APPROACHES TO IDENTIFY BIOTIC STRESS RESISTANCE GENE ANALOGUES IN *HEVEA*

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Abstract *A preliminary attempt has been made to identify possible biotic stress tolerance gene analogues in rubber. An alternate methodology for DNA extraction from rubber leaves was standardised. 18 resistance gene analogues (RGA) were used as primers with a view to identifying biotic stress tolerance in rubber. Two clones, RRII 105 and FX 516, tolerant to Phytophthora leaf fall disease were selected for this study. The RGA-PCR strategy points towards the applicability of identifying and sequencing valuable resistance genes that would be of help in designing suitable primers for future investigations. Identification and cloning of resistant genes could find application in evolving transgenic plants.*

Key words: *Hevea*; biotic stress resistance gene; abiotic stress; tolerance;

INTRODUCTION

Exploitation of plant genetic resistance is one of the main approaches for controlling stresses in plants. Stresses are mainly of two types: biotic, caused by micro-organisms, and abiotic, caused by environmental factors. Breeding for resistance to both biotic and abiotic stresses requires the development of suitable and reliable screening techniques and identification of resistance characteristics that are heritable. Sethuraj (1995) has stated that considerable efforts have been made in recent years to utilise molecular the methods to combat diseases caused by fungi, bacteria and viruses. Amongst the top priorities of the Rubber Research Institute of India has been the development of clones resistant to stresses. Secondary leaf fall disease caused by *Phytophthora* sp. leading to serious crop loss is a major biotic stress in rubber. Because rubber is a perennial tree crop, any attempt to breed and evolve clones takes a long period of 25-30 years. This is a serious limitation encountered by the rubber breeder. It is therefore important to develop molecular techniques to speed up this process and improve the chances of finding solutions to some of the intractable problems faced by the rubber breeder. Low and Gale (1991) have discussed the potential usefulness of RFLP markers in *Hevea* breeding programme. A preliminary attempt has been made to identify possible biotic stress tolerance gene analogues in rubber.

MATERIALS AND METHODS

Plant Material

Two clones of rubber, FX 516, a resistant clone of the cross between *Hevea benthamiana* and *Hevea brasiliensis*, and RRII 105, a tolerant clone of *Hevea brasiliensis*, were selected for

study.

Methods

DNA extraction

2g of lyophilised leaves of each clone were ground to fine powder in liquid nitrogen, using a mortar and pestle. The powder was mixed with 20 ml DNA extraction buffer (0.35 M Sorbitol, 0.1 M Tris (pH 7.5), 5 mM EDTA (pH 8.0) from a stock of 50 ml containing 0.19g sodium bisulphite, in a tube. The solution was centrifuged at 3,300 rpm for 15 min. The supernatant was poured off and the green pellet re-suspended by vortexing in 2.5 ml cold extraction buffer. 3.5 ml nuclear lysis buffer (200mM Tris-HCl (pH 8), 2 M NaCl, 20 mg ml^{-1}CTAB) was added, and 1.2 ml 5% Sarkosyl, the tube capped and inverted 5-10 times. After incubation at 65°C for 20 min., 10 ml Phenol-Chloroform was added, the tube inverted 30-40 times and centrifuged at 3,300 rpm for 5 min. The aqueous supernatant was transferred into another tube and 10 ml isopropanol added. The tubes were inverted 5-10 times until the DNA precipitated out of solution. The DNA was hooked out into a 1.5 ml Eppendorf tube and 400 µl water added. After heated to 65°C for 15 min, the pellet was re-suspended by vortexing, and centrifuged for 5 min in a microfuge at 13,000 rpm to pellet starch. The clear supernatant was then transferred to a fresh tube. 400 µl phenol/chloroform was added, vortexed for 30 sec and centrifuged for 1 min. The upper phase was removed into a fresh tube and 40µl of 3 M sodium acetate and 1 ml absolute ethanol added. After centrifugation, the supernatant was removed and the DNA pellet re-suspended in 50µl water. To remove RNA, 1µl RNAase was added and incubated at 37°C for 1 hr. Following addition of 350µl water and extraction with 400µl phenol/chloroform, the mixture was vortexed and spun in a centrifuge for 1 min. The aqueous supernatant was transferred to another Eppendorf tube, 40µl of 3 M sodium acetate and 1 ml of ethanol added, and the mixture vortexed and centrifuged for 5 min. The ethanol was removed and the pellet re-suspended in 50 µl water. The concentration of DNA extracted was quantified using agarose gel electrophoresis.

Resistance gene analogue - polymerase chain reaction

Three groups of resistance gene analogues (RGA) were used as primers for the PCR reactions. The sequences of the RGAs used are presented below:

Oligo Name	Sequence (5'- 3')
(Group 1)	
RGA 1	AAGAATTCGGNGTNGGNAAAACAAC
RGA 2	AAGAATTCGGNGTNGGNAAAACTAC
RGA 3	AAGAATTCGGNGTNGGNAAAACCAC
RGA 4	AAGAATTCGGNGTNGGNAAAACGAC
RGA 5	AAGAATTCGGNGTNGGNAAGACAAC
RGA 6	AAGAATTCGGNGTNGGNAAGACTAC
RGA 7	AAGAATTCGGNGTNGGNAAGACCAC
RGA 8	AAGAATTCGGNGTNGGNAAGACGAC
Oligo Name	Sequence (5'- 3')

(Group 2)

RGA 9	CTACTGNTNCTNGACGACGT
RGA 10	CTACTGNTNCTNGACGATGT
RGA 11	CTACTGNTNCTNGATGACGT
RGA 12	CTACTGNTNCTNGATGATGT

(Group 3)

RGA 13	AACTCGAGAGNGCNAGNGGNAGGCC
RGA 14	AACTCGAGAGNGCNAGNGGNAGACC
RGA 15	AACTCGAGAGNGCNAGNGGNAGTCC
RGA 16	AACTCGAGAGNGCNAGNGGNAGCCC
RGA 17	AACTCGAGAANGCCAANGGCAATCC
RGA 18	AACTCGAGAANGCCAANGGCAAACC

Group 1 and group 2 RGAs constituted the forward primers and group 3 RGAs constituted the reverse primers. Five disease resistant/tolerant clones of rubber (FX 516, RRII 105, GT 1, RRII 33, PB 217) were subjected to RGA- PCR investigations. Each of primers RGA 1- RGA 8 was used separately in combination with each of the RGAs 13-18. 48 reactions with different primer combinations were set up for each of the above clones. Each reaction mixture contained the following solutions:

30 µl sterile distilled water
5 µl 10 X PCR buffer
1 µl each d NTPs (10 mM)
5 µl each primer (primer combinations as shown below)
1 µl DNA from the resistant / tolerant rubber clone (0.1 µg/ml)
0.25 µl *Taq* DNA polymerase (4 units/µl).

PCR mixture	Primer combinations	PCR mixture	Primer combinations
1.	RGA 1 + RGA 13	25.	RGA1 + RGA 16
2.	RGA 2 + RGA 13	26.	RGA2 + RGA 16
3.	RGA 3 + RGA 13	27.	RGA 3 + RGA 16
4.	RGA 4 + RGA 13	28.	RGA 4 + RGA 16
5.	RGA 5 + RGA 13	29.	RGA 5 + RGA 16
6.	RGA 6 + RGA 13	30.	RGA 6 + RGA 16
7.	RGA 7 + RGA 13	31.	RGA 7 + RGA 16
8.	RGA 8 + RGA 13	32.	RGA 8 + RGA 16
9.	RGA 1 + RGA 14	33.	RGA 1 + RGA 17
10.	RGA 2 + RGA 14	34.	RGA 2 + RGA 17
11.	RGA 3 + RGA 14	35.	RGA 3 + RGA 17
12.	RGA 4 + RGA 14	36.	RGA 4 + RGA 17
13.	RGA 5 + RGA 14	37.	RGA 5 + RGA 17
14.	RGA 6 + RGA 14	38.	RGA 6 + RGA 17
15.	RGA 7 + RGA 14	39.	RGA 7 + RGA 17
16.	RGA 8 + RGA 14	40.	RGA 8 + RGA 17
17.	RGA 1 + RGA 15	41.	RGA 1 + RGA 18

18.	RGA 2 + RGA 15	42.	RGA 2 ÷ RGA 18
19.	RGA 3 + RGA 15	43.	RGA 3 ÷ RGA 18
20.	RGA 4 + RGA 15	44.	RGA 4 ÷ RGA 18
21.	RGA 5 + RGA 15	45.	RGA 5 ÷ RGA 18
22.	RGA 6 + RGA 15	46.	RGA 6 ÷ RGA 18
23.	RGA 7 + RGA 15	47.	RGA 7 ÷ RGA 18
24.	RGA 8 + RGA 15	48.	RGA 8 ÷ RGA 18

The PCR conditions were 35 cycles of 96°C for 1 min, 42°C for 1 min, and 72°C for 1 min. followed by 72°C for 10 min. The PCR amplification was done in a Techne Genius PCR machine. On completion of DNA amplifications the 48 reaction mixtures were pooled in to 6 sets as follows.

Pool 1	92 µl water, 1 µl each of PCR mixtures	1 - 8
Pool 2	92 µl water, 1 µl each of PCR mixtures	9 - 16
Pool 3	92 µl water, 1 µl each of PCR mixtures	17 - 24
Pool 4	92 µl water, 1 µl each of PCR mixtures	25 - 32
Pool 5	92 µl water, 1 µl each of PCR mixtures	33 - 40
Pool 6	92 µl water, 1 µl each of PCR mixtures	41 - 48

A second round of 24 PCRs was run with the above pooled DNA samples. Each PCR mixture contained the following solutions.

37 µl sterile distilled water
5 µl 10 X PCR buffer
1 µl d NTPs (10 m M)
1 µl pooled DNA sample
1 µl each primer 1 and primer 2 (primer combinations as shown below)
0.25 µl. *Taq* DNA polymerase

PCR Mixture	Primer combinations
1.	RGA 9 + RGA 13
2.	RGA 10 + RGA 13
3.	RGA 11 + RGA 13
4.	RGA 12 + RGA 13
5.	RGA 9 + RGA 14
6.	RGA 10 + RGA 14
7.	RGA 11 + RGA 14
8.	RGA 12 + RGA 14
9.	RGA 9 + RGA 15
10.	RGA 10 + RGA 15
11.	RGA 11 + RGA 15
12.	RGA 12 + RGA 15
13.	RGA 9 + RGA 16
14.	RGA 10 + RGA 16
15.	RGA 11 + RGA 16

16. RGA 12 + RGA 16
17. RGA 9 + RGA 17
18. RGA 10 + RGA 17
19. RGA 11 + RGA 17
20. RGA 12 + RGA 17
21. RGA 9 + RGA 18
22. RGA 10 + RGA 18
23. RGA 11 + RGA 18
24. RGA 12 + RGA 18

The PCR conditions were the same as before. The final PCR products were run on an agarose gel (1%) and viewed under a UV transilluminator. PCR products which showed distinct banding patterns were pooled and the pooled products were purified using a Nucleon QC kit for PCR/oligo cleanned up by Amersham Life Sciences.

DNA molecular cloning

Ligation of target DNA in to pGEM T Easy plasmid vector:
The purified PCR products were ligated in to p GEM -T plasmids by combining:

3 µl purified PCR product
1 µl T_4 DNA ligase (3 U/µl)
1 µl 10 X ligase buffer (300 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP)
1 µl pGEM- T Easy vector (50 ng/µl)
4 µl nuclease free water

Reagents were gently mixed and incubated at 4°C overnight for effecting the ligation process.

Preparation of bacterial competent cells for transformation

E. coli, strain NM 522, were grown overnight at 37°C by inoculating one bacterial colony in to 10 ml broth solution. Next morning 1 ml of the overnight culture was inoculated into 50 ml broth and left until OD_{600}=0.5. The cells were centrifuged for 10 min at 3000 rpm at 4°C. The supernatant was removed and re-suspended in 25ml ice cold 0.1 M $MgCl_2$ and again centrifuged at 3000 rpm for 10 min. The supernatant was removed and re-suspended in 25ml 0.1 M $CaCl_2$. After centrifuged again at 3000 rpm, the supernatant was removed and re-suspended in 2ml of ice cold 0.075 M $CaCl_2$ and stored on ice.

Transformation of bacterial cells

10 µl of the rubber DNA, ligated in to the plasmid, was added to an Eppendorf tube kept on ice. To the tube was added 200 µl of the bacterial competent cells and left on ice for 20 min after which it was heat shocked at 42°C for 2 min. The contents of the eppendorf was transferred in to another tube and kept on a shaker at 37°C for 1 hour. 1 ml of this mix was concentrated in an Eppendorf tube giving a quick pulse, discarded 900 ml of supernatant and plated the remaining

100µl on to an agar plate containing the appropriate antibiotic. The plate was left overnight inverted at 37°C.

Colony PCR

Each transformed colony was transferred in to PCR mixture containing the following solutions in appropriate concentrations.

35.8 µl sterile distilled water
5 µl 10 X PCR buffer
5 µl 10mM d NTP mix
2 µl 20 µM Universal M13 forward primer
2 µl 20 µM Universal M13 reverse primer
0.2 µl *Taq* DNA polymerase (4U/µl)

PCR was performed using a denaturing step of 94°C for 5 min followed by 30 cycles of 94°C for 45 sec, 55°C for 1 min, 72°C for 1 min 15 sec. After the PCRs were completed each reaction was checked by agarose gel electrophoresis. PCR products exhibiting the same fragment length were further subjected to restriction digestion using *Taq*1 restriction enzyme and gel electrophoresis using a 2% agarose gel. Selected PCR products were sequenced using an ABI 370 automatic DNA sequencer.

RESULTS

The DNA isolation methodology resulted in obtaining clean good yield of DNA, which is used for further studies.

Resistance Gene Analogue PCR

The pooled PCR products on gel electrophoresis revealed varying DNA banding patterns. Ligation of DNA from FX 516 and RRII 105 in to pGEM-T Easy plasmid vector and transformation of the bacterial strain NM 522 could be successfully effected. Good proliferation of recombinant colonies were detected on cloning of the above two varieties.

DNA inserts were detected for 7 colonies in the case of FX 516 and 16 colonies in the case of RRII 105 (Figs. 1a & b). The 7 colonies of FX 516 revealed two sizes of DNA banding pattern (Fig. 2a). In the case of RRII 105 the 16 colonies appeared to show varying DNA banding pattern (Fig.2b). The restriction digestion using *Taq* 1 revealed different restriction sites for the different colonies. On sequencing 2 representative colony PCR products of FX 516, sample 1 revealed some amino acid homology with kinase enzymes. (Fig.3a). No homology to any known resistance gene sequence was noticed for the second sample (Fig. 3b)

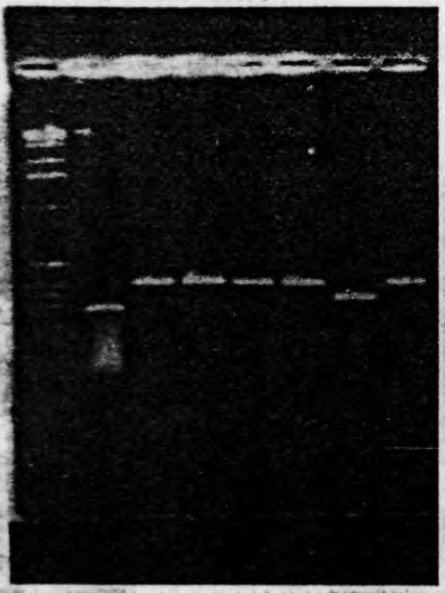

Fig.1a. Colony PCR of FX 516.

(Lane 1 is 1kb ladder marker, lanes 2-8 colonies 1-7)

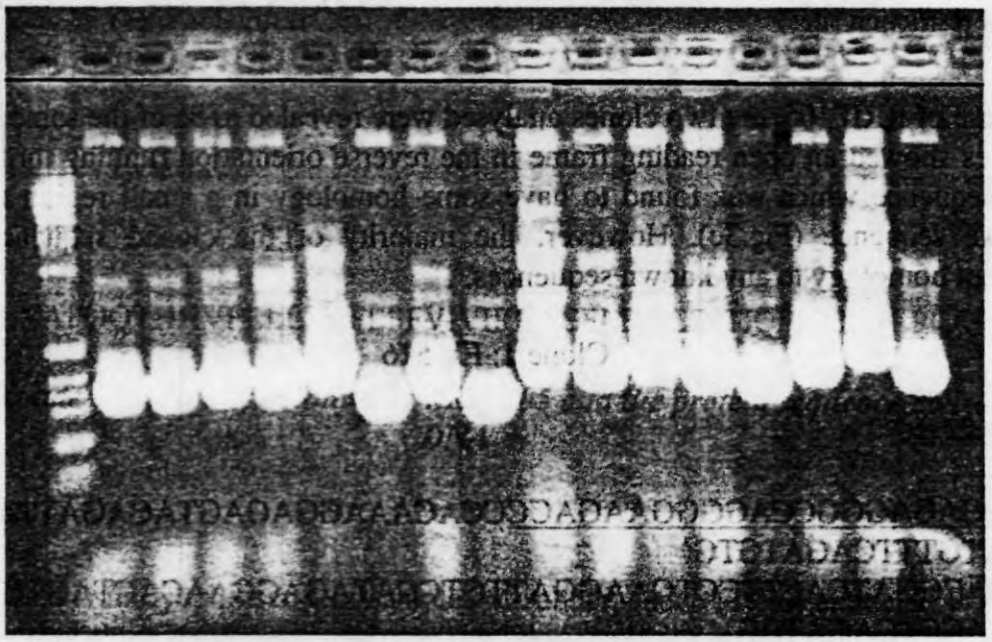

Fig. 1b. Colony PCR of RRII 105.

(Lane 1 is 1 kb ladder marker and lanes 2-17 are colonies 1-16)

Fig.2a Restriction sites of FX 516 colony

(5 samples of same base pairs showing two groups of restriction sites)

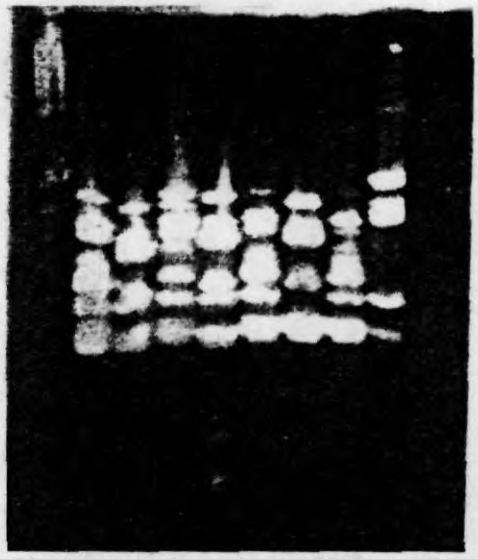

Fig. 2b. Restriction sites of RRII 105 colony

(8 representative samples showing varying restriction sites)

In the case of RRII 105, the two clones analysed were revealed to be of the same sequence. The sequences showed an open reading frame in the reverse orientation running through the entire cloned sequence which was found to have some homology in a small region to a resistance gene like sequence (Fig.3c). However, the majority of the cloned sequence showed no significant homology to any known sequences.

Clone 1: FX 516

Primer 14

AACTCGAGAGGGCCAGGGGAAGACCCCACAAAGGAGAGTACAGATTATTGACGG
GTGGATGTTTCAGATGTG
GGTTTACGGATCACTTCCTGAAGGATTGTCCATAGAGGAACACTAGTGCTCCCACT
GCTCCACCTCAGATGG

Primer 14

AGAGGTCTCCCTCTCGCACTCTCGAGTT

Fig.3a. Nucleotide sequence of colony 1 of FX 516

Clone 2: FX 516 (47)

Primer 5

AAGAATTCGGAGTCGGCAAGACAACTGCAGCTTTCATAGATGTCGTGCGAAGGGA
CAGAGCTAATGGATGTC

Primer 14

TATTCCGGTCTTCCGCTGGCCCTCTCGAGTT

Fig. 3b. Nucleotide sequence of colony 2 of FX 516

Clone 1: RRII105

Primer 17

AACTCGAGAACGCCAAGGGCAATCCTGGGTGGATGCTAAACTGGATCGTATTGAT
GAGTTGATGCAGAAACTCGGCCTCT

GTTATGACGATGACGAAGAAGAGGAAGAAGACGAGAAGCAAGAAGACATGATGC
GTCTGCTGCGGGGCAACTAACGGATT

Primer 17

GCCCTTGGCCTTCTCGAGTT

PROTEIN SEQUENCE ENCODED

LEKAKGNPLVAPQQTHHVFLLLVFFLFFVIVIREAEFLHQLINTIQFSIHPGLPLAFSS

Fig. 3c. Nucleotide sequence of colony 1 of RRII 105 and the protein sequence coded by this DNA

DISCUSSION

DNA was isolated from lyophilised leaf samples of rubber. Jacob (1996) extracted DNA from rubber leaves using three different protocols. The DNA isolation protocol used in the present study could provide another alternative methodology for extraction of pure DNA from rubber. The protocol used for ligation, transformation and development of colonies worked well, establishing the reliability of the methodology in rubber. In the case of FX 516, some resistance gene like homology to kinases revealed by Colony 1 suggested possibilities of identifying resistance genes. Colony 2 could not be matched with any known resistance gene sequences although this may reflect the small size of the cloned insert. In the case of RRII 105, the two clones analysed were revealed to be of the same sequence. It was interesting to note that the sequences showed an open reading frame in the reverse orientation and was found to have some homology in a small region to a resistance gene like sequence (Fig.3c). The results also indicate promising possibilities of identifying possible resistance genes in rubber. *In vitro* DNA amplification is achieved by the thermostable Taq DNA polymerase enzyme via the DNA polymerase chain reaction (Mullis and Faloona, 1987). The strategy of RGA - PCR does work

well in rubber and the technique could be well standardised. These cloned sequences could be further utilised as probes in southern blots to generate RFLP analysis of tolerant and susceptible varieties. The methodology could further be utilised to screen more rubber varieties to locate multiple genes responsible for resistance that would serve as distinct molecular markers for resistance breeding. Reports on molecular markers in *Hevea* are scanty. Varghese *et al* (1997; 1998) reported on evaluation of RAPD markers in rubber for genetic analysis. No successful attempt has so far been made to identify resistance genes in rubber. Shoucai *et al* (1994) reported on the possibility of identifying mildew resistance genes in *Hevea*. Jacob (1996) attempted RAPD-PCR technique for locating markers linked to the resistance to *phytophthora* in rubber plants. In the present study the semi-nested approach of RGA-PCR strategy suggested better scope for identifying markers for screening disease resistance. The primers used in the study were so designed as to bind to specific target nucleotide sequences. The encouraging results of the present investigation points towards the applicability of the strategy for identifying and sequencing valuable resistance genes which would be of help in designing suitable primers for future investigations. The attempt remains as one of the ways to solve problems in the long duration resistance breeding programmes. The strategy could also be tested on susceptible clones and should sequences from resistant clones prove not to be present in susceptible ones. This could be a strong indication that the cloned sequences may play a major role in resistance. Identification and cloning of resistant genes could find application in evolving transgenic plants. The study holds significance that the approach is novel, rapid and feasible in rubber.

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