Legume Research, 38 (1) 2015: 126-130
Print ISSN:0250-5371 / Online ISSN:0976-0571

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
www.arccjournals.com/www.legumeresearch.in

Identification of AFLP markers linked to *Fusarium* wilt disease in pigeonpea [*Cajanus cajan* (L.) Millsp.]

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Received: 14-06-2013 Accepted: 10-02-2014 DOI:10.5958/0976-0571.2015.00021.1

ABSTRACT

An experiment was conducted to identify markers linked to Fusarium wilt disease resistance, Parents namely TTB 7 and ICP 8863 were screened using 151 SSRs markers and 16 AFLP primer combinations. Parental screening revealed five SSR primers and 12 AFLP primer combinations polymorphic between parents. Bulk segregant analysis identified five AFLP primer combinations generating seven markers polymorphic between resistant and susceptible bulks while, none of the SSR markers were polymorphic. This indicates that, these markers are putatively linked to wilt disease. Screening of F_2 segregating population of cross TTB 7 x ICP 8863 with these putatively linked markers revealed four markers (*E*-AAT/*M*-CTG_{850}, *E*-TCG/*M*-CTT_{650}, *E*-TCG/*M*-CTA_{730} and *E*-TCG/*M*-CTT_{230}) which segregated in 3:1 mendelian pattern. Simple linear regression performed on these four markers had identified two markers namely *E*-TCG/*M*-CTT_{650} and *E*-TCG/*M*-CTA_{730} linked to disease.

Key words: AFLP, BSA, *Fusarium* wilt disease, Linkage, Pigeonpea.

INTRODUCTION

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is an important leguminous crop of semi-arid tropics and is grown in an area of 3.73 million hectares in India with production of 3.07 million tons (DES 2010). India is the largest producer of pigeonpea accounting for 77% of the world's production, but productivity is very low in comparison to world's average. This low productivity could be attributed to susceptibility to various pests and diseases.

Fusarium wilt caused by *Fusarium udum* (Butler) is an important soil borne disease which affects plant establishment and seed yield. The loss due to wilt disease is approximately 97,000 tons of grains per year in India (Saxena *et al.*, 2002). Breeding for resistant varieties is considered as one of the most effective method of reducing crop losses. However, long life cycle, out crossing nature, difficulty in accurate phenotyping and linkage drag are the major problems being faced in conventional breeding efforts for wilt resistance in pigeonpea. Identification of markers which are closely linked to wilt resistance would help in quick assessment of susceptibility or resistance at seedling level which in turn will eliminate the need for maintaining susceptible genotypes and repeated phenotyping of segregating populations in the sick plots. Thus it helps in marker-assisted selection (MAS) in breeding programmes.

SSRs and AFLP markers have proved as more reliable, hypervariable and reproducible as compared to RAPD markers. AFLP technique has been used to identify markers linked to sterility mosaic disease in pigeonpea (SMD) (Ganapathy *et al*., 2009 and Gnanesh *et al.*, 2010). However, till date no reliable trait specific markers are available in pigeonpea for *Fusarium* wilt disease except for RAPD markers identified by Kotresh *et al*., (2006). Though RAPD is simple and easy but lacks reproducibility and hence it is not widely used. When SSR markers were limited, AFLP has been used as next alternative for mapping in soybean (Keim *et al*., 1997), *Lens* sp. (Eujayl 1998, Hamwieh *et al.*, 2005) wheat (William *et al*., 2007) and in other crops. Since limited number of SSRs are available in pigeonpea (Odeny *et al*., 2007) and can scan only one locus at a time (Liu *et al.*, 2000; Shen *et al*., 2005) whereas AFLP technique is capable of scanning several loci at once (Arunita *et al*., 2010). Hence as next alternative AFLP markers were used in the present study.

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Single marker analysis (SMA) based on regression is a simplest method to detect association between marker and phenotype without the need for complex linkage maps (Arunita *et al*., 2010). The present study reports identification of two AFLP markers linked to *Fusarium* wilt resistant loci in pigeonpea following SMA.

MATERIALS AND METHODS

The material used for the study comprised of two diverse genotypes selected based on previous reports for their resistance and susceptible levels (Saifulla *et al*., 2005). The resistant parent ICP 8863 differed from susceptible parent TTB 7 with respect to high level of intrinsic resistance to *Fusarium* wilt (Saifulla *et al*., 2005). The mapping populations were developed by crossing TTB 7, a susceptible parent, as female with ICP 8863, a resistant parent, as a male.

The individual flowers of the selected female parents (TTB 7) were hand emasculated and pollinated with the pollen dust from the male parent (ICP 8863). Hybridization was carried out during March 2008 under honeybee proof nylon net to prevent contamination by natural out crossing. Morphological traits such as plant type; flower colour, pod colour and seed colour were used as phenotypic markers to check the trueness of F_1 plants.

F_1 plants were selfed to get F_2 generation during *kharif* 2008. Part of F_1 seeds was retained for disease screening. All the F_2 plants were covered with nylon net to prevent insect pollination. A spacing of 60 cm between rows and 30 cm between plants were followed. Standard package of practices was followed for raising a good and healthy crop. Seeds obtained from each individual F_2 plants were collected and forwarded to F_3 generation for evaluating against *Fusarium* wilt reaction.

Phenotyping of F_3 generation: Screening of parents, F_3's generations were done under wilt sick soils. Ten seeds per family of F_3 population of a cross TTB 7 x ICP 8863 was sown during *kharif* 2009. Plants that are wilted at maturity were classified as susceptible and those, which did not wilt, were recorded as resistant. Average disease incidence was calculated for each family row in F_3 generation. Based on the mean scores of F_3 generation, homozygous resistant and susceptible F_2 plants were identified.

Genotyping of F_2 generation: DNA extraction was done using 0.5g of young leaves from 20-25 day old seedlings were collected in parents and F_2 individuals. DNA was extracted by SDS method involving 3ml of extraction buffer [3X saline sodium citrate (26.31g NaCl, 13.23g trisodium citrate and make up the volume to 1000ml), 50mM EDTA (pH 8.0)] and 150µl of 20% SDS. Quality and quantity of DNA was determined through electrophoresis using 0.8 % agarose gel.

SSR analysis: SSR analysis was performed using 151 pigeonpea specific SSR markers obtained from ICRISAT. Final PCR reaction volume of 10 µl contained 5ng of template DNA, 3 µl of 1mM dNTPs, 0.2 µl of 10pmol of primer mix and 0.2 unit *Taq* polymerase and 1.0 µl of 10X *Taq* buffer. Three different touch-down annealing temperatures namely 55-45ºC, 60-55ºC and 65-60ºC were used. Amplification of primers was tested on agarose (3.5%) gel.

AFLP analysis: AFLP analysis was performed (Vos *et al.* 1995) using 16 primer combinations (Table 1). Genomic DNA (250 ng) was digested with *Eco*RI (20 U) and *MSe*I (10 U) restriction enzymes at 37°C for 3hrs. For 20 µl of digested reaction mix, T4 DNA ligase buffer with 10mM ATP (0.4 µl), *Eco* RI adapter (5pmol /µl), *Mse* I adapter (50 pmol/µl) and T4 DNA ligase (1U/µl) was added and incubated at 37°C for 16- 18 hours overnight. The digested/ligated AFLP templates were then diluted $T_{10}E_{0.1}$ pH 8.0 in the ratio of 1: 5 (i.e 1µl of DNA template: 4µl of $T_{10}E_{0.1}$) and stored at -20°C. Polymerase chain reaction (PCR) was performed in two steps: Pre-amplification and selective amplification. Amplification was performed in Eppendorf master thermal cycler PCR. Components of pre-amplification PCR involved template DNA from ligation step, *Eco* + N* (7.5 ng/µl), *Mse* + N* (7.5 ng/µl), 1 mM dNTP mix, *Taq* buffer (10X) and *Taq* polymerase (5U/µl). Pre-amplification was performed with PCR program 94°C for 30 sec, annealing at 56°C for 60 sec, extension at 72°C for 60 sec repeated for 20 cycles and then at 10°C for 30 min. The pre-amplified products were diluted by adding $T_{10}E_{0.1}$ pH 8.0 in the ratio of 1:5 (i.e. 1µl of DNA template: 4µl of $T_{10}E_{0.1}$). Components for selective amplification PCR were similar to pre-amplification except that primers had 3 nucleotide extensions (*Eco*RI + N*N*N*, *Mse* I + N*N*N*). Selective amplification was performed with PCR program 94°C for 30 sec, annealing at 65°C for 30 sec reducing by 0.7°C/cycle to 56°C, extension at72°C for 60 sec repeated for 11 cycles, followed by 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 60 sec repeated for 24 cycles and then at 10°C for 30 min.

TABLE 1: Details of Polymorphism studies in parents of cross TTB 7 x ICP 8863 using SSR markers

Details	Parents of cross TTB 7 (S) x ICP 8863 (R)	
	Total	Per cent
Good amplification	127	84.11
Poor amplification	24	15.89
Polymorphic	5	3.31
Monomorphic	122	80.79
Total	**151**	-

Bulk Segregant Analysis (BSA): Bulk Segregant analysis (BSA) was carried out as described by Michelmore *et al*., (1991). The DNA (0.5µg) from each of the five resistant and five susceptible F_2 individuals was used for preparing bulks. The resistant and susceptible individuals were identified based on their reaction to *Fusarium* wilt disease in their progeny generation (F_3).

Parental polymorphism and linkage analysis: Two parents TTB 7 and ICP 8863 were screened with 151 SSRs and 16 AFLP primer combinations. Primer combinations, which revealed polymorphism between parents, were used to screen resistant and susceptible bulks. The putatively linked primers from bulk segregant analysis were used for screenings of 72 F_2 individuals of cross TTB 7 x ICP 8863 along with their parents (TTB 7 and ICP 8863) and bulks. Markers which revealed presence of bands in resistant parent (ICP 8863) and resistant bulk but absent in susceptible parent (TTB 7) and susceptible bulk along with their F_2 counterparts were considered for further analysis. Chi- square tests (x^2) (Pearson 1922) were performed to examine the goodness of fit between the expected Mendelian ratio for the segregation data of the linked AFLP markers. Simple linear regression analysis (SLRA) was performed to determine linkage between marker and trait (Ben 1998).

RESULTS AND DISCUSSION

SSR and AFLP analysis: Results of SSR analysis are presented in Table 1. Out of 151 SSR primers screened 84.11 per cent (127) primers could be amplified where as remaining 24 primers could not be amplified. Out of 127 primers only 5 (3.31%) primers showed polymorphism between TTB 7 and ICP 8863. During bulk segregant analysis none of the primers were polymorphic between resistant and susceptible bulks. Screening of parents with 16 AFLP primer combinations revealed 12 combinations polymorphic between two parents (Table 2). Out of 12 AFLP primer combinations, four were found to be polymorphic, while the remaining seven primer combinations did not show polymorphism between bulks. Five primer combinations which showed polymorphism during bulk segregant analysis namely *Eco*RI+TCA/ *Mse*I+CTA, *Eco*RI+TCG/*Mse*I+CTT, *Eco*RI+TCG/ *Mse*I+CTA, *Eco*RI+AGC/ *Mse*I+CTA and *Eco*RI +AAT/ *Mse*I+CTG. were assumed to be linked to disease resistance (Michelmore *et al*., 1991).

Linkage analysis: Four putatively linked AFLP primer combinations during bulk segregant were tested against the genomic DNA of randomly chosen subset of 72 F_2 plants to analyze the segregation pattern of the markers. These four primer combinations generated seven markers (Table 3). Among these seven markers, marker *E*-TCG/*M*-CTT_{650} amplified bands in 23 genotypes out of 72 F_2 plants and was absent in 49 F_2 individuals. Marker *E*-AAT/*M*-CTG_{850} amplified in 21 and absent in 51 individuals out of 72 F_2 individuals. Similarly, markers *E*-TCG/*M*-CTA_{730} and *E*-TCG/*M*-CTT_{230} (Fig 1) showed amplification in 26 and 25 individuals respectively. The remaining three markers namely, *E*-TCG/*M*-$CTA_{900,}$ *E*-TCG/*M*-CTA_{300} and *E*-AGC/*M*-CTA_{520} amplified in 45, 32 and 37 F_2 individuals respectively. Chi-square tests were performed on the above seven markers to examine the goodness of fit between the observed and expected AFLP marker bands. Out of seven markers, only four AFLP markers (*E*-TCG/*M*-$CTT_{650,}$ *E*-TCG/*M*-$CTA_{730,}$ *E*-

TABLE 2: Details of the polymorphic AFLP markers detected in parents and bulks in F_2 of cross TTB 7 (S) x ICP 8863 (R)

Eco RI primer selective nucleotides	*Mse* I primer selective nucleotides	Polymorphism details	
		Parents of cross TTB 7 (S) x ICP 8863 (R)	Resistant and susceptible bulks of F_2
+TCA	+GAC	Polymorphic	Monomorphic
+TCA	+TCG	Polymorphic	Monomorphic
+TCA	+CTT	Polymorphic	Monomorphic
+TCA	+CTG	Monomorphic	Monomorphic
+TCA	+TCA	Polymorphic	Monomorphic
+TCA	+CTA	Polymorphic	Monomorphic
+TCA	+AAT	Polymorphic	Monomorphic
+TCG	+GAC	Polymorphic	Monomorphic
+TCG	+TCG	Monomorphic	Monomorphic
+TCG	+CTT	Polymorphic	Polymorphic
+TCG	+CTG	Polymorphic	Monomorphic
+TCG	+TCA	Monomorphic	Monomorphic
+TCG	+CTA	Polymorphic	Polymorphic
+AGC	+CTA	Polymorphic	Polymorphic
+AAT	+CTG	Polymorphic	Polymorphic
+AAT	+CTA	Monomorphic	Monomorphic
Total	16	12	7

TCG/*M*-CTT$_{230}$ and *E*-AAT/*M*-CTG$_{850}$) had chi-square (calculated) value less than chi-square table value at p<0.01. Hence these four markers were segregating in 3:1 pattern which is typical ratio of any monogenic dominant marker. Of the four markers that showed Mendelian segregation ratio, two markers namely, *E*-TCG/*M*-CTT$_{650}$ and *E*-TCG/*M*-CTA$_{730}$ showed significant linkage with resistance gene when analyzed through SLRA (Table 4).

Identification of stable marker system linked to disease resistance would help in accelerating the breeding programme. The identified markers for *Fusarium* wilt resistance will help in developing Sequenced Characterized Amplified Regions (SCAR) markers or Cleaved Amplified Polymorphic Sequence (CAPS) markers (Paran and Michelmore 1993; Lu *et al*., 2000). These markers can be used for cloning and characterizing *Fusarium* wilt resistance genes and for marker-assisted selection. Since pigeonpea specific SSR markers are very limited efforts are required to bridge this gap. In addition utility of SSR markers from other leguminous species could be explored.

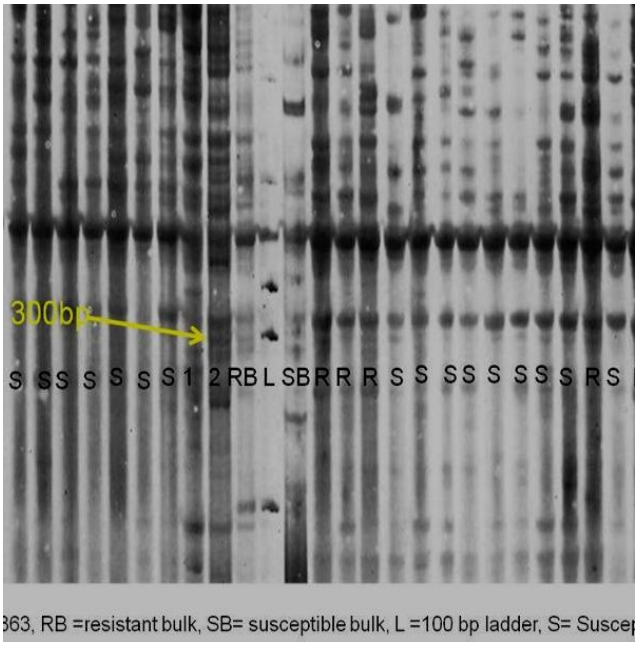

FIG 1: AFLP marker analysis of F_2 mapping population with *E*-TCG/*M*-CTA in Pigeon pea

TABLE 3: Segregation behavior of linked AFLP markers to *Fusarium* wilt resistance in F_2 of cross TTB 7 (S) x ICP 8863 (R)

AFLP marker	Total F_2 plants	Observed Polymorphic bands		Expected polymorphic bands		RatioA:P	x^2(Cal)	x^2(Tab.)
		Present	Absent	Present (P)	Absent (A)			
E-AAT/M-CTG$_{850}$	72	21	51	18	54	3:1	0.667	3.84
E-TCG/M-CTT$_{650}$	72	23	49	18	54	3:1	1.852	3.84
E-TCG/M-CTT$_{230}$	72	25	47	18	54	3:1	3.63	6.64
E-TCG/M-CTA$_{730}$	72	26	46	18	54	3:1	4.75	6.64
E-TCG/M-CTA$_{900}$	72	45	27	18	54	3:1	54.00	6.64
E-TCG/M-CTA$_{300}$	72	32	40	18	54	3:1	14.51	6.64
E-AGC/M-CTA$_{520}$	72	37	35	18	54	3:1	26.74	6.64

TABLE 4: Linkage between *Fusarium* wilt resistant gene and AFLP marker in cross TTB 7 (S) x ICP 8863 (R) by simple linear regression analysis

Marker	b0	b1	-2ln(L0/L1)	F(1,n-2)	pr(F)
E-AAT/M-CTG$_{850}$	72.288	-1.541	0.048	0.047	0.829
E-TCG/M-CTT$_{650}$	62.658	-16.648	5.981	6.064	0.016*
E-TCG/M-CTA$_{730}$	63.67	-14.436	4.282	4.289	0.042*
E-TCG/M-CTT$_{230}$	74.549	1.771	0.058	0.057	0.813

*Significance @ 0.05

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