3

MATERIALS AND METHODS

3.1. Isolation, Cloning and Characterisation of Genomic and cDNA Sequences Coding for β-Glu Gene

3.1.1. Genomic DNA isolation

Young, uninfected leaves were selected from Hevea clones maintained in the germplasm collection nursery of Rubber Research Institute of India. The samples were washed thoroughly in tap water and rinsed with sterile water. DNA extraction was done with a modified protocol of Doyle et al., (1990). This modified CTAB procedure consists of the following steps:

i) Two g leaf tissue was ground to a very fine powder in Liquid Nitrogen using a mortar and pestle.

ii) The ground tissue was homogenized with 20 ml 2X CTAB extraction buffer.

iii) The sample was then kept at 60°C for 30 min in a 50 ml centrifuge tube.

iv) Centrifuged at 8000 rpm for 10 min, pellet was discarded and the supernatant transferred to a new tube.

v) Equal vol of Tris-saturated phenol-chloroform-isoamyl alcohol (25:24:1) was added and mixed gently.

vi) The sample was then spun at 10,000 rpm for 10 min and the aqueous phase was transferred to a new tube. The organic phase containing the denatured proteins was discarded.

2X CTAB buffer: 2% CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl (pH-8.0), 1% Polyvinylpolypyrrolidone and 0.1% β-mercaptoethanol
vii) RNA in the sample was degraded by incubating at 37°C for 1 h after the addition of 5 µl of DNAase free RNAase (10 mg/ml-Sigma).

viii) Proteinase K (20 mg/ml- Bangalore Genei) was added to inactivate the RNAase and other residual proteins. The incubation was continued for another 1 h.

ix) Equal vol of chloroform:isoamyl alcohol was added to the sample, mixed gently and centrifuged at 10,000 rpm for 10 min.

x) The aqueous phase was transferred to a fresh tube and the organic phase contains the lipids and carbohydrates was discarded.

xi) To the sample equal vol of chloroform:isoamyl alcohol was added, mixed gently and centrifuged at 10,000 rpm for 10 min.

xii) Aqueous phase was transferred to a fresh tube and the organic phase was discarded.

xiii) To the sample 0.6 vol ice-cold isopropyl alcohol was added to precipitate the DNA.

xiv) The tube was then kept in ice for 20 min and the precipitated DNA was pelleted by centrifuging at 8000 rpm for 10 min at 4°C.

xv) The DNA was washed twice in 70 % ethanol and once in absolute ethanol.

xvi) The pellet was air-dried and suspended in TE buffer.

xvii) The DNA samples were stored at –20°C.

3.1.1.a. DNA quantification

The quality and quantity of genomic DNA was checked in a UV spectrophotometer (Beckman, USA). The quality was checked by measuring the ratio of absorbance at 260 nm and 280 nm (260/280) respectively. A ratio between 1.7 – 1.8 indicates good quality DNA without protein contamination. DNA quantification was done according to the following formula.

\[ 1 \text{ O.D. at } 260 \text{ nm} = 50 \text{ ng of DNA} \]

The O.D. of each DNA sample at 260 nm was measured and quantified accordingly.
3.1.2. Isolation of RNA from latex

RNA from the latex of rubber tree was extracted through a modified protocol of Kush et al., (1990). All the reagents required were prepared in DEPC (an RNAase inhibitor) treated water. The protocol involved the following steps.

i) Equal vol of latex was collected directly in to a centrifuge tube under ice-cold conditions which contains 10 ml extraction buffer.

Latex RNA extraction buffer: 50 mM Tris-HCl (pH-8.5), 150 mM LiCl, 5 mM EDTA and 2 % SDS

ii) The sample was centrifuged at 12,000 rpm for 30 min at 4°C.

iii) The upper white creamy layer, which consists of rubber particles were removed and the latex serum was transferred to a fresh tube.

iv) It was then treated with an equal vol of extraction buffer saturated phenol, mixed gently and centrifuged at 10,000 rpm for 10 min.

v) The aqueous layer was recovered to fresh tube and phenol extraction was done once more.

vi) The aqueous layer was then treated with an equal volume of choloform:isoamyl alcohol to remove carbohydrates, lipids and traces of phenol; centrifuged and the organic layer was discarded.

vii) RNA in the aqueous layer was precipitated by adding 1/3 vol of 8 M LiCl. The precipitation continued overnight in –20°C.

viii) RNA was pelleted by centrifugation at 10,000 rpm for 20 min. at 4°C.

ix) The pellet was washed with 2 M LiCl, air dried and suspended in 500 µl of DEPC treated H2O.

x) RNA was further purified and concentrated by precipitation with 2.5 vol ethanol in presence of 0.1 vol, 3 M sodium acetate (pH- 5.2).

xi) The RNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C.

xii) The pellet was washed twice in 70% alcohol, air dried and re-suspended in sterile H2O.
xiii) The quantity of RNA was checked using UV spectrophotometer (Section- 3.1.1.a) and its quality and DNA contamination, if any, was checked in 1% agarose gels.

xiv) The isolated RNA samples were stored in 3 vol of 100% ethanol at 

The agarose gel electrophoresis was carried out according to standard protocols (Sambrook et al., 1989).

3.1.3. First strand cDNA synthesis

First strand cDNA was synthesised from the isolated latex RNA by reverse transcription reaction with oligo-(dT) primers using the 'Improm-II™ Reverse Transcription System' (Promega, USA) as follows:

i) Primer annealing to the target RNA and denaturation was performed in a 0.5 ml reaction tube in ice. 1 μl of total latex RNA (1 μg) was combined with 1 μl oligo-(dT) primers (0.5 μg). The reaction was made up to 5 μl by the addition of nuclease free water.

ii) The tube was incubated for 5 min at 70°C in a pre-heated block and immediately chilled in ice for 5 min.

iii) The tube was then spin down for 10 sec in a micro-centrifuge to collect the condensate and maintain the original volume. This RNA-primer combination was kept on ice until the reverse transcription reaction mix gets ready.

iv) The RT-reaction mix was combined in a 1.5 ml tube on ice. 4 μl of reaction buffer supplied by the manufacturer along with 1.5 μl MgCl₂ (1.8 mM), 1 μl dNTP mix (0.5 mM of each dNTP) and 1 μl reverse transcriptase was made up to 15 μl with nuclease free water.

v) The RNA-primer mix (5 μl) was added to the reaction mix to form the final volume of 20 μl.

vi) Annealing was done by incubating the reaction at 25°C for 5 min.

vii) Primer extension was carried out at 42°C for 1 h in a heated block.

viii) The reaction was stopped by inactivating the reverse transcriptase by keeping the tube at 70°C for 15 min.
ix) The synthesised first strand cDNA was stored at -20°C for subsequent PCR amplification.

3.1.4. Design of gene specific primers

Based on a previously published cDNA sequence of β-1,3-glucanase gene (Chye and Cheung, 1995), two gene specific oligonucleotide primers were designed with the help of ‘PrimerSelect’ programme of ‘Lasergene’ software (DNASTAR, USA). The primer sequences and their Tm value are shown below:

Forward primer - 5’ CTT CTT AAT GGC TAT CTC CTC 3’ Tm - 55.9
Reverse primer - 5’ CTC ACA TAT CAC TCT TAA GG 3’ Tm - 53.2

The synthesised primers (Metabion, Deutschland) were dissolved in sterile double distilled water to get a concentration of 100 pmols/μl. The primer stock solutions were stored in -20°C.

3.1.5. PCR amplification of the gene from genomic DNA and cDNA

PCR amplification was performed with 10 ng of genomic DNA or one μl of cDNA from the step 3.1.3. as templates in separate reactions. Amplifications were carried out in 20 μl reactions, which contains the following constituents:

<table>
<thead>
<tr>
<th>Amount</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Reaction buffer (Tris-HCl, pH- 9 – 10 mM, KCl- 50 mM, MgCl2- 15 mM)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Tag DNA polymerase (Amli Taq® from Roche, USA)</td>
<td>0.15 μl</td>
</tr>
<tr>
<td>Sterile dist. Water</td>
<td>12.85 μl</td>
</tr>
</tbody>
</table>

Methodologies
The reaction mix was overlaid with a drop of mineral oil and amplification was carried out in a Perkin-Elmer 480 DNA thermal cycler. The PCR conditions were as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Initial denaturation</th>
<th>4 min-</th>
<th>94°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step II</td>
<td>Denaturation</td>
<td>30 sec.-</td>
<td>94°C</td>
</tr>
<tr>
<td></td>
<td>Annealing</td>
<td>1 min.-</td>
<td>50°C</td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>2 min.-</td>
<td>72°C</td>
</tr>
<tr>
<td>Step III</td>
<td>Repeat step II</td>
<td>35 times</td>
<td></td>
</tr>
<tr>
<td>Step IV</td>
<td>Final elongation</td>
<td>10 min.-</td>
<td>72°C</td>
</tr>
<tr>
<td>Step V</td>
<td>Hold</td>
<td></td>
<td>4°C</td>
</tr>
</tbody>
</table>

The PCR products were analysed in 1.5 % agarose gels. Gel images were captured using ‘EDAS 290’ (Electrophoresis Documentation and Analysis System-Kodak, USA). Molecular weight of the amplified products was determined using Kodak I D Image Analysis software.

3.1.6. Cloning of the PCR products

3.1.6.a. Elution of amplified products from agarose gels

The samples were run in 0.7 % low melting point agarose gels and the DNA bands were cut out from the lane after viewing the gel over long wavelength UV light quickly so as to avoid nicks. The gel slices were taken in a 1.5 ml micro-centrifuge tube and kept at 65°C for 10 min or till the agarose melt completely. To the melted agarose 1/10 vol, 5 M NaCl was added, mixed well and again kept at 65°C for 10 min. Equal vol of Tris-saturated phenol was added, mixed gently and the tube was centrifuged at 8000 rpm for 10 min. The aqueous layer was recovered and treated twice with chloroform. The DNA was precipitated by adding 1/10 vol, 3 M sodium acetate and 2.0 vol ethanol. Precipitation was continued for 30 min at -20°C and was pelleted by spinning at 8000 rpm for 10 min at 4°C. The DNA was washed in 70% alcohol, air-dried and re-suspended in an appropriate quantity of TE buffer.

Elution of DNA bands from agarose gels had also been performed using ‘Clean Genei kit’ (Bangalore Genei) according to the manufacturers instructions.
### 3.1.6.b. Ligation

‘pGEM-T Easy’ vector system (Promega, USA) was used for the cloning of purified PCR products. A vector to insert ratio of 1:3 was used for ligation. The amount of insert required for ligation with 50 ng of the vector was calculated using the following formula:

\[
\text{Amount of insert} = \frac{\text{ng of vector} \times \text{kb size of the insert}}{\text{kb size of the vector}} \times \text{molar ratio of insert: vector}
\]

The ligation mix was prepared as follows.

- 2X rapid ligation buffer (supplied by the manufacturer) - 5.0 µl
- pGEM-T Vector - 1.0 µl (50 ng)
- PCR product - 1.0 µl
- T4 DNA ligase - 1.0 µl
- H₂O - 2.0 µl
- 10 µl

The ligation was performed overnight at 4°C or at 16°C for 4 h. The ligation mix was frozen till the time of transformation.

### 3.1.6.c. Transformation protocols for *E. coli*

The *E.coli* strain *JM 109* was used for the transformation purpose. Competent cell preparation and transformation was done as follows:

i) From a glycerol stock, the *E.coli* strain was streak plated to LB.

ii) Single colonies from the plate was picked and cultured in 3 ml LB overnight at 37°C in an environmental shaker (New Brunswick Scientific, USA).

iii) Next day, 2% of the overnight grown cultures (100 µl) were inoculated to 5 ml LB and grown for 3-4 h (till the OD reaches 0.3 – 0.5).

iv) The cultures were then kept in crushed ice and distributed 1 ml each to 1.5 ml micro-centrifuge tubes.

v) The cells were harvested by spinning at 5000 rpm for 3 min at 4°C.
vi) After discarding the supernatant, the tubes were kept in ice and 200 μl 0.1 M freshly prepared CaCl₂ was added with a pre-cooled pipette tip. The cells were kept suspended in 0.1 M CaCl₂ for 20 min in ice.

vii) The tubes were then spun at 5000 rpm for 3 min at 4°C and the supernatant was discarded.

viii) The cells were re-suspended in 200 μl 0.1 M ice-cold CaCl₂ and either quickly frozen to −70°C for storage or kept in ice for immediate use.

ix) For transformation, 4 μl of the ligated mix was added to the competent cells and incubated in ice for 30 min.

x) Then a heat shock was given at 42°C for 1 min.

xi) After the heat shock, the tube was immediately transferred to ice and allowed to chill for 1 – 2 min.

xii) Then 800 μl LB medium was added and the culture was incubated at 37°C for 90 min with shaking.

xiii) The transformed competent cells (100 μl) were spread over an LB plate (90 mm) containing 50 μg/ml ampicillin coated with 40 μl X-gal (20 mg/ml) and 4 μl IPTG (200 mg/ml).

xiv) The plates were incubated for 12 – 16 h at 37°C.

xv) Transformants containing the insert were selected by blue/white screening.

3.1.7. Confirmation of cloning

3.1.7.a. Through PCR

Both blue and white colonies were cultured overnight in 3 ml LB containing the appropriate antibiotic and were pelleted down quickly by spinning down at maximum speed for 30 sec in a table-top micro-centrifuge. The pellet was suspended in 50 μl sterile double distilled water and boiled for 10 min. The suspension was spun down and the supernatant was used as the template for PCR reactions.

PCR reactions were performed as mentioned in section 3.1.5. Only 30 PCR cycles were performed with 2 μl of the template plasmid DNA. The PCR products from blue and white colonies were analysed in 1.5 % agarose gels.
3.1.7.b. Alkaline lysis procedure for isolation of plasmids in large scale

Plasmid extraction from recombinant bacteria was done according to the alkaline lysis procedure of Birnboim and Doly (1979).

i) An overnight grown 3 ml culture was inoculated into 100 ml of LB broth containing the respective antibiotic and kept under constant shaking for 12 - 16 h at 37°C.

ii) The cells were harvested by centrifugation at 6000 rpm for 5 min at 4°C and the supernatant was drained completely.

iii) The bacterial pellet was washed with 10 ml ice-cold STE buffer

<table>
<thead>
<tr>
<th>STE Buffer</th>
<th>0.1 M NaCl, 10 mM Tris-Cl (pH-8), 1 mM EDTA (pH-8)</th>
</tr>
</thead>
</table>

iv) The washed pellet was re-suspended in 3 ml of solution I.

<table>
<thead>
<tr>
<th>Solution I</th>
<th>50 mM Glucose, 25 mM Tris-Cl (pH-8), 10 mM EDTA (pH-8)</th>
</tr>
</thead>
</table>

v) Cells were lysed by adding freshly prepared solution II (6 ml), mixed thoroughly and kept at room temperature for 5 - 10 min.

<table>
<thead>
<tr>
<th>Solution II</th>
<th>0.2 N NaOH, 1 % SDS</th>
</tr>
</thead>
</table>

vi) Finally 4.5 ml of ice-cold solution III was added to the lysate, mixed thoroughly and kept in ice for 10 min.

<table>
<thead>
<tr>
<th>Solution III</th>
<th>5 M Potassium acetate- 60 ml, Glacial acetic acid- 11.5 ml H₂O- 28.5 ml</th>
</tr>
</thead>
</table>

vii) The precipitated mix which contains chromosomal DNA and high mol.wt. RNA was removed by centrifuging at 6000 rpm for 15 min at 4°C.
viii) The supernatant was treated with 5 μl of RNAase (10 mg/ml) and incubated at 37°C for 1 h.

ix) Supernatant was then washed twice with an equal vol of chloroform:isoamylalcohol and centrifuged at 10,000 rpm for 10 min.

x) Plasmid DNA from the supernatant was precipitated by the addition of 0.6 vol of isopropanol and incubated at room temperature for 10 min.

xi) The plasmid DNA was recovered by centrifugation at 10,000 rpm for 10 min.

xii) Pellet was washed in 70% ethanol, air-dried and dissolved in 100 μl of TE buffer and stored at -20°C.

xiii) The plasmids isolated from recombinant colonies were checked in 0.8% agarose gels and compared with control plasmids.

3.1.7.c. Restriction analysis of the plasmid DNA

To confirm the cloning event, the recombinant plasmids were subjected to restriction digestion. To release the insert, 1.0 U of EcoR I enzyme (Bangalore Genei) was added along with the enzyme buffer supplied by the manufacturer to around 1 μg of the isolated plasmid DNA. The pGEM-T vector is having EcoR I restriction sites on either side of the inserted DNA fragment. The reaction mix was incubated for 3 h at 37°C. Self ligated, non-recombinant plasmids were also analysed as a control. The digested plasmids along with undigested controls were analysed in 0.8% agarose gels.

3.1.8. Sequencing and sequence analysis

3.1.8.a. PEG purification of plasmid DNA

The plasmid DNA isolated through alkaline lysis procedure was purified through PEG precipitation for sequencing purpose.

i) To 32 μl of plasmid DNA, 8 μl 4 M NaCl was added, followed by 40 μl of 13% PEG8000.

ii) After thorough mixing, the sample was incubated on ice for 20 min.

iii) The precipitated plasmid DNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C.
iv) The supernatant was discarded and the pellet was rinsed with 70% ethanol.

v) Pellet was air-dried and re-suspended in 20 μl of sterile double distilled H₂O and stored at -20°C.

3.1.8.b. Sequencing

The sequencing was done at DBT sponsored DNA sequencing facility, Department of Biochemistry, Indian Institute of Science, Bangalore in an automated sequencer (ABI PRISM). Sequence similarity search was done with 'BLASTN' programme of NCBI (National Center for Biological Information), USA (Altschul et al., 1990). The deduced amino acid sequence analysis was performed using the various protein handling tools available on the web at the ExPASy site (Expert Protein Analysis System), which is the proteomics server of the Swiss Institute of Bioinformatics (Gasteiger et al., 2003). Computation of the various physical and chemical parameters of the predicted protein like molecular weight, theoretical pH, amino acid composition, estimated half-life, instability index etc. were carried out using the ProtParam tool. Post translational modification prediction like presence of signal peptides and its cleavage sites were found out with SignalP (Nielsen et al., 1997). Putative glycosylation site in the protein was determined by NetNGlyc protein analysis tool (Gupta et al., 2004).

3.1.9. Southern hybridisation

3.1.9.a. Restriction digestion of genomic DNA

Good quality genomic DNA with sufficient quantity was isolated from a Phytophthora tolerant (RR11 105) and susceptible (RRIM 600) clone of rubber tree and also from another 12 different genotypes. Around 10 μg of genomic DNA was digested with four restriction enzymes namely BamH I, Sau3A I, Xba I and Hind III in separate reactions. The reaction mix (30 μl) was prepared as,

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>15 μl</td>
<td>10 μg</td>
</tr>
<tr>
<td>Enzyme buffer</td>
<td>3.0 μl</td>
<td></td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td>2.0 μl</td>
<td>20 U</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>10 μl</td>
<td></td>
</tr>
</tbody>
</table>

The digestion was continued overnight at 37°C. the fragments were size fractionated in 1% agarose gels.
3.1.9.b. Blotting

The method was based on the standard procedure developed by Southern (1975). The gel was properly documented before the blotting, and processed after electrophoresis as follows:

i) DNA in the gel was depurinated by soaking in a solution of 0.25 N HCl for 12 – 15 min. Then the gel was rinsed twice with dist. H₂O briefly.

ii) Denaturation of the DNA was carried out by treating the gel in denaturation solution for 25 min with gentle shaking. Then it was rinsed with water.

iii) Gel was neutralized by soaking in neutralization buffer for 30 min.

Denaturation Solution

0.2 M NaOH, 1.5 M NaCl

Neutralization Solution

1 M Tris-HCl (pH-8), 1.5 M NaCl

Care was taken to see that gel is completely immersed in all solutions while treatment.

DNA was transferred from the treated gel to nylon membrane (Hybond N*, Amersham, UK) through capillary blotting method (Sambrook et al., 1989).

i) The gel after neutralization was briefly washed in 10X SSC and kept ready.

20 X SSC

3 M NaCl, 0.3 M Sodium citrate
pH adjusted to 7

ii) A tray was filled to a height of 5 cm with 10X SSC. A suitable platform with dimensions bigger than the gel was placed in the tray.

iii) The surface of the platform was covered with Whatman No. 3 filter paper presoaked in 10X SSC, in such way that the ends of the paper are immersed in the SSC. Then 3 sheets of Whatman No.1 filter paper was placed after trimming to the same dimensions of the gel and presoaked in...
10X SSC on top of the platform. Any air bubbles was removed by rolling the surface with a tube.

iv) The gel was placed carefully on top of this and then a Hybond N+ nylon membrane, presoaked in 10X SSC was placed on top of the gel. Any air bubbles were removed by gently rolling a glass rod on the surface.

v) Two sheets of the Whatman No.1 presoaked filter papers were placed on top of this assembly. Three more sheets of clean dry filter paper was then stacked on it over which ordinary filter papers cut to the gel dimensions were stacked to a height of 10 cm. Over this, a suitable weight of around 200-300 g was placed. The weight should not crush the gel but should be sufficient to keep the papers tight.

vi) The transfer was allowed to proceed overnight for a period of 12-16 h.

vii) After transfer, the assembly was disassembled and the nylon membrane was briefly washed in 5X SSC and air-dried.

viii) The membrane was fixed using a UV cross linker (Hoefer, USA) at 12000 J/cm². The membrane was wrapped in Saran and stored between ordinary filter papers in the refrigerator till use.

3.1.9.c. Preparation of labeled probes

The β-1,3-glucanase gene probe was radiolabeled using ‘Multiprime DNA labeling system’ from Amersham, (UK) following manufacturers instructions. It utilizes random hexanucleotides to prime DNA synthesis on denatured template DNA at numerous sites along its length. The procedure was as follows:

i) About 50 ng of template DNA was diluted to 5 μl with dist. H₂O and boiled for 5 min to denature.

ii) Chilled immediately on ice for 5 min and centrifuged briefly.

iii) Added 2.5 μl of buffer, which contains all the dNTPs except dCTP.

iv) Then 2.5 μl of random primer solution was added.

v) To this 2.5 μl of α-³²P labeled dCTP (sp. activity ~ 3000 Ci/mMol or 10 μ Ci/μl) was added.

vi) Then 11.5 μl of nuclease free autoclaved water was added.
Finally 1 µl of the enzyme (Klenow fragment of DNA polymerase I) was added and mixed gently by pipetting up and down.

Spun for few seconds and incubated at 37°C for 30 min.

The reaction was stopped by adding 0.5 µl of 0.5 M EDTA and the probe was diluted to 100 µl with distilled water.

The labeled probe was purified by passing through a Sephadex G-50 column as follows:

Sephadex G-50 was added to dist. water to form a slurry (10 g of dry powder yields around 160 ml of slurry).

Glass wool was placed at the bottom of a 1 ml column and 1 ml of the slurry was added without trapping of air bubbles.

The column was spun at 3000 rpm for 3 min in a swinging bucket rotor.

More slurry was added until the Sephadex tightly packed up to 1 ml level.

The column was equilibrated first with STE buffer and then with dist. water.

The labeled probe was then passed through the column and purified.

The column purified probe was denatured by boiling at 100°C for 3 min and immediately chilled in ice. It was stored in the freezer till use.

Such purification helps to remove all the small nucleotides and other unincorporated nucleotides to avoid background signals in the blot.

3.1.9.d. Hybridisation

Hybridisation of the labeled probe to the nylon membrane was performed according to Sambrook et al., (1989).

The blotted membrane was placed in a hybridisation tube and appropriate amount of pre-hybridisation solution (0.2 ml/cm² of the blot – 25 ml for the 13x10 cm membrane) was added.

Pre-hybridisation solution

6 X SSC, 5 X Denhardt’s reagent, 0.5 % SDS
ii) The pre-hybridisation was carried out at 65°C for 1 h in a hybridisation oven (Amersham, UK) with rotary movement at very low speed.

iii) The pre-hybridisation solution was poured out and hybridisation solution (pre-hybridisation solution containing denatured probe DNA labeled with α-32P) was poured into the tube and then incubated with slow rotation for 12 – 16 h at 65°C.

3.1.9.e. Washing of the blot and autoradiography

After hybridisation, the membrane was washed twice at room temperature for 5 and 15 min respectively with solution I

Washing solution I
2 X SSC, 0.1 % SDS

Then the blot was subjected to two high stringent washes at 65°C for 30 min each with solution II

Washing Solution II
0.1 X SSC, 0.5 % SDS

The membrane was then floated briefly in 0.1 X SSC at room temperature, air-dried and subjected to autoradiography. The membrane was wrapped in a cling film and placed inside the X-ray cassette. An X-ray sheet was placed over it after marking the orientation. An intensifying screen was placed over this assembly and the cassette was closed tightly and placed in -70°C for 1-2 days.

After this, the X-ray sheet was removed and developed in the developer solution under safe red light. As soon as the spots develop, the X-ray sheet was cleaned with water and quickly placed in the fixer for a few minutes. The sheet was extensively washed in water and air-dried.

3.1.10. Isolation of the promoter elements of the gene

Random Amplification of Genomic DNA Ends (RAGE) technique was utilised to amplify the 5' regulatory sequences of the gene (Siebert at al., 1995). At first, the genomic DNA was digested with blunt end cutting enzymes. The
enzymes used in the present study are EcoR V, Stu I, Ssp I, Dra I and Hae III. Around 3-5 μg of DNA were digested overnight at 37°C with 10 U of each enzyme separately in 30 μl reactions. The digestion was checked in agarose gels. Then the DNA fragments were purified by phenol:chloroform:isoamyl alcohol extraction followed by a chloroform extraction and precipitated in 75% ethanol in presence of sodium acetate. The fragments were washed with 80% ethanol and air-dried. Finally, the blunt ended fragments were dissolved in 10 μl TE. Now an adapter sequence was ligated to the DNA fragments.

The adapter consists of a long arm and a short arm, which is amino-linked at 3' end to prevent any erroneous extension during subsequent PCR.

Adapter Sequences

Adaptor long arm
5'-CTA ATA CGA CTA ACT ATA GGG CTC GAG CGG CCG CCC GGG CAG GT-3'  
3'-H2N- CCC GTC CA-5'
Adaptor short arm

The two arms were synthesised separately and the adapter was prepared by mixing 100 μM each of the arms. The mix was kept at 96°C for 2 min and then cooled slowly to room temperature for annealing. The adapter was then ligated to DNA fragments of the 5 restriction enzymes separately as follows:

DNA - 10 μl
Ligase buffer - 2.0 μl
Adapter (100 μM) - 1.0μl (5 μM final concentration)
T4 DNA ligase - 3.33 μl (10 U)
Dist. water - to 20μl

The reaction was performed overnight at 16°C. Then the ligation mix was diluted 1:10 and used as the PCR template with adapter specific forward and gene specific reverse primers.

Adapter specific primer (ASP I)
5'- ATA CGA CTC ACT ATA GGG-3'

Gene specific primer (GSP I)
5'-TAG AGA GCT ATG ACC TCT G-3'
To ensure more specificity, a nested PCR reaction was performed with the products of the first PCR as template.

Primers for the nested PCR

**ASP II** - 5'-ATA GGG CTC GAG CGG C- 3'

**GSP II** - 5'-CAG GTG GAA GGT TGT TGC C- 3'

The binding sites of the second pair of adapter specific and gene specific primers are within the first PCR product. The amplified products of the nested PCR reaction was cloned in pGEM vector and sequenced.

### 3.2. Gene Expression Studies through Northern Hybridisation and RT-PCR Analysis

#### 3.2.1. Plant materials used

All the plant materials used in this study were obtained from the Germplasm collection and nursery of Rubber Research Institute of India. The *Phytophthora* tolerant clone RRII 105 and a highly susceptible clone RRIM 600 were selected for challenge with the pathogen and subsequent northern hybridisation and RT-PCR analysis to study the induction pattern of β-glu gene. Budded stumps of the selected *Hevea* clones were grown in polybags in a glass house, in which the temperature is maintained around 25°C with a high relative humidity.

#### 3.2.2. Fungal culture and inoculation

The fungus used was a highly virulent isolate of *P. meadii*, which was maintained in PDA medium, obtained from Plant Pathology Division of RRII. PDA plates were inoculated with the stock culture and were incubated at 25°C for two days in light. White oats medium was used for sporulation (Rajalakshmy and Joseph, 1986). The oats broth was inoculated with two-day old fresh fungal mycelium from the PDA plates and kept for two days under darkness. The mycelia were then collected in a mesh and washed four times with sterile dist. H₂O in petridishes. All the media components were removed and the mycelia were dispersed in water using forceps and it is then kept in light for 24 h for sporangial growth. The mycelia were observed under microscope to check the sporangial...
growth. The plate containing the sporangia were kept in refrigerator for 10 min and then kept in room temperature. This heat shock will liberate the motile zoospores. Light green leaves of 10–12 days old were inoculated with this zoospore suspension \(10^6\) zoospores/ml. After inoculation, the plants were covered with transparent polythene bags to maintain the required humidity and observed for the development of the symptoms. Control plants were also maintained under similar conditions. Leaf samples were taken at different time intervals (24, 48, 72 and 96 hrs after inoculation) and processed for the RNA isolation.

3.2.3. Isolation of RNA from leaf samples

RNA was isolated from necrotic and near infected zones as well as from control leaves according to the procedure of Venkatachalam et al. (1999) with suitable modifications. All the reagents required are prepared in DEPC treated H\(_2\)O. The protocol involved the following steps:

i) One g of leaf tissue was rinsed with DEPC treated H\(_2\)O and ground to a fine powder in Liquid Nitrogen.

ii) Then 10 ml of extraction buffer was added and the homogenate was transferred to a centrifuge tube.

iii) Equal vol of extraction buffer saturated phenol was also added, mixed gently and centrifuged at 10,000 rpm for 15 min.

<table>
<thead>
<tr>
<th>Extraction buffer</th>
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<tr>
<td>0.2 M NaCl, 0.1 M Tris-HCl (pH-8.5), 0.01 M EDTA, 1.5% SDS, 0.1% β-mercaptoethanol (added immediately before use) and insoluble PVPP (added to the homogenate).</td>
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iv) The upper aqueous phase was transferred to a new tube and re-extracted with equal vol of chloroform.

v) Centrifuged at 10,000 rpm for 10 min and the aqueous phase was recovered.

vi) Then 1/3 vol of 8 M LiCl was added and the precipitation was continued overnight in ice.

vii) The RNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4 °C.
viii) The pellet was washed first with 2 M LiCl followed by 100% ethanol, air-dried and dissolved in 1 ml DEPC treated sterile H₂O.

ix) For further purity, the RNA was re-precipitated with 0.1 vol 3 M sodium acetate (pH- 5.2) and 2.5 vol of ethanol.

x) The precipitated RNA was pelleted at 10,000 rpm for 10 min at 4°C and washed twice with 70% ethanol.

xi) Pellet was air-dried, re-suspended in 200 µl of sterile H₂O.

xii) The quantity of RNA was estimated spectrophotometrically and its quality and contamination, if any, with DNA was assessed in 1% agarose gel.

xiii) The isolated RNA was used immediately for subsequent analysis or stored in 3 vol of ethanol at -70°C.

3.2.4. Amplification of sequences for using as internal controls

3.2.4.a. Amplification of 18S ribosomal RNA gene

The 18S ribosomal RNA sequence was amplified from *Hevea* genomic DNA using gene specific sense, 5’- TAC CTG GTT GAT CCT GCC AG-3’ and antisense, 5’- GCG ATC CGA ACA ACA TTT CAC CG-3’ primers. Amplifications were carried out in 20 µl reactions, which contain the constituents as mentioned in Sec. 3.1.5. The PCR conditions were also the same. The amplified fragment was gel purified, cloned in pGEM vector and sequenced.

3.2.4.b. Amplification of β-actin gene

The partial sequence coding for β-actin in *Hevea* was amplified using gene specific sense, 5’- TCC ATA ATG AAG TGT GAT GT-3’ and antisense, 5’- GGA CCT GAC TCG TCA TAC TC-3’ primers (Kobayashi et al., 2000). The reaction constituents and PCR conditions were as mentioned in the previous section. The amplified product was cloned and sequenced.

3.2.5. Northern hybridisation protocols

3.2.5.a. Electrophoresis of RNA

Agarose (1%) was melted in 31 ml of H₂O and cooled to 60°C. In a fume hood, 10 ml of 5X formaldehyde gel running buffer (FGRB) and 9 ml of
formamide was added to give a final concentration of 1X and 2.2 M respectively. The gel was allowed to set for at least 30 min at room temperature.

5X Formaldehyde gel-running buffer
0.1 M MOPS (pH-7.0), 40 mM sodium acetate, 5 mM EDTA (pH-8.0)

Around 20 μg of RNA (9 μl) was incubated for 15 min at 65°C along with 4 μl FGRB, 7 μl formaldehyde and 20 μl formamide. After a brief spin, 4 μl of formaldehyde gel loading buffer was added to the sample. The six samples analysed include, RNA isolated from uninfected control leaves, infected leaves after 48 and 96 h from the clones RRII 105 and RRIM 600.

3.2.5.b. RNA blotting

Before transfer to the membrane, the gel was washed thrice in DEPC treated H2O to remove the formaldehyde. The nylon membrane (Hybond N+, Amersham, UK) was cut in to the size of the gel and was presoaked in 10 X SSC. Blotting was carried out as described in the Southern protocol (Sec. 3.1.9). After transfer, the membrane was air-dried and UV cross-linked by keeping the RNA side down.

3.2.5.c. Hybridisation and washing

The membrane was kept inside the hybridisation bottle, pre-hybridisation buffer was added and incubated at 42°C for at least 2 h.

Pre-hybridisation solution
5 X SSC, 5 X Denhardt’s, 1 % SDS and 50 % formamide

The probe preparation and purification was as mentioned in the Southern protocol. The α-32P dCTP labeled β-1,3 glucanase gene probe was added and incubated overnight at 42°C. The membrane was washed first with solution I (2X SSC + 0.1 % SDS) for 5 min at room temperature. Then two low stringent washings were given with Solution II for 5 min each at room temperature. This was followed by a stringent washing in pre-warmed solution II at 42°C for 15 min. The membrane was rinsed with 2X SSC and excess liquid was removed with blotting paper. It was then wrapped in a UV transparent plastic wrap (Saran) and
exposed to X-ray film with intensifying screens. The X-ray cassettes were placed at −70° for 2 days and the film was washed and fixed.

3.2.5.d. Stripping of the blot

For re-probing with 18S RNA the β-glu probe was stripped off from the membrane. Prior to re-probing, membranes may be stored in refrigerator (2-8°C), wrapped in Saran. When ready to commence re-probing, membrane was rinsed in 5X SSC for 1-2 minutes. Then a boiling solution of 0.1% SDS was added to membrane and placed on a bench-top shaker for 10 min. The operation was repeated twice, using freshly boiling SDS each time. Finally the membrane was rinsed in 5X SSC, pre-hybridised, probed and detected as described earlier.

3.2.6. RT-PCR assay for differential expression

RNA was isolated from infected zones on the leaves of tolerant clone RR11 105 and susceptible clone RRIM 600 at different time intervals such as 0, 24, 48, 72, and 96 h as well as from uninfected controls as described previously. First strand cDNA was synthesised from total RNA by reverse transcription with oligo-(dT) primers using the ‘Improm II reverse transcription system’ (Promega, USA) according to manufactures protocols. One µl of the first strand cDNA was used to co-amplify the β-glu and β-actin transcripts in a 20 µl reaction. RNA samples were tested for the presence of genomic DNA contamination by using extracted RNA directly as a PCR template, prior to cDNA synthesis, under the same PCR conditions. The PCR reaction products were separated on a 1.5% agarose gel, visualized with Et Br staining under UV and the image was captured using EDAS 290.

3.2.7. Analysis of autoradiograms and RT-PCR products

The relative abundance of β-glu mRNAs with respect to 18S RNA gene was determined by measuring the net intensity of band in northern blots using Kodak 1D image analysis software. Net intensity is the sum of background-subtracted pixel values in the band rectangle. The RT-PCR gel images also were scanned to determine the net intensities of β-glu and actin bands. The net intensity
data for β-glu were corrected for house keeping gene data and then normalized to 0 h. Corrected values were calculated by dividing the β-glu value by its corresponding house keeping gene value and multiplied by the highest house keeping gene value. Normalized values were calculated by designating the 0 h corrected value equal to 1.0, and subsequent corrected values were divided by the 0 h value (Zemanek et al., 2002).

3.2.8. β-1,3-Glucanase enzyme assays

Enzyme assay was done spectrophotometrically according to Pan et al., (1991). The crude enzyme extract (62.5 μl) was added to equal volume of substrate, laminarin (4%) and incubated at 40°C for 10 min. The reaction was stopped by adding 375 μl dinitrosalicylate and the sample was kept in a boiling water bath for 10 min. The coloured solution was diluted 10 times with dist. water and absorbance at 500 nm was measured. One unit of enzyme activity was defined as the amount of enzyme that produce, reducing sugars equivalent to one μM of glucose equivalent per 10 min under above conditions.

3.2.9. Tissue specific expression

RNA was isolated from leaf and latex of an uninfected plant and northern hybridisation was performed as described earlier with β-glu probe to study the tissue specific expression. As constitutive expression was observed in the latex, in order to find out any difference in the expression pattern in different clones, RNA was isolated from the latex of three tolerant (RRII 105, RRII 33, Fx 516) and three susceptible clones (RRIM 600, PB 86 and PR 107) and northern hybridisation was performed with β-glu gene probe.

3.3. Construction of a Functional cDNA Clone and Purification of Recombinant Protein

3.3.1. Design of primers with restriction sites and PCR amplification

Four primers that will amplify the sequences coding for mature functional protein were designed with appropriate restriction enzymes. One set of forward
and reverse primers has an EcoR I site at their 5' end, while the second set possesses a BamH I site.

The sequences of primers were as follows,

1. 5' - GGAATTCCAGGTAGGTGTTGCTATGG-3'
2. 5' - GGAATTCCCAGTTTTTCTGCACC-3'
3. 5' - CGGGATCCAGGTAGTGTTGCTATGG-3'
4. 5' - CGGGATCCAGTTTTTCTGCACC-3'

Primer No.1 and 3 are sense primers, while No.2 and 4 are antisense primers. The first two primers contain an EcoR I site and a BamH I site present in the last couple. Additional nucleotides were added to the 5' end of the restriction sites, since certain endonucleases require additional bases flanking their recognition sequences. This will facilitate the cleavage close to the end of the DNA fragments. The primers were synthesised with Metabion (Deutschland).

PCR amplifications were tried with four different combinations of primers. These were primer No. 1 & 2, No.1 & 4, No. 3 & 2 and primer No. 3 & 4. The constituents of the reactions and PCR conditions were as mentioned earlier.

3.3.2. Cloning in expression vectors

The 963 bp glucanase gene fragment amplified with primer combinations 3 & 2 (BE) and 1 & 2 (Eco) were gel purified. The BE fragment is having a BamH I site at 5’ end and an EcoR I site at 3’ end. The ‘Eco’ fragment has EcoR I sites at both ends. The purified BE fragment was double digested with EcoR I and Bam H I enzymes with multi-core enzyme buffer (Promega, USA) in 20 µl reaction volume. The digestion was performed overnight at 37°C and then the enzyme was heat inactivated by incubating the reaction at 75°C for 10 min. After a chloroform wash, the digested fragments were precipitated with double vol ethanol and 0.1 vol sodium acetate (3 M), washed in 70 % ethanol, air-dried and dissolved in 10 µl H2O. The fragment is now ready for cloning with sticky BamH I and EcoR I sites at 5’ and 3’ ends respectively. The ‘Eco’ fragment is digested with EcoR I and purified as described.
Two expression systems namely pGEX-2T (Amersham, UK) and pET 32a+ (Novagen, USA) were tried for recombinant protein expression. The plasmids were isolated in large quantities and double digested with EcoRI and BamHI enzymes and also with EcoRI alone in separate reactions, to create cohesive ends. The 4.9 kb pGEX and 5.9 kb pET vectors were checked for digestion in 0.8% agarose gels. When the digestion is complete, the vector was dephosphorylated by directly adding one U of calf intestinal alkaline phosphatase to the digestion mix and incubation at 37°C for 30 min. The vector was then gel purified to remove the residual nicked and super-coiled plasmid. The BE fragment with EcoRI and BamHI cohesive ends was used for unidirectional cloning to vectors cut with EcoRI and BamHI. The ‘Eco’ fragment was cloned to vectors opened with EcoRI. The ligation reaction (10 μl) was setup as follows:

- linearised, dephosphorylated vector: 1.0 μl (50 ng)
- DNA fragment: 2.0 μl
- ligase buffer: 1.0 μl
- 10 mM ATP: 1.0 μl
- H2O: 4.0 μl
- T4 DNA ligase: 1.0 μl (3 U)

The reaction was incubated at 16°C overnight. The E.coli strain JM109 was used for transformation and maintenance. Transformants were selected in antibiotic (amp) plate and plasmid was isolated. The cloning was confirmed through PCR and restriction digestion. To determine the insert orientation and size one vector specific forward and insert specific reverse primers were used in PCR reaction.

3.3.3. Sequencing

Sequencing primers for pGEX vectors were brought from Bangalore Genei Ltd. The pET sequencing primers, S-Tag and T7 terminator primers, were synthesised (Metabion). Sequencing of the PEG purified plasmids was carried out to ensure orientation and correct reading frame.

3.3.4. Expression of the target gene

The E.coli strain DH5α was selected as the expression host for pGEX vectors and strain BL 21 (DE 3) was used in the case of pET. These strains were
transferred with their respective plasmids and transformed single colonies were streaked to LB plates. A single colony from a freshly streaked plate was inoculated to 3 ml LB media containing 50 µg/ml ampicillin. The culture was grown overnight and in the following morning 1% of this culture was inoculated to 50 ml LB containing the appropriate antibiotic in a 250 ml flask. Control colonies containing the vector without insert was also inoculated. The cultures were incubated with shaking at 37°C until the OD₆₀₀ reaches 0.6 (around 2-3 h). Then the expression of the target protein was induced by the addition of IPTG to the growing culture. An aliquot of the growing culture was removed to keep as the uninduced control. To the remainder, IPTG was added from a 100 M stock to a final concentration of 1 mM and continued the incubation for 2-3 h. Just prior to harvest, 1 ml of culture from both induced and uninduced samples was removed and the OD₆₀₀ was measured after suitable dilution.

Total protein of the samples were analysed for checking the expression. The cells were harvested from a 1 ml aliquot of both induced and uninduced cultures by centrifuging at 5000 rpm for 5 min at 4°C. The media was completely drained out and 100µl phosphate buffered saline (PBS) was added to yield a concentration factor of 10X (100 µl vs. starting vol of 1 ml culture). Then the cells were lysed with 100µl of 2X SDS sample buffer (80 mM Tris-Cl pH-6.8, 100 mM DTT, 2% SDS, 0.006% bromophenol blue, 15% glycerol) by heating in a boiling water bath for 3 min. The samples were analysed through SDS-PAGE. Host alone and control vector without insert samples were also analysed along with induced and uninduced samples. Equal amount of samples were added to 10% acrylamide gel after measuring the OD₆₀₀ and concentration factors into consideration. Cells were harvested from the remaining of the 50 ml cultures and the pellets were frozen at -70°C for further analysis and purification of the target protein.

### 3.3.5. Purification of the target protein

The frozen bacterial pellet of 50 ml culture was thawed on ice. For the purification of fusion protein cloned in pGEX vector, GST-fusion protein purification kit from Bangalore Genie was utilised. After thawing, the pellets were suspended in 10 ml PBS and sonicated in a tube on ice until the sample is no longer viscous. Sonication was performed 3 times at 40 amplitude for 30 sec with
30 sec interval. Then 0.5 ml wash solution (provided by the manufacturer) was added and centrifuged at 10,000 rpm for 20 min at 4°C. The equilibrated glutathion column was loaded with the supernatant. Then the column was washed with the equilibration buffer till the OD$_{280}$ reaches 0.05. Finally column was eluted with 10 ml of elution buffer and 1 ml fractions were collected. The eluted fractions were assayed by SDS-PAGE with appropriate controls. The fractions containing the maximum amount of protein were desalted by dialysis with PBS.

The Trx (thioredoxin)-fusion proteins of pET vector were purified using the His.Bind purification kit (Novagen, USA). The induced bacterial pellets of 50 ml culture were re-suspended in 4 ml ice-cold binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole pH 7.9). The sample was then sonicated and the soluble proteins were collected by centrifuging at 10,000 rpm for 20 min. Column was prepared with 5 ml of His.Bind slurry to yield a final bed volume of 2.5 ml of settled resin. After washing with 3 vol of sterile deionised water, the column was Ni$^{2+}$ charged with 5 vol of charge buffer (50 mM NiSO$_4$), followed by equilibration with 3 vol binding buffer. The prepared extract was then loaded and the column was then washed with 10 vol binding buffer and 6 vol wash buffer (0.5 M NaCl, 20 mM Tris-HCl, 60 mM imidazole pH 7.9). Finally the bound protein was eluted with 6 vol elute buffer (0.5 M NaCl, 20 mM Tris-HCl, 1 M imidazole pH 7.9) and the elutes were captured in 1 ml fractions. Vector alone samples were also analysed as a control.

3.3.6. Confirmation of the induced target proteins

3.3.6.a. Western blotting

Identity of the induced proteins was confirmed through western blot analysis using the ‘His.Tag AP western reagent’ kit (Novagen, USA). The induced samples were run on 10% PAGE and transferred to nitrocellulose membranes (Sigma, USA) as per standard protocols (Sambrook et al., 1989) using a western blot apparatus (Broviga). The membrane was then washed twice for 10 min each in 15 ml TBS buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5). Then incubated for 1 h in 15 ml blocking solution (3% BSA in TBS). Then the membrane was
washed twice for 10 min each with 20 ml of TBSTT buffer (0.5 M NaCl, 20 mM Tris-HCl, 0.2% v/v Triton X-100, 0.05% v/v Tween -20, pH- 7.5). The primary antibody (His.Tag monoclonal antibody) was then added, diluted in the blocking solution, and incubated for 1 h. Unbound antibodies were removed by washing twice with 20 ml TBSTT followed by a single wash in 15 ml of TBS buffer. Now the secondary antibody (Goat anti-mouse IgG AP conjugate) was added and incubated for another 1 h. Membrane was washed thoroughly for 5 times with 20 ml each of TBSTT. Finally the membrane was developed with BCIP and NBT and incubated in room temperature until the colour develops. When the signals appear, the reaction was stopped by washing the blot thoroughly with deionized water and air-dried.

3.3.6.b. Thrombin Cleavage

A thrombin cleavage site is available on the pET vector that will allow the enzymatic removal of the N-terminal fusion tags. Around 20 µg of the purified recombinant protein from the pET vector was used in the reaction, which contains 1 U of thrombin and 1 X thrombin cleavage buffer (20 mM Tris-HCl, pH-8.4, 150 mM NaCl, 2.5 mM CaCl2). The reaction was incubated overnight at 23°C and the products were analysed in 12% SDS-PAGE gel.

3.3.7. Anti-fungal assay of purified recombinant proteins

All manipulations were carried out under sterile conditions. PDA plates (90 mm) were prepared and a single growing mycelial plug of 4 mm diameter of the Oomycete fungi Phytophthora meadii was inoculated at the center. To allow initial vegetative growth, the plates were incubated at 25 ± 2°C for 24 h. At this time, sterile filter paper discs (5 mm and 9 mm in diameter) were laid on the agar surface and solutions to be tested were applied to the discs. Forty µl of the purified protein solution which contains around 10 µg of recombinant protein were used for each disc. The plates were further incubated and observed for the appearance of inhibition zones and photographed.