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Cloning and characterization of β -1, 3-glucanase gene from different clones of *Hevea brasiliensis*

R. Supriya, A. Saleena and A. Thulaseedharan.*
Biotechnology Division, Rubber Research Institute of India, Kottayam - 686009

Abstract

The relative tolerance of plants towards a particular disease/ pathogen is mainly determined by the interactions between the disease resistant 'R' genes and the associated pathogen avirulence 'avr' genes, affecting the plant. β-1, 3-Glucanase enzyme, which belongs to the category of PR -2 (pathogenesis related proteins) family is known to be involved in the plant defense reactions against pathogens like *Phytophthora* causing abnormal leaf fall disease (ALF) in rubber plantations. The present work was undertaken to characterize the genomic DNA sequences coding for β-1, 3-glucanase from different clones of *Hevea brasiliensis*. High quality genomic DNA was isolated from *Hevea* clones, FX 516, RRII 105, RRII 33, PR 107, PB 86 and RRIM 701. β-1, 3 Glucanase genomic sequences were PCR amplified using oligonucleotide primers designed based on previously reported sequences. A PCR product with approximately 1.3 Kb in size was amplified from all the clones. The isolated gene was later cloned in the Topo TA® cloning vector, sequenced and analysed for sequence variations. The result shows major variations within the intronic sequences. 'CT' repeats within the intronic sequences show deletion/ insertions among the clones studied. Although, the differences occur in the number of deletions, the location is almost similar. Only minor variations in nucleotides were observed in the exons. Though, the exact role of introns in a particular gene is not completely known, it is reported that through alternative splicing and non-sense mediated mRNA decay; the introns play an important role in the expression of a particular gene.

Key words: β-1, 3-glucanase, *Hevea brasiliensis*, abnormal leaf fall disease, genomic sequences, intron deletions

Introduction

Hevea brasiliensis, belonging to the family *Euphorbiaceae*, is the major source of commercial natural rubber. The unique isoprenoid compound, cis – 1, 4- polyisoprene, is present in latex, the milky cytoplasm of specialized cells called laticifers, which are located adjacent to phloem vessels. Many of the recently developed *Hevea* clones are susceptible to a variety of diseases and abiotic stresses leading to heavy yield loss. Among the major fungal diseases affecting rubber plantations, abnormal leaf fall disease (ALF), caused by *Phytophthora* is the most economically significant one in India. Although many promising clones with a fair degree of tolerance to ALF disease were evolved through conventional breeding programmes from the initial germplasm collections of the 19th century, a fully tolerant clone to *Phytophthora* with high yield has not been developed so far.

Plants offer remarkable strategies to counter the pathogen attack using an array of constitutive or inducible biochemical and molecular mechanisms. They exhibit both long and short-term defence responses to immediate challenges like pathogen attacks (Gachomo *et al.*, 2003). The defence systems often depend on the combination of a specific set of dominant avirulence (Avr) genes in the pathogen (Keen, 1990). Expression of the Avr genes triggers plant defense responses governed by the product of the R gene (Bogdanove, 2002). The R genes and genes encoding signal transduction proteins possess loci at their downstream sequences for production of pathogenesis

*Author for correspondence

related proteins (PR's) (Gachomo *et al.*, 2003). Plant â-1, 3-glucanases are PR proteins that are expressed in response to microbial invasion (Boller, 1987). These come under the family of PR – 2 proteins (glucan endo – 1, 3-â - glucosidases) and are able to hydrolyze the 1, 3- â-D glucosidic linkages in â-1, 3-glucans. These are abundant, highly regulated enzymes and are widely distributed in seed plants (Metzger and Meins, 1999). Since the cell wall of *Phytophthora* is mainly made of â-1, 3-glucans, the â-1, 3-glucanase alone produced by the host plant may confer tolerance to *Phytophthora* infection.

Earlier reports on the initial molecular changes associated with *Phytophthora* infection reveals that the genomic sequences encoding for â-1, 3-glucanase is present at equal copy numbers in the tolerant as well as susceptible clones (Thanseem, 2004). But on infection with the fungus, the gene is expressed differentially in the clones. In the susceptible clones, â-1, 3-glucanase transcript level starts decreasing by 48 hrs; while in the tolerant clones a steady state level of transcript abundance is observed. It is also observed that a single protein â-1, 3-glucanase could inhibit the *Phytophthora* mycelial proliferation (Thanseem *et al.*, 2005a, b). The relative expression of the gene is controlled by the structure of the promoter elements and the property and the stability of the proteins is controlled by the amino acid sequence which in turn is determined by the gene sequence. Therefore, it is essential to study whether the sequence variation plays a role in the gene expression as well as in the stability of the protein.

As a preliminary step, in the present study an attempt has been made to study the genomic DNA sequence of â -1, 3 - glucanase gene in the tolerant and susceptible clones of *Hevea brasiliensis*. Three *Hevea* clones, RRII 105, RRII 33 and FX 516 tolerant to abnormal leaf fall disease and three clones RRIM 701, PB 86 and PR 107 susceptible to ALF, were selected for this study.

Materials and Methods

DNA isolation

All plant materials used in the present study were collected from the germplasm nursery of Rubber Research Institute of India. Genomic DNA was isolated from the young uninfected leaves of *Hevea*. The isolation was done according to the modified protocol of Doyle *et al.* (1990). The isolated DNA was checked for quality via agarose gel electrophoresis.

PCR amplification of the β – 1, 3-glucanase gene from the genomic DNA

For the amplification of β-1, 3-glucanase gene, primers were designed based on a previously reported sequence (Thanseem *et al.*, 2004; Gene bank accession no: AY325498). The PCR primers designed was as follows: forward primer – 5' CTT CTT AAT GGC TAT CTC CTC 3' and reverse primer – 5' CTC ACA TAT CAC TCT TAA GG 3'. PCR was carried out in 20 µl reactions, which contained 1 X buffer having 1.5 mM $MgCl_2$ (pH 8.3), 100 µM of each dNTP's, 0.5 Units of *Taq* DNA polymerase enzyme (M/S Bangalore Genei, India), 20 ng of template DNA and 250 nM of Primers in a thermal-cycler (Perkin Elmer 480). The PCR conditions were: initial denaturation of 94°C for 2 minutes, followed by 36 cycles with denaturation at 94°C for 1 minute, annealing temperature of 55°C for 1 minute and an extension at 72°C for 2 minutes. The final extension was carried out at 72°C for 10 minutes. Amplified products were separated on a 1.5 % agarose gel and later eluted out using the DNA gel band purification kit (M/S Amersham Pharmacia Biotech, USA) following the manufacturer's instructions.

Cloning and Sequence Characterization of the β-1, 3-glucanase gene

The amplified product after purification was used directly for cloning. Cloning of the amplified genomic DNA of the selected *Hevea* clones was carried out using TOPO TA® cloning kit for Sequencing (M/S Invitrogen Life Technologies, USA) following the manufacturer's protocol. The cloned gene was later transformed into the chemically competent *E. coli* (DH5α) cells supplied along with the kit. The transformed cells were plated on LB agar plates containing 50 µg/ ml ampicillin.

Confirmation of transformation

A few colonies were selected after 16 hours of incubation and colony PCR was done to identify positive clones. Plasmids were isolated from positive clones using Perfect prep Plasmid mini Kit (M/S Eppendorf, USA). The confirmation of transformation was done by the PCR amplification of the plasmids using β-1, 3-glucanase specific primers. Amplification was checked through 1.5 % agarose gel electrophoresis. The presence of the insert was confirmed by restriction digestion using the *Eco* enzyme to release the inserted fragment.

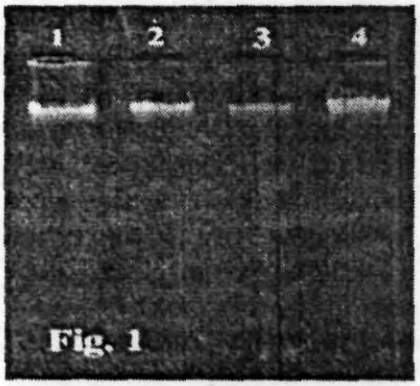
Characterization of the DNA sequence for β-1 3-glucanase gene

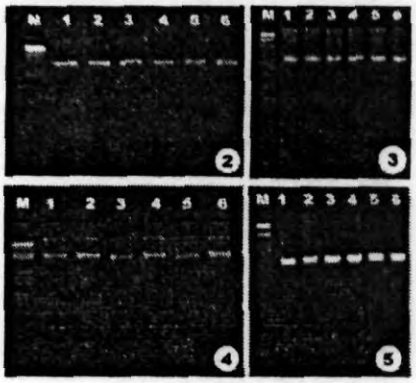
The recombinant plasmid obtained were sequenced at M/S Macrogen, Korea. Sequence of β-1 3-glucanase from different clones were aligned using the CLUSTAL W software at the EMBL EBI site. Homology of the sequence with other sequences in the

ublic database (NCBI) was done to identify similar equences.

Results and Discussion

The present study was conducted in clones three olerant and three susceptible *Hevea* clones with varying legrees of tolerance and susceptibility towards abnormal eaf fall disease. Good quality genomic DNA was isolated rom leaves of the selected clones (Fig.1).





g. 1 Agarose gel electrophoresis of DNA samples isolated from different Hevea clones

g. 2 *PCR amplification of genomic DNA for β–1, 3-glucanase from 6 Hevea clones*

M : Marker DNA ; Lanes: 1 - 6 : 1. RRII 105, 2. RRII 33, 3. FX 516, 4. PB 86, 5. FX 516 , 6. PR 107

. 3 *Colony PCR for the amplification of β–1, 3-glucanase from the transformed bacteria*

M : Marker DNA; Lanes: 1 - 6 : 1. RRII 105, 2. RRII 33, 3. FX 516 4. PB 86, 5. FX 516 , 6. PR 107

. 4 *Agarose gel electrophoresis of recombinant plasmid DNA containing the β-1, 3-glucanase genomic sequences from 6 Hevea clones*

M : Marker DNA ; Lanes: 1 - 6 : 1. RRII 105, 2. RRII 33, 3. FX 516 4. PB 86, 5. FX 516 , 6. PR 107

. 5 *PCR amplified β–1, 3-glucanase from the recombinant plasmids*

M : Marker DNA; Lanes : 1 - 6 : 1. RRII 105, 2. RRII 33, 3. FX 516 , 4. PB 86, 5. FX 516 , 6. PR 107

The primers designed were highly specific for the selective amplification of β-1, 3-glucanase gene in *Hevea*. Under optimal conditions, a single band of approximately 1.3 kb in size was obtained from the genomic DNA of the six clones studied (Fig. 2). The PCR product eluted from the gel was used directly for cloning. All the colonies selected were subjected to colony PCR and a 1.3 kb band was amplified (Figure 3). The plasmids isolated from the liquid cultures of the positive colonies were in good concentration (Fig. 4). Before sequencing, the presence of inserts in all the recombinant plasmids were confirmed through PCR and all the samples showed good amplification as a single band product (Fig. 5). The sequence analysis of all the clones revealed the presence of a single intronic sequence at +103 position starting from the translation initiation codon (TIC). It was interesting to note major variations within the intronic sequences of the β-1, 3-glucanase gene of all the clones. There were not much variation observed in the exons of different clones. The intron sequences of all the clones studied were found to have 'CT' repeats. The number of 'CT' repeats varied from 7-8 duplets in length, in the clones studied. The maximum number of nucleotides in the intron were found in the clones PR 107 and RRII 105. A 131 bp intron was observed in the clones, FX 516, PR 107 and RRII 105, which contained 18 'CT' repeats. The least number was found in RRIM 701, with 115 nucleotides in the intron, comprising 10 'CT' repeats. The sequences with the number of insertions/deletions in different clones are presented in Table 1. It is understood that even though the indel sequences show numerical variations, the location of the particular 'CT' repeats remained more or less the same. Through the sequence similarity search at NCBI site, 146 Blast hits was obtained with the characterized sequences, which includes the already reported sequences of â – 1, 3 – glucanase gene in *Hevea*.

Table 1. Details of intron position and insertion/deletions of 'CT' repeats in the genomic sequence of β-1,3-glucanase from different *Hevea* clones

Name of the clone	Number of nucleotides in the intron	Number of CT repeats	Location of CT deletions
RRII 105	131	18	-
RRIM 701	115	10	126-143
RRII 33	117	11	128-143
PB 86	117	11	128-143
PR 107	131	18	-
FX 516	116	18	128-143

It is necessary to go deep into the functions regarding introns, mainly, the functions due to the presence of microsatellite insertions/deletions. Earlier reports have shown inhibition of gene expression by the presence of dinucleotide repeats in the promoter region of nucleolin gene (Rothenburg *et al.*, 2001). Inhibition of gene expression by dinucleotide repeats has also been observed in tilapia prolactin 1 (Streelman *et al.*, 2002). CA repeat deletions in the intron 1 of HSD11B2 led to a significant decrease (40 %) in the expression of this gene in human cortical collecting duct cells and its influence on expression was dependent upon the location and orientation of the CA repeat (Agarwal, 2001). Since there is no report suggesting the association of deletion of introns with tolerance and susceptibility of an eukaryotic plant towards a pathogen attack, studies should be carried out to understand the role of the intron sequences in gene expression.

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