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Optimization of RQRT-PCR protocols to measure β-1,3-glucanase mRNA levels in infected tissues of rubber tree (*Hevea brasiliensis*)

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RQRT-PCR technique was evaluated for its validity as an alternative to Northern blotting for quantification of plant gene expression in diseased tissues of *Hevea*. Reliable RT-PCR results could be obtained by co-amplification of housekeeping actin gene as the internal control along with the gene of interest. The product of interest was quantified relative to that of the internal control by measuring net intensity of bands. Expression levels of defense-related β-1,3-glucanase gene was studied in the pathogen infected tissues of rubber. The β-1,3-glucanase gene was found to be induced in infected leaf tissues and reached a peak at 48 h after inoculation. The β-1,3-glucanase gene expression during pathogen infection was determined through Northern blot hybridization also, using 18S RNA as the internal control. RQRT-PCR and Northern hybridization showed almost similar results, thereby validating the use of this technique to study the gene expression in rubber.

Keywords: Actin, β-1,3-Glucanase, *Hevea brasiliensis*, *Phytophthora*, Relative RT-PCR

Steady state levels of individual RNAs have been measured commonly by non-PCR based methods like Northern blotting, nuclease protection assay and *in situ* hybridization. These techniques, however, are time consuming and require large quantity of RNA for detection[1]. Reverse transcription-polymerase chain reaction (RT-PCR) overcomes these limitations and is more rapid, sensitive and specific than Northern blot analysis. However, quantification might be difficult because many sources of variation exist, like sampling errors, template quality and amplification efficiency, which may affect the accuracy of RT-PCR reactions. One approach to compensate such variations is the use of multiplex RT-PCR with two primer sets in a single PCR (one set to amplify the cDNA of interest and a second to amplify an invariant endogenous control). The product of the gene of interest could be quantified relative to that of the internal control[2]. For the relative quantitative RT-PCR (RQRT-PCR) to be accurate, specific PCR conditions and appropriate internal control must be determined. In order to obtain meaningful results, the relative RT-PCR must be analysed in the linear range of amplification for both PCR products, before the PCR components become limiting. At some point during the reaction, the amplification efficiency falls and the rate of product accumulation slows or plateaus. Therefore, the point at which the reaction reaches the plateau should be determined.

The internal control selected for the study should show minimum variability in expression levels between tissues and in time course. In this study β-actin was used as the internal control in RQRT-PCR experiments and 18S rRNA was used in Northern as it was not possible to reverse transcribe 18S rRNA transcripts with oligo-(dT) primers.

Induction of β-1,3-glucanase (β-glu) has been well documented as a part of plant's broad generalized defense mechanism against infection by a variety of pathogenic fungi in many plant species. There are several reports on induction of β-glu during pathogen infection in different plant species like tobacco[3], potato[4], tomato[5], soybean[6], bean[7], pepper[8], wheat[9], and peach[10]. β-Glu are hydrolytic enzymes, able to catalyze the endotype hydrolytic cleavage of β-1,3-linked glucans, which is the principal cell wall component in many phytopathogenic fungi. *Phytophthora* disease, like abnormal leaf fall (ALF), is the most destructive disease of rubber trees (*Hevea brasiliensis*) in India. It accounts for almost 40% yield loss in terms of latex in susceptible clones[11]. The causative organism belongs to the class Oomycetes, the cell wall of which is primarily made up of β-1,3 linked glucans.

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In the present study, we have optimized the conditions for the measurement of β-1,3-glucanase mRNA levels in the leaf tissues of *H. brasiliensis* infected with *P. meadii* through RQ-RTPCR. Northern blot analysis was also performed to cross check the results obtained and thus, to make sure the suitability of RQRT-PCR for measuring the changes in gene expression in rubber.

Materials and Methods

Biological materials—Two-week-old leaves of polybag-raised *Hevea brasiliensis* plants (clone RRII 105) were inoculated with a virulent strain of *Phytophthora meadii*. The fungus was cultured for 2 days in dark in oats medium and then the mycelia were kept in distilled water under light for sporulation[12]. Light green leaves (10-12 days old) were inoculated with zoospore suspension, (10^6 zoospores/ml) of *P. meadii*. 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. After inoculation, the leaf samples were collected at regular intervals and processed for further study.

RNA isolation and cDNA synthesis—Around 500 mg of leaf tissue from the infected region was ground to a fine powder in liquid nitrogen and 5 ml extraction buffer [0.2, *M* NaCl; 0.1 *M*, Tris-HCl (*p*H-8.5); 0.01 *M*, EDTA (*p*H 8.0); 1.5%, SDS; 0.1%, β-mercaptoethanol; 1%, insoluble PVPP) was added. Following extraction with an equal volume (vol) of extraction buffer-saturated phenol (centrifugation for 15 min at 10,000 rpm), the aqueous phase was decanted and re-extracted twice with equal vol of chloroform. RNA was precipitated overnight with 1/3 vol of LiCl (8 *M*) at -20°C. The precipitated RNA was recovered by centrifugation for 10 min at 10,000 rpm, washed with 2 *M* of ice-cold LiCl and dissolved in 250 µl sterile distilled water. RNA was then re-precipitated by adding 0.1 vol sodium acetate (3 *M*) and 2.5 vol of 100% ethanol at -20°C. Following centrifugation for 10 min at 10,000 rpm, the pellet was re-suspended in 100 µl sterile distilled water. First strand cDNA was synthesised from 1 µg of total RNA by reverse transcription with oligo-(dT) primers using 'ImProm-II Reverse Transcription System' (Promega, USA) according to manufacturer's protocol.

Relative RT-PCR—The first strand cDNA was diluted 1/10 with water and was used as template for PCR. An upstream TCCATAATGAAGTGTGATGT and a downstream GGACCTGAC TCGTCATACTC primers[13] were used for amplification of the housekeeping β-actin gene. PCR was performed in a final volume of 20 µl containing 1 µl of diluted cDNA, 0.25 µ*M* of each primer, 100 µ*M* each of dNTPs, 1.5 m*M* of $MgCl_2$ and 0.75 U of *Taq* DNA polymerase (Roche, USA). Nineteen to 35 cycles of PCR were carried out, consisting of 30 sec denaturation at 94°C, 1 min annealing at 55°C followed by 2 min extension at 72°C. Cycling was started by 5 min denaturation at 94°C and terminated by 7 min extension at 72°C. The PCR reaction products were resolved on agarose gel (1.5%), visualized by ethidium bromide staining under UV, and the image was captured using EDAS 290 (Electrophoresis Documentation and Analysis System, Kodak, USA). The intensity of bands was determined by densitometric analysis of gels using Kodak 1D image analysis software. The band intensity was plotted against number of cycles to find out the linear range of amplification. β-1,3-Glucanase gene was amplified using the forward CTTCTTAATGGCTATCTCCTC and reverse CTCACATATCACTCTTAA GG primers based on a previously published sequence of *Hevea* β-1,3-glucanase gene[14] (GenBank Acc. No. AY325498). The cycles, in which the product was still in the linear range of amplification, were determined for β-1,3-glucanase and β-actin. For relative quantification, both genes were co-amplified for 30 cycles in a hot start PCR. 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.

Southern hybridization—One µl of the PCR product from each sample was diluted ten times and 1 µl of the diluted samples were run in agarose gels (1%) and blotted on to nylon membranes (Amersham). The probes were labeled with α-^{32}P labeled dCTP using 'Multiprime DNA Labeling System' (Amersham Pharmacia, UK). Hybridisation was performed for 16 h at 65°C in a buffer containing 6X SSC, 5X Denhardt's solution and SDS (0.5%). The membrane was washed with SSC (0.1X) and SDS (0.1%) for 30 min at 65°C and exposed to X-ray film (Kodak).

Northern blot analysis—Around 10 µg of total RNA was isolated from the infected leaves at different time intervals after inoculation (0, 24, 48, 72 and 96 h), separated in 1% of formaldehyde-agarose gel, and transferred to positively charged nylon

membranes (Hybond N^+, Amersham, UK). After transfer, the membrane was air-dried and UV-crosslinked (Hoefer, USA) by keeping the RNA-side down. Hybridisation was performed as described by Sambrook *et al.*[15]. For pre-hybridisation, blots were incubated in 50%, formamide; 5X, SSC; 5X, Denhardt's reagent; and 1%, SDS at 42°C for 2 h. The β-1,3-glucanase probe was PCR-amplified using primers mentioned earlier. 18S rRNA was used as internal control, and the gene fragment was amplified using the primers TAC CTGGTTGATCCTGCCAG and GCGATCCGAACATTTCACCG. α-^{32}P- labeling of the probes by random priming was carried out using 'Multiprime DNA Labeling System' (Amersham, UK) following manufacturer's instructions. Hybridization was performed for 16 h at 42°C. The membrane was washed first with SSC (2X) + SDS (0.1%) at room temperature and then with SSC (0.2X) + SDS (1%) at 42°C. The labeled membrane was then exposed to X-ray film (X-Omat, Kodak) with intensifying screens, at –70°C. The glucanase probe was then stripped off and the membrane was re-probed with 18S rRNA.

Analysis of RT-PCR products and autoradiograms—The RT-PCR gel images were scanned to determine the net intensities of β-glu and actin bands using Kodak 1D image analysis software. Net intensity is the sum of background subtracted pixel values in the band rectangle. The relative abundance of β-glu mRNAs with respect to 18S rRNA transcripts in Northern blots was also determined. The net intensity data for β-glu were corrected for housekeeping gene data and then normalized to 0 h. Corrected values were calculated by dividing the β-glu value by its corresponding housekeeping gene value and multiplied by the highest housekeeping 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[10].

Results

Good quality RNA without degradation and DNA contamination was obtained from the infected as well as control samples. Actin and β-glu genes were amplified separately in order to determine the linear range of amplification. It was observed that between 25 and 33 cycles both the PCR products were in the linear range of amplification (Fig. 1).

In RQRT-PCR, it was observed that, transcript levels of actin gene were almost same at all time intervals. But, β-1,3-glucanase appeared to be differentially regulated. The net intensities of β-glu bands amplified from control uninfected samples and infected samples at 0 h were low. In the infected samples, the band intensities started to increase after 24 h of inoculation and an exponential increase in band intensity was observed at 48 h (Fig. 2). This increase could be due to the quantity of target sequences in cDNA template available in PCR as all other constituents remained the same, and thus can be correlated with the increased gene activity. Almost 25-fold increase compared to their basal levels was observed at 48 h after inoculation. The intensity remained high till 96 h after inoculation.

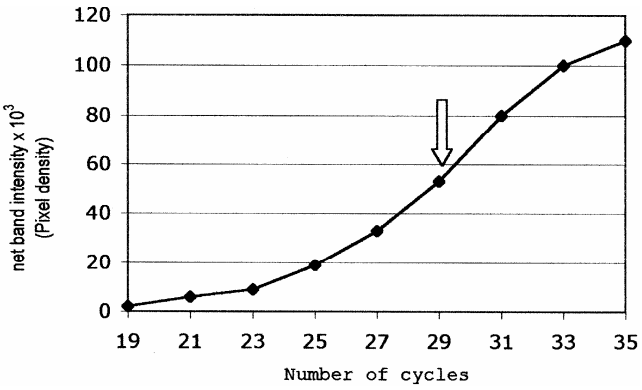

Fig. 1—Identification of the linear range of a PCR reaction. Actin gene fragments were amplified from *Hevea* cDNA using specific primers at different cycles from 19 – 35. PCR products from each cycle were analysed in Et Br stained gel and the band intensity was measured. The band intensity was plotted against number of cycles.(The arrow indicates the cycle number within the linear phase that was chosen to standardize the treatments.)

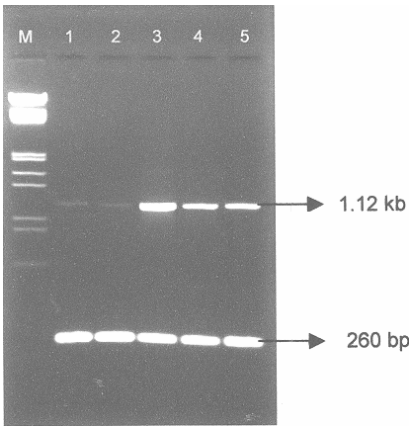

Fig. 2—Comparison of gene expression levels of β-glu and actin using relative quantitative RT-PCR analysis. The 1.12 kb β-1,3-glucanase and 260 bp actin gene fragments were co-amplified using cDNA isolated from infected leaf samples at different time intervals after inoculation (0, 24, 48, 72 and 96 h; lanes 1-5, respectively)

The β-glu band intensity corrected to that of actin and normalized to 0 h was plotted against the hours of post inoculation (Fig. 3). Induction of β-glu in leaf tissues in the infected samples was more evident in this plot. Induction of β-glu transcripts reached a peak at 48 h after inoculation and thereafter it declined.

Detection methods with low sensitivity like ethidium bromide staining of agarose gels may cause some distortion. Hence, the PCR products were analyzed through Southern blotting which also showed similar trends in gene expression pattern (Fig. 4).

Northern blot hybridization was also performed in order to compare the results obtained in RT-PCR. In Northern analysis, 18S rRNA was used as the internal control. RNA, isolated from necrotic zones of infected tissues, control uninfected leaves, and at different time intervals after inoculation, were subjected to Northern analysis with a PCR-amplified genomic fragment coding for β-glu in *Hevea*. To verify the amount of RNA loaded in each well, the β-glu probe was stripped off and the blot was re-hybridized with the 18S rDNA probe. The signals generated indicated that the 18S rRNA content did not vary with the treatments and time course. This uniform expression of rRNA indicated that there was no non-specific shift in the relative amounts of mRNA. As expected, β-glu mRNA levels changed dramatically (Fig. 5). The glucanase probe hybridized to a 1.2 kb mRNA, while 18S rDNA hybridized to a 1.7 kb mRNA. The results of Northern analysis were in concordance with the RT-PCR findings, since at 48 h after inoculation there was an exponential increase in β-glu mRNA transcripts. The expression level remained high till 96 h after inoculation, although there was a slight decrease in hybridizing band intensity after 4 days. No signal was obtained in control and infected samples till 24 h of inoculation. However, in RT-PCR, faint bands were obtained in control samples also.

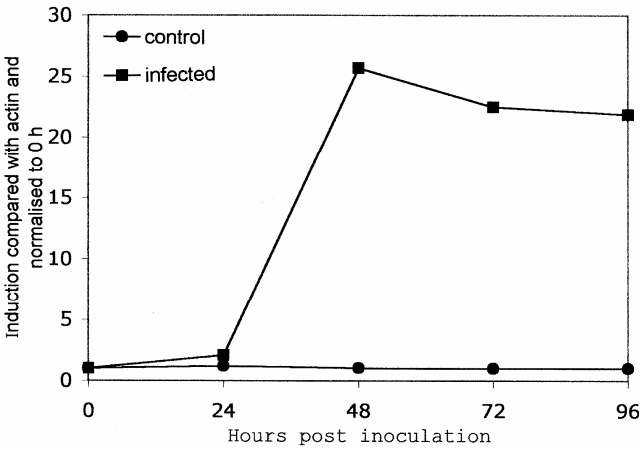

Fig. 3—Graphic plot on the induction of β-glu expression relative to that of actin in control and infected leaves of *Hevea* clone RRII 105 as determined by RQRT-PCR. The β-glu intensity data was corrected for housekeeping gene actin data and normalized to 0 h. Almost 25-fold increase was observed than its basal levels at 48 h after inoculation.

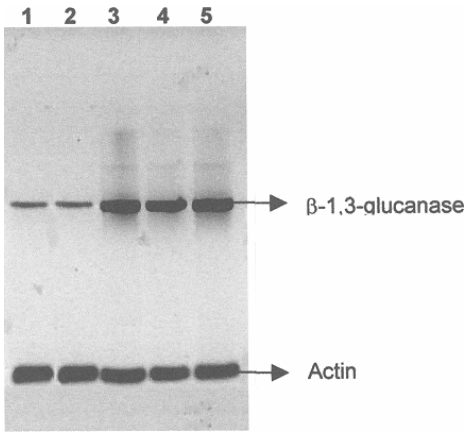

Fig. 4—Southern hybridization with β-glu and actin gene probes to detect the PCR products which are co-amplified in RQRT-PCR. One μl of ten times diluted PCR product was loaded in each well.

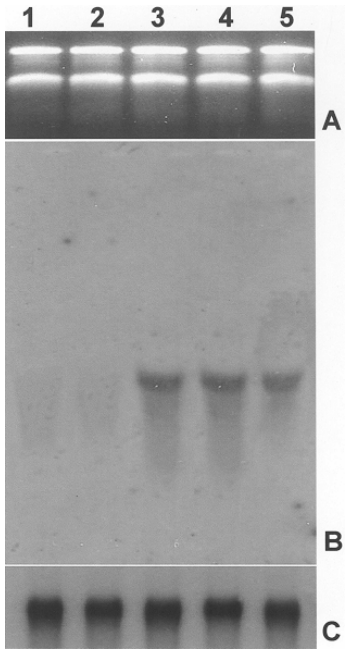

Fig. 5—Northern blot analysis of β-glu expression in *Hevea* leaf tissues – (A)- rRNA bands visualized in RNA gel used to prepare Northern blot.;(B)- Northern blot probed with β-glu showing differential expression as observed in the case of RQRT-PCR; (C)- Northern blot probed with 18S RNA, showing uniform expression. (Lanes 1- 5: 0, 24, 48, 72 and 96 h after inoculation respectively)

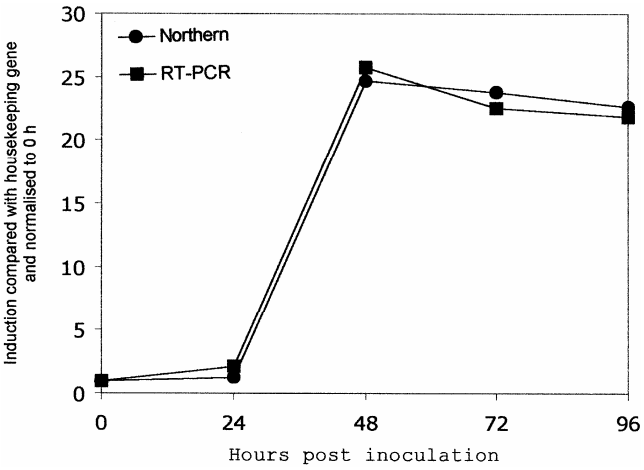

Fig. 6—Comparison of Northern blot hybridization and RQRT-PCR in studying the induction pattern of β-glu in the leaves of *Hevea* infected with the fungus.

The net intensities of hybridising bands in Northern were corrected for 18S RNA and normalized to 0 h. When this value was plotted against the hours post inoculation, the induction levels of β-glu were found to be almost similar to those obtained by relative RT-PCR with the housekeeping actin gene as an internal control (Fig. 6).

Discussion

We tried this PCR-based technique in order to have an alternative method to Northern blotting for the quantification of plant gene expression in the pathogen infected tissues. Although RNA transcript levels can be quantified using Northern blot analysis, it has limitations if the target gene has a low level of expression, or if only limited amounts of tissue are available for RNA extraction. RT-PCR technique can alleviate these limitations, with an advantage that it requires less RNA, takes less time, lesser expensive than Northern blot analysis and requires only standard PCR reagents. However, due to the sensitivity of PCR, considerable variations are theoretically possible. Co-amplification of an internal control with the gene of interest can compensate these variations. In order to test whether this RQRT-PCR technique adequately measured gene expression in diseased leaves, it was compared to Northern blotting using differentially expressing β-glu with housekeeping 18S RNA gene as internal control.

Relative RT-PCR has been successfully employed to study the expression of β-glu in *Nicotiana benthamiana* infected with *Collitotrichum destructivum*[2]. This competitive RT-PCR technique has increasingly been used in animal and human systems also[16,17]. In this study, results from Northern blot and relative RT-PCR analysis of β-glu expression were similar, thereby validating the use of relative RT-PCR with a suitable internal control for measuring the host gene expression during infection. Use of primers to amplify the actin gene fragment in each sample helped to account for variation in template concentration and amplification efficiency.

To be quantitative, RT-PCR must be analysed in the linear range of amplification for both the genes of interest and internal control before reaction components become limiting. The linear range of reaction has been defined as the period of PCR in which the amplification efficiency is at its maximum and remains constant over a number of cycles. When target transcripts are abundant, PCR amplification may plateau after 20 cycles. Therefore, relative RT-PCR conditions must be determined to ensure that both bands are still accumulating at the end of each cycle, and that they are still below saturation. In many animal systems it has been observed that the reaction plateaus below 25 cycles[18]. However, in the present study, it was observed that the amplified products were still below saturation level at the end of 33 cycles and the quantification was done at 30 cycles. This may be due to the relatively low template amount or low amplification efficiency from diseased leaves.

We used actin as the internal standard in RT-PCR experiments, while in most other cases 18S RNA has been tried[19]. Actin is supposed to be a better choice in RT-PCR experiments, since 18S RNA sequences are highly conserved; therefore, the internal control may amplify fungal 18S RNA also, leading to an underestimation of plant gene expression. Absence of genomic DNA contamination in RNA preparations was ensured before cDNA synthesis by using isolated RNA directly as a PCR template, which failed to amplify any products. Actin pseudogenes may contribute to the PCR product in experiments using RNA that is contaminated with genomic DNA, regardless of whether the PCR primers are designed to flank introns. Another important parameter that has to be standardized for a successful relative RT-PCR experiment is the optimal ratio of actin primers to the competimers. Since they compete for available resources, it is critical that the control target (actin) be amplified from RT-PCR at a level roughly similar to the amplicon under study. If one target is present at a significantly higher concentration than the other and

both are amplified at a similar efficiency, the competition for reagents in PCR will result in loss of exponential amplification for the template of lower abundance. We used a 2:8 ratio of actin primers to β-glu primers for amplification of cDNA from the control samples, since the relative abundance of glucanase mRNAs was assumed to be less in the uninfected samples.

Determination of β-glu expression relative to the expression of housekeeping genes using both Northern and relative RT-PCR analyses revealed that the rate of β-glu expression was increased throughout the infection, and it reached a peak in the early stages of infection i.e. at 48 h. The β-glu transcript levels were 15-fold higher than the basal levels even 4 days after inoculation in the *Phytophthora* tolerant rubber clone, RRII 105. The wide spread role of β-glu in plant defense response has been well investigated[20,21]. Many workers have suggested induction of different isoforms of glucanase as a mark of incompatible plant-pathogen interactions[5,22,23]. The level and onset of β-glu expression is often positively correlated to the level of resistance to the pathogen[24]. More rapid, faster and prolonged expression of β-glu has been reported in the case of resistant varieties compared with the susceptible ones, in many plant species[8].

In conclusion, a modified RT-PCR technique was optimised for the study of anti-fungal β-glu gene expression in the leaf tissues of rubber tree. Results were reproducible and reflected the trends in gene expression. This technique produced results that were similar to Northern blotting, which suggested that despite potential sources of error in reverse transcription and PCR reactions, this technique using host actin gene as an internal standard was effective for determining gene expression in the fungal infected plant tissues. To our knowledge, RQRT-PCR technique was used here for the first time in studying the differential gene expression in rubber tree.

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