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Identification of simple sequence repeat markers associated with wilt resistance in pigeonpea

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Abstract

The present study was carried out with the objective of identifying markers for *Fusarium* wilt resistance in pigeonpea using simple sequence repeat (SSR) and bulk segregant analysis (BSA). The wilt resistant (ICPL 87119) and wilt susceptible (T.Vishakha-1) genotypes were crossed and their F_2 population was used for marker analysis. Although, the parents were surveyed with 76 SSR primers to identify the polymorphic markers, only 26 primers were found polymorphic between the parents under study. The polymorphic information content scores of SSR markers ranged between 0.077 to 0.333, with an average of 0.18 per marker. These 26 primers were selected for BSA, which indicated that five SSR primers (PFW 26, PFW 31, PFW 38, PFW 56, and PFW 70) were able to distinguish the resistant and susceptible bulks and parents for wilt resistance. Hence, these five SSR markers can be utilized further for identification of wilt resistant genotypes of pigeon pea and for development of new wilt resistant varieties of pigeonpea.

Key words

Bulk segregant analysis, *Fusarium*, Pigeonpea, Simple sequence repeat, Wilt

Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp] is a major grain legume of the tropical and subtropical regions of the world (Varshney *et al.,* 2007). It is grown over more than 5 million hectares with a production of 4.40 million tonnes (FAOSTAT, 2011). Globally, it is the sixth most important legume food crop and India is the largest producer. Pigeonpea accounts for 4.42 million hectares of area, production 2.86 million tonnes, and yield 647 kg ha^{-1} in India (FAOSTAT, 2011).

We are not able to realize the yield potential of present day pigeonpea cultivars due to several biotic and abiotic constraints. This crop is attacked by a large number of plant pathogens including fungi, bacteria, viruses, mycoplasma like organisms and nematodes (Reddy *et al.,* 2012). *Fusarium* wilt (*Fusarium udum* Butler) of pigeonpea is one of the most important soil borne diseases of pigeonpea, which causes serious yield losses upto 100% in susceptible cultivars (Kiprop *et al.,* 2002). In the Indian subcontinent, the crop loss due to *Fusarium* wilt range from 16-47% (Prasad *et al.,* 2003). In India alone, this disease is estimated to cause production loss of approximately 97,000 tonnes per year (Saxena *et al.,* 2002).

Heavy losses due to this disease warrant for an urgent need to develop pigeonpea cultivars resistant to *Fusarium* wilt. However, it requires the availability of molecular markers for identification of the wilt resistant plants which can be used in the resistance breeding against wilt. The use of genetically linked molecular markers may facilitate marker-assisted selection in such cases. Although, RAPD markers have been used earlier to study the wilt resistance in pigeonpea (Kotresh *et al.,* 2006; Prasanthi *et al.,* 2009), however, non reproducibility of the RAPD fingerprints, low level of polymorphism, low sample throughput, locus-unspecific, and dominance of the RAPD markers are of greater concern. Simple sequence repeat (SSR) markers offer several advantages namely, high reproducibility, polymorphism, rapid automation, PCR-based and readily portable within a species as compared to other molecular markers (Edwards *et al.,* 1996; Shariflou *et al.,* 2001).

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There is a lack of complete understanding about the inheritance of wilt resistance which limit the breeding programmes aimed at efficiently introgressing such resistance into the genotypes having good agronomic traits and adapted to the region (Okiror, 2002a). Bulk segregant analysis (BSA) is a rapid and technically simple alternative for identifying markers linked to the gene of interest (Michelmore *et al.,*1991). The use of BSA, in combination with PCR-based molecular markers, has proven to be a very powerful technique for identifying markers co-segregating with genes underlying monogenic traits by utilizing populations resulting from a cross that segregates for the gene of interest (da Silva *et al.,* 2003). Inspite of the great loss incurred due to the attack of *Fusarium* wilt disease on pigeonpea, very limited research has been undertaken that focuses directly or indirectly on identifying markers for wilt resistance. Therefore this study aims to characterise parents and F_2 population derived from a cross contrasting for wilt reaction, using markers and bulk segregant analysis.

Materials and Methods

Plant material : The F_2 population comprising of 242 plants developed from the cross ICPL 87119 (wilt resistant parent) X T.Vishakha-1 (wilt susceptible parent) were used for this study. ICPL 87119 is a high yielding popular variety in Central and Southern zones of India; whereas the genotype T.Vishakha-1 is a semi spreading, early variety in Central Zones of India. Screening for the wilt resistant and susceptible plants was carried out on the wilt sick plot at the Pulses Research Unit, Dr. Panjabrao Deshmukh Agricultural University, Akola.

DNA extraction : Fresh leaf samples were collected from two weeks old germinated seedlings of parental genotypes and the segregating populations. Samples were used for isolation and purification of total genomic DNA following cetyltrimethyl ammonium bromide (CTAB) protocol with some modifications (Doyle and Doyle, 1990).

SSR markers and PCR : A total of 76 SSR primers, procured from Genetix (Bangalore, India), were used for screening of polymorphism between the two parents. Subsequently, the polymorphic markers between the parents were identified. The two DNA pools or bulk segregants were constructed by combining 50 µl (30 ng μl^{-1}) of DNA from each of the 10 highly resistant and the 10 highly susceptible selected samples. PCR reactions were performed in a 12.5 µl reaction volume [1.25 µl of 10X *Taq* buffer, 1 µl of 25 mM $MgCl_2$, 0.125 µl of 10 mM DNTPs, 0.7 µl of 5 units/µl *Taq* DNA polymerase (MBI fermantas, Germany), 0.75 µl of each primer, 2 µl (60 ng) of template DNA, and 5.925 µl of sterile water].

DNA amplification : DNA amplification was performed in a column gradient PCR programme GeneAmp PCR System 2700 (Applied Biosystem, USA) by using the following thermal profile: 95°C for 5 min (1 cycle); 94°C for 1 min, 57-62°C for 1 min; 72°C for 1 min (45 cycles); with a final extension at 72°C for 10 min (1 cycle).

Electrophoresis of PCR product : About 10 µl of PCR product were separated on 10% polyacrylamide gel (19:1 acrylamide/bisacrylamide, 8 M urea) (Sambrook *et al.,* 1989) at 80V for 12-14 hours in 1X TBE buffer (10X: 0.9 M Tris-base, 0.9 M boric acid and 0.32 M EDTA). 100 bp DNA ladder (MBI fermantas, Germany) was used as the molecular weight standard. After electrophoresis, gels were stained with silver staining (Tagelstrom, 1992) followed by destaining with double distilled water.

Data scoring : The SSR fingerprints were visualized and documented in a gel documentation system (Alpha Imager, Alpha Innotech Corp., California, USA). The banding pattern resolved after electrophoresis of the PCR products were scored for the presence or absence of bands and a locus was considered to be polymorphic if the band was present in one parent and not in the other.

Field evaluation for wilt resistance : 300 seeds of F_2, obtained from the cross ICPL 87119 X T.Vishakha-1, were sown in the wilt sick plot in order to establish the resistant and susceptible bulks that could be used in BSA for wilt resistance in pigeonpea. 242 plants of segregating population, parents and susceptible check were observed and data was recorded for wilt incidence on the basis of: purple band extending upwards from the base of the main stem, dieback symptoms of branches, partial wilting of the plant, visible browning or blackening of the xylem when the main stem or primary branches are split open.

Table 1 : Percent wilt incidence in F_2 population, parents and susceptible check

Type of material	Genotype	Total no. of plants	No. of susceptible plants	No. of resistant plant	Wilt incidence (%)
Segregation population for wilt reaction	F_2 population of crossICPL 87119 X T.Vishakha-1	242	35	206	14.5
Wilt resistant parent	ICPL 87119	40	00	40	00
Wilt susceptible parent	T.Vishakha-1	40	36	04	90
Wilt susceptible check	ICP 2376	100	100	00	100

Table 2 : Polymorphism obtained by SSR markers in ICPL 87119 and T.Vishakha-1 genotypes of pigeonpea

SSR Primers	Sequence of SSR Primer		No. of amplified bands (y)	No. of polymorphic bands (x)	% Polymorphism (x/y X 100)
	Forward Primer (5'→3')	Reverse Primer (5'→3')			
PFW 13	F: GCAAGTGTTCCCTACGTTGC	R: CTCCAACGGCCATAGTAGGA	6	2	33.30
PFW 24	F: TCTTAGCATGTCCTCTATTTTCGT	R: AGTACATTTCAAATCCACACATCC	13	1	7.69
PFW 25	F: TCACAGAGGACCACACGAAG	R: TGGACTAGACATTGCGTGAAG	10	1	10.00
PFW 26	F: GCGCTAAGGGAAAACAAAAA	R: AACTCCCTTGTTGTCATATGGTG	12	1	8.33
PFW 31	F: TCAGGGGTAAATGCGGTATC	R: GAATTGCTTTTTGCTTCCTCA	12	4	33.30
PFW 35	F: TGGGCATGGTAGAGGAAGTT	R: CGTCATGAAGCAACAGGAGA	10	1	10.00
PFW 37	F: AGGCTTTCTCCCTTCAATCC	R: GCCTTTTCAAACTTTTCTCACA	7	2	28.50
PFW 38	F: ACATGTGTGGCGTAGTGTGA	R: GCAAAACCGTTCCATAAAAA	7	1	14.20
PFW 43	F: TCGTGGGAATGCTCTACAAC	R: AACCACAAGTACACCCACACC	12	1	8.33
PFW 44	F: ATGGGCATGGTAGAGGAGGT	R: CGCTCATCATCGTCATCAAA	13	1	7.69
PFW 50	F: AGGTGCAAAGGAAGCACTAAT	R: CAGCTCCACTGTCTTCAACG	10	1	10.00
PFW 52	F: ATCCCAGACTTCATAGGGAGATAG	R: GTCTAGTCCCAGGTACAAAGAGGT	6	2	33.30
PFW 56	F: ATCCTCCAAAAGTTCCACCA	R: CAAAGGAGGATTTCCACCAA	10	2	20.00
PFW 58	F: ACCTTGCTTGTTTCGCTTTT	R: AAGGGAGGTGGACTACAAGGA	11	1	9.00
PFW 59	F: GTGAGTGAGAGTGAGTGTATTTGTG	R: GCTCTGATGCCAAATGTTGA	15	3	20.00
PFW 62	F: TGCTCTAATGGCTAGTTCATCC	R: AAACACTCATGGGTTAGATTCTCC	5	1	20.00
PFW 64	F: CAGGTCTGCTACTGCCATCA	R: AGCCCACTTCTGCATCACTC	13	3	23.00
PFW 65	F: CCACATCCCTCAACCCATAC	R: GAAAAGCCCTTGATGACACC	10	3	30.00
PFW 67	F: GGGAAACAAAATATCCCCTAATC	R: TAATCACACACATCACACCTAGCA	3	1	33.30
PFW 68	F: CACGATTCCATTGGTGGAG	R: ACGGTTTCTGGGAGGGTCTA	10	4	40.00
PFW 69	F: CCACAAGTACACCCACACCA	R: TTCGTGGGAATGCTCTACAA	12	1	8.33
PFW 70	F: ACAAATCCGGTGACCCATAA	R: CCGAGAACAAAAACATTGAACA	11	3	27.20
PFW 71	F: GACTAGAAAATTCACCTCCGTCTG	R: TTACAAAGGCTACATTGATGAGAAC	8	2	25.00
PFW 72	F: TCTTTCAGACGCAATGACCTT	R: CACTTATTTGTGGGGACCATC	9	1	11.10
PFW 73	F: CAAGGAATCACTTAAAAACCAAGC	R: AGATGGCCAAGATTCCACAAC	14	4	28.50
PFW 77	F: AACGATGAAATTCCCAAACG	R: TGTTAGATGCTCAACCCAAGG	8	2	25.00

Bulked segregant analysis : Bulk segregant analysis (BSA) (Shoba *et al.,* 2012) was used in conjunction with SSR analysis to find markers linked to genes of interest. Resistant and susceptible bulks were prepared from F_2 individuals by pooling aliquots containing equivalent amounts of genomic DNA (30 ng μl^{-1}) from each of the selected ten susceptible and ten resistant F_2 plants based on the phenotypic assessments. SSR markers were then screened on the resistant and susceptible parents first. The markers that were polymorphic between the parents were then screened to test the polymorphism between resistant and susceptible bulk.

Results and Discussion

Field evaluation for wilt resistance : Resistance to *Fusarium* wilt is one of the most important objectives of pigeonpea breeding programs in all pigeonpea growing regions of the world. Screening pigeonpea seedlings by artificial inoculation in the greenhouse permits examination of a large number of populations for resistance under uniform disease pressure. The on-field evaluation and screening for wilt resistance is considered as one of the best approach for identifying genes for genetic studies, because there may be differences in the expression of wilt symptom at various growth stages (Karimi *et al.,* 2010).

As expected, the F_2 population, obtained from the cross ICPL 87119 X T.Vishakha-1, segregated for the wilt resistance. Out of the 242 F_2 individuals under observation, 35 were found susceptible till maturity (Table 1). It was because of the fact that wilting of the pigeonpea cultivars have been reported to occur at various growth stages of the plant. Out of 35 susceptible individuals, 12 (PN 13, PN 14, PN 21, PN 23, PN 24, PN 26, PN 55, PN 87, PN 117, PN 124, PN 133, PN 147) showed wilt symptoms till seventy five days after sowing and were grouped separately as more susceptible individuals. Resistance to wilt diseases may

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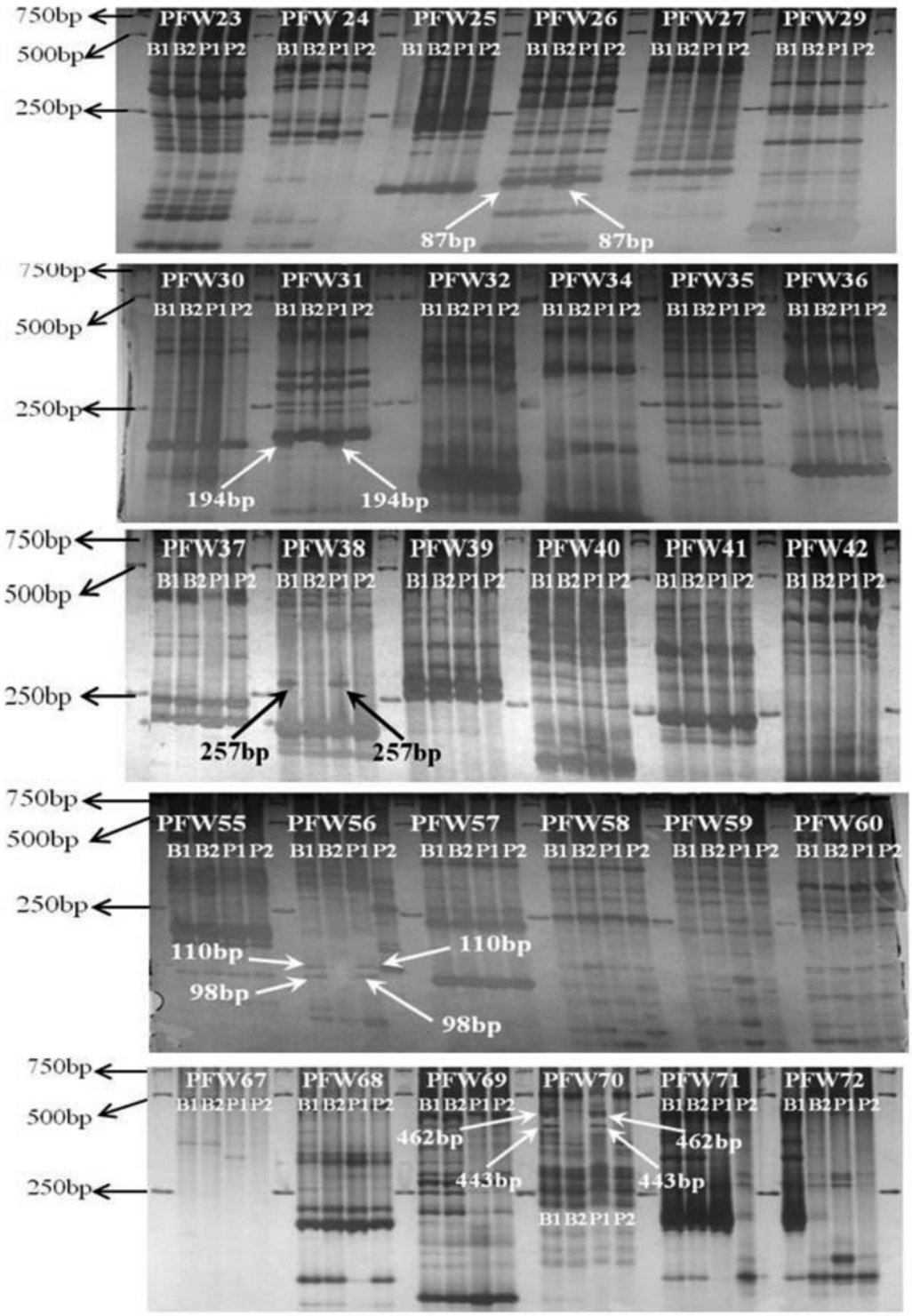

Fig. 1: SSR primers showing polymorphic loci for *Fusarium* wilt resistance

comprise of genes expressed at seedling, adult or maturity stages only, or at more than one stage (Okiror, 2002b). In pigeonpea, resistance to *Fusarium* wilt has been reported to be under the control of single dominant and a recessive gene (Karimi *et al.,* 2010).

SSR primer pairs combined with BSA have been successfully used earlier to identify DNA marker(s) linked to genes of interest (Shoba *et al.,* 2012) and comparing bulk samples is easier than evaluating many individuals in different populations (Sweeney and Dannebeger, 1994). BSA combined with several types of molecular markers have been extensively used to find markers linked to disease resistance genes in number of species (Barakat and Imbaby, 2005; William *et al.,* 2003). However, to ensure optimal cost-effectiveness, molecular markers used for marker assisted selection should permit efficient screening of large populations (Huang and Roder, 2004).

Parental polymorphism : Out of the 76 SSR primer pair tested to see whether they reveal polymorphism between the resistant (ICPL 87119) and susceptible (T.Vishakha-1) parents of the segregating population for wilt, only 26 SSR primers (34.21%) showed polymorphism and a total of 46 polymorphic loci suitable to differentiate between the two parents were identified (Khalekar *et al*., 2014). With the SSR primers showing polymorphism, the total number of bands amplified ranged from 3-15 whereas; the number of polymorphic bands ranged from 1-4. Of the total polymorphic SSRs, 11.54% SSRs showed a maximum of four polymorphic loci, 15.38% SSRs three polymorphic loci, 23.08% SSRs two polymorphic loci (39.13%) and 50.0% of the polymorphic SSRs showed single polymorphic loci (Table 2). The polymorphism information content for these markers ranged from 0.077 to 0.333 with an average of 0.18 per marker.

Very small number of SSR markers are available in pigeonpea (Odeny *et al.,* 2009; Saxena *et al.,* 2010a; Yang *et al.,* 2006) which are most commonly used for the study of genetic diversity in pigeonpea. Saxena *et al.* (2010b) studied 23 marker loci which showed polymorphism with 2-4 alleles and the polymorphism for these markers ranged from 0.12 to 0.65, with an average of 0.43 per marker. They have taken high number of polymorphic markers, higher genetic dissimilarity coefficient and contrasting phenotypic data into consideration for identifying parental combinations for developing mapping populations for *Fusarium* wilt resistance. While using the genic SSR markers for diversity studies, the expansion and contraction of SSR repeats in genes of known function can be tested for association with phenotypic variation or, more desirably, biological function (Ayers *et al.,* 1997).

Screening of bulk : The resistant and susceptible bulks were designed by adding equal quantities of DNA (30 ng μl^{-1}) respectively from the 10 resistant and 10 susceptible individuals from the segregating population. The above twenty six SSRs that were polymorphic between the parents were again used to test polymorphism between the resistant and susceptible bulks. Such an approach of BSA has been suggested to saturate the genomic regions of interest with molecular markers (Shoba *et al.,* 2012) instead of screening a relatively large number of individuals in the population (Lawson *et al.,* 1994).

Finally, only five polymorphic SSRs were able to generate polymorphic loci (seven in number) only in the resistant parent (ICPL 87119) and resistant bulk with molecular size 87 bp for Primer PFW 26; 194 bp for Primer PFW 31; 257 bp for Primer PFW 38; 98 and 110 bp for Primer PFW 56; 443 and 462 bp for Primer PFW 70 (Fig. 1). Hence, these five markers (PFW 26, PFW 31, PFW 38, PFW 56, and PFW 70) were found to be associated with the wilt resistance following the bulked segregant analysis and the seven polymorphic loci mentioned above could be considered as reliable markers for wilt resistance in pigeonpea.

Comparison of bulk samples is easier than evaluating many individuals in different populations (Sweeney and Dannebeger, 1994). BSA, therefore, provides a rapid and technically simple alternative for identifying markers linked to gene of interest and is considered highly efficient because it detects only a small percentage of polymorphisms in F_2 individual progeny tests. The use of BSA in combination with PCR-based molecular markers has proven to be a very powerful technique for identifying markers tightly linked to or cosegregating with genes underlying monogenic traits (Nakamura *et al.,* 2001; Shen *et al.,* 2003). The only prerequisite is the existence of a population resulting from a cross that segregates for the gene of interest (Magwene *et al.,* 2011). Looking into the severe damage caused by wilt disease, the present work suggest informative markers for identification of lines resistant to *Fusarium* wilt and such plants may be used in future molecular breeding programmes.

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