GENERATION OF EXPRESSED SEQUENCE TAGS AND MARKER DEVELOPMENT FOR STERILITY MOSAIC DISEASE RESISTANCE IN PIGEONPEA
[*Cajanus cajan* (L.) Millsp.]

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Bengaluru **April, 2010**

Affectionately dedicated to

my beloved parents

and wife Kavitha

DEPARTMENT OF GENETICS AND PLANT BREEDING
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CERTIFICATE

This is to certify that the thesis entitled **"Generation of Expressed Sequence Tags and Marker Development for Sterility Mosaic Disease Resistance in Pigeonpea [*Cajanus cajan* (L.) Millsp.]"** submitted by **MR. GNANESH B.N, ID NO. PAK 6O39** in partial fulfillment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY (Agriculture)** in **GENETICS AND PLANT BREEDING** to the University of Agricultural Sciences, Bengaluru, is a record of bonafide research work done by him during the period of his study in this university under my guidance and supervision and that no part of the thesis has been submitted for the award of any degree, diploma, associateship, fellowship or any other similar titles.

Bengaluru
April, 2010

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I. INTRODUCTION

Pigeonpea (*Cajanus cajan* L. Millsp.) is an important grain legume crop of rainfed agriculture in the semi-arid tropics. It is the only cultivated food crop of the *Cajaninae* sub-tribe and has a diploid genome with 11 pairs of chromosomes (2n = 2x = 22) and a genome size estimated to be 858 Mbp (Greilhuber and Obermayer, 1998). The genus *Cajanus* comprises 32 species most of which are found in India, Australia and one species is native to West Africa. Pigeonpea is cultivated in more than 25 tropical and sub-tropical countries, either as the sole crop or a mixed crop with sorghum, pearl millet, maize, or with short duration legumes, e.g., groundnut. It plays an important role in food security, balanced diet and alleviation of poverty because of its diverse usages as a food, fodder and fuel (Rao *et al*. 2002).

India is the largest producer of pigeonpea (2.30 mt) followed by Myanmar (0.54 mt) and Malawi (0.16 mt) (FAOSTAT 2007). The Indian sub continent alone contributes nearly 92 per cent of the total world production. Major states in terms of area and production are Maharashtra, Uttar Pradesh, Madhya Pradesh, Karnataka, Gujarat and Andhra Pradesh together contributes for about 90 per cent of area and 93 per cent of production. Although, India leads the world both in area and production of pigeonpea, its productivity is lower than the world average. This is attributed to factors such as various abiotic (e.g. drought, salinity and water-logging) and biotic (e.g. diseases like *Fusarium* wilt, sterility mosaic and pod borers) stresses.

Among the diseases, sterility mosaic disease (SMD) is considered to be the most important disease of pigeonpea in India and at times can cause yield loss upto 95 per cent (Reddy and Nene, 1981; Kannaiyan *et al.* 1984)). The disease is caused by pigeonpea sterility mosaic virus (PPSMV) (Kumar *et al.* 2003) and

transmitted by eriophyid mite (*Aceria cajani*) Channabasavanna. The disease is characterized by the symptoms like bushy and pale green appearance of plants followed by reduction in size, increase in number of secondary and mosaic mottling of leaves and finally partial or complete cessation of reproductive structures. Some parts of the plant may show disease symptoms and other parts may remain unaffected (Kumar *et al.* 2003). The task of developing resistant varieties is complicated in view of the genetic plasticity of the pathogen. This dynamic nature of the SMD pathogen has warranted the use of strain specific sources of resistance in crop improvement. So, there is a need for identifying strain specific sources of resistance and its inheritance pattern for better understanding of the disease.

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. Due to out crossing nature and long life cycle of the crop, there is a problem to screen varieties and breeding population for SMD resistance. 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 marker assisted selection.

With the advent of genomic tools such as molecular markers, genetic maps, etc., conventional plant breeding has been facilitated greatly and improved genotypes/ varieties with enhanced resistance/tolerance to biotic/abiotic stresses have been developed in several crop species (Varshney *et al.* 2005, 2006). Among them, SSR markers have proved as more reliable, hyper variable and reproducible as compared to RAPD markers and less cumbersome and time consuming than the RFLPs. AFLP technique initially developed for fingerprinting plant genomes

(Vos *et al.* 1995) also emerged as an important technique for gene tagging (Maksem *et al.* 1995). In case of pigeonpea, however, a very limited number of genomic tools are available so far (Varshney *et al.* 2009a, 2009b) and only a few microsatellite or simple sequence repeat (SSR) markers were developed. For instance, 156 SSR (Burns *et al.* 2001, Odeny *et al.* 2007, 2009, Saxena *et al.* 2009a) and 908 expressed sequence tags (ESTs) (**NCBI** www.ncbi.nlm.nih.gov), at the time of undertaking the study, were available in pigeonpea. However, because of the lower level of polymorphism in the cultivated pigeonpea germplasm, there is a need to develop genomics tools at the appropriate scale.

Expressed sequence tag (EST) projects have generated vast amount of publicly available sequence data from plant species; these data can be mined for simple sequence repeats (Varshney *et al.* 2005). These SSRs are useful as molecular markers because their development is inexpensive, they represent transcribed genes and a putative function can often be deduced by a homology search. For enhancing the genomic resources in pigeonpea, transcriptome sequencing to generate ESTs should be a fast approach. ESTs, which are generated by large-scale single pass sequencing of randomly picked cDNA clones, have been cost - effective and valuable resource for efficient and rapid identification of novel genes and development of molecular markers (Sreenivasulu *et al.* 2002). Further, ESTs have been employed to identify the genes that are differentially expressed in various tissues, cell types, or developmental stages of the same or different genotypes (Ogihara *et al.* 2003, Ronning *et al.* 2003).

In recent years, a number of practical examples have demonstrated the power of SSRs in development of genetic maps in legumes such as soybean (Song *et al.* 2004), common bean (Blair *et al.* 2003) and peas (Loridon *et al.* 2005). Mapping in pigeonpea has been hampered by the lack of appropriate and sufficient molecular markers. Microsatellites are the markers of choice for the development

of a pigeonpea linkage map due to the genetic complexity of breeder's populations and high levels of heterozygosity in individual genotypes.

Being a perennial crop, development of superior lines in pigeonpea using conventional methods has been very slow. Most of the important agronomic characters are controlled by several genes (quantitative traits). The genetic factors responsible for a part of the observed phenotypic variation for a quantitative trait are called quantitative trait loci (QTL). In other legumes, SSR markers have been used as a tool to identify major genes and QTLs and also to introduce new characters in elite germplasm (Asensio-S. - Manzanera *et al.* 2005). Availability of adequate SSRs in pigeonpea would enable breeders to know the location of specific genes and QTLs making it possible to improve the efficiency of breeding through marker assisted selection (MAS). Use of molecular markers in precise mapping of genes in the genome will be pivotal for MAS in breeding programmes.

Conventional breeding has helped in identifying varieties but selection process are difficult, time consuming and most times disease symptoms are elusive because of the complexity of the disease and its occurrence. Due to this, selection of the right genotypes with resistance may not be accurate. However, DNA markers may help in selecting the right genotypes precisely and the associated markers for the trait helps in tracking the genes responsible for resistance in F_1's, segregating populations, backcross populations and in germplasm lines. These markers when used in combination with the available and proven breeding methods may be helpful for precision breeding as well as enhancing the process of breeding. Realizing the importance of such an investigation, the present study was carried out with the following objectives.

1. Development of F_2 and $F_{2:3}$ mapping populations.
2. Phenotyping and detection of inheritance pattern of sterility mosaic disease resistance.
3. Construction of c-DNA library of pigeonpea and generation of expressed sequenced tags (ESTs).
4. Sequence analysis of selected expressed sequenced tags (ESTs) and development of EST-based simple sequence repeat (SSR) markers.
5. Construction of genetic linkage map and identification of marker or QTL associated with SMD resistance.

II. REVIEW OF LITERATURE

In the present investigation the literature pertaining to the objectives has been reviewed and presented under the following headings.

2.1 Molecular markers studies in pigeonpea.

2.2 Inheritance studies for sterility mosaic disease (SMD).

2.3 Construction of c-DNA library and generation of expressed sequenced tags.

2.4 Linkage mapping and QTL identification.

2.1 Molecular markers studies in pigeonpea

Conventional plant breeding have had limited success in enhancing genetic resistance against diseases due to lack of genetic information and complexity of genome. Genetic studies on SMD revealed that resistance is mostly controlled by recessive genes hence necessitating more generations and large population to identify resistant segregants. Transfer of resistance to SMD from land races and wild relatives to cultivated background is difficult due to linkage drag *viz.*, undesirable traits, low yield, poor adaptability and long duration associated with resistance. Under these circumstances, newly emerging biotechnological tools like marker assisted selection can play crucial role in the success of disease resistance breeding.

Molecular markers are useful in disease resistance breeding as they can substitute phenotypic screening in the early phase of breeding program and to identify resistant lines at juvenile stage to save time and cost of screening. It helps in easy identification and transfer of recessive genes and to monitor alien gene introgression, reduces the linkage drag and aids in eliminating undesirable traits in much shorter time frame than those expected through conventional breeding programs. It facilitates map-based cloning of disease resistance genes and pyramiding of genes for multiple disease resistance in a single cultivar, faster recovery of the recurrent parent genome in the backcross breeding

programme (Tanksley *et al.* 1989). It could also reduce the need for phenotypic selection that may be inappropriate in identifying genotypic differences and in selection of rare recombinants between tightly linked resistance genes.

Molecular markers offer great scope for improving the efficiency of conventional plant breeding. The essential requirements for developing MAS system are (i) availability of germplasm with substantially contrasting phenotypes for the traits of interest, (ii) highly accurate and precise screening techniques for phenotyping mapping population for the trait of interest,(iii) identification of flanking markers closely associated with the loci of interest and the flanking region on either side and (iv) simple and robust DNA marker technology to facilitate rapid and cost-effective screening of large population (Paterson *et al.* 2004).

2. 1.1 Molecular diversity studies in pigeonpea

Varietal identification is important for the documentation of genetic resources. Traditional techniques like morphometric traits observation and biochemical techniques based on protein and isozyme polymorphism have been used. But for differentiation and characterization of varieties at molecular level, fingerprinting of crop varieties using DNA markers are very useful and this is found to be more reliable than traditional markers (Vasconcelos *et al.* 1996).

Microsatellite or simple sequence repeat markers are short tandem repetitive DNA sequences with a repeat length of a few (1-5) base pairs (Litt and Luty, 1989). Microsatellite markers have been increasingly used to assess the genetic diversity and population structure among plants (Li *et al.* 2000, Pillen *et al.* 2000). The high variability of repeat numbers among individuals has led to the use of microsatellite markers for the development of genome specific DNA fingerprints (Weising *et al.* 1992; Zavodra *et al.* 2000).

The amplified fragment length polymorphic (AFLP) marker (Vos *et al.* 1995) is one of the important technique that has been used for genetic characterization of plant pathogens. AFLP techniques 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 and Panguluri *et al.* 2005).

AFLP technique initially developed for fingerprinting plant genomes (Vos *et al.* 1995) has emerged as an important technique for genome mapping (Becker *et al.* 1995; Maheshwaran *et al.* 1997), gene tagging (Maksem *et al.* 1995), assessment of genetic diversity (Paul *et al.* 1997; Zhu *et al.* 1998; Aggarwal *et al.* 2002; Bensnard *et al.* 2002), phylogenetic analysis of closely related plant species (Hill *et al.* 1996; Sharma *et al.* 1996; Aggarwal *et al.* 1999), and to assess somaclonal variation (Polanco and Ruiz, 2002).

Restriction fragment length polymorphism (RFLPs) have been used in pigeonpea, to overcome the problems associated with phylogenic grouping such as inconsistencies in taxonomic relationships based on data from morphology, cytology and crossability. RFLP analysis has revealed that accessions of cultivated species *Cajanus cajan* shared more DNA fragments with *Cajanus scarabaeoides* than with *C. cajanifolius* (Nadimpalli *et al.* 1992).

RFLP markers have been utilized to study the cytoplasmic variation in the lines of pigeonpea developed by interspecific crosses using four probes from maize mitochondrial DNA- atp α, atp β, cox -I and cox- II (Sivaramakrishnan *et al.* 1996).

Rathnaparkhe *et al.* (1995) reported high levels of polymorphism among the wild species using RAPD markers, while little polymorphism was found within cultivated *Cajanus cajan* accessions.

RAPD markers were used for investigating quantitative trait loci (QTLs) in two strains of pigeonpea and in the F_1 and F_2 progeny (Tyagi, 1997). However, the level of polymorphism among parents was very low. The F_1 hybrid was intermediate between two parents, but F_2 showed little variation, indicating that both parents were different morphologically, but with little genetic variation at DNA level.

The somaclonal variants of pigeonpea line ICPL 87 were distinguished at the molecular level by RAPD analysis using specific arbitrary sequences of 19 decamer primers. A high level of polymorphism was evident with the primer OPA-20. Whereas, a low level was observed with the primer OPA-07 and these served as molecular markers for specific somaclonal variants thereby, providing a method for selecting somaclones with better agronomic performance (Prasannalatha *et. al*. 1999).

Burns *et al.* (2001) reported a set of 10 simple sequence repeat (SSR) markers in pigeonpea. Ten loci exhibited polymorphism when 20 primer pairs were screened across 12 diverse pigeonpea accessions.

Lohithaswa *et al.* (2003) studied the genetic divergence in 11 pigeonpea genotypes using RAPD markers. Decamer oligonucleotides primers were initially screened to identify the most promising primers for detecting polymorphism. Eight primers were selected for screening and 52 bands were detected. Of the 52 bands, 33 (63.46 %) bands were polymorphic between the genotypes. The genotype ICPL 87, TS 3, GS 1 and GS 3 had high genetic diversity between them. The primer OPB 15 produced unique banding pattern

specific to different varieties, whereas the primer OPB 19 produced specific banding pattern profiles in ICP 8863 and GS 1.

Souframanien *et al.* (2003) used RAPD markers for identification of two pigeonpea cytoplasmic male sterile (CMS) lines derived from crosses between the wild (*Cajanus scarabaeoides* and *C. sericeus*) and the cultivated species (*Cajanus cajan*). The male sterile (A) line and its maintainer (B) line could be easily differentiated with certain random primers. Amplification product of 600 bp amplified by primer OPC-11 was observed in both the cytoplasmic male sterile lines (288 A and 67 A), which was absent in the maintainer lines (288 B and 67 B) and the putative R-line (TRR 5 and TRR 6). Dendrogram constructed based on the similarity index showed that considerable genetic variation exists between CMS lines, two putative R lines and wild species studied.

Panguluri *et al.* (2005) detected DNA polymorphism in the cultivated pigeonpea and two of its wild relatives *Cajanus volubilis* and *Rhynchosia bracteata* using amplified fragment length polymorphism (AFLP) fingerprinting. The two wild species shared only 7.15% bands with the pigeonpea cultivars, whereas 86.71% common bands were seen among cultivated cultivars. Similarly, 62.08% bands were polymorphic between *C. volubilis* and pigeonpea cultivars in comparison to 63.33% polymorphic bands between *R. bracteata* and pigeonpea cultivars, and 13.28% polymorphic bands among pigeonpea cultivars. The cluster analysis revealed low polymorphism among pigeonpea cultivars and very high polymorphism between cultivated pigeonpea and its wild relatives.

Wasike et *al.* (2005) used AFLP markers to study the genetic relatedness between Asia and African pigeonpea cultivars and recorded limited genetic variability among the genotypes used for the study. AMOVA at continent wide hierarchial level, revealed significantly weak population structure but when treating the cultivars as samples from a panmictic population revealed a stronger genetic structure. Estimates of average gene diversity were higher for Indian

genotypes suggesting East African pigeonpea cultivars are less genetically diverse than Indian cultivars. The study also demonstrated AFLP markers as a suitable tool for DNA fingerprinting and genetic studies in pigeonpea.

Dendrogram constructed by combined RAPD and SSR data depicted that the SMD susceptible genotypes TTB 7 and ICP 8863 clustered together while, the resistant genotypes Hy 3c and BRG 3 subclustered with ICP 7035 indicating ICP 7035, BRG 3, HY 3C are differing at molecular level from the SMD susceptible genotypes TTB 7 and ICP 8863 (Gangadhara, 2006).

Diversity array technology (DArT) markers revealed low level of genetic diversity in cultivated pigeonpea as compared to wild relatives. Most of the diversity was among the wild relatives of pigeonpea or between the wild and the cultivated species (Yang *et al.* 2006).

Datta *et al.* (2007) studied genetic relatedness among 16 varieties representing eight different pulse crops namely, pea, lentil, lathyrus, chickpea, pigeonpea, frenchbean, urdbean and mungbean using 40 RAPD markers. From the clustering analysis, chickpea and lentil grouped together whereas, pea and lathyrus were close to each other. The two crops of the genus vigna, (mungbean and urdbean) grouped together with their varieties forming subclusters. Frenchbean and pigeonpea were grouped into distinct clusters showing relative divergence of these crops from other pulse crops.

Choudhury *et al.* (2007) identified 21 RAPD markers for identification of specific genotypes and assessment of genetic relatedness among the pigeonpea cultivars. Among these, 16 primers were found to be unique, producing 40 genotype specific bands in 16 different genotypes. An average of 12.6 bands per primer was obtained with 89.4 per cent polymorphism.

Odeny *et al.* (2007) identified 19 SSR primers to be polymorphic among 15 cultivated and nine wild pigeonpea accessions providing evidence for cross species transferability within the genus *Cajanus*. A total of 98 alleles were detected at the 19 polymorphic loci with an average of 4.9 alleles per locus. Less alleleic variation (31 alleles) was observed within the cultivated species than across the wild species (92 alleles) and suggested for development of more microsatellite markers for future genomic studies in pigeonpea.

108 RAPD markers were screened to identify CMS lines derived from crosses between wild (*Cajanus scarabaeoides* and *C. sericeus*) and cultivated pigeonpea (Choudhury *et al.* 2008). A set of RAPD primers were identified that could distinguish the CMS systems of GT 288 A/B and 67 A/B. Moreover, specific primers differentiating the CMS lines (GT 288/67A), maintainers (GT288B/67B) and putative restorers (ICP 41 and DPPA 85-7) were identified for use in heterosis breeding.

Singh *et al.* (2008) used 21 SSR markers obtained from different crop species to assess polymorphism in 16 cultivated pigeonpea genotypes. Based on SSR fingerprinting, 16 genotypes were grouped into two groups as early and late duration genotypes indicating that SSR markers could be used as a good choice to classify the pigeonpea genotypes.

Odeny *et al.* (2009) used 113 pigeonpea genomic SSRs, 73 of which amplified interpretable bands. Thirty-five of the primers revealed polymorphism among 24 pigeonpea breeding lines. The number of alleles detected ranged from 2 to 6 with a total of 110 alleles and an average of 3.1 alleles per locus. GT/CA and GAA class of repeats were the most abundant dinucleotide and tri-nucleotide repeats respectively. Additionally, 220 soybean primers were tested in pigeonpea, 39 of which amplified interpretable bands.

Saxena *et al.* (2009a) identified 13 polymorphic SSR markers to be polymorphic amongst 32 cultivated and eight wild pigeonpea genotypes representing six *Cajanus* species. These markers amplified a total of 72 alleles ranging from two to eight alleles with an average of 5.5 alleles per locus. The polymorphic information content for these markers ranged from 0.05 to 0.55 with an average of 0.32 per marker. These markers should be useful for genome mapping, trait mapping, diversity studies and assessment of gene flow between populations in pigeonpea.

In order to maximize polymorphism in the mapping populations for mapping loci for fusarium wilt (FW) and sterility mosaic disease (SMD) resistance in pigeonpea, a set of 32 pigeonpea lines were screened using 30 SSR markers by Saxena *et al.* (2009b). A total of 23 marker loci showed polymorphism with 2-4 alleles and the polymorphism information content for these markers ranged from 0.12 to 0.65 with an average of 0.43 per marker.

2.1.2 Identification of trait specific molecular markers

Till date very little literature pertaining to identification of DNA markers linked to pigeonpea sterility mosaic disease is reported. Very few reports regarding identification of trait specific markers in pigeonpea are available. Hence, literature pertaining to identification of trait specific markers in related crops is also reviewed.

The use of DNA marker systems, such as random amplified polymorphic DNAs (Williams *et al.* 1990), amplified fragment length polymorphisms (Vos *et al.* 1995), and simple sequence repeats (Akkaya *et al.* 1992), has contributed greatly to the development of genetic linkage maps for many important crop species including cowpea (Fatokun *et al.* 1993; Waugh *et al.* 1997).

In combination with the bulked segregant analysis (BSA) method, (Michelmore and Meyers, 1998) the use of RAPDs, AFLPs, and SSRs has made it possible to rapidly identify molecular markers linked to genes of agronomic importance (Lee 1995; Young, 1999). The development and use of molecular marker technologies has also facilitated the subsequent cloning and characterization of disease, and pest resistance genes from a variety of plant species (Meyers *et al.* 1999; Hammond and Jones, 1997).

Tiwari *et al.* (1998) identified coupling and repulsion phase RAPD markers linked to powdery mildew resistant gene *er*-1 in pea using bulk segregant analysis of F_3 individuals. Marker OPO-18 was found to be linked in coupling phase while, the markers OPE 16 and OPL 6 were in repulsion phase to resistant gene *er* -1.

Quedraogo *et al.* (2001) identified three AFLP markers (E-AAC/M-CAA_{300} (2.6 cM), E-ACT/M-CAA_{524} (0.9 cM), and E-ACA/M-$CAT_{140/150}$ (0.9 cM), tightly linked to *Rsg2-1* which appears to be codominant. Segregation analysis of a different F_2 population resulting from a cross of the striga susceptible line IT84S-2246-4 with Tvu 14676, a *S. gesnerioides* race 3 resistant line, showed that resistance to *S. gesnerioides* race 3 was controlled by a single dominant gene, designated as *Rsg4-3*. The identification of AFLP markers linked to striga resistance provides a stepping stone for a marker assisted selection program and the eventual cloning and characterization of the gene(s) encoding resistance to this noxious parasitic weed.

Quedraogo *et al.* (2002) identified seven AFLP markers linked to *Striga gesnerioides* gene *Rsg3* from the F_2 population of cross Gorom x Tvx 3236 using bulk segregant analysis. From the linkage analysis the distance between the marker and *Rsg3* locus ranged from 9.9 to 2.5 cM, with two markers E-AGA/M-CAG_{300} and E-AGA/M-CTA_{460} flanking the *Rsg3* locus at 2.5 and 2.6 cM respectively.

Kotresh *et al.* (2006) identified RAPD markers associated with pigeonpea wilt using F_2 population derived from contrasting parents GS 1 (susceptible), ICPL 87119 (resistant) and ICP 8863 (resistant). PCR testing revealed presence of two amplicons at 704 bp and 500 bp linked with susceptibilty. Analysis of individual F_2 plants showed a segregation ratio of 3:1 for the presence: absence of amplicons in the crosses.

Selvi *et al.* (2006) identified three RAPD markers in mungbean *viz.*, OPT16, OPS7 and OPAK 19 specific to MYMV resistant parent and resistant bulk but absent in MYMV susceptible parent and susceptible bulk. From linkage analysis, one RAPD marker $OPS7_{900}$ was identified to be associated with mungbean yellow mosaic virus resistance.

Blair *et al.* (2007) developed a co-dominant SCAR marker SR2, tightly linked to *bgm-1* resistance gene using commonbean RIL population derived from the cross DOR 476 x Sel 1309. The polymorphism between the resistant and the susceptible genotype was based on 37 bp insertion event in the SR2 allele associated with susceptibility.

Ganapathy *et al.* (2009) used two AFLP primer pairs generating 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 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, 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.

2.2 Screening techniques for sterility mosaic disease resistance

Three methods are being used for evaluating resistance to SMD. An effective technique called "Leaf stapling technique" for screening pigeonpea germplasm and breeding material for resistance to SMD was developed by Nene and Reddy (1977). It is the most commonly used method under field and glass house conditions. This technique involves stapling of SMD infected pigeonpea leaves on the healthy seedlings at 2-4 leaf stage. Mites infected with virus from the stapled leaf migrate and transmit the virus to the test plants. This technique was shown to facilitate inoculation at primary leaf stage and to express disease symptoms rapidly.

2.2.1 Resistant source material

Rangaswamy *et al.* (2005) evaluated ICP 7035, along with the two local varieties, TTB 7 and Hy 3c, in SMD nursery at the Gandhi Krishi Vignana Kendra (GKVK), Bangalore. Average SMD incidence in susceptible cultivars ranged from less than 2.0 to 90.3% during various years, but ICP 7035 remained free from SMD.

Among 79 genotypes screened against SMD, seven genotypes *viz.,* ICP 7035, BAD 2001-6, NDA 98-8, Hy 3c, MAL 24, MAL 23 and BRG 3 showed moderately resistant reaction with 11- 30 per cent SMD incidence while, the remaining genotypes including the susceptible check TTB 7 were found to be susceptible with disease incidence more than 50 per cent (Saifulla *et al.* 2005)

Four genotypes *viz*., BRG 3, ICP 7035, Hy 3c and ICP 8863 were screened against SMD for three consecutive years from 2002-03 to 2005-06. BRG 3 and ICP 7035 recorded resistant reaction while, the genotype HY 3C recorded moderate resistant reaction to SMD. The susceptible check ICP 8863 recorded 100 per cent disease incidence (Saifulla *et al.* 2006).

Ganapathy, (2009) has confirmed the resistant levels of four genotypes *viz*., BRG 3, ICP 7035, TTB 7 and ICP 8863 before using them as parents in his crossing programme. BRG 3 and ICP 7035 showed 100 per cent resistance with no mosaic symptoms while, the susceptible genotypes TTB 7 and ICP 8863 showed 100 per cent susceptibility with severe mosaic symptoms

2.2.2 Inheritance studies for SMD resistance

Singh *et al.* (1983) studied the inheritance of resistance to SMD in 15 crosses, involving five resistant and three susceptible genotypes and reported resistance to be controlled by four independent non-allellic genes. The symbols Sv1, Sv2, Sv3 and Sv4 were assigned to four resistant genes. Sv1 and Sv2 were reported to exhibit duplicate dominant epistasis while, Sv3 and Sv4 exhibited duplicate recessive epistasis and concluded that presence of at least one dominant allele at locus 1 or 2 and homozygous recessive genes at locus 3 or 4 was essential for resistant reaction.

Sharma *et al.* (1984) reported both 9 (resistant): 7 (susceptibility) and 1 (resistant): 3 (susceptible) segregation ratios in different crosses and explained basis of inheritance of SMD controlling two genes and more than two alleles per locus.

Reddy *et al.* (1995) reported that in the SMD affected genotypes, leaf cuticle and the epidermal cell wall thickness were found to be less compared to resistant genotypes. Thick leaf cuticle and epidermal cell wall of resistant genotypes prevents the mites to feed on them which might give resistance against the disease. Resistance is therefore attributed to the thick cuticle of the resistant lines through which the mite vectors cannot penetrate into the living epidermal cells to transmit the SMD pathogen.

Murugesan *et al.* (1997) reported monogenic inheritance of resistance to SMD. They studied F_1 and F_2 generation of Vamban 1 (resistant) x Gulbarga 1 (susceptible). The F_1 hybrid was resistant indicating resistance being dominant over susceptibility. The F_2 population segregated in 3 resistant: 1 susceptible ratio indicating monogenic control.

Srinivas *et al.* (1997) studied the inheritance of resistance and allelic relationship in three resistant pigeonpea sources for strain 2 of sterility mosaic pathogen. The resistant genotypes ICP 7035, ICP 7349 and ICP 8850 were crossed with susceptible genotypes BDN 1 and LRG 30 to determine the inheritance of resistance. The resistant and susceptible genotypes were crossed among themselves to obtain information on their allelic relationship. Parents, F_1 and F_2 generations were sown in pots and screened using "Infector hedge row technique". Observations obtained from parents, F_1 and F_2 generations, indicated dominance of resistance in certain crosses and the dominance of susceptibility in others.

Amala Balu and Rathnasamy (2003) studied the pattern of inheritance of the sterility mosaic resistance in pigeonpea. Two susceptible parents (Prabath and Co 5), two resistant parents (ICPL 83024 and ICPL 83027), their F_1 hybrids and F_2 progenies were screened for sterility mosaic disease. F_1s were moderately resistant to SMD while, in F_2 the rating scale for SMD ranged from 3 to 9. The F_2 generation of the four combinations fitted well with the segregating ratio of 13:3 for susceptibility and resistance indicating that the resistance was being controlled by non-allelic interaction of two factors.

Nagaraj *et al.* (2004) studied the inheritance to the Bangalore strain of sterility mosaic virus in crosses involving two resistant lines (ICP 7035 and MAL 14) with no apparent symptoms and susceptible lines (TTB 7, ICP 8863, and BDN 1) with severe mosaic symptoms. The F_1, F_2, BC_1 and BC_2 generations were sown in the field and screened following infector hedge and leaf stapling

techniques to study the inheritance pattern. Resistance was recessive and appeared to be governed by two independent non-allelic genes exhibiting complementary epistasis. The presence of at least one allele in homozygous recessive condition was found to be necessary to express resistant phenotype.

One hundred and fifteen wild *Cajanus* accessions from six species (*C. albicans, C. platycarpus, C. cajanifolius, C. lineatus, C. scarabaeoides* and *C. sericeus*) were evaluated against three SMD isolates prevailing in peninsular India. Evaluations were done under greenhouse conditions in endemic locations of each isolate through mite-mediated virus inoculation and graft inoculation techniques. Fifteen wild accessions showed resistance to all three isolates of SMD. Most of the wild accessions did not support mite multiplication. The majority of the accessions resistant to SMD following inoculations with viruliferous mites were susceptible by graft inoculation, suggesting that vector resistance is conferring resistance to infection with PPSMV (Kumar *et al.* 2005).

Ganapathy (2009) reported F_1's of the resistant x susceptible cross were susceptible indicating susceptiblity to be dominant over resistance. A digenic ratio of 7 resistant: 9 susceptible was obtained in the F_2 population of cross ICP 8863 (S) x ICP 7035 (R) indicating complementary nature of two genes for resistance. In contrast, a monogenic ratio of 1 resistant: 3 susceptible was obtained for the cross TTB 7 (S) x BRG 3 (R) indicating single gene control.

2.3 Construction of c-DNA library and generation of expressed sequenced tags.

Expressed sequence tag (EST) libraries and databases have proven to be powerful tools for gene discovery, gene mapping and for the analysis of quantitative traits. ESTs are generated by large-scale sequencing of randomly picked clones from cDNA libraries constructed from mRNA isolated at a particular development stage and/or tissue.

2.3.1 EST development

Plants are known to have developed integrated defence mechanisms against viral infections by altering spatial and temporal transcriptional changes. The EST approach was successfully utilized in identification of disease-responsive genes from various tissues and growth stages in plants.

Asamizu *et al.* (2000) studied comprehensive analysis of genes expressed in a model legume, *Lotus japonicus*, a total of 22,983 5' end expressed sequence tags (ESTs) were accumulated from normalized and size-selected cDNA libraries constructed from young plants. The EST sequences were clustered into 7137 non-redundant groups. Of these 3302 sequences showed similarity to genes of known function, 1143 sequences to hypothetical genes and 2692 sequences were novel sequences.

Alkharouf *et al.* (2004) constructed cDNA libraries made from mRNA extracted from roots of the resistant soybean *Glycine max* L. Merr. Expressed sequence tag analysis of the libraries provided rapid discovery of genes involved in the response of soybean to the nematode. A total of 3454 cDNA clones were examined from the three libraries, of which 25 cDNAs were derived from nematode RNA. To provide resources for barley genomics, Zhang *et al.* (2004) generated 110981 expressed sequence tags (ESTs) from 22 cDNA libraries representing tissues at various developmental stages. Of these, 17.5% showed no significant similarity to other barley ESTs present in dbEST.

Buhariwalla *et al.* (2005) constructed an EST library after subtractive suppressive hybridization (SSH) of root tissue from two very closely related chickpea genotypes (*Cicer arietinum*). A total of 106 EST-based markers were designed from 477 sequences with functional annotations and these were tested on *C. arietinum*. Forty-four EST markers were polymorphic when screened across nine *Cicer* species (including the cultigen). The EST markers generated in

this study have detected high levels of polymorphism amongst both common and rare alleles.

Coram and Pang (2005) generated ESTs which were clustered and assembled into 516 unigenes, of which 4% were defence-related, encoding lignin and phytoalexin biosynthesis enzymes, pathogenesis-related proteins, signalling proteins, and putative defensive proteins. These unigenes may be involved in chickpea defence against ascochyta blight. The generation of an EST library represents the first step in a functional genomics approach aimed at elucidating the function of genes involved in ascochyta blight resistance and the pathway of their action.

To better understand the molecular basis of the defense response against the rice blast fungus, Jantasuriyarat *et al.* (2005) used large-scale expressed sequence tag (EST) sequencing approach to identify genes involved in the early infection stages in rice. Comparison of the pathogen-challenged libraries with the uninoculated control library revealed an increase in the percentage of genes in the functional categories of defense and signal transduction mechanisms and cell cycle control, cell division, and chromosome partitioning. The large cataloged collection of rice ESTs in this study provides a solid foundation for further characterization of the rice defense response and is a useful public genomic resource for rice functional genomics studies.

Luo *et al.* (2005) developed expressed sequence tag (EST) libraries for cultivated peanut (*Arachis hypogaea* L.) from two cDNA libraries constructed by means of mRNA prepared from leaves of peanut line C34-24 (resistant to leaf spots and *Tomato spotted wilt virus*) and immature pods of peanut line A13 (tolerant to drought stress and preharvest aflatoxin contamination). 44 EST-derived simple sequence repeat (SSR) markers have been characterized for cultivated peanut, in which over 20 % of the SSRs produced polymorphic markers among 24 cultivated peanut genotypes. Ji *et al.* (2006) sequenced 2003

ESTs generated from salinity-treated *Glycine soja* cDNA library, putatively representing 1071 unigenes. Comparison of *Glycine soja* ESTs with those of Gl*ycine max* revealed the potential to investigate the wild soybean's expression profile using the soybean's gene chip. Through analysis of the ESTs with putative functional annotations, a large number of putative stress-regulated genes were identified.

Fusarium equiseti causes a discoloration on ginseng roots that significantly affects their marketability. The cellular and biochemical changes in affected roots that lead to this symptom, as well as differential gene expression following pathogen inoculation were studied. Accumulation of phenolics, cell disruption, and development of a zone of lignified cells were observed in affected tissues. A number of genes involved in host defense responses were up-regulated, particularly those induced by jasmonic acid and genes mediating phenolic production and detoxification (Goswami and Punja, 2008).

Mian *et al.* (2008) generated 41,516 ESTs from nine cDNA libraries of tall fescue. *In silico* gene expression studies using these ESTs were performed to understand stress responses in tall fescue. A large number of ESTs of known stress response gene were identified from stressed tissue libraries. These ESTs represent gene homologues of heat-shock and oxidative stress proteins, and various transcription factor protein families. Highly expressed ESTs representing genes of unknown functions were also identified in the stressed tissue libraries.

2.3.2 EST-SSR markers

Conventional SSR marker development is a costly and time-consuming process. Thanks to the availability of genomic or EST/genic sequences in public databases and the recent advent of bioinformatics tools, SSR marker development has become easier and more cost-effective (Jayashree *et al.* 2006). In the past, SSR markers have been successfully developed by mining EST

databases in several crops reviewed including monocots (Varshney *et al.* 2005; Jayashree *et al.* 2006; Ellis and Burke 2007) and dicots (Kumpatla and Mukhopadhyay 2005, Jayashree *et al.* 2006). EST-SSRs were reported in many cereals/grass species, including rice (Cho *et al.* 2000), sugarcane (Cordeiro *et al.* 2001), durum wheat (Eujayl *et al.* 2002; Gupta *et al.* 2003; Yu *et al.* 2004; Zhang *et al.* 2006), rye (Hackauf and Wehling 2002; Studer *et al.* 2008), barley (Thiel *et al.* 2003), tall fescue grass (Saha *et al.* 2004), finger millet (Dida *et al.* 2007) and pearl millet (Senthilvel *et al.* 2008).

The nature and frequency of SSRs in EST collections have been comprehensively discussed in Kantety *et al.* (2002), Varshney *et al.* (2002), La Rota *et al.* (2005) and Jayashree *et al.* (2006) for monocots and Kumpatla and Mukhopadhyay (2005) and Jayashree *et al.* (2006) for dicots. These EST-SSR markers were used for diversity analysis in rice (Cho *et al.* 2000), wheat (Leigh *et al.* 2003, Gupta *et al.* 2003, Zhang *et al.* 2006), barley (Thiel *et al.* 2003; Varshney *et al.* 2007, 2008) pigeonpea (Raju *et al.* 2010) and for mapping in wheat (Gupta *et al.* 2003, Yu *et al.* 2004; Balyan *et al.* 2005), barley (Varshney *et al.* 2006), finger millet (Dida *et al.* 2007) and pearl millet (Senthilvel *et al.* 2008).

Generally, the EST-derived SSR markers are found to be less polymorphic than genomic SSRs as these are derived from transcribed regions of the genome (Cho *et al.* 2000; Eujayl *et al.* 2002; Thiel *et al.* 2003; Varshney *et al.* 2002, 2005; Jayashree *et al.* 2006; Ellis and Burke 2007). Most transcribed regions are greatly conserved across species. In rice, the model organism for cereals, Cho *et al.* (2000) reported 54% of polymorphism using EST-SSR markers across seven mapping population parents. EST-SSR markers developed in durum wheat identified only 25% polymorphism (Eujayl *et al.* 2002), whereas Thiel *et al.* (2003) reported 8-54% polymorphism on three different mapping population parental line pairs in barley. Even though EST-SSR markers exhibit a lower percentage of polymorphism than their genomic SSR counterparts, this

marker system has been greatly exploited in the transition from structural genomics to functional genomics.

EST-SSR markers are superior in terms of cross-species transferability, as they were derived from the most conserved regions of genome, and thus are well suited for application in phylogenetic analysis and comparative genome mapping (Zhang *et al.* 2006). Wang *et al*. (2005) developed a small number of EST-SSR markers (30) in sorghum along with wheat, rice and maize. The transfer rate of EST-SSR markers from sorghum to paspalum (*Paspalum* spp.) and to maize was 68% and 61%, respectively. Saha *et al.* (2004) reported about 57% transferability across six grass species using tall fescue EST-SSRs. EST-SSR markers developed in wheat (Yu *et al.* 2004) found 62% transferability across four species including wheat, rice, maize and barley. Thiel *et al.* (2003) identified 40% transferability of barley EST-SSRs to rice.

2.4 Linkage mapping and QTL identification.

Construction of genetic linkage map is necessary to apply marker assisted selection tool in crop improvement programme but it was a hard task for pigeonpea researchers because of its low level of genetic polymorphism .Till date no literature pertaining to linkage mapping and QTL identification in pigeonpea sterility mosaic disease is reported. Hence literature pertaining to linkage mapping and QTL identification in related crops is reviewed.

Hayashi *et al*. (2001) developed a genetic linkage map of the model legume *Lotus japonicus* based on amplified fragment length polymorphism (AFLP), simple sequence repeat polymorphism (SSRP) and derived cleaved amplified polymorphic sequence (dCAPS). The F_2 mapping population used was derived from a cross between two *L. japonicus* accessions Gifu B-129 and Miyakojima MG-20. The framework of the linkage map was constructed based on co-dominant markers, and then dominant markers were integrated separately

in each linkage group of the parents. The resulting linkage groups correspond to the six pairs of chromosomes of *L. japonicus* and consist of 287 markers with 487.3 cM length in Gifu B-129 and 277 markers with 481.6 cM length in Miyakojima MG-20.

Thoquet *et al*. (2002) obtained an F_2 segregating population of 124 individuals between the cultivar Jemalong and DZA315 using an efficient manual crossing technique established for *Medicago truncatula* to construct a genetic map. This map spans 1225 cM (average 470 kb/cM) and comprises 289 markers including RAPD, AFLP, known genes and isoenzymes arranged in 8 linkage groups (2n = 16). Markers are uniformly distributed throughout the map and segregation distortion is limited to only 3 linkage groups. By mapping a number of common markers, the eight linkage groups are shown to be homologous to those of diploid alfalfa (*M. sativa*), implying a good level of macrosynteny between the two genomes. Using *M. truncatula* map and the derived F_3 populations, they mapped the *Mtsym6* symbiotic gene on linkage group 8 and the *SPC* gene, responsible for the direction of pod coiling, on linkage group 7.

The chickpea landrace ILC 3279 has resistance to pathotypes I and II of the ascochyta blight pathogen. Udupa and Baum (2003) identified and mapped a major locus (*ar1*, mapped on linkage group 2), which confers resistance to pathotype I, and two independent recessive major loci (*ar2a*, mapped on linkage group 2 and *ar2b*, mapped on linkage group 4), with complementary gene action conferring resistance to pathotype II. Out of two pathotype II-specific resistance loci, one (*ar2a*) linked very closely with the pathotype I specific resistance locus, indicating a clustering of resistance genes in that region of the chickpea genome.

Zhang *et al*. (2003) constructed genetic linkage map with 89 SSR marker loci covering 1543 cM on ten chromosomes with an average interval of 17.3 cM

using maize population consisting of 184 F_2 individuals. The $F_{2:3}$ families was phenotyped and 3 QTLs conferring resistance to sugarcane mosaic virus (SCMV) were detected on chromosomes 3,5 and 10 at seedling stages; four QTLs on chromosomes 3,5,6 and 10 at elongation and anthesis stage and five on chromosomes 1,3,5,6,10 at grain filling stages.

Moretzsohn *et al*. (2005) screened, 433 SSR markers against *Arachis duranensis*, accession K7988 and *A. stenosperma*, accession V10309, 204 (46.8%) were polymorphic, with 170 codominant and 34 dominant markers. The 80 codominant markers segregating 1:2:1 ($P<0.05$) were initially used to establish the linkage groups. Distorted and dominant markers were subsequently included in the map. The resulting linkage map consists of 11 linkage groups covering 1,230.89 cM of total map distance, with an average distance of 7.24 cM between markers.

Chaerani *et al*. (2007) performed QTL mapping study for early blight disease in F_2 and F_3 populations derived from the cross between the susceptible *Solanum lycopersicum* cv. 'Solentos' and the resistant *Solanum arcanum* LA2157 and genotyped with AFLP, microsatellite and SNP markers. A total of six QTL regions were mapped on chromosomes 1, 2, 5–7, and 9 with LOD scores ranging from 3.4 to 17.5. Three early blight QTL also conferred resistance to stem lesions in the field. All QTL displayed significant additive gene action; in some cases a dominance effect was found. Additive × additive epistatic interactions were detected between one pair of QTL. For two QTL, the susceptible parent contributed resistance alleles to both early blight and stem lesion resistance.

Phan *et al*. (2007) reported the first genetic linkage map of white lupin. An F_8 population developed from Kiev mutant × P27174 was mapped with 220 amplified fragment length polymorphism and 105 gene based markers. The genetic map consists of 28 main linkage groups (LGs) that varied in length from

22.7 cM to 246.5 cM and spanned a total length of 2951 cM. There were seven additional pairs and 15 unlinked markers, and 12.8% of markers showed segregation distortion. Two quantitative trait loci (QTLs) with significant effects were identified for anthracnose resistance on LG4 and LG17.

Silva *et al*. (2008) used two $F_{2:3}$ populations of soybean, derived from the crosses between the resistant lines PI 230970 (*Rpp2*), PI 459025 (*Rpp4*) and the susceptible cultivar BRS 184. The mapping populations and parental lines were inoculated with a field isolate of *P. pachyrhizi* and evaluated for Asian soybean rust and were screened with SSR markers, using the bulk segregant analysis (BSA) to expedite the identification of linked markers. Both resistance genes showed an expected segregation ratio for a dominant trait. This study allowed mapping *Rpp2* and *Rpp4* loci on the linkage groups J and G, respectively.

De Souza *et al*. (2008) mapped and characterized quantitative trait loci (QTL) associated with resistance to sugarcane mosaic virus (SCMV) in a maize population consisting of 150 $F_{2:3}$ families from the cross between two tropical maize inbred lines, L520 (resistant) and L19 (susceptible). F_2 individuals were genotyped with SSR markers, and the derived $F_{2:3}$ families were evaluated for their response to artificial inoculation with SCMV under field conditions. Multiple interval mapping was used for QTL detection with a linkage map based on 19 SSR markers. Three QTLs for SCMV resistance were identified with two QTLs (Scm2a and Scm2b) clustered on chromosome 3, and one QTL (Scm1) on chromosome 6, explaining 13.34, 41.85 and 7.66% of the phenotypic variation for SCMV resistance, respectively.

Taleei *et al*. (2008) mapped fifty-eight SSR markers and one morphological marker (flower color) on F_2 individuals and $F_{2:3}$ families of chickpea derived from the cross ICC 12004 (resistant) times Bivanij (susceptible local variety). The linkage map comprised eight linkage groups, excluding flower color which didn't assign to any linkage group. Area under disease

progress curve was used to evaluate the F_2 population and F_3 families. Using composite interval mapping, three genomic regions were detected, which were in association with reaction to Ascochyta blight. These QTLs on LG3, LG4 and LG6 accounted for 46.5% of the total estimated phenotypic variation for reaction to Ascochyta blight

An intraspecific linkage map of cultivated chickpea was constructed by Kottapalli *et al*. (2009), using an F_2 population derived from a cross between an Ascochyta blight (AB) susceptible parent ICC 4991 and resistant parent ICCV 04516. The resultant map consisted of 82 SSR markers and 2 EST markers covering 10 linkage groups, spanning a distance of 724.4 cM with an average marker density of 1 marker per 8.6 cM. Three quantitative trait loci (QTLs) were identified that contributed to resistance to an Indian isolate of AB, based on the seedling and adult plant reaction. QTL1 was mapped to LG3 linked to marker TR58 and explained 18.6% of the phenotypic variance for AB resistance at the adult plant stage. QTL2 and QTL3 were both mapped to LG4 close to four SSR markers and accounted for 7.7% and 9.3%, respectively, of the total phenotypic variance for AB resistance at seedling stage.

Varshney *et al*. (2009c) developed a genetic linkage map for tetraploid cultivated groundnut. A total of 1,145 microsatellite or simple sequence repeat (SSR) markers were screened on two genotypes, TAG 24 and ICGV 86031 that are parents of a recombinant inbred line mapping population. As a result, 144 (12.6%) polymorphic markers were identified and these amplified a total of 150 loci. A total of 135 SSR loci could be mapped into 22 linkage groups, covering 1,270.5 cM of total map distance.

III Material and Methods

The details of material used and the methods adopted in the present investigation are described in this chapter under the following headings

3.1 Development of F_2 and $F_{2:3}$ mapping population and inheritance studies.

3.2 Statistical data analysis.

3.3 Construction of c-DNA library and generation of expressed sequenced tags.

3.4 Construction of genetic linkage map and identification of QTL associated with SMD resistance.

3.1 Development of F_2 and $F_{2:3}$ mapping population.

3.1.1 Experimental material

The mapping population was developed at the All India Co-ordinated Research Project on Pigeonpea, University of Agricultural Sciences, Bengaluru by crossing a susceptible parent TTB 7 with a resistant parent ICP 7035. Salient morphological features of parental lines are presented below.

Line	Origin	Pedigree	Characteristics
ICP 7035	India (Madhya Pradesh)	Germplasm line	Mid late, indeterminate with semi spreading growth habit. Flowers are red colour with denser purple streaks. Purple colour pods, with reddish brown and bold pea shaped seeds. Resistant to SMD.
TTB 7	India (Karnataka)	Local Selection	Mid late, indeterminate with spreading growth habit, Yellow flowers with red streaks. Green colour pods with purple streaks. Produces light brown oval seeds. Susceptible to SMD.

3.1.1.1 Hybridization and development of F_1's

The individual flowers of the selected female parent TTB 7 were hand emasculated and pollinated with the pollen dust from the male parent ICP 7035 in the cool hours of the day to get sufficient F_1 seeds (*Kharif* 2006). F_1s plants along with their parents were grown during summer 2007 and true F_1 plants were selfed by covering nylon net to prevent out crossing through honey bees and other insect pollinators.

3.1.1.2 Development of F_2 population

Seeds from the F_1 plants were collected and used for raising F_2 generation during *Kharif* 2007. All the F_2 plants (224) were covered with nylon net to prevent insect pollination. At 2nd leaf stage, DNA samples from all the F_2 plants were collected for genotyping. Seeds obtained from F_2 plants were collected to forward $F_{2:3}$ generation for phenotyping against SMD (Plate 1).

3.1.1.3 Phenotyping of $F_{2:3}$ population

During *kharif* 2008, all the 224 $F_{2:3}$ populations with 15 plants per family along with their parents, F_1s and susceptible check (ICP 8863) were raised in poly bags with two replications for phenotyping against SMD (Plate 2).

3.1.2 Resistance screening techniques for SMD

A comprehensive study of variability in the sterility mosaic pathogen of pigeonpea (Reddy *et al*. 1993), revealed the occurrence of five different isolates of the pigeonpea sterility mosaic virus (PPSMV) in India. Amongst them, three distinct isolates have been characterised, *viz*., Bengaluru (B), Patancheru (P) and Coimbatore (C). Patancheru and Coimbatore isolates are mild strains of PPSMV while the Bengaluru isolate was most virulent one (Kulkarni et *al*. 2003). This

has necessitated for phenotyping of SMD at different locations to identify strain-specific resistance sources.

Phenotyping of SMD was done at two locations namely UAS, Bengaluru and ICRISAT, Patancheru to screen against Bengaluru and Patancheru isolates of SMD following "Leaf Stapling Technique" (Nene and Reddy, 1977). SMD infected leaves were stapled to leaves of test plants at 2 - 3 leaf stage as shown in the Plate 3. As the stapled leaflets from the infected plants gets dried, mites from the infected leaves migrate to healthy leaf and inoculates the virus. At both the locations, plants were scored for incidence of SMD at 15 days interval up to 75 days by counting the healthy plants (no mosaic symptoms) and diseased plants (with mosaic symptoms) as per the criterion followed in All India Co-ordinated Research Project on improvement of pigeonpea lines in sterility mosaic screening nursery and grouped them following the standard scale (Singh *et al.* 2003) and the same is given below.

Per cent disease incidence	**Reaction scale**
0-10% of plants infected	Resistant
10.1-30% of plants infected	Moderately Resistant
30.1-100% of plants infected	Susceptible

3.2 Statistical data analysis

3.2.1 Analysis of Variance (ANOVA)

The analysis of variance for scoring of sterility mosaic disease at two different locations is performed to test the significance of differences between genotypes on the basis of the model given by Panse and Sukhatme (1961).

ANOVA

Source of variation	Degree of freedom	Sum of squares	Mean sum of squares	'F' Ratio
Replications	r - 1	RSS	Mr	Mr/Me
Genotypes	t - 1	VSS	Mv	Mv/Me
Error	(r - 1) (t - 1)	ESS	Me	
Total	(rt - 1)	TSS		

Where,

r = Number of replications
t = Number of genotypes

3.2.2 Estimation of genetic parameters

In order to assess and quantify the genetic variability among the genotypes, different parameters were estimated as given below:

3.2.2.1 Estimation of variance components

Phenotypic and genotypic variances were estimated using the following formula.

$$\text{Genotypic variance } (\sigma_g^2) = \frac{\text{MSS (genotypes)} - \text{MSS (error)}}{\text{No. of replications}} = \frac{M_2 - M_3}{r}$$

$$\text{Phenotypic variance } (\sigma_p^2) = \sigma_g^2 + \text{MSS error} = \frac{M_2 - M_3}{r} + M_3$$

Where,

σ^2_p = Phenotypic variance

σ^2_g = Genotypic variance

σ^2_e = Environmental variance

3.2.2.2 Coefficient of Variability

Both genotypic and phenotypic coefficients of variability were estimated as per the method suggested by Burton and Devane (1953).

a) Genotypic Coefficient of Variation (GCV)

$$GCV = \frac{\sqrt{\sigma_g^2}}{\overline{X}} \times 100$$

Where,

σ^2_g = Genotypic variance

$\overline{X}$ = Mean of the characters

b) Phenotypic Coefficient of Variation (PCV)

$$PCV = \frac{\sqrt{\sigma_p^2}}{\overline{X}} \times 100$$

Where,

σ^2_p = Genotypic variance

$\overline{X}$ = Mean of the characters

GCV and PCV were classified as suggested Sivasubramanian and Menon (1973) into low (0 - 10%), moderate (10.1% - 20%) and high (>20%).

3.2.2.3 Heritability ($h^2_{(b.s.)}$)

Heritability in broad sense was computed as the ratio of genotypic variance to the total phenotypic variance as suggested by Hanson *et al.* (1956) and expressed as percentage.

$$h^2 = \frac{\sigma_g^2}{\sigma_p^2} \times 100$$

Where,

σ^2_g = Genotypic variance

σ^2_p = Phenotypic variance

Heritability (broad sense) estimates were categorized into low (5-10%), medium (10-30%), high (30-60%) and very high (> 60%) by Robinson *et al.* (1966).

3.2.2.4 Genetic advance (GA)

Genetic advance was estimated by using the formula given by Johnson *et al.* (1955).

$$GA = h^2 \times K \times \sigma_p$$

Where,

h^2 = heritability estimate

σ_p = Phenotypic standard deviation

K = Selection differential at 5% is equal to 2.06 of selection

3.2.2.5 Genetic advance as percent of mean (GAM)

$$GAM = \frac{GA}{\overline{X}} \times 100$$

Where,

$\overline{X}$ = Grand mean of the trait

GA = Genetic advance

The genetic advance as per cent of mean was categorized as suggested by Johnson *et al.* (1955) and the same is given below.

Low (0 - 10%), Moderate (10.1% - 20%) and High (20% and above)

3.2.2.6 Standard Error (S.E.m)

$$S.E.m = \sqrt{\frac{(N - 1)}{N}} \sqrt{\frac{(Error\ MS)}{r}}$$

Where,

N = Number of Individuals

Error MS = Error mean sum of square

r = Number of replications

3.2.2.7 Coefficient of Variation (C.V.)

$$CV = \frac{\sqrt{\text{Error MS}}}{GM} \times 100$$

Where,

Error MS = Error mean sum of square

GM = Grand mean

3.2.2.8 Coefficients of Skewness and Kurtosis

Skewness, the third degree statistics and kurtosis, the fourth degree statistics were estimated (Snedecor and Cochran, 1994) to understand the nature of distribution of quantitative traits. Genetic expectations of skewness (-3/4 d^2 h) reveal the nature of genetic control of the traits (Fisher *et al.* 1932). The parameter 'd' represent additive gene effects and 'h' represents dominance gene effects. Kurtosis indicates the relative number of genes controlling the traits (Robson, 1956). The adjusted mean values of quantitative trait were used to estimate coefficients of skewness and kurtosis using 'STATISTICA' software program.

3.3 Construction of c-DNA library and generation of expressed sequenced tags (ESTs)

Investigations on the construction of c-DNA library of pigeonpea and generation of expressed sequenced tags were carried out at the Centre of Excellence in Genomics (CEG), ICRISAT, Patancheru.

3.3.1 Plant material and Growth Conditions

Two pigeonpea genotypes namely ICP 7035 (resistant to SMD) and TTB 7 (highly susceptible to SMD) were used for constructing the cDNA libraries and generating the ESTs. Forty seeds from each genotype were sown in plastic bags filled with sterilized soil and were maintained in a glass house at 23 ± 3^{o}C

under 80% relative humidity (Plate 4). Fifteen days after sowing, leaves of ICP 7035 and TTB 7 were stapled with SMD infected leaves. Leaves from both susceptible and resistant parents were harvested at 45 and 60 days after sowing (DAS) for construction of cDNA libraries.

3.3.2 RNA isolation

RNA was isolated from the leaf tissue of ICP 7035 (resistant to SMD) and TTB 7 (highly susceptible to SMD) according to the protocol described by Schmitt *et al.* (1990). The leaf tissues which were stored in -80^{o}C were finely powdered using pestle and mortar in liquid nitrogen. This powder was then homogenized in extraction buffer (10 mM Tris, pH 8.0, 10 mM EDTA, 0.5% SDS) and saturated phenol before thawing and vortexed for 5 min in 50 ml centrifuge tubes. The sample was then mixed with Chloroform-Isoamyl alcohol (CIA) and briefly vortexed. These samples were centrifuged at 3000 rpm for 20 min at 4^{o}C, to settle down the debris. Supernatant was carefully transferred into a fresh 50 ml centrifuge tube and was mixed with equal volume of CIA. This was then centrifuged at 3000rpm for 15min. This step was repeated till clear layer of supernatant was obtained. The clear upper aqueous phase was transferred into a fresh 15 ml centrifuge tube and one third volume of 8 molar lithium chloride (Li Cl) was added and incubated overnight at 4^{o}C. The RNA pellet was obtained by centrifugation at 3000 rpm for 10 min at 4^{o}C, followed by 70% (v/v) ethanol wash twice. The pellet was air dried and resuspended in 0.1% Diethyl pyrocarbonate (DEPC) treated water and quantified by UV spectrophotometer at A_{260}:A_{280}. The integrity was assessed by e (ctrophoresis in 1.2% (w/v) equilibrated formaldehyde agarose gel (Sambrook *et al.* 1989) (Plate 5)

3.3.2.1 mRNA isolation

Following total RNA isolation, mRNA was isolated using the PolyA Tract® system 1000 kit (Promega, USA) according to manufacturer's instructions.

3.3.2.2 cDNA synthesis

cDNA was constructed using Super SMART™ PCR cDNA Synthesis kit (Clontech®, USA) as described in the manufacturer's instructions. The resulting cDNA was size fractioned on 1.2% agarose gel (Plate 4). cDNA fractions containing fragments >500 bp were eluted using GFX™ PCR DNA and gel band purification kit (GE Healthcare, UK) according to manufacturer's instructions.

3.3.2.3 Ligation and transformation

The eluted cDNA was ligated into the pGEM®- T Easy Vector (Promega, USA) and was purified using butanol precipitation. The resulting ligation mix was electroporated into One Shot® Top 10 ElectrocompTM cells (Invitrogen, USA). The transformants were spread on Luria agar plates containing ampicillin (100 µg/ml) for direct picking up of colonies by blue/white selection. The inserts were checked by digesting the insert with restriction enzyme, ECoRI for randomly selected cDNA clones. Clones were prepared in Nunc-Immuno™ 96 MicroWell™ plates ((Nunc™, Denmark)) containing Luria broth with 100 µg/ml ampicillin and grown for overnight at 37^{o}C on a rotary shaker at 220 rpm. Glycerol stocks in 96-well format were prepared by combining 38 µl of 60% glycerol with 150 µl of culture and frozen at -80^{o}C. The plasmid DNA from these clones (i.e. colonies) was extracted using a 96-well alkaline lysis method for sequencing (Sambrook *et al.* 1989).

3.3.3 EST sequencing, editing and assembly

Clones were randomly selected and on an average of 1000 clones per library were prepared. Plasmid DNA sequencing was performed by commercial DNA sequencing service provider (Macrogen Inc., Korea) using the standard M13 forward primer. The FASTA files containing the raw sequences were edited by the software Sequencher^{TM} 4.0 (Gene Codes Corporation, Ann Arbor. MI, USA) to remove the vector sequences. The vector trimmed sequences were subjected to EST trimmer **(EST trimmer)**, to trim poly-A ends and low quality sequences using perl script. High quality sequences of >100 bp were selected for further sequence analysis. ESTs were clustered and aligned into contigs and singletons using the CAP3 program (Huang and Madan, 1999). In order to assess the number of unique and overlapping transcripts among the four libraries one data set was generated between SMD-responsive genotypes. In addition to the above assembly of unigene sets, CAP3 analysis was also performed to libraries derived from SMD- resistant genotype and from SMD susceptible genotype individually.

3.3.3.1 Homology search and functional annotation

The unigene sequences were also characterized for nucleotide homology search against the EST datasets of selected legume species [pigeonpea (*Cajanus cajan*)-908*,* chickpea (*Cicer arietinum*)-7,097*,* soybean (*Glycine max*)-880,561*, Medicago* (*Medicago truncatula*)-249,625*,* common bean (*Phaseolus vulgaris*)-83,448, cowpea (*Vigna unguiculata*)-183,757 and *Lotus* (*Lotus japonicus*)-183,153] and selected model plant species [rice (*Oryza sativa*)-1,240,613*, Arabidopsis* (*Arabidopsis thaliana*)-1,527,298 and poplar *(Populus alba*)-418,223] available at National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) using BLASTN algorithm (**NCBI EST database**). A match was considered significant at E-value ≤1E-05. Each unigene dataset was subjected to BLASTX analysis against the non-redundant protein database of

UniProt to deduce a putative function. Sequence similarity was considered as significant at E-value ≤1E-08. Each unigene was assigned a putative cellular function based on the significant database hit with the lowest e-value. Subsequently, unigenes that showed a significant BLASTX hit were used for functional annotation based on Gene Ontology categories from UniProt database (UniProt-GO). This process allowed assignment of unigenes to the GO functional categories of biological process, cellular component and molecular function. Distribution of unigenes was further investigated in terms of their assignment to sub-categories of the main GO categories.

3.3.3.2 In *silico* expression

In order to identify the differentially expressed genes between SMD-responsive genotypes, 328 contigs coming SMD-responsive genotypes were analyzed by using IDEG.6 web interface tool (**IDEG.6 analysis tool**, Romualdi *et al.* 2003). The IDEG.6 web tool allows running six different statistical analysis for the detection of differentially expressed genes in multiple tag experiments. For pair-wise comparisons, the Audic and Claverie test, Fisher exact test and chi-square tests (χ^2) were used and in multiple comparisons R-statistics test, Greller and Tobin test and chi-square tests (χ^2) were used.

3.3.3.3 Identification and development of SSR markers

A total of 5,085 unigenes were searched for SSR mining by an assembly of 3,788 ESTs generated in the present study and 5,680 ESTs generated from *Fusarium* wilt disase responsive study (Raju *et al.* 2010) and 908 sequences downloaded from public domain. SSRs were searched using a perl script program, *MISA* (*MIcroSAtellite*) (**SSR identification tool,** Thiel *et al.* 2003, Skiba *et al.* 2005**)**. The SSR motifs, with repeat units more than five times in di-, tri-, tetra-, penta- and hexa- nucleotides were considered as SSR search criteria in *MISA* script. The Primer3 programme (Rozen *et al.* 2000) was used for

designing the primer pairs for SSRs and were custom synthesized by MWG (MWG-Biotech AG, India).

3.4 Construction of genetic linkage map and identification of QTL associated with SMD resistance

3.4.1 DNA isolation from Parents and F_2 lines

DNA was extracted from the young tissues of parents and F_2 using standardized high throughput mini DNA extraction method (Cuc *et al*. 2008).

3.4.1.1 Sample preparation:

- Leaves were harvested from 15 days old seedlings.
- Leaf tissue of 70-100mg was placed in 12 x 8-well strip tube with strip cap (Marsh Biomarket, USA) in a 96 deep-well plate together with two 4mm stainless steel grinding balls (Spex CertiPrep, USA)

3.4.1.2 CTAB extraction

- Each sample was mixed with 450µl of preheated (65ºC) extraction buffer (100 mM Tris-HCl (pH-8, 1.4 M NaCl, 20mM EDTA, CTAB (2-3%w/v), β- mercaptoethanol) was added to each sample and secured with eight strip caps.
- Samples were processed in a Geno Grinder 2000 (Spex CertiPrep, USA), following the manufacturers instructions, at 500 strokes/min for 5 times at 2 min interval.
- Plate was fitted into locking device and incubated at 65ºC for 10 min with shaking at periodical intervals.

3.4.1.3 Solvent extraction

- Each of the sample were mixed with 450µl of chloroform-isoamylalcohol (24:1) by inverting twice.
- Plate was centrifuged at 5500 rpm for 10 min. The aqueous layer (300µl) is transferred to fresh strip tubes (Marsh Biomarket, USA)

3.4.1.4 Initial DNA precipitation:

- 0.7 vol (210µl) of isopropanol (stored at –20ºC) was added to each sample and inverted once to mix.
- Plate was centrifuged at 5000 rpm for 15 min.
- Supernatant was decanted from each sample and pellet was air dried for 20 min.

3.4.1.5 RNase treatment:

- 200µl low salt TE (10 mM Tris EDTA (pH-8)) and 3µl RNase was added to each sample and incubated at 37ºC for 30 min.

3.4.1.6 Solvent extraction:

- 200µl of phenol-chloroform-isoamylalcohol (25:24:1) was added to each sample and inverted twice to mix.
- Plate was centrifuged at 5000 rpm for 5 min.
- Aqueous layer was transferred to a fresh 96 deep-well plate (Marsh Biomarket, USA).
- 200µl chloroform-isoamylalcohol (24:1) was added to each sample and inverted twice to mix.
- Plate was centrifuged at 5000 rpm for 5 min.
- Aqueous layer was transferred to a fresh 96 deep-well plate.
- 315µl ethanol-acetate solution (30ml ethanol, 1.5ml 3M NaOAc (pH-5.2)) was then added to each sample and placed in –20ºC for 5 min.
- Plate was again centrifuged at 5000 rpm for 5 min.
- Supernatant was decanted from each sample and pellet was washed with 70% ethanol.
- Plate was centrifuged at 6000 rpm for 10 min.
- Supernatant was again decanted from each sample and samples were air dried for approximately 1 hour.
- Pellet was resuspended in 100µl low-salt TE and stored at 4ºC.

3.4.2 Quantification of DNA

DNA quality was checked and quantified on 0.8% agarose gel with known concentration of uncut lambda DNA standard (Plate 6).

3.4.3 Analysis of parents and F_2 using SSR markers

Initially the parents, TTB 7 and ICP 7035 were screened for polymorphism by using 3236 pigeonpea genomic specific SSR markers available at ICRISAT and 84 newly developed EST SSRs.

3.4.4 PCR Amplification

All PCR reactions were performed in 5 µl reaction mixture using ABI thermal cycler using a touchdown amplification profile (Table 3). The reaction mixture consisting of 5ng DNA template, 0.25µl of 2mM dNTPs, 0.5µl of (1pmole/µl M13 tailed forward: 2 pM/µl reverse) SSR primer, 0.5µl of 2pmole/µl of M13 tailed primer, 10X PCR buffer which includes 15mM $Mgcl_2$ (Zonaki), and 0.3U (0.06µl of 3U/µl) of *Taq* DNA polymerase (Zonaki). The details on touch down PCR profile for the targeted microsatellite loci is presented below.

60-55

Steps	Temperatures (^{0}C)	Time	
Initial denaturation	95	3 min	
Denaturation	94	20 sec	5 cycles
Annealing	60	20 sec	
Primer extension	72	30 sec	
Denaturation	94	20 sec	30 cycles
Annealing	56	20 sec	
Primer extension	72	30 sec	
Final extension	72	20 min	
Store at	4		

3.4.5 Electrophoresis

Before loading PCR Products in the sequencing gel, amplification was checked on 1.2 per cent agarose gel. For the separation of DNA fragments, capillary electrophoresis was used.

3.4.5.1 Capillary electrophoresis (ABI 3730 DNA sequencer)

After confirming the PCR amplification on 1.2 per cent agarose gel, Amplified products were separated by using capillary electrophoresis. For post PCR multiplexing, 1.2µl PCR products of each of FAM, VIC, NED and PET-labeled products were pooled and mixed with 7µl Hi-Di formamide, 0.05µl of Liz-500 size standard (Applied Biosystems, USA) and 2.95 µl of double distilled water (adjusted as per dye and number of primers used for multiplexing). The pooled PCR amplicons were denatured and size fractioned using capillary electrophoresis on an ABI-3730 automatic DNA sequencer (Applied Biosystems, USA). Allele sizing (A, B, H and missing (-) peak patterns) of the electophoretic data was carried out using GeneMapper® version4.

3.4.6 Genotyping data analysis

3.4.6.1 Linkage Analysis

Eighty four polymorphic SSR markers were used for genotyping 130 F_2 individuals. Chi-square (χ^2) test was performed on the genotypic data to test the null hypothesis of expected 1:2:1 Mendelian segregation on all the scored markers. Of these, 5 markers showed segregation distortion (SD). Due to less number of polymorphic markers, even the distorted markers were also used for linkage map construction and QTL analysis.

The linkage analysis was performed using MAPMAKER/ EXP V 3.0 (Lander *et al.* 1987, Lincoln *et al.*1992). A minimum LOD score of 3.0 and maximum recombination fraction (θ) of 0.4 were set as threshold values for linkage group determination. Eleven linkage groups were defined with the "Make Chromosome" command and a set of markers were used as anchors. The most likely marker order within each linkage group was estimated by using three point analyses ("three point" command). Marker orders were confirmed by comparing the Log-likelihood of the possible orders using multipoint analysis ("compare" command) and by permuting all the adjacent triple orders ("ripple" command). In the second step, LOD score was set to 3.0 in order to include new markers in the linkage groups. The "try" command was used to determine the exact position of the new marker orders. The new marker orders were again confirmed with the "first order", "compare" and or "ripple" commands. Recombination fraction was converted into map distances in centiMorgans (cM) using Kosambi mapping function (Kosambi, 1944). The intermarker distances calculated from mapmaker were used to construct linkage map by using MAPCHART version 2.2 (Voorrips, 2006).

3.4.6.2 Quantitative trait (QTL) Analysis

Genotyping and phenotyping data against SMD were analyzed for mapping QTLs by using the method composite interval mapping (CIM) proposed by Zeng (1994) in the Windows QTL Cartographer, version 2.5 (Wang *et al.* 2007). CIM analysis was performed using the Model 6, scanning the genetic map and estimating the likelihood of a QTL and its corresponding effects at every 1 cM, while using significant marker cofactors to adjust the phenotypic effects associated with other positions in the genetic map. The number of marker cofactors for the background control was set by forward–backward stepwise regression. A window size of 10 cM was used, and therefore cofactors within 10 cM on either side of the QTL test site were not included in the QTL model. Thresholds were determined by permutation tests (Churchill and Doerge, 1994;

Doerge and Churchill, 1996) using 1,000 permutations and a significance level of 0.05. QTLs were determined significant if the corresponding likelihood ratio (LR) score was greater than 11.5 (equal to a LOD score of 2.5).The per cent phenotypic variance (PV) explained by a QTL was estimated at the highest probability peaks.

IV. EXPERIMENTAL RESULTS

The results obtained from the present investigation are furnished under the following headings.

4.1 Development of F_2 and $F_{2:3}$ mapping population and inheritance studies.

4.2 Generation of expressed sequenced tags (ESTs) and development of EST-based simple sequence repeat (SSR) markers.

4.3 Construction of genetic linkage map.

4.4 Identification of QTL associated with SMD resistance.

4.1 Development of mapping population and inheritance studies

4.1.1 Development of F_2 and $F_{2:3}$ mapping population

Growing of parents for crossing and raising of mapping population was carried out under nylon net coverings to avoid cross pollination through insect pollinators. TTB 7 a highly susceptible cultivar to SMD was crossed with a resistant parent ICP 7035 and the resultant F_1 was raised. F_2 seeds collected from a single F_1 plant were used to obtain 224 F_2 plants. All the 224 F_2 plants were selfed to obtain the $F_{2:3}$ mapping population and used for phenotyping against SMD at two different locations *viz*., UAS, Bengaluru and ICRISAT, Patancheru.

4.1.2 Inheritance studies against SMD

Study of variability in the pigeonpea sterility mosaic pathogen by Reddy *et al*. (1993), revealed the occurrence of five different isolates of the pigeonpea sterility mosaic virus (PPSMV) in India. Amongst them, Bengaluru isolate was most virulent compared to other isolates (Kulkarni et *al*. 2003). This has necessitated for phenotyping of SMD at two locations to identify strain-specific

resistance sources. Further, information on genetics and mode of inheritance of strain-specific resistance is also lacking for the disease. The present investigation was done to elucidate the strain-specific inheritance pattern of resistance for two isolates of the sterility mosaic pathogen in pigeonpea.

4.1.2.1 Reaction of parents and F_1s to SMD

Reaction of parents and F_1s to SMD for Bengaluru and Patancheru isolates is presented in tables 1 and 2 respectively. For both the isolates susceptible control (ICP 8863) exhibited 100% disease incidence.

4.1.2.1.1 Bengaluru Isolate

At Bengaluru, the resistant parent ICP 7035 showed 6.6 per cent disease incidence where as the susceptible genotype TTB 7 showed 100 per cent disease incidence with severe mosaic symptoms. All the F_1s of the susceptible × resistant cross (TTB 7 × ICP 7035) were susceptible.

4.1.2.1.2 Patancheru Isolate

At Patancheru, ICP 7035 showed zero per cent disease incidence with no apparent symptoms while the susceptible genotype TTB 7 showed 100 per cent susceptibility with severe mosaic symptoms. The F_1s of the susceptible × resistant cross (TTB 7×ICP 7035) were susceptible.

4.1.2.2 Reaction of $F_{2:3}$ segregating population to SMD

The SMD phenotyping which was destructive was avoided in F_2, so that seeds can be harvested from these plants to obtain F_3 generation. The phenotyping

data against SMD recorded in $F_{2:3}$ progenies of TTB 7 × ICP 7035 for Bengaluru and Patancheru isolates is presented in table 3. The mean disease reactions are presented in Appendix I.

At Bengaluru, out of 224 $F_{2:3}$ families screened against SMD, only two were resistant, 9 were moderately resistant and 213 families were susceptible. While at Patancheru, out of 219 $F_{2:3}$ families screened against SMD, 44 were resistant, 11 were moderately resistant and 164 were susceptible. Resistant families were relatively low for Bengaluru isolate as compared to Patancheru isolate.

4.1.3 Analysis of variance (ANOVA)

The mean SMD percentage disease reaction of 15 plants for each $F_{2:3}$ progeny against Bengaluru and Patancheru isolates were subjected to analysis of variance (ANOVA). The ANOVA obtained by completely randomized design for Bengaluru and Patancheru isolates is presented in table 4 and 5 respectively. The F calculated value was significant at 1% level of significance, suggesting that the genotypes under consideration showed considerable variation for the SMD disease reactions.

4.1.4 Genetic components of variation

The data on genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), heritability and genetic advance as percent of mean (GAM) for SMD is presented in table 6.

In general, PCV estimates are higher than GCV estimates. GCV and PCV estimates for SMD were high for Patancheru isolate (57 and 63.5 %) as compared

to Bengaluru isolate (21.5 and 30.19 % of GCV and PCV respectively) indicating higher magnitude of variation. Heritability was high for both Bengaluru isolate (51%) and Patancheru isolate (80.2%). Genetic advance as per cent of mean was very high for Patancheru isolate (105.03) while it was 31.68 % for Bengaluru isolate.

4.1.5 Frequency distribution of $F_{2:3}$ segregating population to SMD

Descriptive statistics of mean SMD incidence in $F_{2:3}$ population of TTB 7 × ICP 7035 is presented in table 7. The SMD incidence for Bengaluru isolate ranged between 4.1 to 100% with a mean of 78.94 %. The coefficient of skewness was -1.45 while that of kurtosis was 2.11. SMD incidence for Patancheru isolate ranged between 0 to 100% with a mean of 55 %. The coefficient of skewness was -0.49 while that of kurtosis was -1.09.

The variation existed in the $F_{2:3}$ population for SMD incidence is represented graphically using frequency distribution of means for two different isolates (Fig. 1 and 2). The disease scores were plotted on X-axis against genotype frequency on Y - axis with equal class intervals. The resulting histogram showed near normal curves for both the isolates with skewed towards susceptibility for SMD. In general the distribution of $F_{2:3}$ were within the parental limits for both the isolates.

4.2 Generation of expressed sequenced tags (ESTs) and development of EST-based simple sequence repeat (SSR) markers.

4.2.1 Generation of SMD - responsive ESTs

A total of four unidirectional cDNA libraries were constructed from the two genotypes (ICP 7035 and TTB 7) which represent parents of mapping population segregating for SMD. Details of EST generated from different cDNA libraries is presented in figure 3. Using Sanger sequencing approach, 1920 ESTs were generated from cDNA libraries of each SMD-responsive genotypes, resulting in 3840 raw ESTs. All the 3840 raw ESTs were subjected to stringent screening for shorter (<100 bp) and poorer quality sequences and resulted with 3,788 high quality ESTs. With an objective to minimize redundancy, clustering and assembly was done for 3,788 high quality ESTs to define unigenes for SMD-responsive ESTs. This has resulted 1,308 unigenes with 328 contigs and 980 singletons. All the EST sequences (3788) were deposited in the dbEST of GenBank (NCBI www.ncbi.nlm.nih.gov).

4.2.2 Frequency and distribution of pigeonpea ESTs.

Frequency and distribution of pigeonpea ESTs among the assembled contigs is presented in figure 4. The cluster analysis of 3,788 ESTs resulted in 1,308 unigenes with 328 contigs and 980 singletons. The number of ESTs in a contig ranged from 2 to 282, with an average of 9 ESTs per contig. As expected, contigs with two EST members exhibited a higher percentage (28%) than contigs with three or more EST members.

4.2.3 Comparison of pigeonpea unigenes with other plant EST databases

Detailed results of BLASTN similarity for all the unigenes sets (1,308) are given in table 8. All the unigenes were analyzed for BLASTN similarity search against available EST datasets of legume species namely chickpea (*Cicer arietinum*), pigeonpea (*Cajanus cajan*), soybean (*Glycine max*), cowpea (*Vigna unguiculata*) medicago (*Medicago truncatula*), lotus (*Lotus japonicus*), common bean (*Phaseolus vulgaris*) and three model plant species namely *Arabidopsis* (*Arabidopsis thaliana*), rice (*Oryza sativa*) and poplar (*Populus alba*). An E-value significant threshold of ≤1E-05 was used for defining a hit.

Analysis of unigenes found highest similarity of 72.3% with soybean, followed by cowpea (62.4%), medicago (61.3%), common bean (59.9%), lotus (56.4%), and the least similarity was observed with chickpea (38.7%). Comparative BLASTN analysis of pigeonpea unigenes with EST databases of model plant species showed, high similarity with poplar (51.8%), followed by Arabidopsis (50.9%) and the least similarity with rice (39.7%). Of 1,308 unigenes, 1,015 (77.5%) showed significant similarity with ESTs of at least one plant species analysed, while 114 (8.7%) showed significant similarity across all the plant EST databases. It is also interesting to note that 4 unigenes did not show any homology with the legume species or any plant species examined.

4.2.4 Functional categorization of pigeonpea unigenes

To identify the putative function of unigenes compiled in this study, the unigenes designed were compared against the non-redundant UniProt database, using the BLASTX algorithm and presented in figure 5. Out of 1,308 unigenes, 1,257 (96.1%) showed hits against UniProt data base using BLASTX algorithm and 51 (3.89%) were showing no hit. Among 1,257 unigenes, only 638 unigenes

(48.77%) were considered as significant hits at a threshold of 1e-08 and the remaining 568 unigenes (47.34%) were non-significant.

The unigenes (638) which showed significant hit (≤1E-08) against the UniProt database were categorized according to the UniProt Gene Ontology (GO) functional category denomination and presented in figure 6 and 7. It was observed that one gene could be assigned to more than one principal category, which exceeded the number of unigenes analyzed. Out of 638 unigenes which showed significant similarity with known proteins, only 448 were successfully annotated among three main principal GO categories i.e. biological process, molecular function and cellular component. A total of 44 were grouped under biological process, 48 under molecular function and 43 under cellular component. Under the biological process, cellular process accounted to 33, followed by metabolic process (30), development process (8) and response to stimulus (2). In the cellular component category, 46 unigenes coded for cell part, 35 to organelle, and 23 to organelle part, while majority of the unigenes in molecular function category were involved in binding (32) and catalytic activity (14). The remaining 190 unigenes which could not be categorized were classified as "unclassified". Enzyme IDs were retrieved for the unigenes from the UniProt database and were distributed into one of the six major enzyme classes such as oxidoreductases (40) followed by transferases (30), hydrolases (30), lyases (18), ligases (7) and isomerases (10).

4.2.5 *In silico* expression analysis

Differentially expressed genes between libraries of SMD resistant (ICP 7035) and susceptible (TTB7) genotypes are presented in the figure 8 and cells covering different degrees of blue color represent extent of gene expression. The identification of these differentially expressed genes among specific cDNA libraries of SMD-responsive genotypes based on EST counts in each contig was

done using a web statistical tool IDEG.6. A total of 20 genes were differentially expressed between ICP 7035 (SMD- resistant) and TTB 7 (SMD- susceptible) genotypes.

4.2.6 Identification and development of genic microsatellite markers

EST based markers can assay the functional genetic variation compared to other class of genetic markers and hence were targeted for marker development. The unigene set based on generated ESTs were used for development of simple sequence repeats (SSR). By using 10,376 ESTs (3788 ESTs generated in this study and 5680 ESTs generated in *Fusarium* wilt disease responsive study (Raju *et al.* 2010) and 908 available in public domain ESTs), 5085 pigeonpea unigenes were developed and were searched for SSRs using a perl script program of MISA tool (MIcroSAtellite) and the results obtained is presented in table 9. Out of 5,085 pigeonpea unigenes, 3,583 SSRs were identified at the frequency of 1/800 bp in coding regions, 698 ESTs contained more than one SSR and 1,729 SSRs were found as compound SSRs.

In terms of distribution of different classes of SSRs i.e. mono-, di-, tri-, tetra-, penta- and hexa-nucleotide repeats, mononucleotide SSRs (3,498) contributed to the largest proportion (97.6%) (Table 9). Only a limited number of SSRs of other classes were found (figure 9) like di and tri- nucleotide SSRs accounted for 40 (1.1%) and 33 (0.9%) respectively, followed by nine tetrameric, two pentameric and one hexameric microsatellites.

In general, mononucleotide SSRs are not included for primer designing and synthesis. However, due to limited number of SSR markers currently available for pigeonpea in public domain and in a separate study some mononucleotide SSRs were found polymorphic (Saxena *et al.* 2009a), primer pairs were designed for 383

SSRs including mononucleotide SSRs. From these 383 SSRs a total of 94 primer pairs were considered for validation after excluding the primers for monomeric SSR motifs and compound SSRs with mononucleotide repeats. However based on repeat number criteria, such as five minimum for di-, tri-, tetra, penta-nucleotides, primer pairs were synthesized only for 84 SSRs. The details of 84 newly developed pigeonpea EST-SSR primers along with corresponding SSR motif, primer sequence, annealing temperature and product size are provided in Appendix II.

4.3 Construction of genetic linkage map

4.3.1 Genotyping

Genotyping of parents and F_2 were carried out at ICRISAT, Patancheru. A total of 3236 pigeonpea genomic SSR markers available at ICRISAT along with newly synthesized 84 EST-SSR (genic SSR) in the present study were analyzed on two genotypes ICP 7035 and TTB 7 which represent parents of mapping population segregating for SMD and the details is presented in table 10. A snapshot showing capillary electropherogram for P1 (TTB7), P2 (ICP7035) and the resulting hybrid is presented in figure 10.

Out of 3236 genomic SSRs screened for the parents, 2055 primer pairs provided scorable amplified products. From these 2055 primer pairs, 354 primer pairs produced a number of faint bands indicative of non-specific amplifications, 1618 were monomorphic and 83 primer pairs showed clear polymorphism, while 1181 primer pairs were not amplified (table 10).

Out of 84 genic SSR markers screened for the parents, 52 primer pairs provided scorable amplified products. From these 52 primer pairs, 31 primer pairs produced a number of faint bands indicative of non-specific amplifications and 20

were monomorphic. Only one marker (ICPeM0075) showed clear polymorphism (table 10). Over all, out of 3320 SSR markers (3236 genomic+84 genic), 2107 (63.5%) could amplify and 84 (83 genomic + 01 genic) SSR markers were found to be polymorphic (2.5%).

4.3.2 Linkage map construction

Since, there is no comprehensive linkage map developed in pigeonpea, linkage map construction is one of the major objective of the present investigation. The linkage map was constructed using software MAPMAKER/EXP V 3.0 (Lander *et al.,* 1987; Lincoln *et al.,* 1992). Multipoint analysis with minimum LOD scores of 3.0 and maximum recombination fraction (θ) of 0.5 were set as threshold for linkage group determination.

Only 84 markers which were polymorphic are used for genotyping and linkage map construction in the F_2 mapping population of the cross TTB 7×ICP 7035. The chi-square (χ^2) test was conducted to test the Mendelian segregation ratio (expected 1:2:1) and five markers showed segregation distortion (SD). But due to paucity of polymorphic markers, all the 84 markers were used for linkage map construction and presented in the table 11 and figure 11.

A total of 82 markers were mapped on 11 linkage groups (LGs) spanning 539.5 cM and two markers remained ungrouped. The number of markers mapped per linkage group ranged from three (LG 11) to twelve (LG 7). The lengths of linkage groups were ranging from 4.2 cM (LG 11) to 104.2 cM (LG 3) with an average distance of 6.1 cM. The linkage map constructed based on TTB 7 × ICP 7035 F_2 mapping population was used for identification and mapping of QTL for resistance to SMD.

4.4 Identification of QTL associated with SMD resistance.

4.4.1 QTL Mapping

The foremost step towards QTL mapping is to have linkage map with good coverage of markers. The map developed from the F_2 cross of TTB 7 × ICP 7035 was used for QTL analysis by using phenotyping data of SMD derived from $F_{2:3}$ family means. In order to take care of distribution abnormalities, arc-sine transformed means for SMD were utilized for QTL identification. QTLs associated with resistance to Bengaluru and Patancheru isolates of SMD is presented in table 12 and figure 12 and graphically represented in figure 13 and 14.

4.4.1.1 Bengaluru Isolate

For Bengaluru isolate of SMD, two QTLs flanked by the markers *CcM2337-CcM0416* and *CcM0970-CcM2485* with LOD score 2.82 and 3.56, respectively were identified. The first QTL (*CcM2337-CcM0416*) positioned on LG 3 explained 10.39 per cent of the phenotypic variation with 0.17 additive effect. The second QTL (*CcM0970-CcM2485*) located on LG 7 accounted for 15.74 per cent of phenotypic variation with an additive effect of 0.22 (Table 12, Figure 12 and 13).

4.4.1.2 Patancheru Isolate

Two QTLs at marker interval *CcM2149 - CcM0468* (LG 2) and *CcM1825-CcM1895* (LG 11) were detected with 3.95 and 6.89 LOD scores, respectively for Patancheru isolate of SMD. The QTL (*CcM2149- CcM0468)* explained 12.3 per cent phenotypic variation with 0.24 additive effect. The second QTL (*CcM1825-CcM1895)* explained 24.69 per cent of phenotypic variation having an additive effect of 0.33 (Table 12, Figure 12 and 14).

V. DISCUSSION

The results obtained from the present investigation are discussed under the following headings to arrive at valid conclusions.

5.1 Development of F_2 and $F_{2:3}$ mapping population.

5.2 Inheritance studies for sterility mosaic disease (SMD) resistance.

5.3 Generation of expressed sequenced tags (ESTs) and development of EST-based simple sequence repeat (SSR) markers.

5.4 Construction of genetic linkage map.

5.5 Identification of QTL associated with SMD resistance.

5.1 Development of F_2 and $F_{2:3}$ mapping population.

In plants, QTL mapping is commonly performed using F_2 or BC individuals derived from the cross involving two inbred lines. Typical QTL mapping statistics assume that each F_2 individual is genotyped for the markers and phenotyped for the trait. For plant traits, Zhang and Xu (2004) suggested the use of average phenotypic values of F_3 progeny derived from selfing F_2 plants in place of the F_2 phenotype itself. All F_3 progenies derived from the same F_2 plant belong to the same $F_{2:3}$ family, denoted by $F_{2:3}$. If the size of each $F_{2:3}$ family (the number of F_3 progeny) is sufficiently large, the average value of the family will represent the genotypic value of the F_2 plant, and thus the power of QTL mapping may be significantly increased. The strategy of using F_2 marker genotypes and F_3 average phenotypes for QTL mapping in plants is quite similar to the daughter design of QTL mapping in dairy cattle (Zhang and Xu, 2004).

In the present study, a population of 224 F_2 plants were selfed to obtain the $F_{2:3}$ mapping population and used for phenotyping against SMD at two different locations *viz*., UAS, Bengaluru and ICRISAT Patancheru to

understand the nature of inheritance to SMD and to identify markers linked to the disease. The mapping population consisting of 224 $F_{2:3}$, exhibited significant variation for resistance to SMD. The magnitude of variation was high as revealed by phenotypic coefficient of variation with high heritability and the population revealed substantially high heritable variation.

5.2 Inheritance studies for SMD resistance

A basic knowledge of inheritance and number of genes governing the traits are essential for efficient selection. There are conflicting reports about the genetics of resistance to sterility mosaic disease claiming both susceptibility and resistance to be dominant. However in most cases, susceptibility was shown to be dominant and resistance to be under the control of recessive genes (Singh *et al*. 2003). The task of developing resistant varieties has been complicated in view of the reported genetic variability of the pathogen. This dynamic nature of the SMD has warranted the identification and use of isolate specific sources of resistance in the crop improvement programmes. Hence, the present investigation was undertaken to elucidate the mode of inheritance for Bengaluru and Patancheru isolate of sterility mosaic disease resistance.

The present study was carried out by crossing a promising resistant line ICP 7035 with the susceptible line TTB 7. All the F_1s screened for SMD infection for Bengaluru and Patancheru isolates were found to be susceptible indicating the susceptibility to be dominant over resistance. Similar observations on susceptibility being under the influence of dominant genes have been reported in pigeonpea (Singh *et al.* 1983; Sharma *et al.* 1984; Amala balu, 1992; Nagaraj *et al.* 2004 and Ganapathy *et al.* 2009). On the contrary, susceptibility under the influence of recessive genes was reported by Murugesan *et al.* (1997). In another study on inheritance of resistance to two isolates of SMD, Srinivas *et al.* (1997) used three crosses and observed that resistance was dominant in two crosses and susceptibility in the other cross.

The phenotyping against SMD was carried out in $F_{2:3}$ progenies of TTB 7 × ICP 7035 for two different isolates at two locations. At Bengaluru, SMD incidence varied from 4.1 to 100 per cent with wide range of variability. Out of the 224 $F_{2:3}$ families, none of the plants were immune, only 2 plants were resistant, nine were moderately resistant and 213 families were susceptible. Absence of immune plants and rare occurrence of extreme phenotypes indicates polygenic control of SMD inheritance and higher level of virulence to SMD isolate prevailing in Bengaluru location and the same was reported by Kulkarni *et al.* (2003). Inheritance of SMD seems to be complex and does not fit a simple gene- for-gene interaction. While at Patancheru, 0 to 100 per cent SMD incidence was recorded with high variability. Out of 219 $F_{2:3}$ families, more number of resistant plants (44) were recorded as compared to Bengaluru. For Patancheru, relatively more number of plants showed resistance to SMD because of lower level of virulence to SMD isolate prevailing in Patancheru location (Kulkarni *et al.* 2003).

In the present study, for both Bengaluru and Patancheru isolates, susceptibility is controlled by dominant genes, there fore number of plants with high level of resistance to SMD are fewer in $F_{2:3}$ generation. Resistance to SMD in general is controlled by recessive genes and the causal organism has higher level of virulence, hence it is very difficult to realize plants with resistance to SMD in Bengaluru location. There is a need to search sources with high level resistance from either primary or tertiary gene pools.

The pattern of frequency distribution of SMD incidence in the $F_{2:3}$ were found to be continuous depicting quantitative nature of SMD resistance for both Bengaluru and Patancheru isolates. However, large number of plants could be classified into categories of moderately resistant and susceptible class. Only few plants were classified in to resistant group. In the present study, frequency distribution of SMD was platykurtic and negatively skewed

indicating involvement of large number of segregating genes with majority of them having increasing effects.

Genetics of SMD has been studied earlier and depending on the resistance source, SMD isolate and scoring method, resistance to SMD in pigeonpea appears to be complex (Saxena, 2008). The present study reveals quantitative inheritance of SMD for both the Bengaluru and Patancheru isolates. In contrast, resistance to SMD has been reported to be controlled by single gene (Murugesan *et al.* 1997; Srinivas *et al.* 1997), oligogenic (Singh *et al.* 1983; Sharma *et al.* 1984; Amala Balu and Rathnasamy 2003; Nagaraj *et al.* 2004; Ganapathy 2009).

5.3 Generation of expressed sequenced tags (ESTs) and development of EST-based simple sequence repeat (SSR) markers.

Plants are known to have developed integrated defence mechanisms against viral infections by altering spatial and temporal transcriptional changes. The EST approach was successfully utilized in identification of disease-responsive genes from various tissues and growth stages in chickpea (Coram and Pang, 2005), lathyrus (Skiba *et al.* 2005), soybean (Iqbal *et al.* 2005), rice (Jantasuriyarat *et al.* 2005) and ginseng (Goswami and Punja 2008). Many earlier studies have shown that resistant genotypes have efficient mechanisms for stress perception and enhanced expression of defence-responsive genes, which maintain cellular survival and recovery (Reddy *et al.* 2008). Hence, the present study was undertaken to identify catalog of defence related genes in response to SMD infection in pigeonpea by generating ESTs from stress challenged leaf tissues at various time intervals.

5.3.1 Generation of cDNA libraries and unigene assemblies

Plants are encountered with many biotic stress factors which includes bacterial, fungal and viral infection. Roots and leaves are the primary sites of infection by these organisms. Therefore, a total of 4 cDNA libraries were generated from specifically targeted leaf tissue of ICP 7035 and TTB 7 infected with SMD at time intervals of 45 and 60 days after sowing. In total 3,788 high quality ESTs were generated from SMD challenged genotypes. Sequence clustering and assembly process of all assembled 3,788 high quality ESTs resulted in 1,308 unigenes.

5.3.2 Functional annotation of pigeonpea unigenes

Homology searches (BLASTN and BLASTX) against other plant ESTs and functional characterization were done for all the 1,308 unigenes. Of the 1,308 unigenes assembled from all the pigeonpea ESTs (Table 8), 1,015 (77.5%) had significant similarity with ESTs of at least one plant species analyzed, 114 (8.7%) unigenes showed significant similarity with ESTs of all analyzed plant species, while 4 (0.3%) were found to be novel to pigeonpea. A high significant similarity was observed with soybean (72.3%), followed by EST databases of other legumes such as cowpea (62.4%), *Medicago* (61.3%), common bean (59.9%), lotus (56.4%) and model plant species i.e. poplar (51.8%), *Arabidopsis* (50.9%), rice (39.7%) and the least percentage of similarity was observed with chickpea (38.7). These observations are in accordance with phylogenetic relationships of legumes (Wojciechowski *et al.* 2000).

The pigeonpea ESTs showed higher similarity to legume ESTs databases (38.7-72.3%) of the legume species than model species (39.7-51.8%). Comparative analysis of pigeonpea ESTs with monocot species like rice (39.7%) showed that the percentage of significance is much lower

compared to any other legume species, inspite of larger EST repository. This is clearly attributed to phylogenetic divergence between dicots and monocots in course of evolution. These comparisons also indicate that several unigenes that were absent in analysed non-legumes but present in all legume species may be specifically confined to legumes.

BLASTX analyses indicated that those ESTs without significant similarity to any other protein sequences in the existing database may be novel and involved in plant defence responses. In this study, 51 novel ESTs which represented a significant addition to the existing pigeonpea EST resources provides valuable information for further predictions / validation of gene functions in pigeonpea.

A large number of unigenes were involved in cell part, organelle, binding, organelle part, metabolic and cellular process among the significantly annotated ones. These observations are consistent with the earlier reports of functional categorization studies on host-pathogen interactions in rice (Jantasuriyarat *et al.* 2005), soybean (Alkharouf *et al.* 2004), barley (Zhang *et al.* 2004) and tall fescue (Mian *et al.* 2008). However, the sequences encoding activities related to categories such as regulation of biological process and response to stimulus are 7 and 2 respectively. This was possibly due to the fact that the ESTs generated from SMD stress are chloroplast binding proteins. Earlier studies of Lee *et al.* (1998) and Ablett *et al.* (2000), also reported that photosynthesis-related proteins were the most prevalent from aerial parts of the plant, which would help to make energy related activities such as cell division, growth, elongation and development. In this study also, photosynthesis related genes were identified in larger proportion of SMD-responsive cDNA libraries derived from leaf tissues.

5.3.3 *In silico* differential gene expression

The invasion of pathogen not only results in expression of novel genes/ transcripts, but also in altering the abundances of different ESTs resulting in induction or repression. This was evident from differential expression of 20 genes between SMD-responsive genotypes. Significant number of unigene sequences related to proteins like kinases, phosphatases, peroxidases, ribonucleases, endochitinases, glucanases and hormones like Abscisic acid responsive (ABA) genes were identified to be differentially expressed and are known to play a vital role in defence mechanism. The protein coding for ABA-responsive protein (Figure 8, UniProt ID: Q06930- ABR18), which is involved in stimulus mechanism and cell localization etc. during plant development and one of the vital roles is in defence mechanism during biotic stress signaling was identified to be expressed relatively higher in SMD resistant pigeonpea genotype ICP 7035 compared to the susceptible genotype TTB 7. During pathogen infection ABA inhibits the transcription of a basic β-1, 3-glucanase that can degrade the β-1, 3-glucan callose, forming a physical barrier to viral spread through plasmodesmata. This down regulation of β-1, 3-glucanase by ABA can be termed as a resistance factor in plant pathogen interactions (Mauch-Mani and Mauch 2005). In the present study, the significant expression level was observed in SMD resistant genotype ICP 7035 during viral infection. This positive correlation between the ABA levels and disease resistance was reported in plant species like common bean (Mayek-Perez *et al.* 2002), rice (Koga *et al.* 2004) and tobacco (Whenham *et al.* 1986).

5.3.4 Development of functional markers

The primary goal of the present study is to develop molecular markers based on expressed sequences and screen them for polymorphism. During the last decade, microsatellites or SSRs have proven to be useful markers in plant genetic research and have been used for marker-assisted breeding purposes.

The presence of SSRs in the coding region suggests their importance as functional or gene based markers (Varshney *et al.* 2007, 2009a, Kota *et al.* 2001). Unfortunately, development of microsatellite markers is expensive, labor intensive and time consuming if they are being developed from genomic libraries (Gupta and Varshney 2000). The data mining of microsatellites markers from EST data can be a cost effective option. The cost of mining EST libraries is far lower than other traditional methods, and SSR development from ESTs has been successful in EST data mining (Varshney *et al.* 2005, 2002, Thiel *et al.* 2003, Cordeiro *et al.* 2001, Kantety *et al.* 2002).

SSR motifs with repeats of more than eight for di-nucleotides, six for tri-nucleotides and five for tetra-nucleotides were considered. Dimeric repeat motifs were relatively abundant than trimeric repeats (Mian *et al.* 2008). In addition to this, tetra-, penta- and hexameric repeat motifs were considerably less represented. In the present study, a total of 94 SSR markers have been synthesized and characterized for polymorphism survey. However, there are some distant contrasts in frequency and distribution of SSRs in ESTs and in genomic survey sequences (GSSs). In the present study, di-nucleotide repeats are more abundant than tri-nucleotide repeat motifs. Similar kind of results was reported by Yu *et al.* (2006) and Quilang *et al.* (2007). In contrast, Varshney *et al.* (2002, 2005); Thiel *et al.* (2003) and Luo *et al.* (2005) reported that di-nucleotide SSRs of all repeat lengths are more common in GSSs and tri-nucleotide SSRs are common in the ESTs. However this observation is not unexpected as the frequency and distribution of SSR depends on several factors such as size of dataset, tools and criteria used for SSR discovery (Varshney *et al.* 2005).

In this study, a total of 84 EST-SSRs primer pairs were validated and used for screening two genotypes ICP 7035 and TTB 7 which represent parents of mapping population segregating for SMD. Out of 84 genic SSR markers screened for the parents, 52 primer pairs provided scorable amplified products.

From these 52 primer pairs, 31 primer pairs produced a number of faint bands indicative of non-specific amplifications and 20 were monomorphic, only one marker (ICPeM0075) showed clear polymorphism (table 10). The low genetic variability amongst cultivars suggests that natural and artificial selection has contributed to the selection of specific alleles and to changes of allelic frequencies at specific loci (Odeny *et al.* 2007). EST-SSR markers developed in this study complement the currently available or ongoing efforts on development of genomic SSRs that will be a valuable resource for linkage mapping and marker assisted selection in pigeonpea (Varshney *et al.* 2009b).

5.4 Construction of genetic linkage map

Pigeonpea is an important grain legume crop of rainfed agriculture in the semi-arid tropics. SMD is considered to be the most important disease of pigeonpea and causes yield loss upto 95 per cent (Kannaiyan *et al*. 1984)**.** Development of cultivars with resistance to SMD is the best strategy to diminish cost of cultivation, soil and environment pollution. Majority of the wild species harbor resistance to these diseases but the introgression is thwarted due to cross compatibility barrier and linkage drag. Hence limited success has been achieved in pigeonpea resistance breeding.

The development of genetic linkage map will greatly expedite the ability of breeders to tag and follow the introgression of specific chromosome segments linked to desirable traits from wild species into breeding lines of cultivated pigeonpea. Without the availability of a genetic map, it is difficult to utilize molecular markers or to combine molecular and conventional genetic techniques in pigeonpea improvement programs. Simple sequence repeats (SSRs) are the markers of choice because they are ubiquitous throughout the genome, multi-allelic, co-dominant and breeder friendly (Gupta and Varshney, 2000). Since, there is no comprehensive genetic map in the cultivated pigeonpea; the present investigation emphasizes linkage map construction

based on SSR markers and identification of QTL contributing to resistance in TTB 7 × ICP 7035 population.

Out of 3320 SSR markers (3236 genomic+84 genic), 2107 (63.5%) could amplify and 84 markers were found to be polymorphic (2.5%). Very low polymorphism observed between the parents TTB 7 and ICP 7035 revealed their narrow genetic base. Similar kinds of features were observed in earlier SSR based studies in pigeonpea (Odeny *et al.* 2007; Ganapathy *et al.* 2009; Saxena *et al.* 2009b). However, in this study EST-SSRs were less polymorphic (1.2 %) than genomic SSRs (2.5%). This is due to greater DNA sequence conservation in transcribed regions (Scott *et al.* 2000). Hence, while developing mapping populations for the traits of interest, screening of different genotypes or germplasm using molecular markers and the combination of genotypes which gives higher polymorphism could be a better approach (Anderson *et al.* 1993; Mace *et al.* 2006).

Segregation distortion affects the estimation of map distances and the order of markers when many distorted markers are present. In this study, a total of five markers (5.95%) out of 84 markers showed segregation distortion which is relatively less compared to lupin (12.8%) (Phan *et al*. 2007). This could be due to more similarity and less genetic diversity between the parents in the present investigation as compared to use of wild species leading to sterility.

The linkage map obtained consists of 82 markers mapped on 11 linkage groups and spanning 539.5 cM with an average distance of 6.1 cM; only 2 markers remained unlinked. There were no earlier reports on construction on the genetic linkage map based on SSRs in pigeonpea and the present study constitutes the first attempt on development of linkage map using SSR markers. As a result, no comparison can be made on linkage map obtained in this study with other studies in pigeonpea. Similar kind of results were obtained by Kottapalli *et al*. (2009) in cultivated chickpea where the resultant map

consisted of 82 SSR markers and 2 EST markers covering 10 linkage groups, spanning a distance of 724.4 cM with an average marker density of 1 marker per 8.6 cM.

Though highest numbers of markers (3320 SSRs) were screened in the present study but limited polymorphism (84 SSRs) remained the biggest constraint in the construction of a good skeletal / framework map. Alternatively use of a larger number of highly polymorphic markers like SNPs (single nucleotide polymorphisms) and DArTs (Diversity Array Technologies) could be utilized in the development of framework map which could be later enriched with co-dominant SSRs (Paterson *et al.,* 2004).

5.5 Identification of QTL associated with SMD resistance.

The present investigation is the pioneering attempt to identify QTLs associated with SMD and it was carried out by using genotypic and phenotypic segregation data based on 130 F_2 population and $F_{2:3}$ progenies. QTL analysis revealed two QTLs associated with resistance to SMD each contributing 10.39 and 15.74 per cent of the phenotypic variation for Bengaluru isolate and two QTLs each contributing 12.30 and 24.69 per cent of the phenotypic variation for Patancheru isolate. Identification of QTLs for SMD trait in pigeonpea is the first of its kind. As a result, no comparison could be made on QTLs identified in this study with other studies in pigeonpea, but was compared with other diseases in other crops.

There were no common QTLs identified sharing Bengaluru and Patancheru isolates, indicating both the isolates are different from each other. All the four QTLs identified for Bengaluru and Patancheru isolates inherited the resistant allele from the susceptible parent TTB 7. This is not uncommon and has been reported in many plant species (e.g., Young *et al.* 1993; Lefebvre and Palloix 1996; Pilet *et al.* 1998). For early blight resistance in tomato,

Zhang *et al.* (2003) also detected a QTL on chromosome 3 for which the resistance allele was inherited from the susceptible parent.

It is, however important to mention here that though four QTLs were identified for Bengaluru and Patancheru SMD isolates, one QTL for Patancheru isolate on linkage group 3 explained a high phenotypic variation (24.69 %) with LOD value of 6.89 which could be used for marker assisted breeding. Where as for Bengaluru isolate QTL on linkage group 7 explained 15.7 4% phenotypic variation with LOD value of 3.56 which is lower when compared to Patancheru isolate. On the contrary, Phan *et al.* (2007) detected two regions significantly associated with anthracnose resistance on LG 4 and LG 17 at an LOD of > 3. These QTLs explain over 31 and 26% of the phenotypic variance respectively, and were inherited from the resistant parent P 27174. Based on QTL mapping studies in other species, it can be generalized that higher phenotypic variation for the given trait in the mapping population and high/reasonable marker density genotyping data are the pre-requisites to identify the major QTLs explaining higher phenotypic variation.

For breeding purposes, QTL with large additive effect which are stable across environments and which do not depend on epistatic interactions, are most desirable. Unfortunately, due to limitation of seed quantity, stability of the QTLs was not possible across different seasons for each isolate. Nevertheless, it would be useful for breeders to make use of the QTL on linkage group 7 and 11 for Bengaluru and Patancheru isolates respectively.

Future line of work

1. The $F_{2:3}$ families developed in the present study needs to be forwarded to develop RILs in order to obtain homozygous lines, each containing a unique combination of chromosomal segments from the original parents.

2. In the present study, 84 SSR markers which were polymorphic were detected. However, more number of polymorphic markers needs to be identified to get a fine saturated map of pigeonpea.

3. The identified QTLs associated with SMD resistance needs to be validated across populations, seasons and environment before using in MAS to introgress the QTLs resistant to SMD into desirable genetic backgrounds.

VI. SUMMARY

The present investigation was carried to develop F_2 and $F_{2:3}$ populations, to know the nature of inheritance to SMD, to construct c-DNA library of pigeonpea and generation of expressed sequenced tags (ESTs), sequence analysis of selected expressed sequenced tags (ESTs), development of EST-based simple sequence repeat (SSR) markers, to construct genetic linkage map and to identify QTL associated with SMD resistance. The summary of the findings are as follows.

224 F_2 plants were selfed to obtain the $F_{2:3}$ mapping population and used for phenotyping against SMD at two different locations *viz.,* UAS, Bengaluru and ICRISAT, Patancheru. The mapping population exhibited significant variation for resistance to SMD. The magnitude of variation was high as revealed by phenotypic coefficient of variation with high heritability and the population revealed substantially high heritable variation.

All the F_1s of the resistant x susceptible cross were susceptible for both Bengaluru and Patancheru isolates indicating susceptibility to be dominant over resistance. At Bengaluru, SMD incidence varied from 4.1 to 100 per cent with wide range of variability. Out of the 224 $F_{2:3}$ families, only 2 plants were resistant, nine were moderately resistant and 213 families were susceptible. Absence of immune plants and rare occurrence of extreme phenotypes indicates polygenic control of SMD inheritance for Bengaluru isolate. While at Patancheru, 0 to 100 per cent SMD disease incidence was recorded with high variability. Out of 219 $F_{2:3}$ families, more number of resistant plants were recorded as compared to Bengaluru isolate. Inheritance of SMD seems to be complex and does not fit a simple gene- for-gene interaction.

The pattern of frequency distribution of SMD incidence in the $F_{2:3}$ was found to be continuous depicting quantitative nature of SMD resistance for

both the Bengaluru and Patancheru isolates. However large number of plants could be classified into categories of moderately resistant and susceptible class. Only few plants were classified in to resistant. In the present study, frequency distribution of SMD was platykurtic and negatively skewed indicating involvement of large number of segregating genes with majority of them having increasing effects.

A total of four cDNA libraries were generated from specifically targeted leaf tissue of ICP 7035 and TTB 7 infected with SMD at time intervals of 45 and 60 days after sowing. In total 3,788 high quality ESTs were generated from SMD challenged genotypes. Sequence clustering and assembly process of all assembled 3,788 high quality ESTs resulted in 1,308 unigenes. Detailed analysis of these datasets have provided several important features of pigeonpea transcriptome such as conserved genes (across legumes and model plant species) as well as possible pigeonpea specific genes, assignment of pigeonpea genes to different GO categories, identification of differentially expressed genes in response to SMD.

Out of 3320 SSR markers (3236 genomic+84 genic), 2107 (63.5%) could amplify and 84 markers were found to be polymorphic (2.5%), indicating low level of polymorphism. A total of 82 markers were mapped on 11 linkage groups (LGs) spanning 539.5 cM and two markers remained ungrouped. The number of markers mapped per linkage group ranged from three (LG 11) to twelve (LG 7). The lengths of linkage groups were ranging from 4.2 cM (LG 11) to 104.2 cM (LG 3) with an average distance of 6.1 cM.

The present study yielded two QTLs flanked by the markers *CcM2337-CcM0416* and *CcM0970-CcM2485* with LOD score 2.82 and 3.56, respectively for Bengaluru isolate. The first QTL (*CcM2337-CcM0416*) positioned on LG 3 expressed 10.39 per cent of the phenotypic variation with 0.17 additive effects and the second QTL (*CcM0970-CcM2485*) located on LG 7 accounted for

15.74 per cent of phenotypic variation with an additive effect of 0.22. For Patancheru isolate, two QTLs were identified at marker interval *CcM2149* (LG 2) and *CcM1825-CcM1895* (LG 11) and were detected with 3.95 and 6.89 LOD scores, respectively. The QTL (*CcM2149)* explained 12.3 per cent phenotypic variation with 0.24 additive effect and the second QTL (*CcM1825-CcM1895)* explained 24.69 per cent of phenotypic variation having an additive effect of 0.33.

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Appendix I: Mean SMD disease reaction (%) in $F_{2:3}$ population of TTB 7 × ICP 7035.

Sl.No	Bengaluru	Patancheru	Sl.No	Bengaluru	Patancheru	Sl.No	Bengaluru	Patancheru
1	100.0	56.4	**41**	68.9	79.1	**81**	91.7	0.0
2	100.0	0.0	**42**	77.3	56.4	**82**	76.4	49.2
3	4.2	0.0	**43**	74.1	90.0	**83**	76.2	0.0
4	77.5	6.7	**44**	85.7	36.4	**84**	100.0	92.3
5	93.8	73.3	**45**	94.4	16.7	**85**	100.0	91.7
6	87.5	0.0	**46**	90.9	100.0	**86**	80.6	55.0
7	66.3	74.6	**47**	87.3	29.5	**87**	95.8	31.4
8	100.0	0.0	**48**	100.0	61.7	**88**	91.7	83.0
9	88.9	0.0	**49**	60.7	6.7	**89**	45.8	0.0
10	70.2	0.0	**50**	70.7	0.0	**90**	100.0	0.0
11	95.0	100.0	**51**	95.0	76.2	**91**	17.4	65.1
12	100.0	96.2	**52**	89.4	75.0	**92**	12.5	39.8
13	83.3	3.3	**53**	91.7	12.5	**93**	77.1	62.5
14	92.9	0.0	**54**	31.4	83.3	**94**	66.3	79.8
15	88.9	0.0	**55**	61.1	68.3	**95**	55.0	70.0
16	77.9	44.4	**56**	95.5	78.4	**96**	92.9	83.3
17	67.5	47.8	**57**	100.0	63.3	**97**	100.0	96.7
18	75.0	89.2	**58**	58.3	78.3	**98**	100.0	11.3
19	93.8	0.0	**59**	100.0	53.3	**99**	75.0	58.3
20	93.8	72.4	**60**	65.2	40.1	**100**	66.0	100.0
21	87.5	73.9	**61**	63.1	0.0	**101**	92.9	100.0
22	81.8	0.0	**62**	93.8	93.3	**102**	68.1	100.0
23	66.7	74.3	**63**	87.5	76.3	**103**	100.0	30.8
24	86.7	35.6	**64**	77.9	100.0	**104**	94.4	100.0
25	87.5	71.4	**65**	95.8	40.0	**105**	32.5	100.0
26	78.6	83.5	**66**	100.0	100.0	**106**	65.5	53.1
27	82.5	51.7	**67**	91.2	58.6	**107**	81.3	85.0
28	85.5	89.3	**68**	71.4	86.7	**108**	100.0	75.0
29	79.5	69.2	**69**	47.3	46.4	**109**	75.9	0.0
30	90.5	100.0	**70**	50.9	48.6	**110**	41.1	60.0
31	74.5	4.5	**71**	49.4	60.8	**111**	72.5	47.5
32	70.0	77.8	**72**	53.2	53.0	**112**	100.0	50.0
33	85.5	87.5	**73**	100.0	25.0	**113**	68.2	100.0
34	80.0	85.7	**74**	100.0	79.0	**114**	100.0	80.0
35	73.9	82.4	**75**	24.3	10.0	**115**	100.0	100.0
36	53.6	96.2	**76**	96.4	47.7	**116**	100.0	77.4
37	63.5	89.9	**77**	20.0	76.4	**117**	67.8	81.8
38	90.9	73.9	**78**	85.0	60.0	**118**	100.0	10.7
39	100.0	66.7	**79**	65.9	52.8	**119**	100.0	22.5
40	93.8	43.3	**80**	87.3	45.0	**120**	65.3	76.8

Contd…

Sl.No	Bengaluru	Patancheru	Sl.No	Bengaluru	Patancheru	Sl.No	Bengaluru	Patancheru
121	100.0	76.0	**161**	84.3	82.6	**201**	55.8	83.3
122	87.9	38.1	**162**	91.9	4.5	**202**	73.5	80.0
123	92.9	10.0	**163**	70.0	96.7	**203**	72.9	70.7
124	100.0	76.2	**164**	91.7	0.0	**204**	83.0	76.8
125	100.0	3.6	**165**	16.7	88.8	**205**	67.3	88.9
126	96.4	0.0	**166**	95.5	69.2	**206**	85.7	41.5
127	90.0	73.3	**167**	61.9	71.4	**207**	79.5	7.7
128	64.6	31.7	**168**	86.1	71.4	**208**	92.9	0.0
129	90.0	66.7	**169**	100.0	61.1	**209**	91.7	7.7
130	6.3	90.8	**170**	83.8	69.7	**210**	36.4	69.5
131	82.5	78.5	**171**	70.8	90.0	**211**	17.9	78.4
132	80.9	34.1	**172**	100.0	48.1	**212**	100.0	54.2
133	65.7	52.7	**173**	81.7	61.4	**213**	92.3	0.0
134	66.7	88.9	**174**	88.9	66.7	**214**	87.5	81.7
135	75.0	0.0	**175**	89.3	5.6	**215**	75.0	16.7
136	75.0	85.5	**176**	60.7	59.3	**216**	75.0	70.8
137	89.9	67.3	**177**	85.7	100.0	**217**	72.5	75.0
138	100.0	79.8	**178**	66.7	49.2	**218**	92.9	100.0
139	100.0	100.0	**179**	77.3	89.3	**219**	73.9	37.5
140	73.3	75.0	**180**	90.6	71.4	**220**	100.0	-
141	76.2	0.0	**181**	77.4	66.9	**221**	83.3	-
142	75.0	0.0	**182**	100.0	0.0	**222**	100.0	-
143	94.4	91.7	**183**	95.8	39.6	**223**	100.0	-
144	74.6	100.0	**184**	91.3	75.0	**224**	83.3	-
145	43.8	85.4	**185**	56.3	60.3			
146	100.0	40.4	**186**	57.4	13.9			
147	80.0	0.0	**187**	95.0	85.7			
148	62.5	57.1	**188**	85.7	33.3			
149	29.0	51.8	**189**	50.0	0.0			
150	76.7	3.8	**190**	78.6	85.7			
151	86.1	66.5	**191**	49.4	61.8			
152	90.0	88.5	**192**	81.5	0.0			
153	86.1	0.0	**193**	100.0	68.8			
154	90.0	95.8	**194**	100.0	5.6			
155	93.8	100.0	**195**	53.3	60.0			
156	83.3	90.0	**196**	80.0	11.5			
157	77.4	60.0	**197**	16.7	69.7			
158	100.0	60.0	**198**	90.0	92.3			
159	95.0	96.7	**199**	100.0	58.3			
160	28.8	16.7	**200**	88.9	3.8			

Appendix II: List of newly developed pigeonpea EST-SSRs

Sl. No.	Primer ID	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')	Tm(°C)	P.S
1	ICPeM0001	ATGGTGCAAGTCTGAGATCG	ATTCCCTTGGGGTTAAGCAG	60.312	240
2	ICPeM0002	GGAATTCCATTGTTGGTGATTT	TTGGGGGCCCCTTAAAAA	62.381	279
3	ICPeM0003	TGCCACATCTTTTGCAAATACTA	GCCCCAAAAGAGATACCACA	59.933	264
4	ICPeM0004	TCTCCACAAATTTATCATGCCA	TTTTCCATATGGTTGACCTGC	59.815	253
5	ICPeM0005	TTGAAATGATGTGAGGTGCG	AATTTGTCCCCAGTTTTCCC	60.032	269
6	ICPeM0006	CCCGGAATTTCTTTTGGTTT	GGTTTGTTGAATCTTCATTCTTTTG	60.25	246
7	ICPeM0007	GGCCCCTTAGAAAAATCCAA	TTTGCCGCAATCTTTATTGA	59.286	279
8	ICPeM0008	ATAATATTGTTGGTTCAATTTTGGT	AAAAACCCAAACATGTCCCC	60.817	272
9	ICPeM0009	CGGGGCGTTTAAATGAATAC	GGGAATTTCCTTGGGGTTTA	59.992	208
10	ICPeM0010	CCCCTTTAGGATGGTCCAAT	CTCCATGGAAGGCTAGGTTG	59.688	266
11	ICPeM0011	TGGTAATCGATTTGAAGTTCTTG	AATTCCAAGTTTGGCTTCCC	60.299	280
12	ICPeM0012	TTTCTAGTGCAATGTCTTTTATGGA	AAGTTTCCCCGGTTTTCTTC	59.438	272
13	ICPeM0013	AGACACCGGGCTCATTCA	TTGGGGGCCCCTTAAAAA	62.381	279
14	ICPeM0014	CGTGGAAGAAAAATTTGGGA	TAAAGAAAGGGCCCCAAAAG	60.404	232
15	ICPeM0015	TGGTGGATTAGGGATGGTGT	CCTTTTGGAAAATCCCAGGT	60.159	256
16	ICPeM0016	CATGGTTGCTGTCCTTTTAGC	CCTAGGGGTTTAAACAGGGG	59.693	140
17	ICPeM0017	CGGGGACTGATTAGCACAGA	CCTGATAGCCACCTTCTTTCTT	58.914	206
18	ICPeM0018	CTTGAACTTAGTGGGCCAGG	TGTTGTTTTGGTTATTATTGAGAGC	58.729	201
19	ICPeM0019	CGCTGACTTCAAATCTGCCT	AAAAATGCCATCGCCATAAG	59.928	236
20	ICPeM0020	TTGCCAAAATGGATTTGAATTA	CGTGTTTCCCCCAATTTTT	59.648	280
21	ICPeM0021	AAAAATTGGGGGAAACACG	CCCAAAAAGAAAACCCCTTT	59.325	237
22	ICPeM0022	AATTTTTAAAGGGCCCCCA	TTGGGAGGAAAAGGGAAAAG	60.395	279
23	ICPeM0023	CGGTTCATTGTATATAAAGAGAGATTG	TTTGGGGCCCCTTAAAAA	60.231	279
24	ICPeM0024	CCCCCGTTTAAACCAAAAA	CCCAAATAAAGGGGGTTTGT	59.918	246
25	ICPeM0025	AAAAGTTTTTCCGGGGGA	GGAAAAGCCCCCAATAACAA	61.011	223
26	ICPeM0026	AAAATTGGGGAAAAACAGGG	ACCCAAATAAAGGGGGTTTG	59.918	141
27	ICPeM0027	CGGGGAAAACAAACCTTGA	ATTAAATGGGGTTTGGTCCC	59.756	177
28	ICPeM0028	TGTAGCTTTTGTCCTCTCCG	CCGGATTTCCTTTGGGTTTA	61	239
29	ICPeM0029	TTTGTGGTTTGCAGCTCTTG	CCCGAATTTCCTAGGGGTTA	60.136	184
30	ICPeM0030	TGATTGCTGGAAGCTTGTTG	AAGTATGCCTTCCACGGGTT	60.743	252
31	ICPeM0031	TTTGGTAAAATCACGTTGGC	GAAAACCCAAGTGGCCTTC	59.517	279
32	ICPeM0032	TAACCCCTAGGAAATTCGGG	CCCAGTTTTCCCCTTTGAA	59.888	275
33	ICPeM0033	TTTTTAAAAGGCCCCAAAAA	GAAGGAAGGAAAGGGGTCAA	60.414	272
34	ICPeM0034	GAAAGCCCATCCAAAACAAA	GCTCTTGCATTTGCCATTCT	60.361	236
35	ICPeM0035	AGGCGGATCTATCCACACAG	GCGTACGATATTTTTCCAGCA	60.109	218
36	ICPeM0036	AGAGAAAAGGAGGGTGTGGG	CATTCCCATTACTCGCCCTA	59.916	234
37	ICPeM0037	CGTCAATCTGTGCTTGGTGT	CAATTGGTAACCTGCAAGGG	60.357	237
38	ICPeM0038	CCCTCCCCCTTTTACACATT	GAGGAAGAGGGAGTATGGGG	59.89	266
39	ICPeM0039	CCCCTTAAAAATCCCCAAGT	GGCTCAGAGAAAATTGAAGAGAA	59.146	262
40	ICPeM0040	GTGTGGGCGTTGTTGGTT	AAGCATTAATGGAACGCACC	59.967	193
41	ICPeM0041	TCCACAAATCCATCCGTACA	CTCCAAGACATCCACCACCT	59.962	272
42	ICPeM0042	GTGGCAGTAATGGCGAGAAT	CAGTCCGACCCTCTTGAAAA	60.224	233
43	ICPeM0043	TGGCTGTAAATCACTTGAGGA	TCCCGGCGCTTGTTAAAG	62.199	277
44	ICPeM0044	TAGGGTACGCTGGATCTTGG	TTTCTCTACGGGGGTGAATG	59.926	253
45	ICPeM0045	TCTAGCCTTTAGGGCGTTCA	TGAGAAGCTCCCATTCACAA	59.369	273
46	ICPeM0046	TTTCTGAGTTTTTCAGGGGC	ATTGGATTCGCTACACAGCC	60.103	155
47	ICPeM0047	AGGTACATTGGCGTGATCG	GGTGGTGATATCTAGGCGGA	59.917	213

Contd..

Sl. No.	Primer ID	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')	Tm(°C)	P.S
48	ICPeM0048	CGCGTTTCTGAATTGCCTAT	TTGGGGGCCCCTTAAAAA	62.381	276
49	ICPeM0049	GAATTTTTAAAAGGGCCCCA	CACAAGTTTTTCCCCTTGGA	59.942	201
50	ICPeM0050	TTTGCTTAAATTGAAAATCTCCG	TTTTTGGGGGCCCTTTTG	62.955	264
51	ICPeM0051	GGTCACAGACCAAATAGTTTTAGGA	AAAAACCAAGGTTTGCCCC	61.065	279
52	ICPeM0052	ATACACACCAGCCCACCC	GCAGCATGCAGCTGGTAATA	60.006	233
53	ICPeM0053	CTCCCTCTCCCTCCCTCCT	GGCAACAAAAGGCGATAAGA	60.209	136
54	ICPeM0054	GTGGATAACACCCAACACCC	AAACGGCCAAAATTCAAATG	59.81	137
55	ICPeM0055	GCCTCCCCATTACCCTCTT	GCGGCCTTTCGTCTTTTT	60.331	179
56	ICPeM0056	TATTCAAGGCGACGACCC	TTATTGCGGGGCTATTCAAG	60.054	271
57	ICPeM0057	TGAGTGTCTCGGAATGGACA	GGGTTGTTTTGTGGTTTGGT	59.592	223
58	ICPeM0058	GAGCCGGTCACGTGTTTAAT	CGGTTTGTCGTTAGGTCACA	59.609	259
59	ICPeM0059	TGCGATATTCCTTGGTTTCG	GGAGCCCTGTTTTGTTGTTT	59.077	203
60	ICPeM0060	GGCTCTGAGGTTGTGGAAAA	TTGAGGTAAGGCAACCTGCT	59.875	280
61	ICPeM0061	CATGCATTTTTGCCGCTAAT	TCAGAGCGATACCAAATGGA	59.226	243
62	ICPeM0062	CCCATTATTCATGCAATCCC	CCACGGTGAATTTGGAGTTC	60.353	195
63	ICPeM0063	ACTCTGGATGATCGAGTCGG	AACCGCACACTTTTGGGTAG	60.03	246
64	ICPeM0064	GTTGGGATCTTCCGTTACCA	CCCACGCGAAAGCTATTATT	59.234	239
65	ICPeM0065	TTGCAAGAAGAATTGCATCG	TCTAGGGATCCCCTTTTTGG	60.25	278
66	ICPeM0066	ACGAGTCCTTTCTCTCCGGT	TCCTTTACCACGCACCCTAC	59.993	162
67	ICPeM0067	CGGGGATCATCACAAACAG	CTCTTACCTGCGCAACATCA	60.011	167
68	ICPeM0068	CAACTGCCGTGTTTTCAATG	TCATCCACAAATAGCACCCA	59.924	211
69	ICPeM0069	GGTGACAGAGGCATAACAAACA	TTTTGAGGACCCCATTATGC	59.762	110
70	ICPeM0070	AAGCTTCCAACCTTACAGCG	TAAACGAGGCAATGAATGGG	60.827	270
71	ICPeM0071	CCGTTGTGCTCTCAATGCTA	CGGTTCCGCTGCAATAATAA	60.95	244
72	ICPeM0072	CAGATACGCACGCTGTATGTTT	CCATGTAGGATCAAGCCTCAA	60.081	101
73	ICPeM0073	GAAAGACAGCCCCATTTTCA	AATGTCTCCACAAAGGACGG	59.966	133
74	ICPeM0074	TCATGGGTATGGAGAGCACA	AAAAGGCACACCCCTCACTA	59.592	234
75	ICPeM0075	AGAGAATGGCTCAGGCAATG	GCAAGCACAGCTTGAACAAA	60.18	271
76	ICPeM0076	TCTTTGCATCCCTTAGCACC	AGGCTTCAGATGATGGGATG	60.034	276
77	ICPeM0077	ACATTGGCCTGATGGGAATA	TAGCCGCGCTAGTCTCTTTC	59.889	278
78	ICPeM0078	GGGAACAGGAAACCAAGACA	CGTCGTGAAGGTGGATACAA	59.566	238
79	ICPeM0079	AACGTACATGGCACTGATCG	TCCTTCCTCCTCAATGGTTG	60.042	199
80	ICPeM0080	AGGGGCACACGTAAAGTGAC	GCCGAAAACACTAGAAGGCA	60.386	127
81	ICPeM0081	GCCATTTTCTACAACCCCAA	GTGCAAACAAGTCCCGATCT	60.119	276
82	ICPeM0082	TGGAGATGGTGGTACGTTGA	CGTCCCTATACACAAATGGGA	59.693	263
83	ICPeM0083	CTTGAGCGAAGCGTAAAAGC	GCTTCCAGAGCGTACTCCAC	60.02	160
84	ICPeM0084	GCAAAAGCTTAGGAACCTGC	TGAATCTCAGCCTCGCTTTT	60.096	204

Table 1: Reaction of parents and F_1 against pigeonpea sterility mosaic disease at 75 DAS for Bengaluru isolate

	Genotypes	Total no. of plants	Resistant plants	Susceptible plants	Per cent disease incidence	Disease reaction
P_1	TTB 7	30	-	30	100	Susceptible
P_2	ICP 7035	30	28	2	6.6	Resistant
F_1	TTB 7 X ICP 7035	25	-	25	100	Susceptible
Control	ICP 8863	100	-	100	100	Susceptible

Table 2: Reaction of parents and F_1 against pigeonpea sterility mosaic disease at 75 DAS for Patancheru isolate

	Genotypes	Total no. of plants	Resistant plants	Susceptible plants	Per cent disease incidence	Disease reaction
P_1	TTB 7	40	-	40	100	Susceptible
P_2	ICP 7035	40	40	-	0	Resistant
F_1	TTB 7 X ICP 7035	30	-	30	100	Susceptible
Control	ICP 8863	100	-	100	100	Susceptible

Table 3: Reaction of the $F_{2:3}$ segregating generation to pigeonpea sterility mosaic disease at 75 DAS

Per cent disease incidence	Reaction	No of $F_{2:3}$ family under SMD incidence (Bengaluru isolate)	No of $F_{2:3}$ family under SMD incidence (Patancheru isolate)
0-10% of plants infected	Resistant	2	44
10.1-30% of plants infected	Moderately Resistant	9	11
30.1-100% of plants infected	Susceptible	213	164
	Total	224	219

Table 4: Analysis of variance for pigeonpea sterility mosaic disease reaction in $F_{2:3}$ plants Bengaluru isolate

Source of Variation	Degree of freedom	Sum of squares	Mean Sum of squares	F ratio
Replication	1	937.64	937.64	
Treatment	223	191326.75	857.98	3.07**
Error	223	62193.10	278.89	
Total	447	254457.50	569.25	

Table 5: Analysis of variance for pigeonpea sterility mosaic disease reaction in $F_{2:3}$ plants Patancheru isolate

Source of Variation	Degree of freedom	Sum of squares	Mean Sum of squares	F ratio
Replication	1	552.39	552.39	
Treatment	218	486694.00	2232.54	9.11**
Error	218	53411.35	245.00	
Total	437	540657.75	1237.20	

**** Significance at 1% level.**

Table 6: Estimates of variance components, broad sense heritability and genetic advance for pigeonpea sterility mosaic disease reaction in $F_{2:3}$ population of TTB 7 × ICP 7035

Isolate	Mean	Range	GCV (%)	PCV (%)	h^2b.s (%)	GAM (%)
Bengaluru	78.94	4.1-100	21.55	30.19	50.94	31.68
Patancheru	55.37	0-100	56.92	63.55	80.22	105.03

GCV – Genotypic coefficient of Variation, PCV-Phenotypic coefficient of Variation, h^2b.s- heritability in Broadsense
GAM – Genetic Advance as per cent of Mean

Table 7: Descriptive statistics of mean pigeonpea sterility mosaic disease reaction in $F_{2:3}$ population of TTB 7 × ICP 7035

Isolate	Sample Size	Mean	Range	Standard error	Standard deviation	Coefficient of variance	Skewness	Kurtosis
Bengaluru	224	78.94	4.1-100	1.38	20.71	26.23	-1.45	2.11
Patancheru	219	55.37	0-100	2.25	33.41	60.33	-0.49	-1.09

Table 8: BLASTN analyses of pigeonpea unigenes against legume and model plant ESTs

High quality ESTs generated	3,788	
Unigenes	1,308	
Legume ESTs	**Total**	**%**
Pigeonpea (*Cajanus cajan*) (908)	224	17.1
Chickpea (*Cicer arietinum*) (7,097)	507	38.7
Soybean (*Glycine max*) (880,561)	946	72.3
Cowpea (*Vigna unguiculata*) (183,757)	817	62.4
Medicago (*Medicago truncatula*) (249,625)	803	61.3
Lotus (*Lotus japonicus*) (183,153)	738	56.4
Common bean (*Phaseolus vulgaris*) (83,448)	784	59.9
Significant similarity with ESTs of at least one legume species	1,001	76.5
Significant similarity across legume ESTs	156	11.9
No similarity with legume species	4	0.3
Model plant ESTs		
Arabidopsis (*Arabidopsis thaliana*) (1,527,298)	667	50.9
Rice (*Oryza sativa*) (1,240,613)	520	39.7
Poplar (*Poplus alba*) (418,223)	678	51.8
Significant similarity with ESTs of at least one Model plant species	763	58.3
Significant similarity across ESTs of all model plant species	460	35.1
Significant similarity with ESTs of at least one plant species analyzed	1,015	77.5
Significant similarity across ESTs of all plant species analyzed	114	8.7
No similarity with ESTs of any plant species	4	0.3

Table 9: EST-SSRs generated in Pigeonpea

Total number of sequences examined	5,085
Total length of examined sequences (bp)	2,878,318
Number of ESTs containing SSRs	1,365
Number of identified SSRs	3,583
Number of ESTs containing more than 1 SSR	698
Number of SSRs present in compound formation	1,729
Frequency of SSR	1/0.8 kb
Number of mononucleotide repeats	3,498

Table 10: Details of the SSR primers tested in parents of pigeonpea mapping population TTB 7 × ICP 7035

Sl. No.	Details	Genomic SSR		Genic SSR		Total SSR	
		Total	Per cent	Total	Per cent	Total	Per cent
1.	**Total primers**	3236		84		3320	
2	**Total Amplified**	2055	63.5	52	61.9	2107	63.5
a	**Non specific amplification**	354	10.9	31	36.9	385	11.6
b	**Polymorphic**	83	2.5	01	1.2	84	2.5
c	**Monomorphic**	1618	50.0	20	23.8	1638	49.3
3	**Not amplified**	1181	36.4	32	38.0	1213	36.5

Table 11: Genetic markers assigned to linkage groups in pigeonpea

Linkage group (LGs)	Number of Microsatellites	Likelihood ratio (LOD)	Length (cM)	Average distance (cM)
LG 1	8	-240.90	70.6	8.8
LG 2	7	-181.72	31.6	4.5
LG 3	8	-314.34	104.2	13.0
LG 4	8	-244.20	61.4	7.7
LG 5	8	-210.69	60.4	7.6
LG 6	7	-199.29	44.4	6.3
LG 7	12	-341.15	94.2	7.9
LG 8	6	-160.98	26.8	4.5
LG 9	7	-158.39	23.4	3.3
LG 10	8	-146.91	18.3	2.3
LG 11	3	-80.44	4.2	1.4

Table 12: QTLs associated with resistance to pigeonpea sterility mosaic disease for two different isolates in TTB 7 × ICP 7035 $F_{2:3}$ population

Bengaluru SMD Isolate

QTL	LG	Position (cM)	Marker interval	LOD	R^2 (%)	Additive effect	Dominant effect
1	LG 3	103.41	CcM2337-CcM0416	2.82	10.39	0.17	-0.10
2	LG 7	92.41	CcM0970-CcM2485	3.56	15.74	0.22	-0.03

Patancheru SMD Isolate

QTL	LG	Position (cM)	Marker interval	LOD	R^2 (%)	Additive effect	Dominant effect
1	LG 2	0.01	CcM2149-CcM0468	3.95	12.30	0.24	-0.02
2	LG 11	2.01	CcM1825-CcM1895	6.89	24.69	0.33	-0.10

X

F_2 (Genotyping)

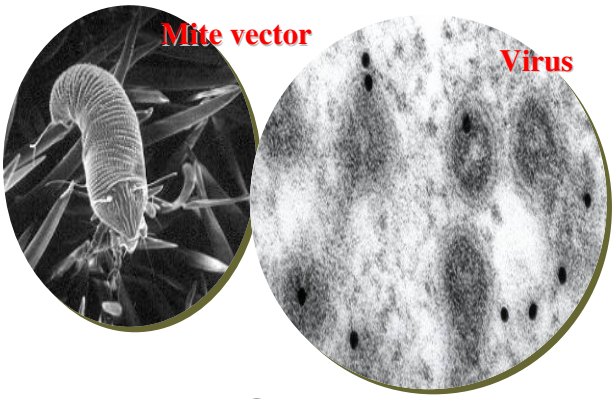
$F_{2:3}$ (Phenotyping)

Plate 1: General view of developing F_2 and $F_{2:3}$ mapping population of pigeonpea for sterility mosaic disease genotyping and phenotyping

Screening of pigeonpea $F_{2:3}$ populations against sterility mosaic disease at Bengaluru

Screening of pigeonpea $F_{2:3}$ populations against sterility mosaic disease at Patancheru

Plate 2: Experimental view for pigeonpea sterility mosaic disease screening at Bengaluru and Patancheru locations



a

b

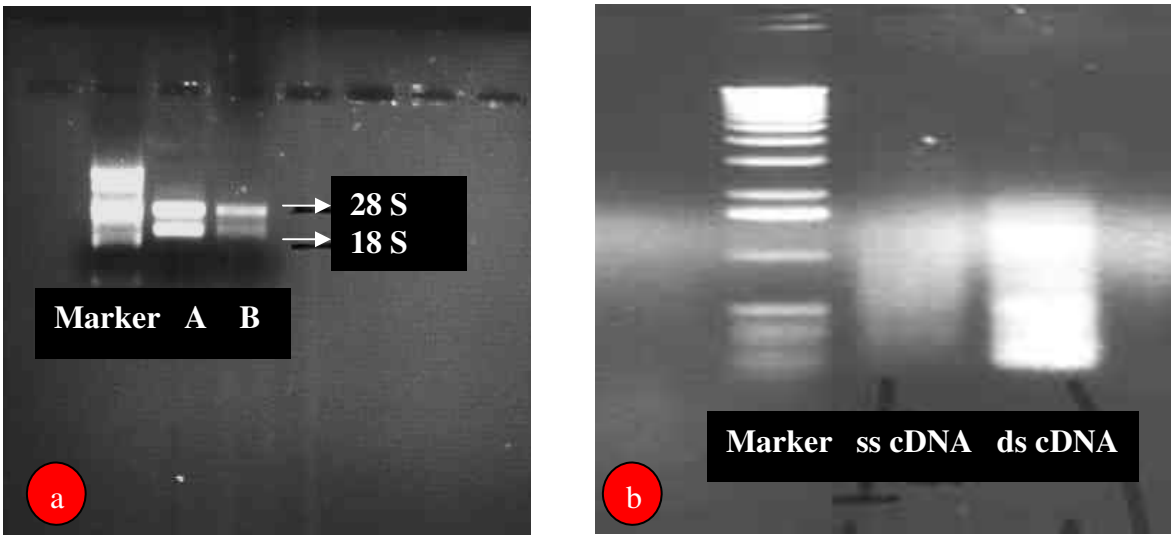
c

Plate 3: General view of leaf stapling technique

a: SMD vector eriophyid mite and pigeonpea sterility mosaic virus (PPSMV)

b: Infected SMD source plants ICP 8863

c: Leaf stapling technique



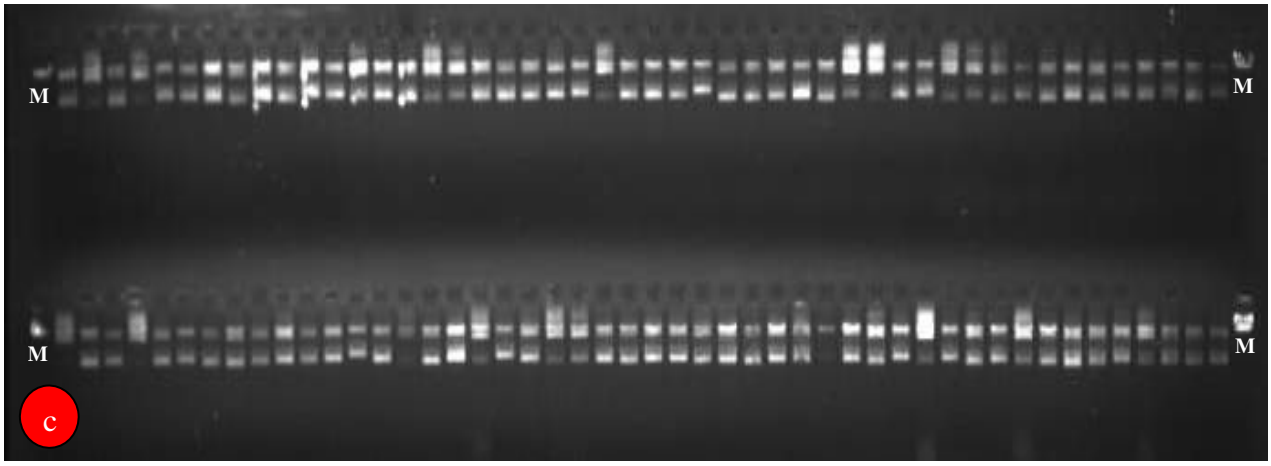


Plate 5: cDNA synthesis

a: Formaldehyde agarose gel showing total RNA samples A (TTB 7) and B (ICP 7035) along with RNA marker.

b: Agarose gel showing first and second strand cDNA along with 1 Kb DNA marker.

c: Quantification of Plasmid DNA on agarose gel with 100ng standard marker.

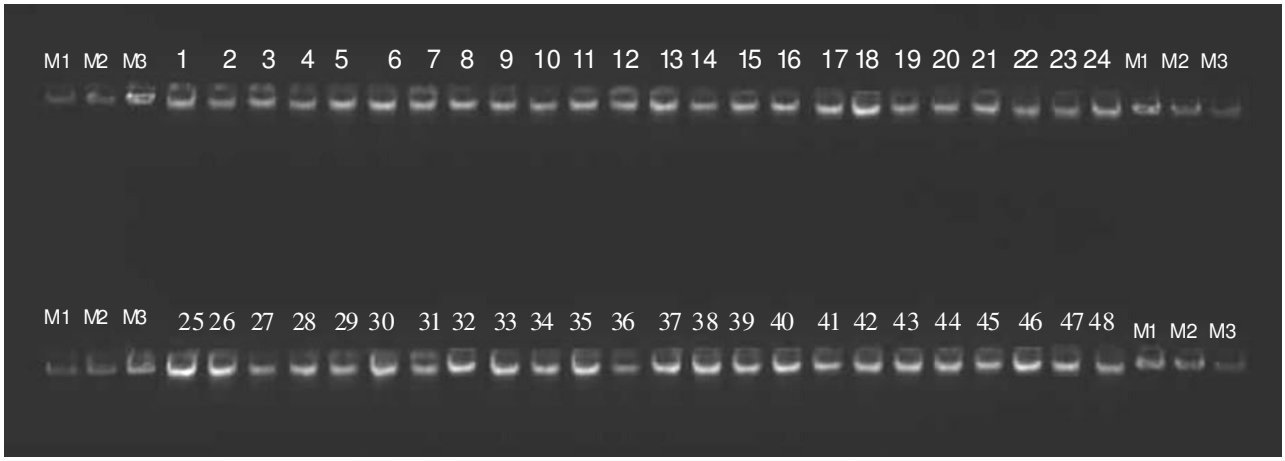


Plate 6: Quantification of DNA samples on 0.8% agarose gel
M1:50ng/ul, M2:100ng/ul, M3:200ng/ul and Lane 1-48 representative F_2 lines

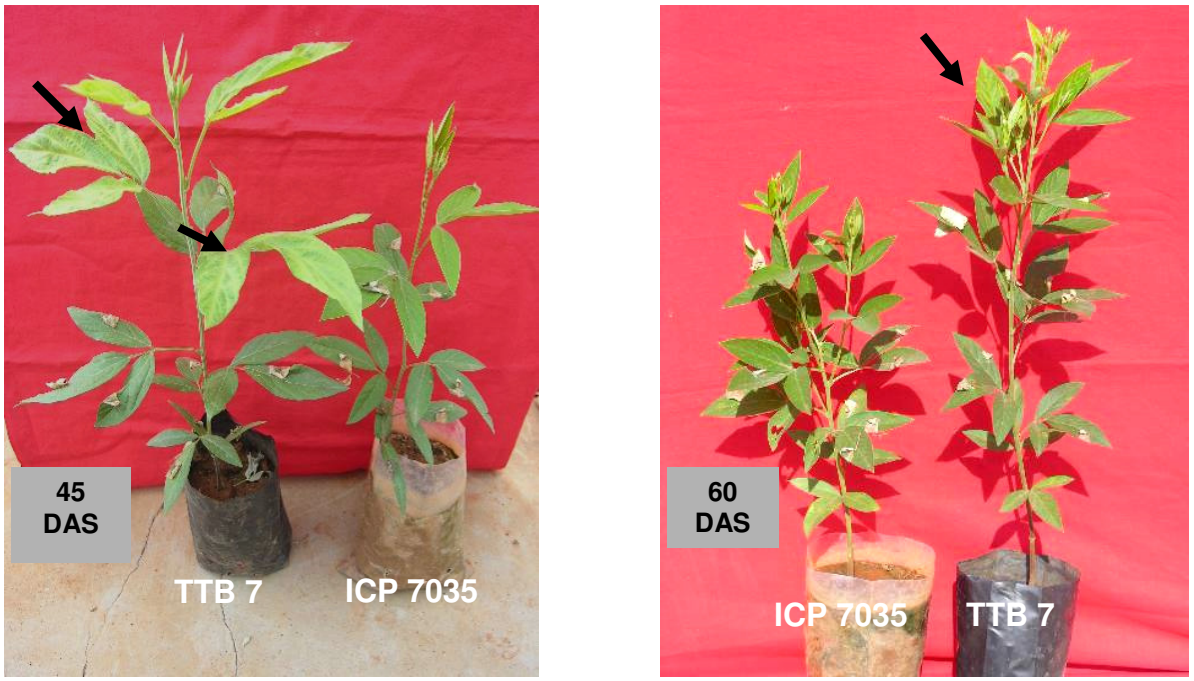


Plate 4: Sterility mosaic disease responsive pigeonpea genotypes for cDNA construction

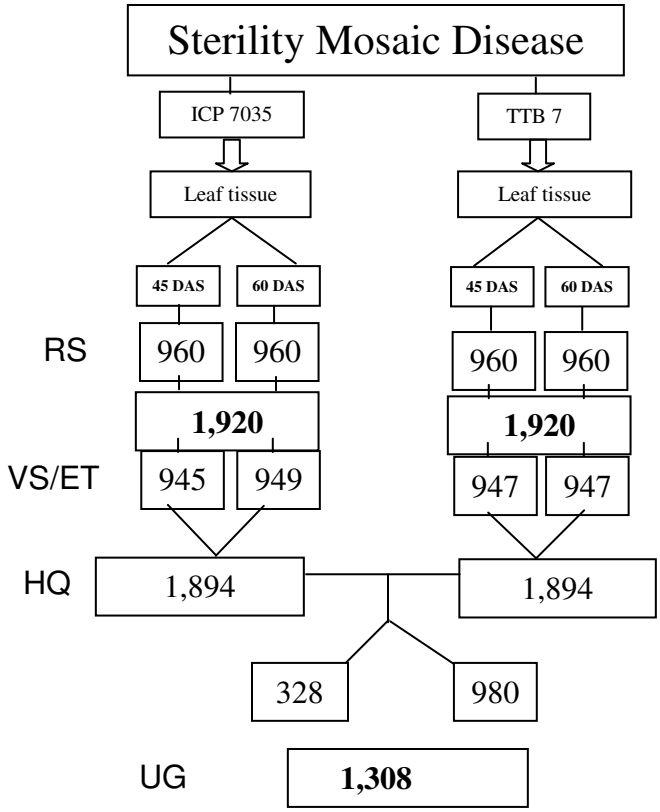


Figure 3: Summary of total ESTs generated from SMD responsive pigeonpea genotypes

RS: Raw sequences, VS/ET: Vector trimmed/ EST trimmed sequences

HQ: High quality sequences, UG: unigenes

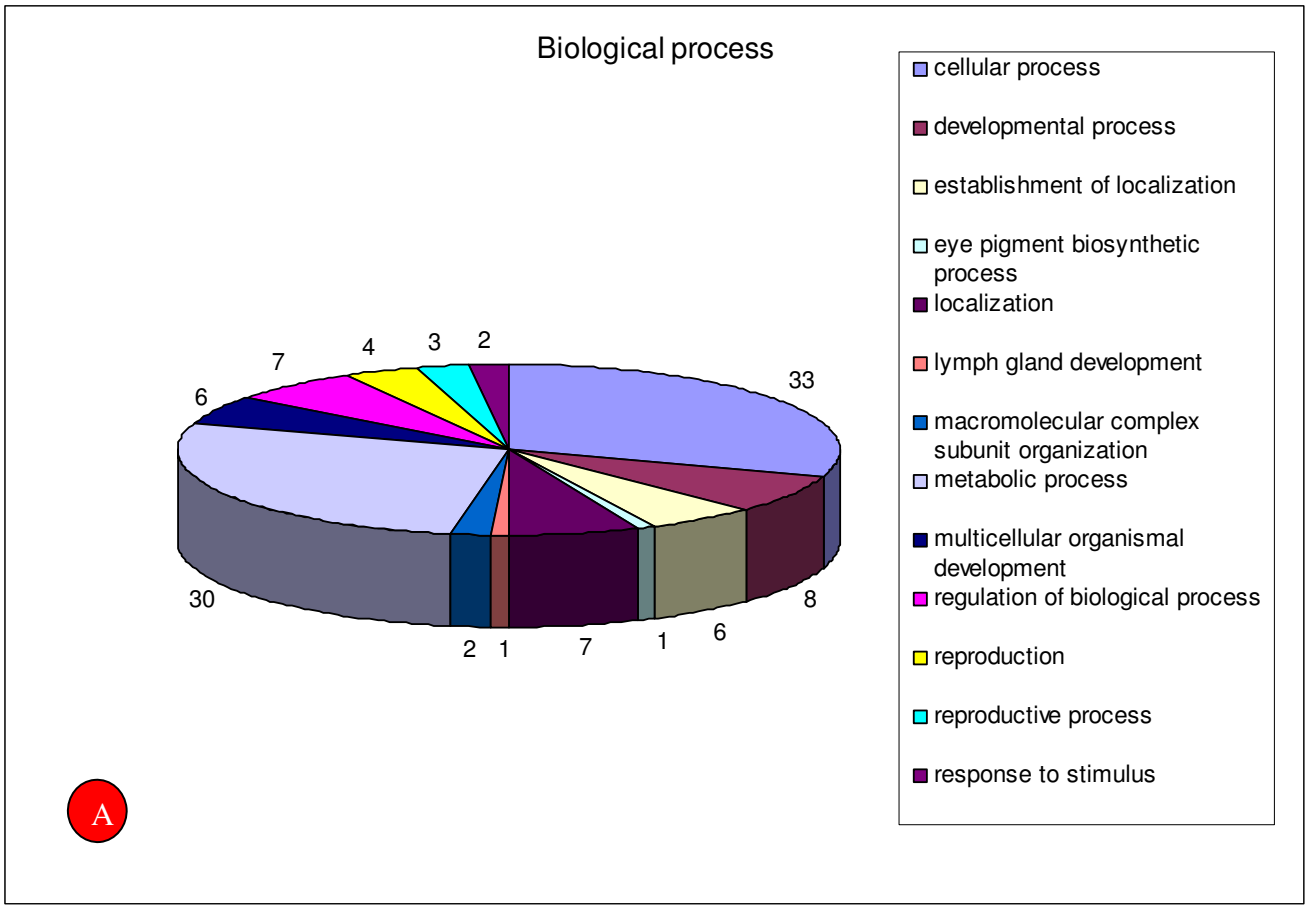
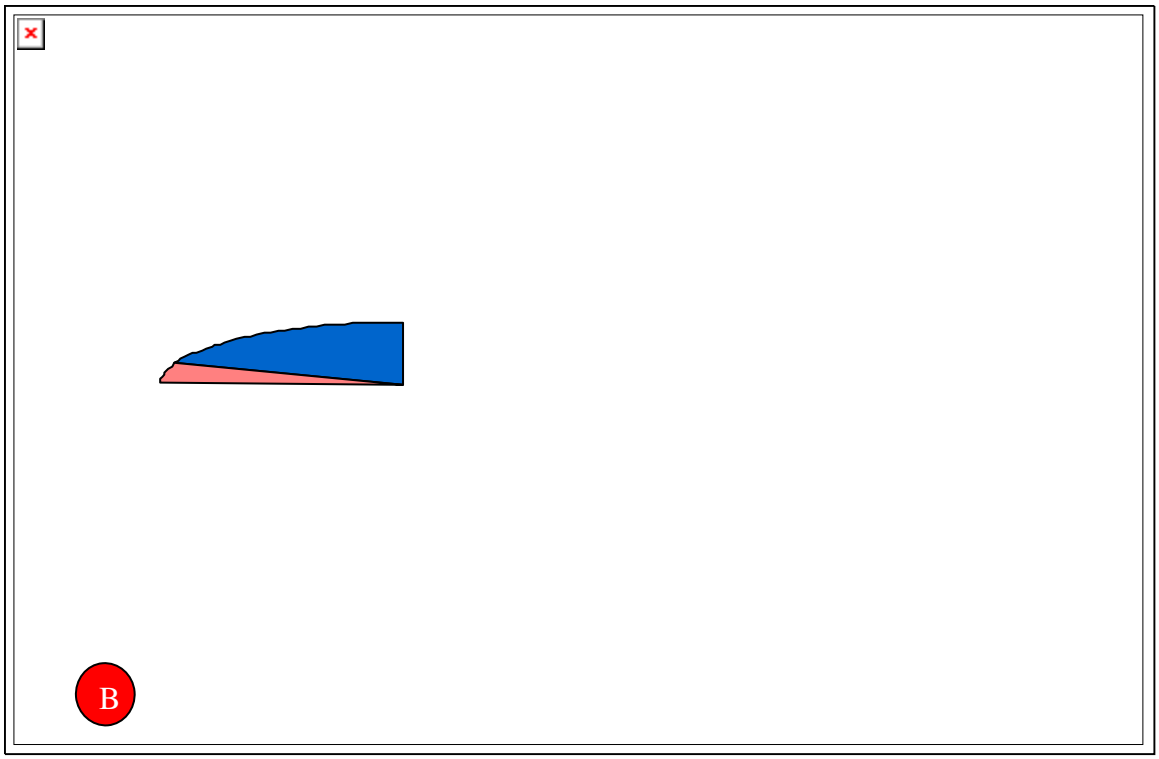


Figure 6: Gene Ontology (GO) assignment of pigeonpea unigenes by GO annotation

A- Biological process



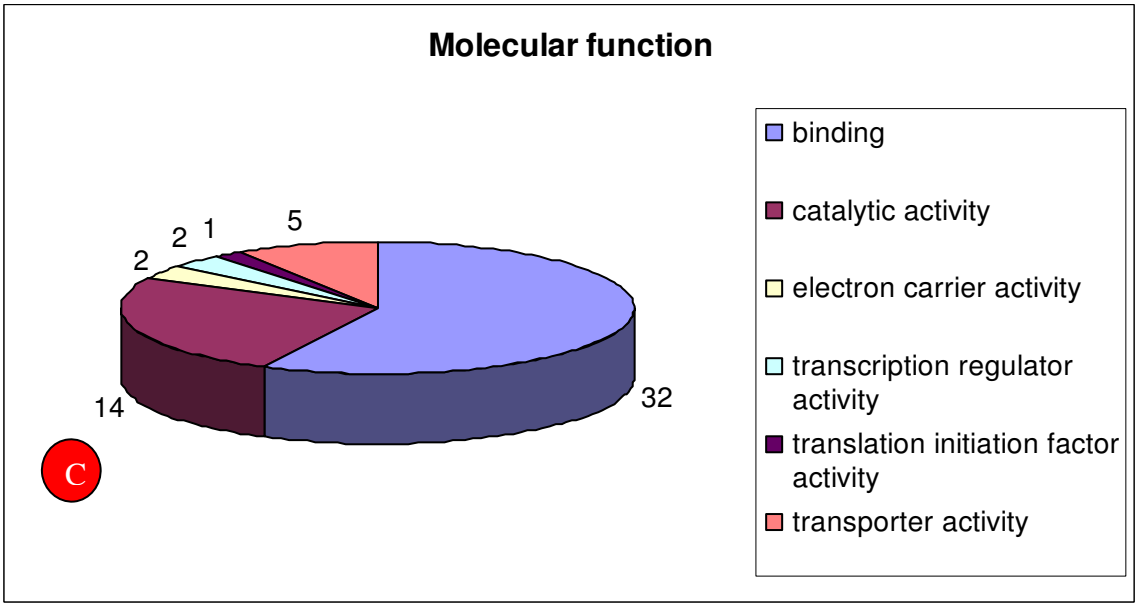


Figure 7: Gene Ontology (GO) assignment of pigeonpea unigenes by GO annotation

B -Cellular component and C- Molecular function

UNIQID	Description	Lib1	Lib2	ICP 7035	TTB7
Contig1	>Q9FY64|RS154_ARATH 40S ribosomal protein S15-4 – Arabidopsis	13	0	68.6	0
Contig5	>P40620|HMGL_VICFA HMG1/2-like protein - Vicia faba (Broad bean)	19	0	100.3	0
Contig7	>Q6BK66|CCS1_DEBHA Superoxide dismutase 1 copper chaperone -	17	1	89.8	5.3
Contig9	>Q9XF89|CB26_ARATH Chlorophyll a-b binding protein CP26, chloroplast	21	2	110.9	10.6
Contig15	>Q43517|FER1_SOLLC Ferredoxin-1, chloroplast precursor - Solanum	43	8	227	42.2
Contig16	>P43399|MT1_TRIRP Metallothionein-like protein 1 - Trifolium repens	45	12	237.6	63.4
Contig20	>Q05502|HHEX_CHICK Homeobox protein PRH - Gallus gallus (Chicken)	40	5	211.2	26.4
Contig30	>P49107|PSAN_ARATH Photosystem I reaction center subunit N,	21	0	110.9	0
Contig44	>Q93VI8|TLP7_ARATH Tubby-like F-box protein 7 - Arabidopsis thaliana	15	0	79.2	0
Contig49	>Q06930|ABR18_PEA ABA-responsive protein ABR18 - Pisum sativum	13	0	68.6	0
Contig55	>P17067|CAHC_PEA Carbonic anhydrase, chloroplast precursor - Pisum	24	3	126.7	15.8
Contig57	>Q9XFB0|YAB2_ARATH Axial regulator YABBY 2 - Arabidopsis thaliana	13	0	68.6	0
Contig81	>Q5XJD3|FIP1_DANRE Pre-mRNA 3-end-processing factor FIP1 - Danio	22	1	116.2	5.3
Contig87	>Q9XEX2|PRX2B_ARATH Peroxiredoxin-2B - Arabidopsis thaliana	14	0	73.9	0
Contig177	>P93276|M030_ARATH Uncharacterized mitochondrial protein AtMg00030 -	0	26	0	137.3
Contig188	>Q9ULL4|PLXB3_HUMAN Plexin-B3 precursor - Homo sapiens (Human)	41	125	216.5	659.1
Contig198	>Q6CQE5|TAR1_KLULA Protein TAR1 - Kluyveromyces lactis (Yeast)	1	44	5.3	232.3
Contig203	>Q9MTN0|YCX6_OENHO Uncharacterized 6.9 kDa protein in psbD-trnT	4	32	21.1	168.1
Contig217	>Q8TGM7|ART2_YEAST Uncharacterized protein ART2 - Saccharomyces	6	57	31.7	300.1
Contig294	>Q59296|CATA_CAMJE Catalase - Campylobacter jejuni	188	94	992.6	496.3

Figure 8: Differential gene expression between pigeonpea sterility mosaic disease responsive genotypes using IDEG.6 web tool

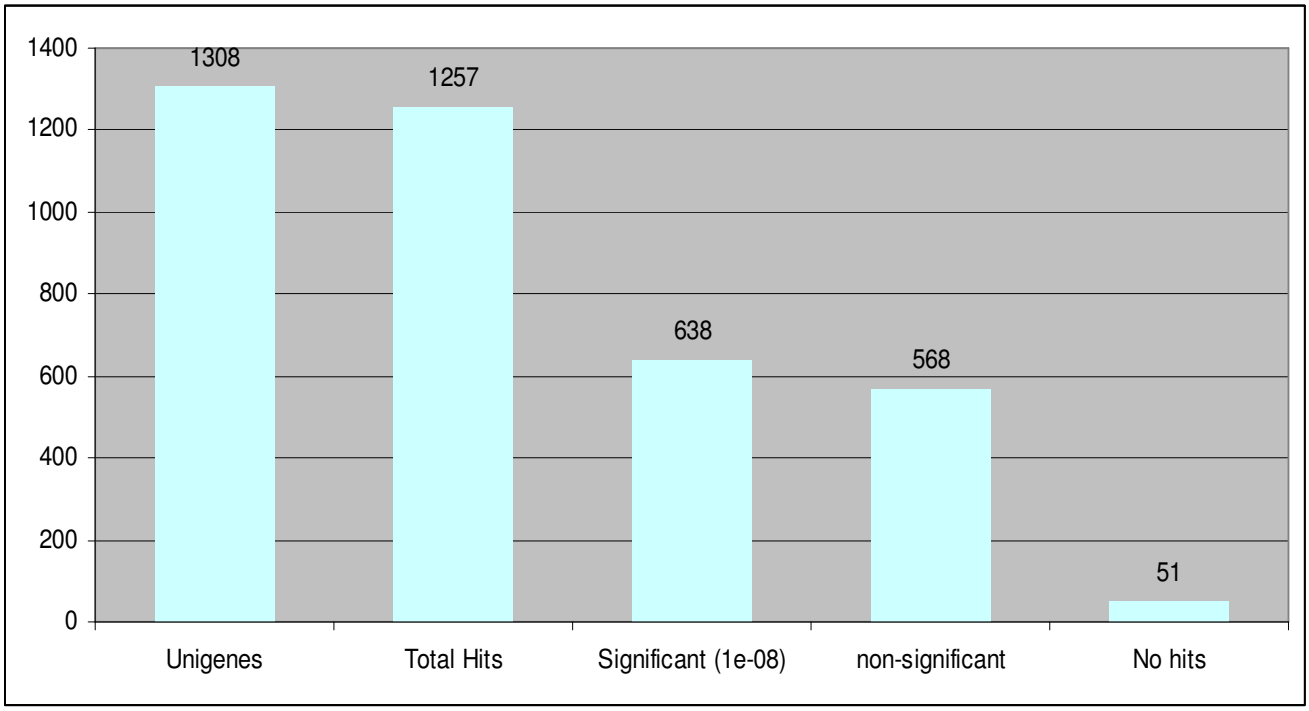


Figure 5: BLASTX similarity search for pigeonpea unigene sets against uniprot database

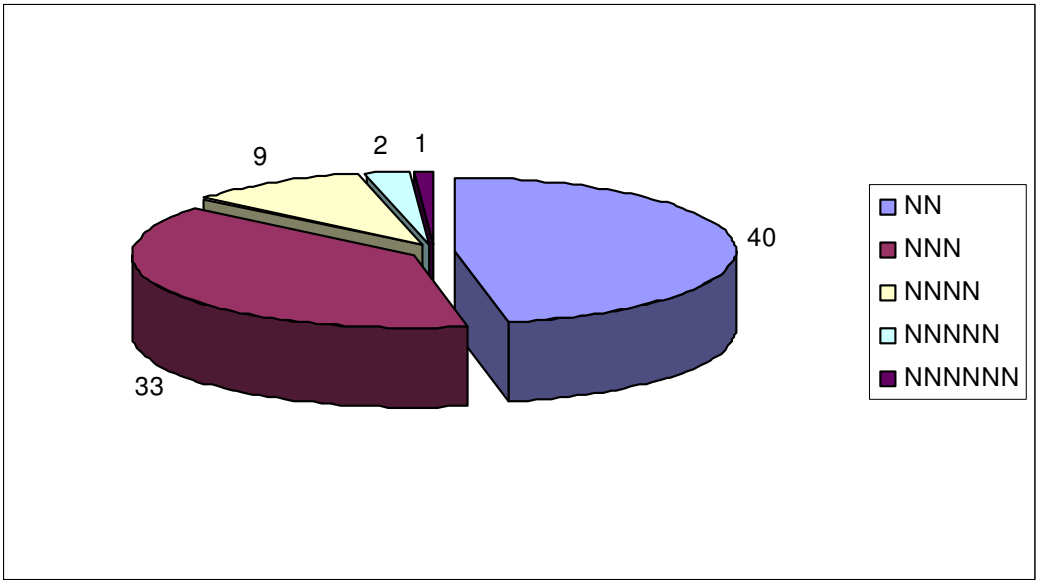


Figure 9: EST-SSR motifs derived from pigeonpea unigenes

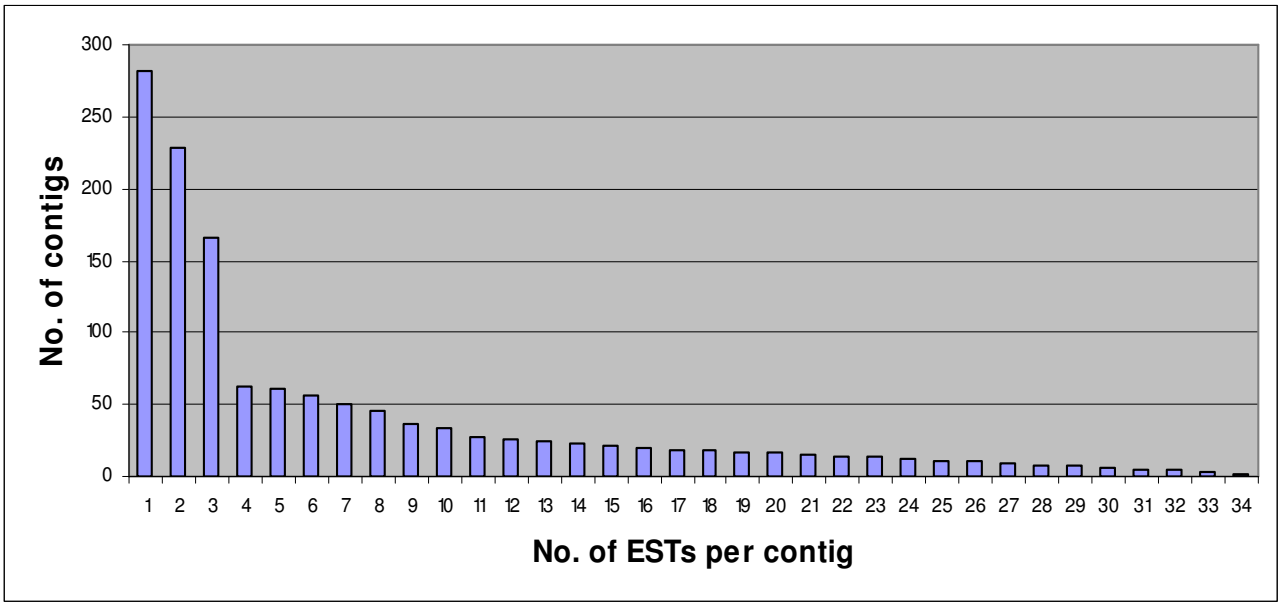


Figure 4: Frequency and distribution of pigeonpea ESTs among the assembled contigs

Figure 10: A snapshot showing capillary electropherogram for P1 (TTB7), P2 (ICP7035) and hybrid with ICPem0075 and CcM2456 SSR primers respectively

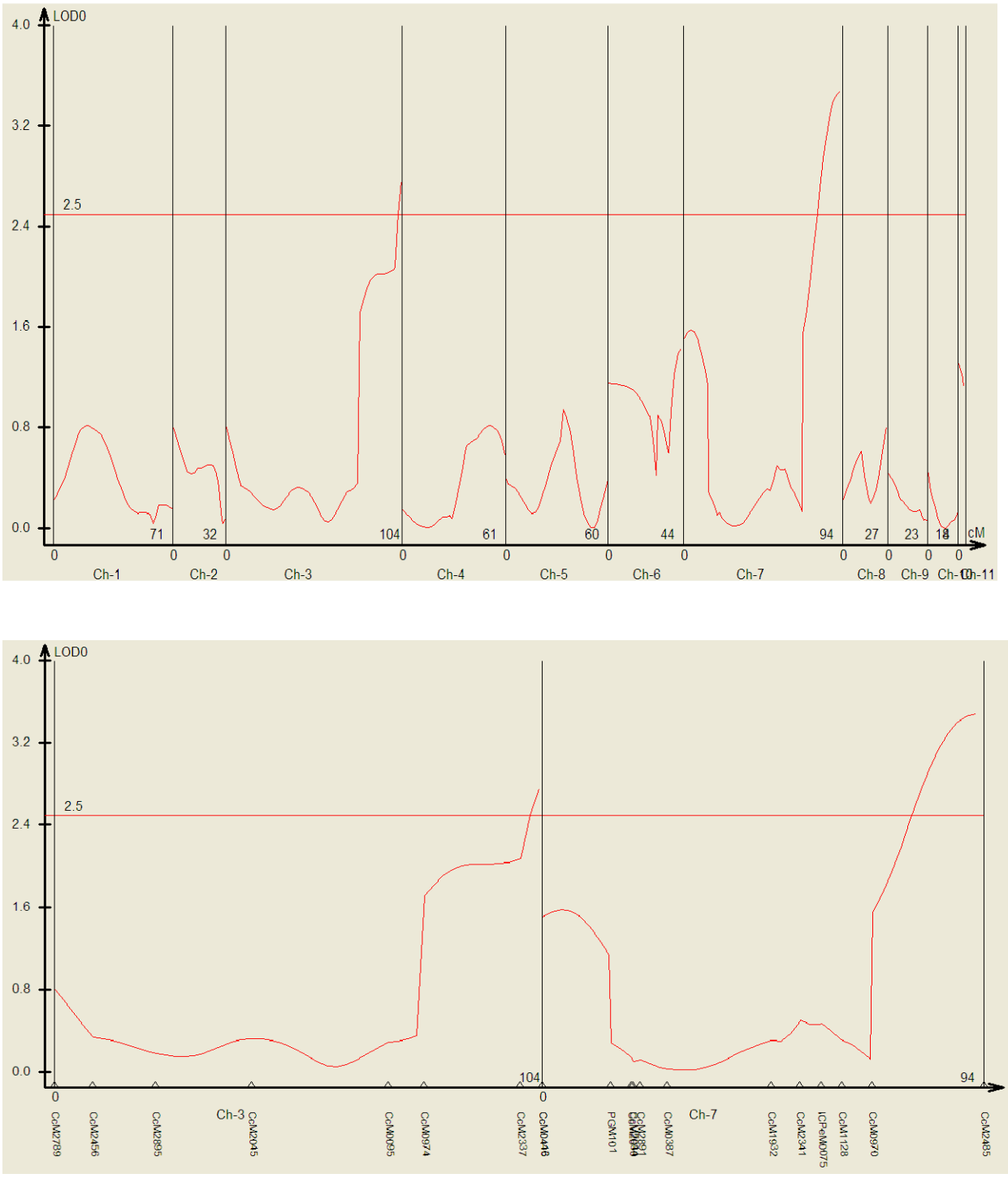
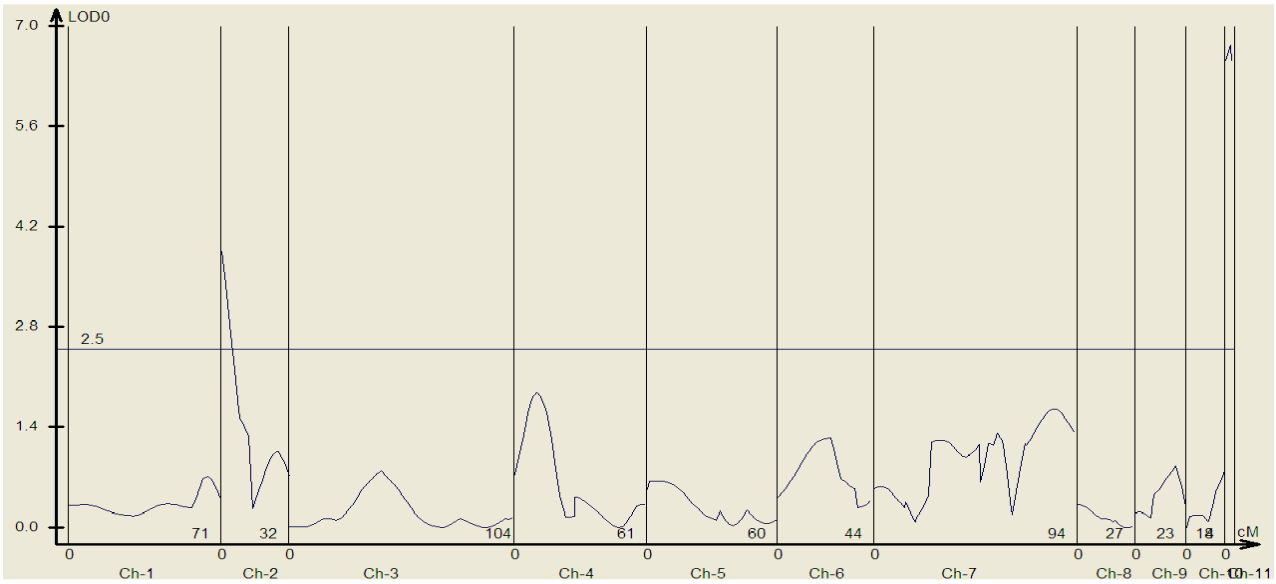


Figure 13: Graphical representation of QTLs associated with resistance to sterility mosaic disease for Bengaluru isolate in TTB 7 × ICP 7035 population



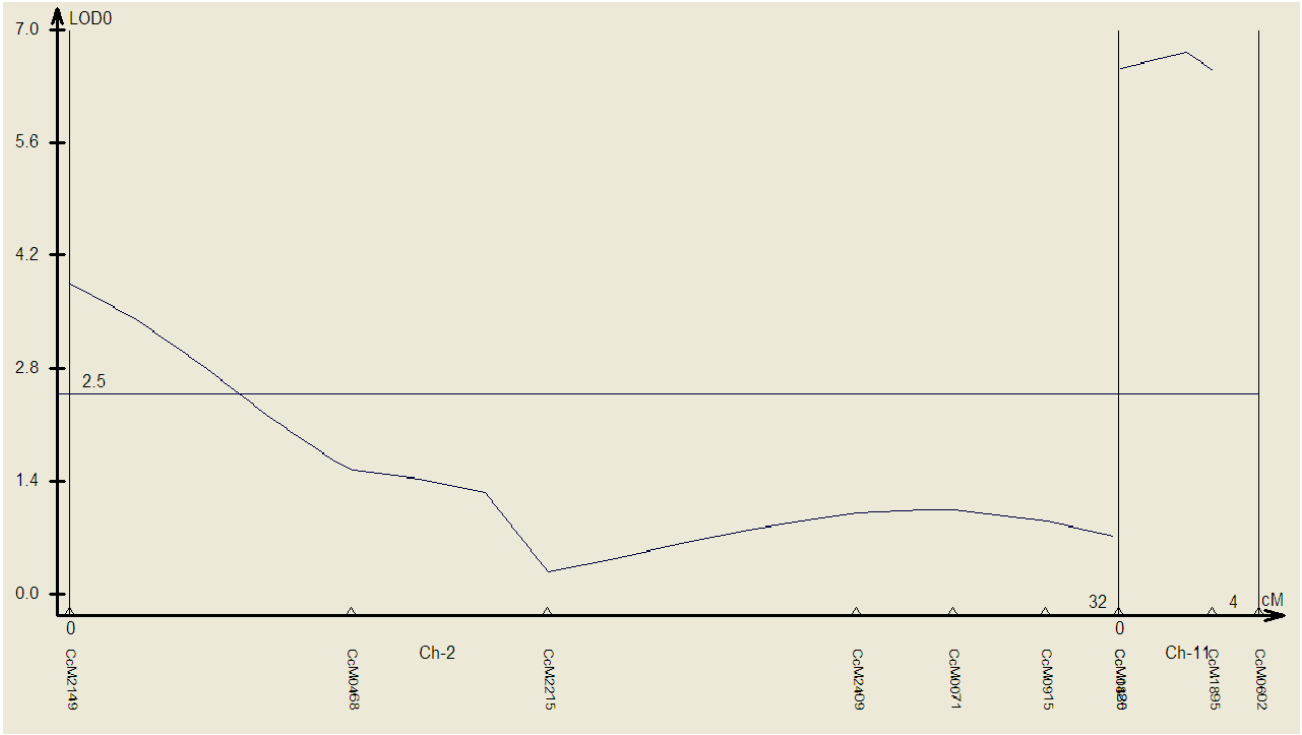


Figure 14: Graphical representation of QTLs associated with resistance to sterility mosaic disease for Patancheru isolate in TTB 7 × ICP 7035 population

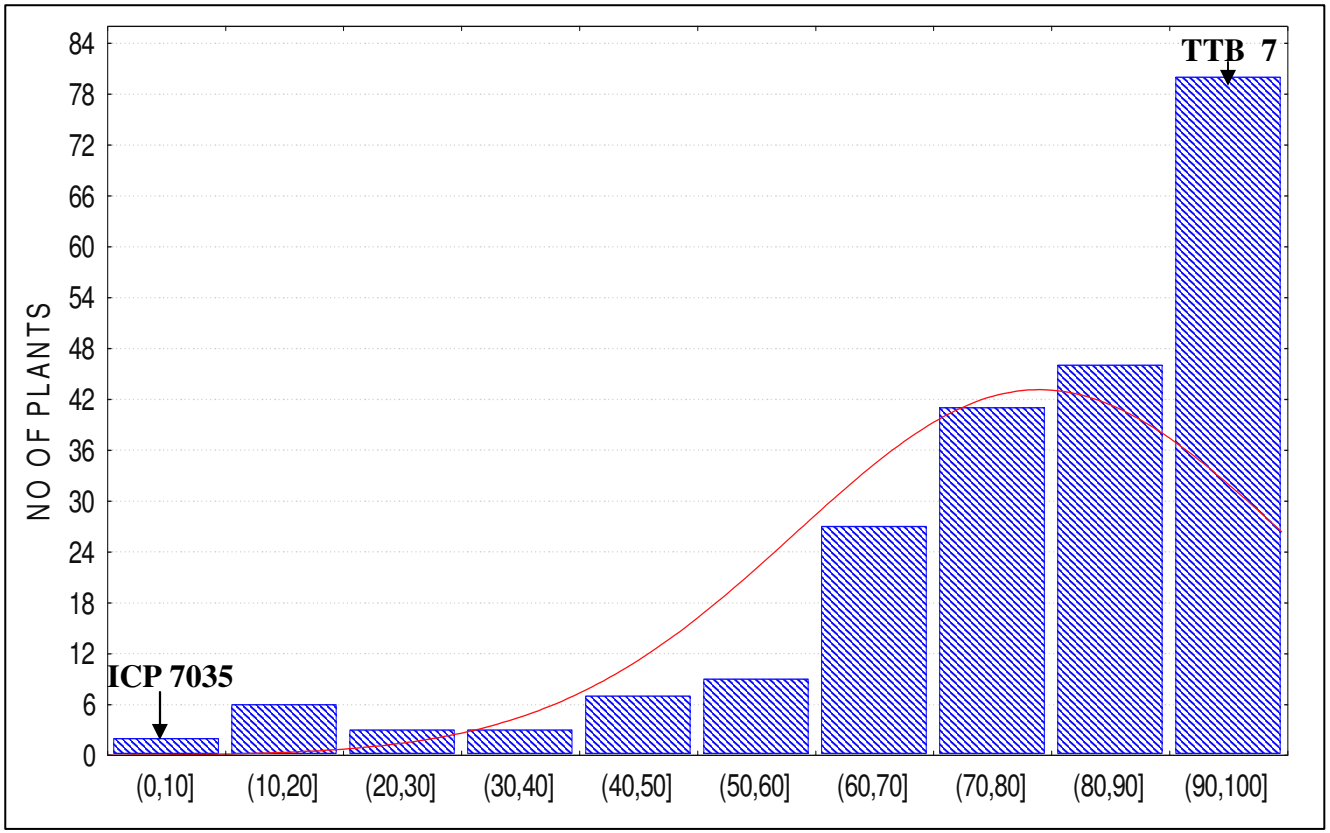


Figure 1: Frequency distribution of pigeonpea sterility mosaic disease incidence in $F_{2:3}$ mapping population (TTB 7 × ICP 7035) for Bengaluru isolate

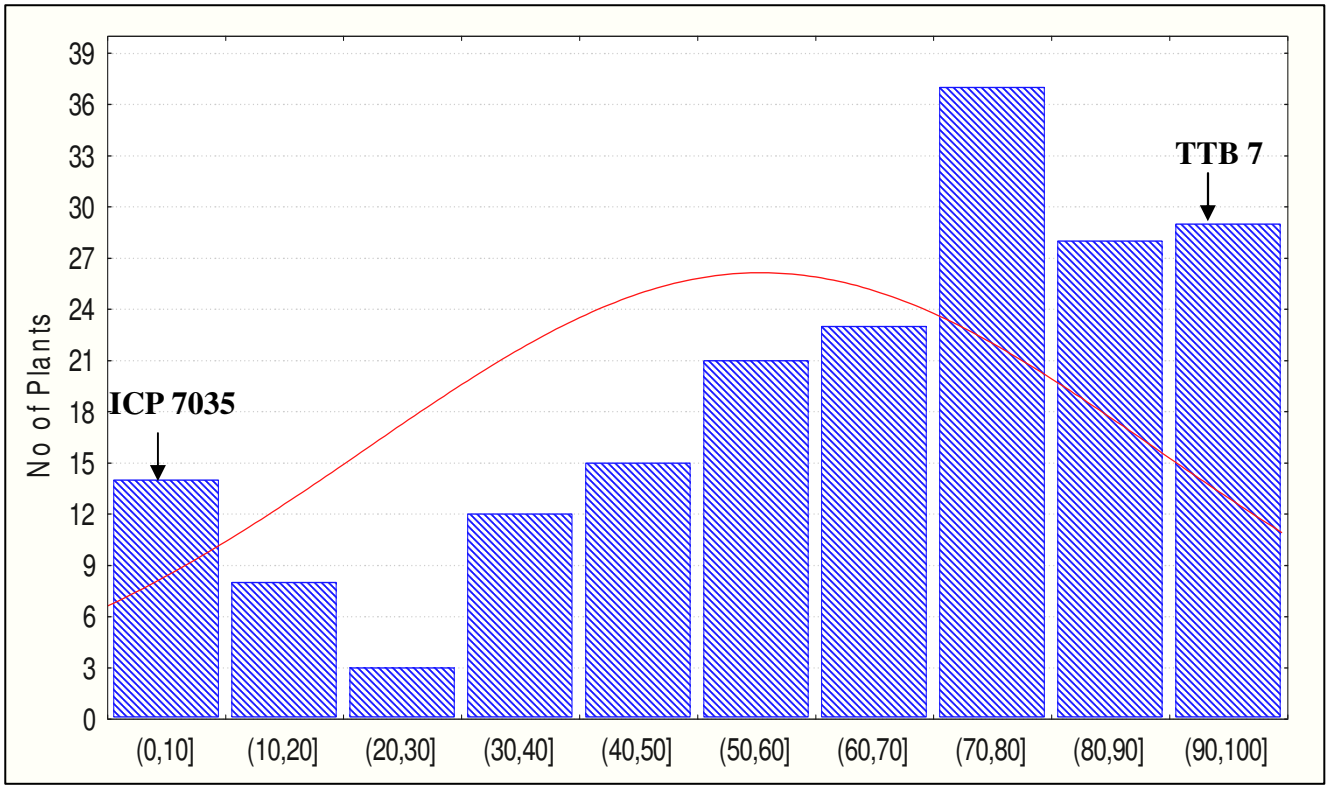


Figure 2: Frequency distribution of pigeonpea sterility mosaic disease incidence in $F_{2:3}$ mapping population (TTB 7 × ICP 7035) for Patancheru isolate

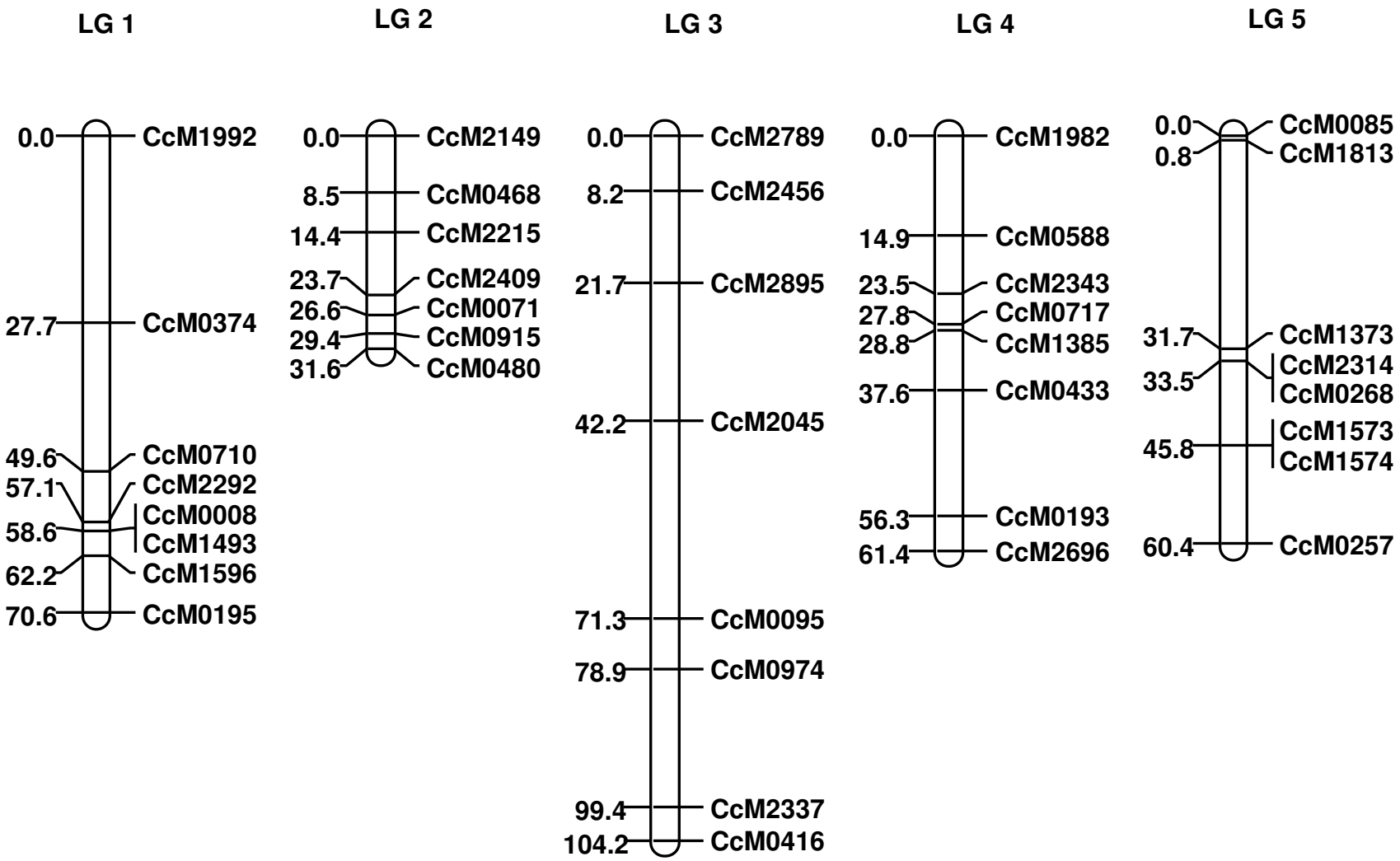
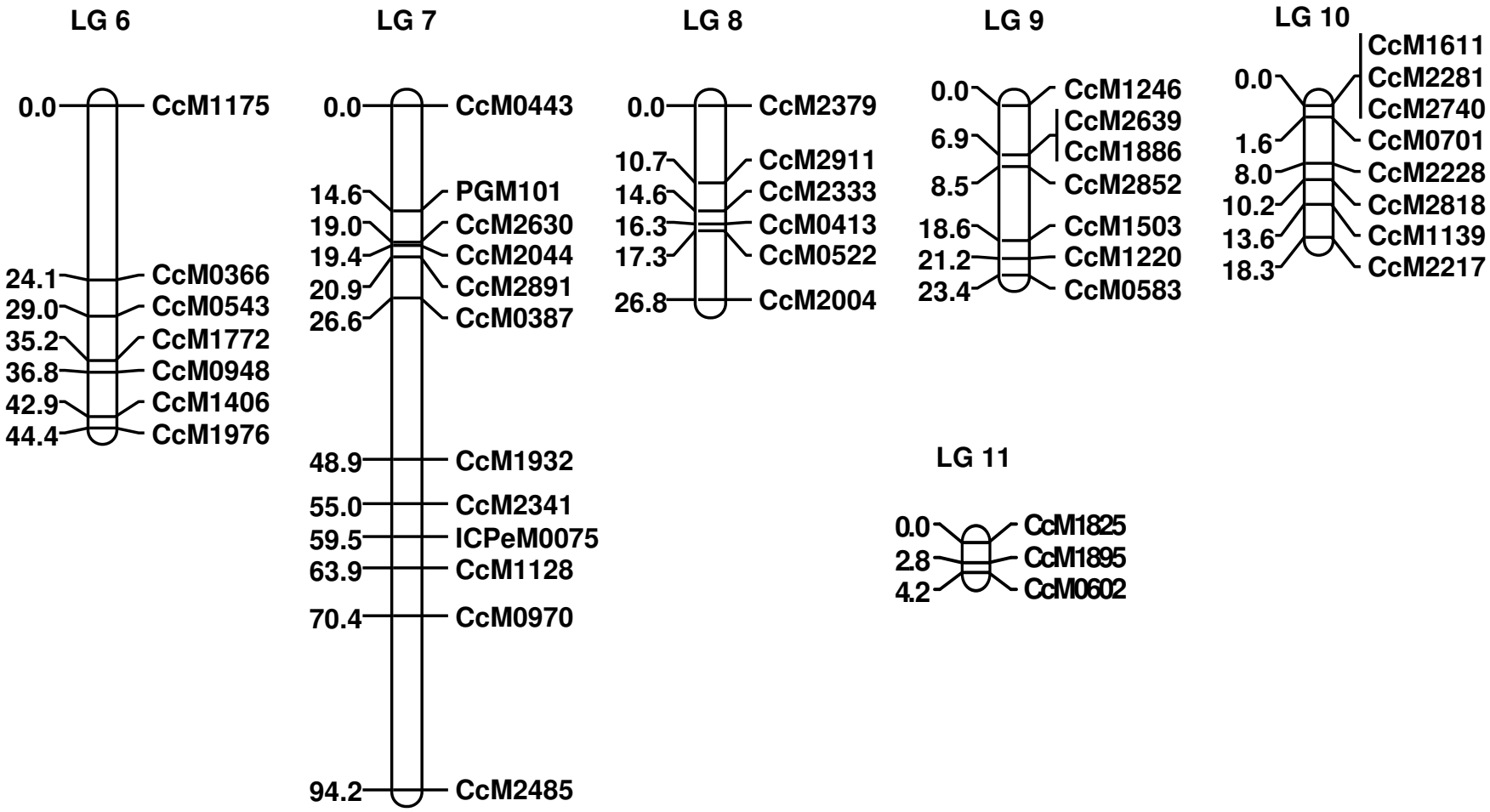


Figure 11: Genetic linkage map of pigeonpea cross TTB 7 × ICP 7035 F_2 population

Contd...

Figure 11:Contd...



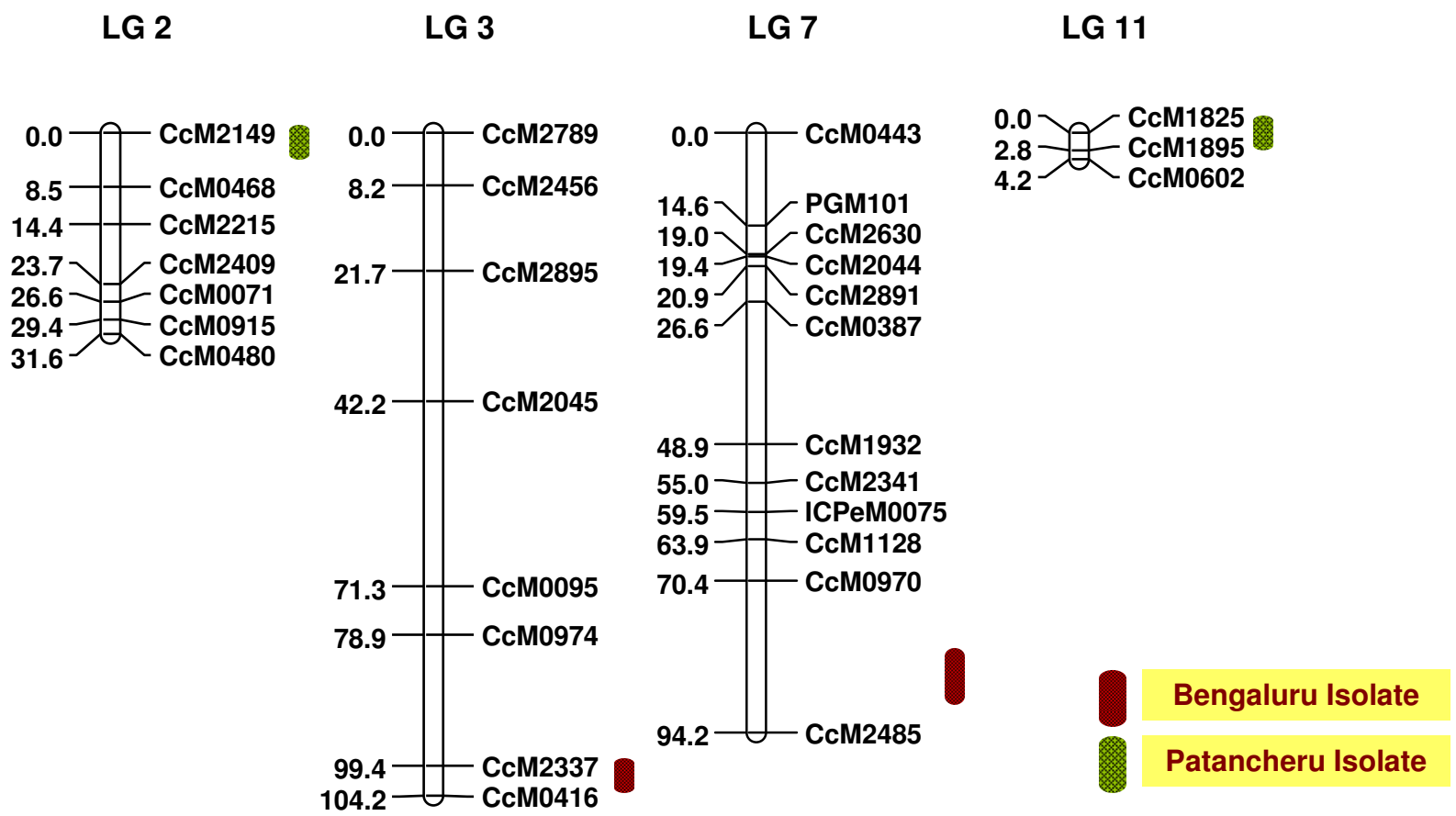


Figure 12: QTLs associated with resistance to pigeonpea sterility mosaic disease for Bengaluru and Patancheru isolates in TTB 7 × ICP 7035 population