Development of RAPD Markers linked to Fusarium Wilt Resistance Gene in Castor Bean (*Ricinus communis* L)

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Accepted: September 1, 2011; Published: November 11, 2011

Abstract

Wilt, caused by *Fusarium oxysporum* f. sp. *ricini*, is the most severe disease of castor bean (*Ricinus communis*). For the development of marker for wilt-resistant gene, 200 F_2 individuals from a cross between the resistant cultivar 48-1 and the susceptible cultivar VP-1 were inoculated with a race of *Fusarium oxysporum* f. sp. *ricini*. Bulked segregant analysis was used to search for random amplified polymorphic DNA (RAPD) markers linked to wilt resistance in F_2 progeny derived from the above cross. Three markers (RKC 23_{1375}, RKC 21_{1080} and OPBE 18_{900}) flanking the wilt resistance gene were identified. The developed markers segregated from the screened *Fusarium* wilt resistant progenies of F_2 and F_3 families confirmed their linkage with *Fusarium* wilt resistance and the resistant castor bean genotypes were successfully identified among thirteen cultivars screened. Linkage analysis was carried out using the three markers on the 200 F_2 individuals which showed that the genetic distance for the three markers at the wilt resistance gene was 5 cM, 10.7 cM and 7.6 cM respectively. The predicted protein 3D model of the translated amino acid sequence from RKC 23 showed characteristic features of DNA binding protein (2BIN). This study provides a base for probable mining of Fusarium wilt resistance gene in castor bean and simultaneously gives valuable insights into the characterization of *R-gene* subclass in castor genome.

Keywords: Castor bean; Marker-Assisted Selection (MAS); protein modeling; RAPD marker; susceptible.

1. Introduction

India is the leading country among the vegetative oil producing countries in the world, accounting for over 64 % of the global production followed by China and USA. Castor bean (*Ricinus communis* L, family Euphorbiaceae) contains 50-55 % oil in seeds. The crop holds impressive position in the global oilseed scenario accounting for 19 % of the total area and 9 % of the total vegetative oil production [1]. Wilt disease of castor bean (*Ricinus communis*) is caused by *Fusarium oxysporum* f. sp. *ricini*. It is a major disease of the crop often resulting in loss of more than 80 % crop yield in India. Hybrid breeding approach is sometimes practiced to compact such yield, India has the castor bean improvement programmes under Directorate of Oilseed Research (DOR), Hyderabad, for developing high-yielding disease resistant varieties. Breeding of pathogen-resistant castor is believed to be a better option to restore the yield of this commercially important crop plant. Traditional breeding approaches in developing wilt-resistant genotypes are time consuming as well as costly and it requires screening of the plants with test fungus, up to 7-8 generations. The time and cost may be reduced by adopting DNA marker assisted selection (MAS) process. Application of MAS leads to a quick and reliable analysis for susceptibility. Therefore, use of MAS, coupled with traditional breeding technique is the best to combat plant diseases.

Pathogen avirulence (*Avr*) genes have coevolved with the resistance genes. So, prevention of disease by individual R-gene is not feasible. In this regard, tight linkage between a DNA marker and a disease trait has led to the successful transfer of resistant gene to a susceptible genetic background, often by building resistant genes-pyramid in crop plants [2]. Though no genetic or linkage map is available for castor bean but, homozygous breeding lines with respect to wilt disease resistance are available at various breeding stations across the globe. They may help to tag resistant gene with molecular (DNA) markers. A number of DNA markers such as RFLP, RAPD, AFLP, and SCAR are available for MAS, to tag disease resistant genes [3-5]. Amongst prominent MAS application are stem rust in barley [6], mosaic virus of tomato [7], gall midge

resistance in rice [8, 9] downy mildew in soybean [10], anthracnose resistance in common bean [11], etc. In addition, RFLP markers were used to select fusarium wilt disease in chickpea [12], rust disease of crucifers [13], head smult of sorghum [14], and AFLP markers for leaf mould disease of tomato [15]. Work of Williams *et al.* (1990) paved the path for use of RAPD technique in marker-assisted molecular breeding [16-18] and detecting DNA polymorphism in crop plants [19, 20].

RAPD markers are simple, rapid and have the advantage of no prior knowledge of genome sequence and also have certain advantage over other methods because they are easy to generate and do not require the use of isotopes [21]. This has been used successfully to generate markers for various organisms and thus RAPD markers have been widely used in the construction of genetic maps. The technique provides a large number of multi-loci markers that can be used for high-resolution discrimination of samples. Criticism for RAPD-based trait selection and diversity study based on its reproducibility of producing amplicons has been restricted in the past few years by stringent and improved laboratory practices and development of more accurate scoring procedures [22-26]. The RAPD analysis has been used widely in phylogenetic studies of bacteria, fungi and plants [27].

The RAPD markers can be of several orders of high magnitude due to the unlimited number of such primers that can be designed for the selection of any traits. This leads to a higher level of markers saturation on genetic map. In order to develop DNA markers for screening fusarium-wilt resistance castor bean cultivars, MAS was applied utilizing RAPD markers coupled with bulked segregant analysis (BSA) of parents (48-1 and VP-1) and their segregating populations of fusarium wilt resistant hybrid GCH-4.

2. Methods

2.1. Plant materials

To link DNA markers to fusarium-wilt resistant locus, seeds of resistant (48-1) and susceptible (VP-1) parents, their F_1 hybrid (GCH-4), and its inbreeds up to F_3 generations were germinated and maintained in the experimental plots of the Directorate of Oilseeds Research (DOR), Hyderabad and at the Calcutta University experimental garden, Kolkata, India. The F_2 population was raised by selfing the F_1 plants. Five seeds were randomly collected from each 200 individual selfed resistance F_1 plants separately. All these seeds represent F_2 progenies. Similarly, for F_3 population five seeds were randomly collected from 20 F_2 selected resistant plants. Two other cultivars DCS-9 and Aruna were chosen as resistant and susceptible checks.

2.2. Phenotyping populations segregating for fusarium-wilt reaction by forced inoculation techniques

Fusarium oxysporum f. sp. *ricini,* the causal agents of wilt in castor bean, was a gift from Dr. M. A. Raoof, Principal Scientist, Pathology, DOR (ICAR), Hyderabad, India. This race was used for inoculation of all the castor cultivars as well as 200 F_2 plants, 20 F_3 progenies and parents (48-1 and VP-1) under study according to the modified method of [28].

2.3. Isolation of genomic DNA

DNA was isolated from young healthy leaves of one month old non-inoculated castor plants according Keim *et al.* (1998) [29] with some modifications**.** Quality and quantity of DNA preparation were checked by standard spectrophotometer method. The DNA samples were diluted to 25 ng/µl for experimental use.

2.4. Bulk Segregant Analysis (BSA) using RAPD

A total of 64 arbitrary decamer primers were used, twenty each of the OPA, OPJ and OPBE kits (Operon Technologies, USA) DNA and four selfed designed primers (RKC 20 to RKC 23), to screen the genotypes 48-1 and VP-1, GCH-4 (F_1, F_2, F_3). PCR were performed on 48 well plates in a Perkin Elmer thermal cycler (Perkin Elmer 2400). RAPD protocol was optimized in 25 µl reaction mixture with varying concentrations of $MgCl_2$ (0, .5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM), Taq DNA polymerase (0.5, 1.0, 1.5, 2.0, 2.5 U), genomic DNA (12.5, 25, 50, 75, and 100 ng) and primers. On optimizing the PCR amplification conditions, the optimal PCR mixture was genomic DNA 25 ng, $MgCl_2$ 1.5 mM and Taq DNA polymerase 0.75 U, dNTP mixture and 10x Taq polymerase buffer (100 mM Tris-Cl buffer, 500 mM KCl and Gelantin 0.01 %) were 50 pMol, 200 µM and 1x respectively. Deionized water was added in the reaction mixture to make the volume to 25 µl. Optimized performance condition was maintained for all the experiments. To minimize errors, same cocktail mixture was used for all experiments at a given time.

Four DNA bulks were prepared from equimolar amounts of DNA from 200 individual plants as follows: (1) resistant F_2 (F_2^R) plants, (2) susceptible F_2 (F_2^S) plants, (3) resistant F_3 (F_3^R) plants, (2) susceptible F_3 (F_3^S) plants. Uniform PCR amplification conditions were maintained. Among the 64 primers screened, primers showing polymorphism were selected to find out markers linked to the target locus using resistant and susceptible bulks along with parental and check DNA. Segregation for number of diseased vs normal F_2 plants and their respective RAPD markers were evaluated by the χ^2 – test ($p = 0.05$) according to the Mendelian segregation ratio of a single dominant inheritance fashion (3:1).

Using the above mentioned RAPD primers, 13 castor bean cultivars were analyzed for polymorphism to discriminate them into resistant and susceptible genotypes. Then they were tested in the sick plots to confirm the use of these linked markers in identifying disease reaction in castor bean cultivars.

2.5. Linkage analysis

A preliminary screening for polymorphism of the molecular markers among the resistance parent (48-1) and susceptible parent (VP-1), and their progenies, was carried out using 64 arbitrary decamer primers (Operon). Linkage map was constructed with the developed three RAPD markers data using MAPMAKER (Version 3.0) [30] Linkage groups were identified using minimum LOD value of 3. The mapping population consists of 200 F_2 plants. Recombination values were transformed to linkage distances in centiMorgans (cM) by Haldane's mapping function.

2.6. Elution of DNA fragments from gel

Amplified DNA fragments of interest were eluted from the low melting agarose gel by using Bangalore Genei Elution Kit (Cat # KT 02) as recommended by the manufacturer for further amplification and sequencing.

2.7. DNA sequencing and characterization of the markers

RAPD was done with eluted DNAs using respective primers and the products were confirmed on a 1.6 % (w/v) agarose gel. An aliquot of the products were PCR-sequenced in an automated sequencer (ABI PRISM 377, PE).

The sequences were analyzed by searching GenBank and EMBL accessions using BLASTN [31] at the website http://www.ncbi.nlm.nih.gov/BLAST/. The sequences were subjected to the biocomputing tool, Vector NT1 Suite 6.0, and ExPASY Proteomics tools for further analyzing and compilation. The amino acids sequences of the proteins were deduced from the nucleotide sequences using Expasy Server. Alignment of protein sequences with the sequences deposited in the databases was done by Clustal W program. The predicted protein sequence from *FOR1* sequence was used to build the three dimensional structures of the proteins using Rossetta server by *ab inito* method, as there was no detectable sequence homology between proteins of known 3D structures. Nucleotide sequence of the characterized DNA marker was deposited as GenBank accession number BankIt1359615Seq2HM748464.

3. Results

3.1. Phenotyping of F_2 and F_3 populations segregating for Fusarium-wilt reaction

A total of 200 F_2 plants were forcibly inoculated by root-tip inoculation method and screened for wilt disease in glasshouse conditions, and the results were illustrated in Table 1**.** The observed segregating pattern fits almost perfectly a 3:1 ratio, for resistant and susceptible progenies, which were in agreement with the presence of single dominant gene in the fusarium-wilt resistant parent and F_2 individuals. Fusarium-wilt resistant of F_3 plants were analyzed, almost all the F_3 plants that were from the randomly selected resistant F_2 plants were designated as phenotype resistant and plants which were derived from 3 resistant F_2 plant seeds segregated into resistant and susceptible genotypes. These results showed all F_2 plants from which F_3 population was formed were homozygous, except for the 3 F_2 plants which segregated for the fusarium-wilt resistant gene. The 3 F_2 plants were heterozygous for the same gene.

Table 1: Co-segregation of Fusarium-wilt reaction on forced inoculated F_2 individuals (200) of castor bean hybrid, GCH-4 and the three RAPD markers.

	F_2 Population				$\chi 2$	Probability	Recombination Fraction	
	Resistance (R)		Susceptible (r)		(3:1)			
				Phenotyping of F_2				
F_2 Individuals	144		56		0.91	0.05		
				Genotyping of F_2				
RAPD Marker	**RR**	**Rr[a]**	**rR[b]**	**rr**			**Rr[a]**	**rR[b]**
OPBE 18	146	6	4	44	0.42	0	0.015	0.010
RKC 21	137	9	6	48	0.61	0	0.023	0.015
RKC 23	140	5	10	45	0.59	0	0.013	0.025

r[a] Phenotyically resistant but devoid of DNA markers.
R[b] Phenotyically susceptible but contains DNA markers.

3.2. RAPD screening

A total of 64 arbitrary decamer primers were used for RAPD analyses for detecting variability amongst the parents. The number of amplified products by each primer varied from 0 to 31 with an average of 13.8 bands per primer, and the amplified products ranges from 200 bp to 2100 bp. Only the easily scorable and consistent profile of the RAPD was analyzed for all the primers used. The percent polymorphism was from 0–100 %. The F_2 and F_3 segregating populations of GCH-4 along with the two parents viz. 48-1 and VP-1 were screened with 104 RAPD primers for fusarium-wilt reaction under forced inoculation conditions. Three replica analyses for each experiment were performed. Among the primers showing polymorphism between the two genotypes, only three primers, viz OPBE 18, RKC 21 and RKC 23, were selected for further studies, since they generated amplification products that were present in resistant parents and resistant bulks but absent in susceptible parent and susceptible bulks. The polymorphism was confirmed by repeated amplification experiments by comparing with parents, resistant and susceptible bulks. The F_2 and F_3 bulk segregated analysis for the resistance locus, resulted to, 1:1 segregation ratio.

3.3. Genotyping of F_2 and F_3 populations segregating for Fusarium-wilt reaction

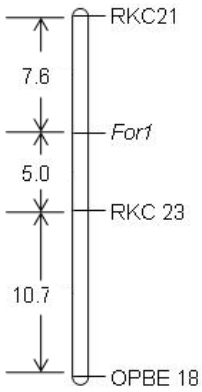
The polymorphic primers OPBE-18, RKC 21 and RKC 23 were used to know the genotypes of the individuals of known phenotypes (fusarium-wilt resistant) from the F_2 population and F_3 population. The OPBE18 primer generated 900bp DNA band for all the fusarium wilt resistant 146 out of 200 F_2 individuals, which followed the segregation ratio of almost 3:1 (Table 1). This primer generated 900bp band in 92 F_3 resistant individuals out of 100 plants tested for fusarium wilt reaction. The 3 randomly selected F_2 plants whose progenies were segregated for the resistant gene were heterozygous and the non-segregated seeds of rest F_2 plants were homozygous for the fusarium-wilt resistance gene. Similarly, RKC-21 and RKC-23 generated 1080 bp and 1375 bp DNA bands in F_2 and F_3 individuals respectively whose segregation followed the simple Mendelian inheritance (Table 1). Linkage analysis carried out on the 200 F_2 individuals showed the genetic distance for the three markers (RKC $23_{1375,}$ OPBE 18_{900} and RKC 21_{1080}) linked to the wilt resistance gene as 5, 10.7 and 7.6 cM respectively, calculated as described in materials and methods (Figure 1).

3.4. DNA marker sequence analysis

Development of DNA marker is essential in both marker-assisted selection and characterization of any important gene, so the three markers developed in our report were sequenced and characterized. Homology searches using GenBank and EMBL accessions using BLASTN showed that the three markers do not show any significant similarities to any of the accession in either of the databases. The sequences were modified by eliminating noncoding fragments by biocomputing tools. Among the three markers sequences, the modified 900 bp sequence of RKC 23 was characterized in details and was designated as *FOR1*. The modified sequence was subjected to Translation tool of the NCBI, to get the different frames. To assign possible biological function of the putative amino acid sequence of the modified RKC 23 marker, it was compared with the protein sequences submitted in the publicly accessible databases and have shown significant similarities to the putative proline rich protein (*Oryza sativa*), PRP gene (*Zea mays*), cell division protein (*Arabidopsis thaliana*), nodule protein (ENOD2) (*Pisum sativum*). Secondary structure and fold recognition of the translated $3^{/}5^{/}$ frame 2 by Phyre databases gave some similarity with defensive-like protein (d1eLqa) from defensive family. Multiple sequence alignments and phylogram were generated using Clustal W alignment of the translated protein with other eight protein sequences. Translated amino acid sequence of marker RKC 23 and DNA binding protein

shared the same cluster in the phylogram. Enhancer sequence (GCTGTGT) is present at the position 735 bp. The predicted protein 3D model showed characteristic features of DNA binding protein (2BIN). The detailed and predicted 3D structure of that protein showed both α helices and β sheets. α helices have particular importance in DNA binding motifs. This is because of the structural coincidence of size of α helix being the same as the width of the major groove in the B-DNA (Figure 2).

Figure 1: Linkage map of random amplified polymorphic DNA (RAPD) markers around *FOR1* gene that confers resistance to fusarium wilt in castor bean (*Ricinus communis*). Molecular markers names containing the primer ID are on the right, and distances indicated on the left side are estimated percent recombination between the markers (in cM), which are depicted as the maximum-likelihood solutions provided by MAPMAKER (version 3.0).



3. Discussion

In this report, the classical breeding methods and MAS worked together to tag the fusarium wilt markers linked to the wilt resistant gene in *Ricinus communis* L. The F_2 segregating GCH-4 progenies, out of a cross between 48-1 (resistant) and VP-1 (susceptible), segregated into resistant and susceptible bulks. The results showed expression of one dominant resistance gene in the F_2 resistant individuals under both natural and forced inoculation-screening conditions with the test fungus**.** The segregation of the locus for fusarium wilt resistance was true to Mendelian ratio of 3:1 (resistance: susceptible). Dweikat *et al.* (1997) [32] used RAPD markers for isolating insect resistance genes in wheat. Reports of a single dominant gene inheritance of anthracnose resistance [33, 34] and downy mildew resistance [15] were also reported. Using RAPD primers we assume here fusarium-wilt of *R. communis* L is controlled by a single dominant gene. The findings confirm the linkage of DNA markers to fusarium-wilt resistance**.** It helps the development of linked RAPD markers ($OPBE18_{900}$, $RKC21_{1080}$ and $RKC23_{1375}$). Recombinants were identified by the absence and presence of the above said 900, 1080, and 1374 bp amplicons in the resistant and susceptible F_2 progenies respectively. It confirms the findings of Choudhury *et al.* (2002) on downy mildew disease of soyabean. The developed markers always segregated with the screened fusarium wilt resistant progenies of F_2 and F_3 families confirming their linkage with fusarium wilt resistance. This study has established a linkage between three RAPD markers and fusarium-wilt 13 cultivars, which were screened here (Table 2).

To obtain high accuracy of MAS for an agronomic trait, it is essential to have a high linkage with flanking markers [35, 36]. The presence of three DNA markers, which flank the fusarium wilt resistant gene, is a good start for MAS for castor bean fusarium wilt resistance breeding program. This is perhaps the first report of castor bean wilt resistant gene that is linked DNA markers. Short genetic distance of these markers from wilt resistance gene, designated as *FOR 1*, makes them potentially useful in marker-assisted selection. The detailed sequence analysis of *FOR1* showed that it is a fragment of resistant gene. The homology search showed similarities with proteins like DNA binding Protein, PRP protein of *Zea mays* and early nodule protein. The seedlings of the castor bean which were resistant to the test fungus, showed presence of nodule-like structure at the root-shoot transition region (result not shown). The similarities of *Zea mays* nodule protein sequence with the predicted protein sequence of castor bean showed the role of nodule-like structure in defense mechanism.

Figure 2: PCR amplification of a 1375 bp band with RAPD primer RKC 23 of resistance lines of *Ricinus communis* and its sequence characterization. **A**. Amplification obtained from RKC 23 RAPD primer to identify 1375 bp DNA fragment linked to the fusarium wilt resistance gene in *Ricinus communis*. *Lane M*, DNA marker 100 bp; *Lanes 1-6* represents Resistance parent (48-1), Susceptible parent (VP-1), F_2 resistance bulk, F_2 susceptilbe bulk, F_3 resistance bulk and F_3 susceptible bulk. **B**, Elution of 1375 bp DNA from 1.5% agarose gel. **C**, Reamplification of purified eluted DNA fragment with primer RKC 23. **D**, Phylogram of the translated amino acid sequence of the marker RKC 23 linked to wilt resistance gene, *FOR1* and the eight homologous amino acid sequences. **E** and **F**, Ribbon structure and spacefilled models predicted 3D structure of protein translated from the sequence.

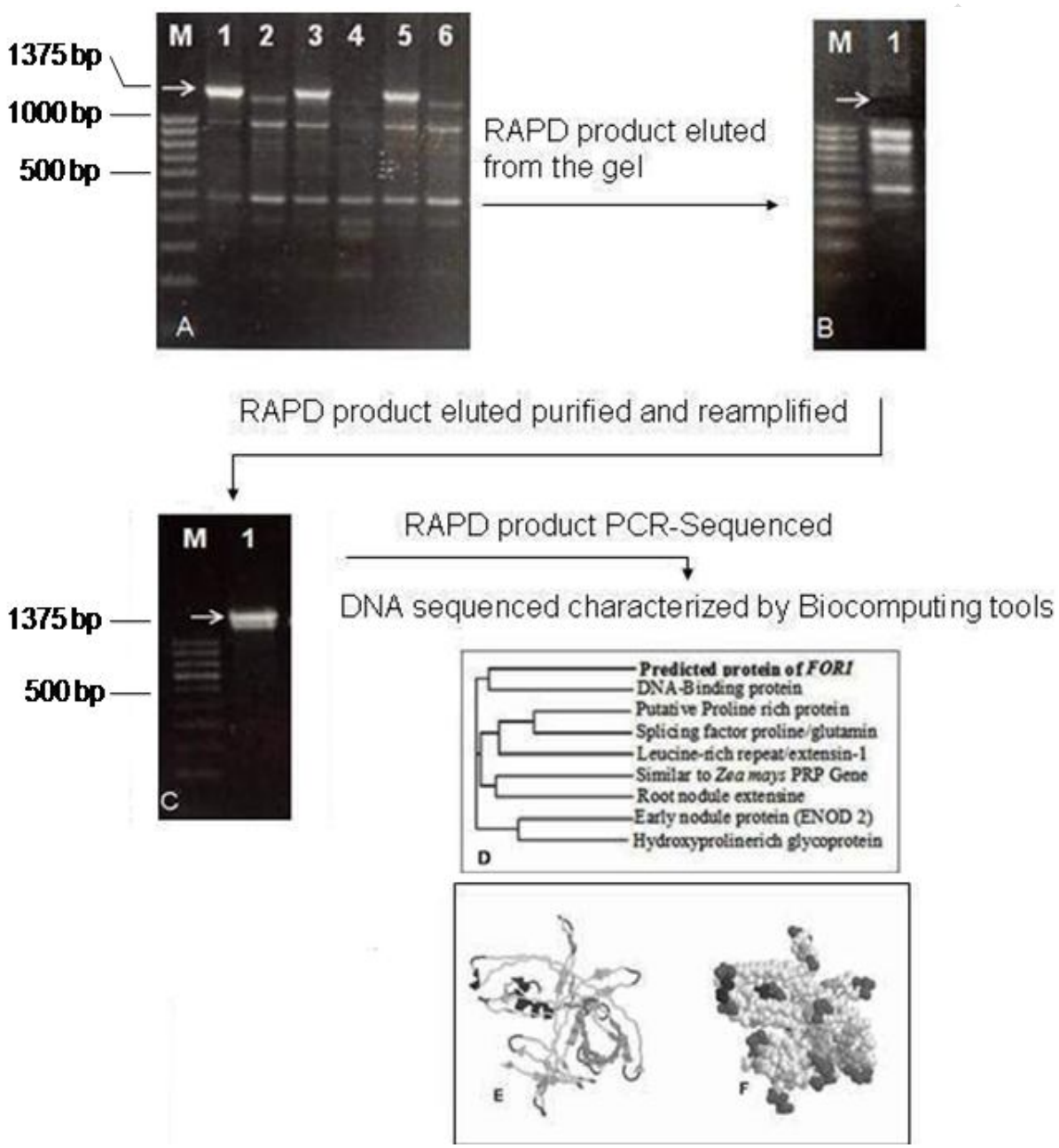


Table 2: Detection of RAPD markers linked to fusarium wilt resistance gene in thirteen castor bean (*Ricinus communis*) cultivars to validate the developed three markers (RKC $23_{1375,}$ RKC 21_{1080} and OPBE 18_{900}).

Cultivars	RAPD Markers			
	RKC 23	RKC 21	OPBE 18	Reaction to wilt
GCH 5	-	-	-	Susceptible
RG 954	+	-	-	Susceptible
M619	+	+	+	Resistance
Aruna	-	-	-	Susceptible
DCS 9	+	+	+	Resistance
DCH 177	+	+	+	Resistance
DCS 178	+	+	+	Resistance
Kranti	-	-	-	Susceptible
ER 2	-	-	-	Susceptible
Local Red	+	+	+	Resistance
ER 5	-	-	-	Susceptible
GCH 4	+	+	+	Resistance
DPC 9	+	+	+	Resistance

+ presence of DNA markers; - absence of DNA markers

4. Conclusion

Young (1999) [10] reported to the plant breeders that it is more attractive to select desirable lines based on genotype rather than analyzing phenotypes. If the three markers are detected in an individual, selection efficiency for the gene will be almost cent percent, given that multiple cross-over between the markers occur at very low level of frequency. Markers linked to more resistant genes, obtained from different genetic backgrounds, will enable one in pyramiding disease resistance found in different germplasm. Cloning and sequencing of these markers will make the development of sequence characterized amplified region (SCAR) markers feasible, to allow tagging of the resistance genes by PCR. The information on the sequences of these markers will facilitate further investigations of wilt-resistant gene identification as well as MAS in castor plant. Not only this but also this result has a potential in the development of kit for quick detection of fusarium-wilt resistance gene in castor bean germplasms for crop improvement programmes.

Abbreviations

BSA: Bulk Segregant Analysis
RAPD: Random Amplified Polymorphic DNA

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

The work is original and it has been carried out by MS for PhD dissertation under supervision of RKC and SKM in Jadavpur University, India. IC helped during the field and experimental works.

Acknowledgement

Authors wish to acknowledge the materialistic support offered by DOR (ICAR, India), DBT and UGC (Government of India) in the form of partial financial support. We also thank the scientists of DIC (Bioinformatics Centre), Bose Institute, Kolkata (India) for generating the 3D structure of predicted protein.

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