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Development of Sequence Characterized Amplified Region (SCAR) Marker for Fusarium Wilt Resistance Gene in Pigeon Pea (*Cajanus cajan* L. Millsp.)

Lakki Reddy Prasanthi* • **Bommu Veera Bhaskara Reddy** • **Kokkanti Rekha Rani** • **Yeturu Siva Prasad** • **Thovi Rajeswari** • **Kondreddy Raja Reddy**

Acharya N.G.Ranga Agricultural University, Regional Agricultural Research Station, Tirupathi -517502, Andhra Pradesh, India

Corresponding author: * **prasanthi64@rediffmail.com**

ABSTRACT

Fusarium wilt resistance in pigeon pea is controlled by a single dominant gene. Bulked segregant analysis (BSA) was applied to identify molecular markers linked to a major resistant gene using the F_2 population of two crosses i.e. LRG-41 x ICPL-87119 and ICPL-7035 X ICPL-8863. A total of 195 random oligonucleotide primers were surveyed. Primer $OPG08_{950}$ was found to produce a consistent marker, which differentiated resistant from susceptible parent and bulk. Co-segregation analysis of the putative marker in the F_2 population confirmed the association of $OPG08_{950}$ produced by primer OPG08 with the resistance gene. An identified random amplified polymorphic DNA (RAPD) marker, OPG08, linked to fusarium wilt resistance in pigeon pea was cloned and sequenced. Their end sequences were used to design an allele-specific sequence characterized amplicon region (SCAR) primer SCAR937(14f/r), which was found promising in all generations. The marker designed was amplified at a specific site of 937 bp in resistant parents, F_1s and only in resistant F_2 plants. This would help to identify the transfer of the Fusarium wilt resistance gene to susceptible lines.

Keywords: bulked segregant analysis, dominance, polymorphism, recessive, selection

INTRODUCTION

Pigeon pea (*Cajanus cajan* L. Millsp.) is a grain legume belonging to the *Cajaninae* sub-tribe of the economically important leguminous tribe *Phaseoleae*. Pigeon pea is the only cultivated food crop of the *Cajaninae* sub-tribe and has a diploid genome comprising 11 pairs of chromosomes (2n = 22). India is the world's largest pigeon pea producer and grows over 77% of the total world production (Saxena 2008). It has an inherent ability to withstand environmental stress (especially drought) making it one of the most sought after crops in plant introduction trials aimed at bringing new areas under cultivation. Wilt caused by *Fusarium udum* is very severe and destructive disease from seedling to adult stages and the fungus spreads about 3 m through the soil in *kharif* crop season along the roots, leading to progressive chlorosis of leaves, branches, wilting and collapse of the root system (Butler 1906). This disease is especially prevalent in India and East Africa, where field losses of 50-70% are common. In India alone, the loss due to this disease was estimated to be US $71 million (Kotresh *et al.* 2006). Identification and development of a Fusarium resistant line with higher seed yield is an essential need in the improvement of productivity in this crop. Resistance to wilt is governed either by a single dominant gene (Pandey *et al*. 1996; Kotresh *et al*. 2006) or a recessive gene (Jain and Reddy 1995) in different crosses. More than one gene has also been reported in some crosses (Shaw 1936; Joshi 1957).

The search for sources of resistance to wilt in pigeon pea began as early as 1905 at Poona, India. Varieties such as Asha (ICPL-87119) and Maruthi (ICP-8863) have shown high stable resistance. In breeding for wilt resistance in pigeon pea traditional methods are based on crosses between susceptible and resistant genotypes followed by phenotypic selection for wilt resistant genotypes in sick plots. The identification and development of a *Fusarium*-resistant line either by growing lines in a sick plot or through artificial inoculation is time consuming because sick plots need to be developed and cultures need to be prepared. The outcrossing nature, long life cycle, difficulty in accurate phenotyping and linkage drag are also some of the problems faced in traditional pigeonpea breeding (Saxena 2008). Molecular markers linked to wilt resistance will be useful in marker-assisted selection (MAS) breeding. By means of molecular markers linked to a trait of interest, selection can be performed at early seedling stages of development, and true breeding genotypes identified with relative ease. Hence, identification of molecular markers closely linked to *Fusarium* wilt resistance facilitates the identification and breeding of new lines by reducing the time required.

New exigencies and challenges for increasing the yield of crops have led to the generation of new genetic tools like genetic engineering and biotechnology through the use of molecular markers. DNA marker technology has permitted the establishment of linkage maps in crop plants. In addition, these linkage maps allow breeders to conduct applied research to identify, characterize and use genetic variability in economically important plants. Markers can also be used as a replacement for phenotyping, which allows selection in off-season nurseries making it more cost effective to grow more generations per year. The proposed uses for molecular markers in plant improvement are their application in the selection of traits with low heritability, identification of disease resistance, abiotic and biotic stress tolerance and the gene introgression coming from native or exotic germplasm. Advantages such as rapidity, non-interference by the environment and accuracy during selection results in a target genotype (Bertrand *et al.* 2008).

Using near isogenic lines (NILS) or bulked segregant analysis (BSA) strategies a number of random amplified polymorphic DNA (RAPD) markers linked to *Rf* genes have been identified in sunflower (Maivannan and Muralidharan 2003). Molecular markers closely linked to a major resistance gene will facilitate the breeding of new resistant

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Table 1 Phenotypic segregation ratios for reaction to Fusarium wilt in F_2 of LRG-41 x ICPL-87119 and ICPL-7035 x ICPL-8863.

Crosses		No. of plants		Total	Ratio (R:S)	Chi square value
		Resistant	Susceptible			
LRG-41 X ICPL-87119	Observed	89	35	124	3:1	0.688 (0.384)
	Expected	93	31	124		
ICPL-7035 X ICPL-8863	Observed	78	30	108	3:1	0.443 (0.384)
	Expected	81	27	108		

Figures in parenthesis are the table chi-square values at 5% level of significance.

lines in pigeon pea. To overcome the problems associated with RAPD analysis and to improve their utility in MAS application, RAPD markers can be converted into SCAR markers. SCAR markers are generally allele-specific and their amplifications is much less sensitive to reaction conditions.

In the present study a dominant RAPD marker $OPG08_{950}$ linked to resistance to Fusarium wilt was identified by means of BSA of an intraspecific F_2 population developed from two crosses, LRG-41 x ICPL-87119 and ICPL-7035 x ICPL-8863. Cloning and characterization of this RAPD marker and development of an allele-specific SCAR, which can be used to quickly and accurately identify dominant genotypes for wilt resistance, was done.

MATERIALS AND METHODS

Plant materials

Fusarium wilt-susceptible and resistant parents were crossed (LRG 41 x ICPL 87119 and ICPL7035 x ICPL 8863). The female parents are susceptible and male parents (ICPL-87119 and ICPl-8863) are known resistant sources to Fusarium wilt, collected from ICRISAT, Hyderabad, India. The F_1 and F_2 generations were subsequently selfed to raise F_3 progeny rows. A mapping population of 124 and 108 F_2 plants was used and RAPD marker OPG08 linked to the resistant gene was generated. These materials provided the foundation for SCAR marker development.

Phenotypic classification of resistant and susceptible plants

The F_2 individuals were classified as resistant and susceptible based on the health or wilting of plants at different stages of the crop after growing in a sick plot (**Table 1**). Individual F_2 and parents were scored for disease incidence at three stages corresponding to seedling, mid and late stages of crop. The reaction of individual plants was recorded based on typical wilt symptoms. A plant without any symptoms was treated as resistant and if plant showed the symptoms at any stage was noted as susceptible. Seeds from each resistant F_2 were planted to establish 30 plants for each F_3 progeny row in the sick plot in order to identify F_2 plants homozygous for dominant resistant gene. Progeny rows of F_3 population with 0% incidence of *Fusarium* wilt in sick plot will be concluded to have come from F_2 plants homozygous for resistance.

DNA extraction and amplification conditions

Genomic DNA was extracted from the leaves of parents and 124 and 108 F_2 plants of crosses at 20 days after sowing in sick plot through cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson 1986) and this DNA was further purified from carbohydrates, proteins and RNA before actual use (Sambrook *et al*. 1989). Quantification and quality checking were done on a 0.8% agarose gel. Individual F_2 plants and the parents were scored for disease incidence at different stages corresponding to seedling, mid and late stages of crop. The reaction of individual plants was recorded based on typical wilt symptoms. A plant was considered as resistant if it did not show any symptoms of the disease (**Table 1**).

Polymerase chain reaction

The PCR reactions were performed in a 25 µl volume in 0.2 ml PCR tubes (Axygen Pvt. Ltd., India). The reaction mixture contained 25 ng of template DNA, 1X amplification buffer (10 mM of Tris-HCL pH 8 (Fermentas), 2.5 mM of $MgCl_2$, 0.2 mM of DNTPs, 1 µM Primer (Operon) and 1U *Taq* DNA polymerase. The reactions were performed in a Master Cycler Gradient (Corbett Research, Australia). The reaction had an initial denaturation step at 94°C for 2 min, followed by 45 cycles of denaturation at 92°C for 1 min, 37°C for 1 min, 72°C for 2 min. The final extension step was at 72°C for 5 min. Amplified products were separated by electrophoresis using a 1% (w/v) agarose gel containing ethidium bromide (10 mg/ml) in 1X TBE buffer and visualized under UV light using an Alpha Innotech Corp. gel doc system and the results were documented.

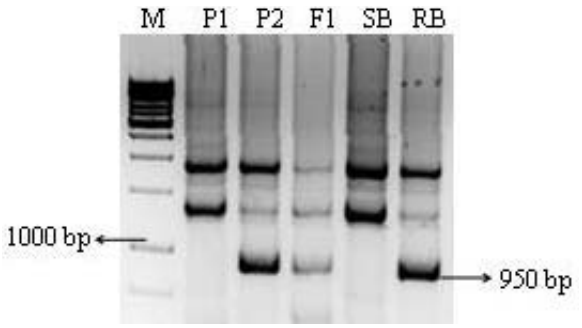

Fig. 1 Amplification profile of the RAPD primer OPGO8 in parents and F_1s of cross LRG 41 x ICPL 87119. Lanes: M, 1 Kb DNA ladder; 1, LRG 41 (susceptible parent); 2, ICPL 87119 (resistant parent); 3, F_1 hybrid from the cross LRG 41 x ICPL 87119; 4, LRG 41 (susceptible bulk); 5, ICPL 87119 (resistant bulk).

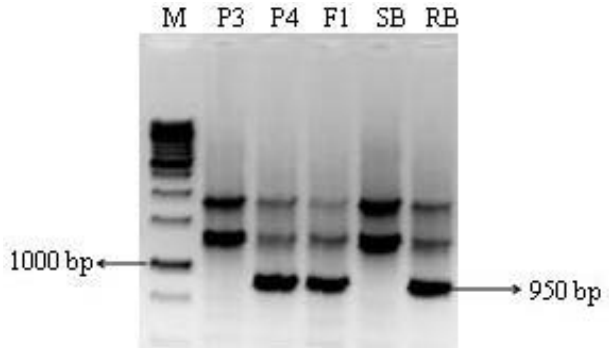

Fig. 2 Amplification profile of OPGO8 primer in parents and F_1s of cross ICPL 7035 x ICPL 8863. Lanes: M, 1Kb DNA ladder; 1, ICPL 7035 (Susceptible parent); 2, ICPL 8863 (resistant parent); 3, F_1 hybrid from the cross ICPL 7035 x ICPL 8863; 4, ICPL 7035 (Susceptible bulk); 5, ICPL 8863 (resistant bulk).

A total of 195 random oligonucleotide primers were surveyed for potential polymorphism between the DNA bulks of resistant and susceptible parents. Primer OPG08 was found to produce a putative marker which differentiated resistant parent and bulk from susceptible parent and bulk (**Figs. 1, 2**). Co-segregation analysis of the putative marker in the F_2 population confirmed the association OPG08 primer with the Fusarium wilt resistance gene.

Constitution of DNA bulk and evaluation of polymorphism

Equal quantities of DNA were bulked from 10 resistant and susceptible F_2 plants. These bulks along with parents were screened

Table 2 Ratio of F_2 plants for wilt reaction and marker status in cross ICPL-7035 x ICPL-8863

Marker		No. of plants		Total plants	Ratio (R:S)	Chi square value
		Present	Absent			
OPG08	Observed	75*	30	108	3:1	0.443 (0.384)
SCAR 14f/r	Observed	78	30	108	3:1	
	Expected	81	27			

Figures in parenthesis are the table chi-square values at 5% level of significance.
*3 plants did not amplify the marker OPG08.

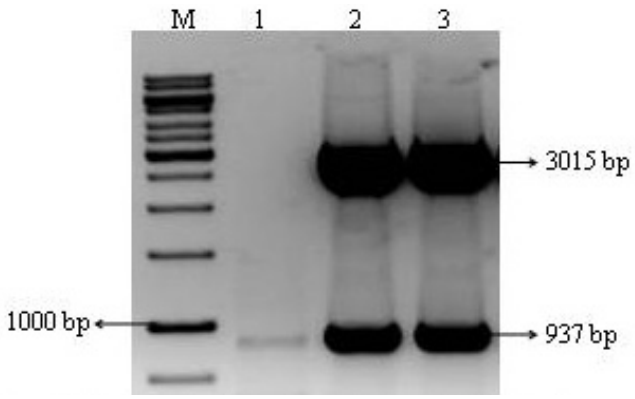

Fig. 3 Confirmation of clones by restriction digestion. Lanes: M, 1 KB DNA ladder; 1, Control – Gel eluted PCR amplified DNA of ICPL 87119; 2, 3 Restriction digested positive clone of ICPL 87119.

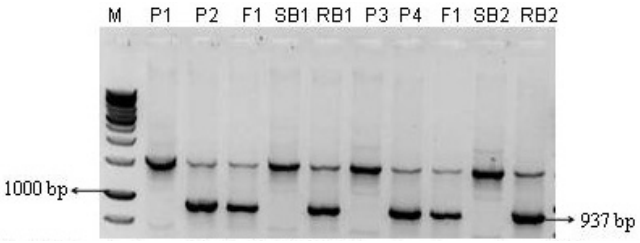

Fig. 4 Amplification profile of the marker SCAR 937 (14 f/r) in parents and F_1s. Lanes: M, 1 Kb DNA ladder; 1, LRG-41 (susceptible parent); 2, ICPL 87119 (resistant parent); 3, F_1 hybrid from the cross LRG 41 x ICPL 87119; 4, LRG 41 (susceptible bulk); 5, ICPL 87119 (resistant bulk); 6, ICPL 7035 (susceptible parent); 7, ICPL 8863 (resistant parent); 8, F_1 hybrid from the cross ICPL 7035 x ICPL 8863; 9, ICPL 7035 (susceptible bulk); 10, ICPL 8863 (resistant bulk).

with primer to identify the polymorphic marker which is present in resistant parent as well as resistant bulk and not in the susceptible parent and susceptible bulk (**Table 2**). The linkage of the polymorphic marker was confirmed by using an individual segregating population from which the bulks were generated.

Cloning and sequencing of RAPD markers

RAPD primer OPGO8 (Operon Technologies Inc., Alameda, CF, USA) was used to amplify genomic DNA of the resistant parents ICPL-87119 and ICPL-8863. Amplified fragments were separated on a 1% (w/v) low melting point agarose gel before being excised and purified by means of the QIAquick Gel Extration Kit (Qiagen, Valencia, CA, USA). The purified DNA fragments were ligated and transformed with the pGEM-T Easy Vector System. Cloned RAPD fragments were identified via Colony PCR with RAPD primer OPG08 and confirmed by restriction digestion with *Eco*RI (**Fig. 3**).

SCAR primer design and amplification

On the basis of the sequence of cloned RAPD product (NCBI Gene Bank Accession No FJ215868) oligonucleotide primer pairs of 14 to 21 bases were designed using Oligo Explorer 2.0 software for specific amplification of the loci identified by selected RAPD marker. Each primer contained the original 10 bases of the RAPD primer sequence plus the next 4, 7, 10 and 11 internal bases (**Table 4**). Care was taken to avoid possible primer dimer or secondary structure formation. Primers were synthesized by Operon Technologies, Inc. SCAR amplification of ICPL-87119 and ICPL-8863, LRG-41 and ICPL-7035 genomic DNA was performed in 25 µL reaction volumes containing the same components used for RAPD analysis except for the concentration of $MgCl_2$ (2 mM). PCR amplification consisted of 35 cycles of 30 min at 94°C, 1 min at 51°C, and 1 min at 72°C followed by a final extension of 10 min at 72°C. Agarose electrophoresis was performed as described for RAPD analysis.

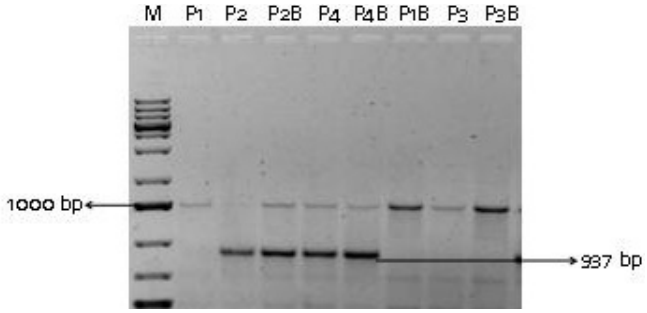

Fig. 5 Amplification profile of the marker SCAR 937 (14f/r) in parents and bulks. Lanes: M, 1 Kb DNA ladder; 1,LRG 41(susceptible); 2, ICPL 8863 (resistant); 3, ICPL 8863 bulk; 4, ICPL 87119 (resistant); 5, ICPL 87119 bulk; 6, LRG 41 bulk; 7, ICPL 7035(susceptible); 8, ICPL 7035 bulk.

RESULTS AND DISCUSSION

A total of 195 random oligonucleotide primers were surveyed for potential polymorphism between the DNA bulks of resistant and susceptible parents. Primer OPG08 was found to produce putative markers which differentiated resistant parent and bulk from susceptible parent and bulk (**Figs. 1, 2**). Co-segregation analysis of putative marker in the F_2 population confirmed the association of $OPG08_{950}$ primer with the Fusarium wilt resistance gene (**Table 2**). $SCAR_{937}$ marker with 14 base pairs designed from the sequence of resistant RAPD DNA fragrant also produced consistent results in parents and F_1s (**Figs. 4, 5**) and F_2 plants (**Table 3**).

The inheritance pattern of resistance to wilt was studied in two F_2 populations based on a segregation ratio of alternative phenotypes (resistant and susceptible). A set of 124 F_2 plants of LRG-41 X ICPl-87119 and 108 F_2 plants of ICPL-7035 X ICPL-8863 were individually evaluated for the incidence of wilt. Of the 124 F_2 plants of first cross, 89 were resistant to wilt disease at all stages and the remaining 35 were susceptible at different stages of the crop. Similarly out of 108 F_2 plants of second cross, 78 were resistant and the remaining 30 were susceptible (**Table 1**). The goodness of fit to Mendelian segregation of resistant and susceptible plants in the segregation population was tested by a Chi-square test (Panse and Sukhatme 1961).

This segregation ratio fits well with the expected ratio of 3 resistant and 1 susceptible and thus confirms that the resistance was controlled by single dominant gene (**Table 1**).

The F_2 segregation pattern of the susceptible x resistant cross revealed a monogenic ratio of 3 resistant and 1 susceptible and thus confirms that the disease was controlled by single gene. Similar observations on resistance being

Table 3 Amplification status of resistant parent specific amplicon by SCAR 14f/r in F_2 individual plants of two crosses (LRG-41 x ICPL-87119, ICPL-7035 x ICPL-8863).

F_2 plant no	Presence/Absence of SCAR 14f/r		F_2 plant no	Presence/Absence of SCAR 14f/r		F_2 plant no	Presence/Absence of SCAR 14f/r	
	Cross 1	Cross 2		Cross 1	Cross 2		Cross 1	Cross 2
1	P	P	46	P	P	91	P	P
2	P	A	47	P	P	92	P	P
3	P	A	48	P	A	93	P	P
4	P	P	49	P	P	94	A	A
5	A	P	50	P	P	95	A	P
6	P	P	51	P	P	96	P	P
7	P	P	52	P	A	97	A	P
8	P	P	53	P	P	98	A	P
9	P	P	54	P	P	99	P	P
10	P	P	55	P	A	100	P	A
11	P	A	56	P	P	101	A	P
12	P	P	57	P	P	102	P	P
13	A	P	58	P	P	103	A	P
14	P	A	59	P	A	104	P	P
15	P	P	60	P	P	105	P	P
16	A	P	61	A	P	106	A	P
17	P	P	62	A	P	107	A	A
18	P	P	63	A	P	108	P	P
19	P	P	64	P	A	109	A	
20	P	P	65	A	P	110	P	
21	P	A	66	A	P	111	A	
22	A	A	67	A	A	112	P	
23	P	P	68	P	P	113	P	
24	P	P	69	A	P	114	P	
25	P	P	70	P	P	115	A	
26	P	A	71	A	P	116	A	
27	P	A	72	P	A	117	A	
28	A	P	73	P	P	118	A	
29	A	P	74	P	P	119	P	
30	P	P	75	P	P	120	P	
31	P	P	76	P	A	121	P	
32	P	P	77	P	P	122	P	
33	P	A	78	P	A	123	P	
34	P	P	79	A	P	124	A	
35	A	P	80	A	P			
36	P	P	81	P	A			
37	P	A	82	P	P			
38	A	A	83	P	A			
39	P	P	84	P	P			
40	P	P	85	P	P			
41	P	P	86	A	P			
42	P	P	87	P	A			
43	P	A	88	P	A			
44	P	P	89	P	P			
45	A	A	90	P	A			

Marker status: A- Absent, P-Present

Table 4 SCAR primers designed based on the sequence of RAPD marker and the size of amplicon observed.

Primer	Primers sequence (5′-3′)	Length (mer)	Annealing temp. (°C)	Size (bp)
SCAR1-F	TTC ACG TCC ACA CT	14	51	937
SCAR1-R	TTC ACG TCC ACC AT	14		
SCAR2-F	GCA AAT GCA CTC CAA ATG AA	20	59	504
SCAR2-R	TTC ACG TCC ACC ATG AAG AA	20		
SCAR3-F	GGT GTT TGG TGC TCA CA	17	53	600
SCAR3-R	TTG GAC CTT AGC TCC TA	17		
SCAR4-F	TTC ACG TCC ACA CTT CTA GCA	21	54	200
SCAR4-R	GTG CCT CAA ATC CTC TTC CT	20		

under the influence of dominant gene over susceptible in different cross combinations involving a resistant and a susceptible parent was reported and the F_2 segregation pattern in above findings revealed monogenic ratio of 3 resistant: 1 susceptible as resistant parent was differed from susceptible parent in respect of single gene by Pandey *et al*. (1996) and Kotresh *et al*. (2006).

Tight linkage of a marker to a gene can be exploited for indirect selection of traits. To avoid screening in sick plots and artificial inoculation in identification of disease resistance line by tagging of resistance gene with molecular marker will help in the screening of genotypes for the presence of resistance character. It will also help to transfer disease resistance gene to susceptible lines.

In this study, BSA was followed to identify the markers linked to resistance to Fusarium wilt in the F_2 population of two crosses i.e. LRG-41 X ICPL-87119 and ICPL-7035 X ICPL-8863 (**Figs. 6, 7**). This approach provides information simultaneously on polymorphism of parents and possible linkage between the marker and targeted gene using only the parents and extreme genotype bulks, thereby reducing the cost and workload by several fold.

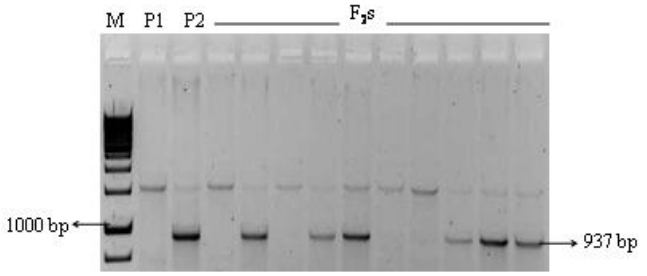

Fig. 6 Amplification profile of the marker SCAR 937 (14 f/r) in parents and F_2 individual plants of cross LRG 41 x ICPL 87119. Lanes: M, 1 Kb DNA ladder; 1, LRG 41 (susceptible); 2, ICPL 87119 (resistant); 3 to12, F_2 plants of LRG 41 x ICPL 87119.

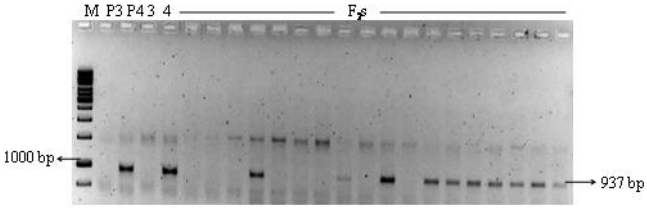

Fig. 7 Amplification profile of the marker SCAR 937 (14 f/r) in parents and F_2 individual plants of cross ICPL 7035 x ICPL 8863. Lanes: M, 1 Kb DNA ladder; 1, ICPL 7035 (susceptible); 2, ICPL8863 (resistant); 3, F_2 Susceptible bulk; 4, F_2 resistant bulk; 5-22, F_2 individual plants.

SCAR developed from RAPD markers have the advantages of cost effectiveness and technical simplicity. The results obtained from this study proved that using RAPD/ SCAR marker analysis in combination with BSA of F_2 population provides a highly efficient strategy to tag the gene of interest. This was already reported in fertility restorer gene in sun flower (*Helianthus annuus* L.), stem rust gene in oat (*Avena sativa* L.), in rice (*Oryza sativa* L.) for blast resistant gene (Naqvi and Chattoo 1996), beta gene in tomato (*Lycopersicon esculentum* L.) (Zhag and Stommet 1996).

A major problem associated with RAPD technology is the reproducibility of the profiles and it has been the subject of considerable debate among various investigators. To ameliorate the utility of RAPDs, SCARs that have greater reliability than simple RAPDs were developed. SCARs have several advantages over RAPD markers in MAS. Because more stringent reaction conditions are used, SCAR markers are generally more allele specific and SCAR amplifications are more stable and reliable and more easily reproduced in different laboratories with various thermal cycles Selvi *et al*. (2006). Thus OPG08 was converted to a SCAR for increasing reproducibility. This may help in monitoring Fusarium wilt resistance by early screening of pigeon pea genotypes and breeding material. These markers may be utilized in marker-assisted breeding programs to track the introgression of the resistant gene into breeding lines and develop lines with Fusarium wilt resistance and increased seed yields.

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