysis of 121 F2 individuals of cross TTB 7 x BRG 3 segregating for SMD. Chi-square tests ($\chi^2$) were performed to examine the goodness of fit between the expected Mendelian ratio for the segregation data of the four linked AFLP markers. Linkage analysis between putatively linked AFLP markers and the SMD resistance was performed using the MAPMAKER 3.0 program (Lander et al., 1987). The Kosambi mapping function (Kosambi, 1944) was used to convert the recombination frequency into map distances in centimorgans (cM).

RESULTS

Inheritance of SMD resistance

All the F1s of cross TTB 7 x BRG 3 were susceptible to SMD, suggesting susceptibility to be dominant over resistance. The F2 segregating pattern showed 88 susceptible plants and 33 resistant plants fitting to expected monogenic segregation ratio of 3:1 for single dominant gene (data not shown).

Parental polymorphism studies and bulk segregant analysis using SSR and AFLP markers

Out of 156 SSR primers initially screened on the parental lines (BRG 3 and TTB 7), 10 primers recorded reproducible polymorphism between the parents (Table 1 [Supplementary data]). Out of 10 SSR markers that were polymorphic between the parents, none of the SSRs were polymorphic between the resistant and susceptible bulks.

Out of 16 AFLP primer pairs tested, 13 AFLP primer pairs recorded detectable polymorphic bands between the parents. Of the 13 AFLP primer combinations polymorphic between the parents, two AFLP primer combinations (Eco RI -CAA/ Mse I - GTG and Eco RI - CAG / Mse 1 - GCC) generating four (E-CAA/M-GTG60, E-CAA/M-GTG150, E-CAG/M-GCC120 and E-CAG/M-GCC150) markers were found to be polymorphic between the resistant and susceptible F2 bulks. Markers (E-CAA/M-GTG60, E-CAA/M-GTG150) segregation in susceptible phenotype were linked in the cis phase to the SMD susceptible allele (Fig. 1 and Table 2 [Supplementary data]) and were observed mostly in susceptible individuals and not in resistant individuals. Whereas, markers segregating with SMD resistance (E-CAG/M-GCC120 and E-CAG/M-GCC150) were linked in trans phase and were present in both resistant and susceptible individuals (Table 2).

Segregation ratio and linkage analysis

The four linked AFLP markers obtained from bulk segregant analysis were subjected to individual AFLP analysis of 121 F2 individuals to analyse the segregation pattern of the markers (E-CAA/M-GTG60, E-CAA/M-GTG150, E-CAG/M-GCC120 and E-CAG/M-GCC150). Chi-square tests were performed to examine the goodness of fit between the observed and expected
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AFLP marker bands. All the four AFLP markers viz., (E-CAA/M-GTG60, E-CAA/M-GTG150, E-CAG/M-GCC120 and E-CAG/M-GCC150) exhibited mendelian segregation ratio of 3:1 which is typical ratio of any dominant marker (Table 3 [Supplementary data]).

The AFLP markers, E-CAA/M-GTG150 and E-CAA/M-GTG60 generated approximately 150 bp and 60 bp fragment size respectively linked in cis phase to the SMD locus. Based on the recombination frequency, the map distance between the AFLP markers viz., E-CAA/M-GTG150 and E-CAA/M-GTG60 to the SMD locus is 5.7 and 4.8 cm respectively. The AFLP markers E-CAG/M-GCC120 and E-CAG/M-GCC150 produced fragment size of 120 bp and 150 bp respectively located at a distance of 5.2 and 20.7 cM respectively from the SMD locus and were found to be linked in trans phase.

**DISCUSSION**

Considerable efforts have been made to identify sources of resistance to sterility mosaic disease because of its importance worldwide. Our study showed that SMD resistance was under the control of single recessive gene. Similar results showing SMD under the influence of single gene is reported by Nagaraj et al., 2004. Marker assisted selection is a powerful tool for breeding programs, since it provides significant advantages to traditional phenotypic screening. Marker assisted selection is rapid, relatively inexpensive and is not hampered by pathogen unavailability. There is no environmental limitation, since MAS can be performed off-season and it allows simultaneous screening for many diseases. The combination of bulk segregant analysis (Michelmore et al., 1991) and high polymorphic PCR based markers permits the identification and mapping of useful molecular markers for breeding programmes. AFLP markers have been reported for linkage analysis in several plant species (Lacape et al., 2003).

None of the SSR markers that were polymorphic in parents of the cross TTB 7 x BRG 3 was polymorphic between resistant and susceptible bulks indicating the markers are not linked to SMD. This indicates lack of adequate polymorphic SSR markers used for the study. Overall, the number of SSR primers polymorphic between the parents of two different cross combinations is not sufficed to tag the genes of interest. More number of SSR markers needs to be screened in order to get sufficient number of polymorphic SSR markers to tag the genes of interest. But due to lack of adequate SSR markers available in the crop species the objective could not be fulfilled using SSR markers. As a next alternative to SSR markers, AFLP markers were employed to screen the parental genotypes to identify polymorphic AFLP markers. AFLP were more efficient in detecting polymorphism among closely related cultivars that could not be detected by other marker systems. AFLP markers have been proved as more reliable and reproducible as compared to RAPD markers and less cumbersome and time consuming than the RFLPs (Okori et al., 2003).

Linkage analysis of 121 F2 individuals of cross TTB 7 x BRG 3 revealed four AFLP markers to be linked to sterility mosaic disease. The four AFLP markers covered a total spanning length of 38.6 cM. Two of them were linked in coupling phase (cis) and two were linked in repulsion phase (trans) to the susceptible dominant allele. The markers linked in coupling phase include the closely linked marker E-CAA/M-GTG150 and E-CAA/M-GTG60 were located at a distance of 4.8 cm and 5.7 cm from the SMD locus. Since these two markers are linked at a distance less than 10 cm from the target locus, these can be used for effective indirect selection (Weber and Wricke, 1994). There is however a need to saturate the locus with more markers to obtain tightly linked markers for SMD resistance. Markers linked in coupling phase to the susceptible dominant allele differentiates homozygous and heterozygous susceptible individuals increasing the selection efficiency and can be effectively used for MAS whereas, markers linked to recessive resistant allele cannot differentiate homozygous resistant and heterozygous susceptible individuals and can be less used for marker assisted selection. However, the four AFLP markers E-CAA/M-GTG150, E-CAA/M-GTG60, E-CAG/M-GCC150 and E-CAG/M-GCC120 found associated with SMD resistance can be used for MAS, helping to introgress the recessive resistant allele of this gene into desired genetic backgrounds. The identification of markers linked to SMD resistance facilitates numerous future endeavours including development of sequence characterized amplified region or cleaved amplified polymorphic sequence markers (CAPS) (Paran and Michelmore, 1993 and Lu et al., 2000). Further more in future, such markers would prove useful in initiating the cloning and characterization of SMD resistance genes using one or more of the currently based methods for map based cloning. The study described above represents the first step towards reaching the goals for improvement of pigeonpea for sterility mosaic disease using DNA markers.

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