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AN APPROACH TO IDENTIFY DISEASE RESISTANCE GENE ANALOGUES IN *HEVEA*

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A preliminary attempt was made to identify possible disease resistance gene analogues in rubber. A procedure was developed for the extraction of high quality DNA from rubber leaves, which can be used for the polymerase chain reaction (PCR) amplification of DNA. Eighteen primers, designed based on homologies between known resistance genes, were used in various combinations to amplify sequences from rubber cultivar FX 516, which is resistant to *Phytophthora* leaf fall disease and cultivar RRII 105, which is tolerant. The PCR products were cloned into plasmid vectors and the cloned inserts were sequenced. Although none of the clones obtained had high homology to resistance gene sequences, the putative protein encoded by one sequence had some homology to hem N gene.

Key words : Cloning, *Hevea*, *Phytophthora* leaf fall, Polymerase chain reaction, Resistance genes.

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INTRODUCTION

Exploitation of genetic resistance is one of the main approaches for controlling stresses in plants. Breeding for resistance to both biotic and abiotic stresses requires the development of suitable and reliable screening techniques and identification of heritable resistance characters. Considerable efforts have recently been made to utilize molecular methods to combat diseases caused by fungi, bacteria and viruses. Abnormal leaf fall disease caused by *Phytophthora* spp. leading to serious crop loss is a major biotic stress in rubber (Jacob *et al.*, 1989). Rubber being a perennial tree crop, any attempt to breed and evolve clones takes a long period of 25 to 30 years. It is therefore important to develop techniques to speed up the process. Low and Gale (1991) have discussed the potential usefulness of RFLP markers in *Hevea* breeding programme. More recently, a number of genes that confer resistance against a diverse range of diseases have been cloned from a range of plant species (Hammond-Kosack and Jones, 1996). Many of these genes contain conserved domains that are postulated to encode functional features such as kinase activity, nucleotide binding

sites and leucine-rich repeats (Bent *et al.*, 1994). Based on these conserved regions, primer sequences that have the potential to amplify similar sequences from plants using the polymerase chain reaction (PCR) have been designed (Collins *et al.*, 1998). These primers have been successfully used to identify the *rpl* gene for rust resistance from maize. Resistance gene analogues (RGAs), which are sequences that are similar to resistance genes but for which no function has as yet been determined could also be amplified using these primers. In this study, a preliminary attempt has been made to identify possible resistance gene analogues in rubber.

EXPERIMENTAL

Plant material

Two clones of rubber, FX 516, a *Phytophthora* resistant hybrid of *Hevea benthamiana* and *H. brasiliensis* and RRII 105, a tolerant *H. brasiliensis* clone were selected for the study.

DNA extraction

Two grams of lyophilised leaves of each clone were ground to a 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 0.19 g sodium bisulphite. The solution was centrifuged at 3,300 rpm for 15 min. The green pellet collected was resuspended by vortexing in 2.5 ml cold extraction buffer. Nuclear lysis buffer (200 mM Tris-HCl (pH 8), 2 M NaCl, 20 mg/ml CTAB (3.5 ml) and 1.2 ml 5% sarkosyl were added and the tube capped and inverted 5 to 10 times. After incubation at 65°C for 20 min., 10 ml phenol-chloroform mixture (1:1) was added, the tube inverted 30 to 40 times and centrifuged at 3,300 rpm for 5 min. Isopropanol (10 ml) was added to the aqueous supernatant collected and the tubes were inverted 5 to 10 times until the DNA precipitated. The DNA was hooked out into a 1.5 ml eppendorf tube and 400 µl water added. After heating to 65°C for 15 min, the pellet was resuspended by vortexing and centrifuged for 5 min in a microfuge at 13,000 rpm to remove starch, 400 µl phenol-chloroform mixture was added to the clear supernatant, vortexed for 30 sec and centrifuged for 1 min. The upper phase was collected into a fresh tube and 40 µl of 3 M sodium acetate pH 6.5 and 1 ml absolute ethanol were added. After centrifugation, the DNA pellet collected was resuspended in 50 µl water. One µl RNAse was added and incubated at 37°C for 1 h. Following this, 350 µl water was added and DNA was extracted with 400 µl phenol-chloroform mixture. The aqueous supernatant was transferred to another eppendorf tube after the mixture was vortexed and spun in a microfuge for 1 min. To this, 40 µl of 3 M sodium acetate (pH 6.5) and 1 ml of ethanol were added and the mixture was again vortexed and centrifuged for 5 min. The pellet was resuspended in 50 µl water. The concentration of DNA extracted was quantified using agarose gel electorphoresis.

Resistance gene analogue polymerase chain reaction

The primers used for amplification of resistance gene analogues were essentially as described by Collins *et al.* (1998), except that only the primers for the GVGKTT, LVLDDV and GLPLAL conserved domains were used. These primer sequences were as given in Table 1.

Table 1. **Primers used for resistance gene analogue PCR**

Oligo name	Sequence (5′ - 3′)
GVGKTT group	
RGA 1	AAGAATTCGGNGTNGGNAAAACAAC
RGA 2	AAGAATTCGGNGTNGGNAAACTAC
RGA 3	AAGAATTCGGNGTNGGNAAAACCAC
RGA 4	AAGAATTCGGNGTNGGNAAAACGAC
RGA 5	AAGAATTCGGNGTNGGNAAGACAAC
RGA 6	AAGAATTCGGNGTNGGNAAGACTAC
RGA 7	AAGAATTCGGNGTNGGNAAGACCAC
RGA 8	AAGAATTCGGNGTNGGNAAGACGAC
LVLDDV group	
RGA 9	CTACTGNTNCTNGACGACGT
RGA 10	CTACTGNTNCTNGACGATGT
RGA 11	CTACTGNTNCTNGATGACGT
RGA 12	CTACTGNTNCTNGATGATGT
GLPLAL group	
RGA 13	AACTCGAGAGNGCNAGNGGNAGGCC
RGA 14	AACTCGAGAGNGCNAGNGGNAGACC
RGA 15	AACTCGAGAGNGCNAGNGGNAGTCC
RGA 16	AACTCGAGAGNGCNAGNGGNAGCCC
RGA 17	AACTCGAGAANGCCAANGGCAATCC
RGA 18	AACTCGAGAANGCCAANGGCAAACC

PCR reactions were performed in mixtures comprising 30 µl sterile distilled water, 5 µl 10X PCR buffer, 1 µl each d NTPs (10 mM), 5 µl each primer (20 µl), 1 µl DNA from the resistant/tolerant clone (0.1 mg/µl) and 0.25 µl Taq DNA polymerase (4 units/µl). In the first round of PCR reactions, each of the primers RGA 1-8 was used in every possible combination with each of the primers RGA 13-18. The products from those reactions that used RGA 13 were then combined into a pool, diluted 100-fold in water and a second round of PCR reactions was performed using each of the primers RGA 9-12 in conjunction with RGA 13. Similarly, those products that used RGA 14 were combined, diluted and the second round performed using primers RGA 9-12 with RGA 14 and similarly with RGA 15, RGA 16, RGA 17 and RGA 18.

The PCR conditions for both rounds of amplifications were 35 cycles of 1 min each at 96°C, 42°C and 72°C followed by 72°C for 10 min. The final PCR products were run on an agarose gel (1%) and viewed over 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 clean (Amersham Life Sciences, UK).

Cloning PCR fragments

Purified PCR products were checked by gel electrophoresis and the fragments were ligated into a pGEM-T easy plasmid vector using the pGEM-T easy cloning kit (Promega, UK) according to the manufacturer's instructions. Plasmids were transformed into the *Escherichia coli* strain JM109 (Promega, UK) and colony-PCR performed with M13 universal primers amplified the

cloned insert sequence from the recombinants.

Colony PCR

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

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

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 and 15 sec. After the PCRs were completed each reaction was checked by agarose gel electrophoresis. PCR products which exhibited the same fragment length were further subjected to restriction digestion using *Taq* 1 restriction enzyme and gel electorphoresis using a 2% agarose gel.

Sequence analysis

PCR products were purified using the Nucleon QC kit and DNA concentration was estimated on an agarose gel. For each fragment 200 ng DNA was sequenced using an Applied Biosystems 373A laser sequencer with M13 primers. Sequence data was analysed using DNASTAR and on-line data bases.

RESULTS AND DISCUSSION

DNA extraction

Using the DNA extraction procedure, it was found that a good clean yield of DNA could be prepared from freeze-dried rubber leaves. Furthermore, the DNA was so clean that PCR could be performed without inhibition of the Taq polymerase.

Resistance gene analogue PCR

Using the resistance gene analogue PCR strategy, agarose gel electrophoresis of the products following the second round of PCR revealed varying DNA banding patterns. However, the bands obtained were in the predicted size range for resistance gene analogues of 300-500 base pairs. Ligation of DNA from FX 516 and RRII 105 into pGEM-T easy plasmid vector and transformation of the bacterial strain JM 109 resulted in a number of colonies that contained recombinant DNA inserts. Fig. 1a shows the sizes of fragments present in

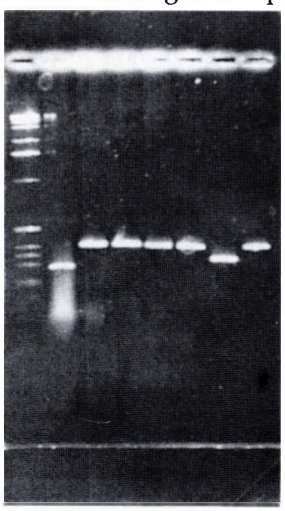

Fig. 1 a. Colony PCR of FX 516 (Lane 1 is 1kb ladder marker, lanes 2-8 clones 1-7)

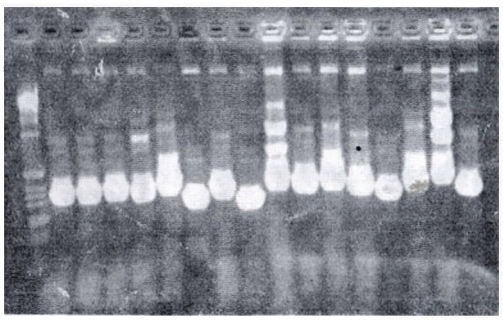

Fig. 1 b. Colony PCR of RRII 105 (Lane 1 is 1kb ladder marker, lanes 2-17 are clones 1-16)

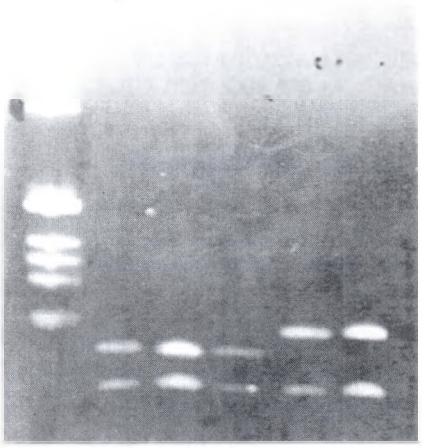

Fig. 2a. Restriction sites of FX 516 clones

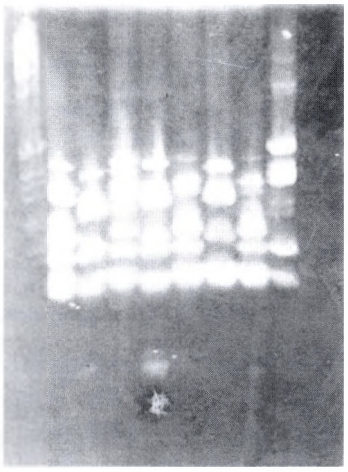

Fig. 2b. Restriction sites of RRII 105 clones

seven clones obtained from cultivar FX 516 and Fig. 1b shows fragments present in 16 colonies from RRII 105.

To determine whether the cloned inserts in the different colonies were identical, they were digested with the restriction endonuclease Taq I. In the case of the five clones from FX 516, two distinct bands (Fig. 2a) were obtained. In the case of RRII 105, the 16 clones appeared to show varying DNA banding pattern (Fig. 2b).

The products of two representative colonies from FX 516 were sequenced, and the results of the sequencing are shown in Figs. 3a & b. No significant homology was noticed with the database sequence search. This could be a new gene product for resistance to *Phytophthora*. However, further work is required to confirm this.

It was interesting to note, however, that the sequence from RRII 105 showed an open reading frame in the reverse orientation and was found to have 86 per cent homology in a small region to coproporphyrinogen 3 oxidase (hem N) gene (Fig. 4). The results also indicate prom-

Clone 1. FX 516

AACTCGAGAGGGCCAGGGGAAGACCCCACAAAGGAGAGTACAGATTA
TTGACGGGTGGATGTTTCAGATGTGGGTTTACGGATCACTTCCTGAAG
GATTGTCCATAGAGGAACACTAGTGCTCCCACTGCTCCACCTCAGATG
GAGAGGTCTCCCTCTCGCACTCTCGAGTT

Fig. 3a. Nucleotide sequence of colony 1 of FX 516 (sequence underlined is that of RGA primer 14)

Clone 2. FX 516

AAGAATTCGGAGTCGGCAAGACAAC.TGCAGCTTTCATAGATGTCGTGCGA
AGGACAGAGCTAATGGATGTCTATTCCGGTCTTCCGCTGGCCCTCTCG
AGTT

Fig. 3b. Nucleotide sequence of colony 2 of FX 516 (sequence underlined is that of RGA primer 5)

Clone 1. RRII 105

AACTCGAGAACGCCAAGGGCAATCCTGGGTGGATGCTAAACTGGATC
GTATTGATGAGTTGATGCAGAAACTCGGCCTCTCTTATGACGATGACG
AAGAAGAGGAAGAAGACGAGAAGCAAGAAGACATGATGCGTCTGCTG
CGGGGCAACTAACGGATTGCCCTTGGCCTTCTCGAGTT

LEKAKGNPLVAPQQTHHVFLLLVFFLFFVIVIREAEFLHQLINTIQFSIHPG
LPLAFSS

Fig. 4. Nucleotide sequence of clone 1 of RRII 105 (sequence underlined is Primer RGA 17). The sequence in bold is the amino acid sequence encoded by the putative open reading frame running through this sequence

ising possibilities of identifying possible resistance genes in rubber. This suggests that the strategy of RGA – PCR does work in rubber and the technique could well be modified and optimised for this species. These cloned sequences could be further utilized as probes in southern blots to generate RFLPs of tolerant and susceptible varieties. The methodology could further be utilized to screen more rubber clones to locate multiple genes responsible for resistance that would serve as distinct molecular markers for resistance breeding. Klein – Lankhorst *et al.* (1991) identified RAPD markers closely linked to Mi gene (nematode resistance gene) in tomato. Reports on molecular markers in *Hevea* are scanty. Varghese *et al.* (1997; 1998) reported on evaluation of RAPD markers in rubber for assessing the extent of genetic variability. Chen *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 resistance to *Phytophthora* in rubber plants. Chen *et al.* (1999) obtained RAPD marker OPV-10_{390} linked to mildew-resistance gene in 11 resistant varieties of rubber. 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 point towards the applicability of the strategy for identifying and sequencing valuable resistance genes which would be helpful in designing suitable primers for future investigations. 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 resistance 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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