IN VITRO ANTIFUNGAL ACTIVITY OF RECOMBINANT CHITINASE PROTEIN AGAINST *CORYNESPORA CASSIICOLA* INFECTING *HEVEA BRASILIENSIS*

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Corynespora leaf disease caused by *Corynespora cassiicola* has emerged as a major disease of rubber in South East Asia. During plant-pathogen interactions, various novel proteins called pathogenesis related (PR) proteins which play a major role in plant disease resistance mechanism are induced. The chitinase (PR3) is one of the most widely studied groups of PR proteins in plants. *C. cassiicola* induced chitinase from *Hevea brasiliensis* was characterized in the present study. The cDNA was developed from the RNA of *C. cassiicola* infected leaves of the clone GT 1. A 978 bp chitinase gene was obtained from *H. brasiliensis* and over expressed in the pET 32a^{+} expression system. The *in vitro* studies of purified recombinant *Hevea* chitinase showed antifungal activity against *C. cassiicola*. The chitinase gene expression in *H. brasiliensis* during *C. cassiicola* infection was quantified through qPCR and increased expression of chitinase transcripts was observed. In clone GT1 chitinase gene was induced up to 24th hour after *C. cassiicola* infection and eventually it came down in later hours, where as in clone RRII 105 chitinase level had been lesser in induced plants than control. The polyclonal antibody was raised with the recombinant chitinase and the induced clone GT I showed a prominent band in western blot, while a minor band was observed in RRII 105 in induced condition.

Keywords: *Corynespora cassiicola*, Chitinase, *Hevea brasiliensis*, Recombinant protein

INTRODUCTION

Corynespora leaf disease of rubber (*Hevea brasiliensis)* caused by *Corynespora cassiicola* is now considered to be a serious problem in all rubber growing countries in South East Asia. In India it is a serious threat for rubber plantations in South Karnataka and adjacent places of Kasaragod district of Kerala (Rajalakshmi and Kothandaraman, 1996). Chemical control is being followed to control the disease in the field (Manju *et al*., 2002). As rubber trees are deciduous in nature, fungicide application has to be repeated every year. Repeated application of fungicide may lead to resistance build up in pathogen in addition to other environmental problems. Protection against disease by inducing the resistance mechanism in

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plants is getting importance recently (Vidhyasekaran, 2008).

In plant-pathogen interactions, damage caused by pathogen remains restricted due to the plant defense response. In biological stresses induced by fungi, bacteria and viruses the host plant synthesizes a wide variety of compounds (Linthrost, 1991). In addition various novel proteins are induced which are collectively called as pathogenesis related proteins (PR proteins) (Van Loon and Van Sterin, 1999). The PR proteins like β-1,3 glucanase (PR 2), chitinase (PR 3) and thaumatin like protein (PR 5) were reported to have antifungal activity in *in vitro* assays (Kombrik *et al*., 1998).

Pathogenesis related proteins have been reported in *H. brasiliensis* also during various fungal infections (Philip *et al*., 2001, Chacko *et al*., 2005 and Thanseem *et al*., 2005). The chitinase (PR-3) is the most widely studied group of PR protein. The role of chitinase in relation to tolerance has been studied in detail in different plant species. Chitinase gene codes for antifungal chitinase, which catalyze the hydrolysis of 1,4 linkages of N-acetyl –d-glucosamine polymer (chitin) found in the cell wall of higher fungi. *C. cassiicola* is a chitinacious fungus belonging to the family deuteromycetes. The objectives of the present study were to amplify and express the chitinase gene from the tolerant rubber clone GT 1 and test the antifungal activity of the expressed protein against *C. cassiicola.*

MATERIALS AND METHOD

Budded stumps of GT 1, a clone relatively tolerant to *Corynespora* and a susceptible clone RRII 105 were raised in polybags in the glass house. Plants with light green leaves were artificially inoculated with spore suspension ($7x10^4$ spores mL^{-1}) and were covered with transparent polythene bags for maintaining moisture. Control plants were sprayed with sterilized distilled water and maintained under similar conditions. Leaves from the inoculated plants were collected at different hours and used for assays.

RNA was isolated from the tissue near the necrotic zone of *C. cassiicola* infected leaves of GT 1 and RRII 105 by lithium chloride ($LiCl_2$) precipitation. Total RNA from the leaves were used as template for synthesis of cDNA using RT-PCR kit (Improm-II Reverse Transcription System, Promega, USA). The conserved primers designed for chitinase gene, Chit.F-5′-ATGGCCAAAAGAAA CCCAAGCCATCC -3′ and Chit.R- 5′- CCGAATAATT TCTCC CAAT -3' were used for the amplification of chitinase gene. Chitinase specific PCR was performed in 20 µL of PCR reaction mixture in a PCR thermal cycler, (M.J. Research Bio Rad USA) programmed with initial denaturation at 94 ^{0}C for 4 min followed by 35 cycles of denaturation at 95 ^{0}C at 30 sec, annealing at 58 ^{0}C for 1 min and extension at 72 ^{0}C for 2 min. A final extension was performed at 72 ^{0}C for 7 min.

The amplified PCR product was gel purified and cloned in pGMT Easy Vector system II (Promega ,USA). After ligation, the products were transformed in *Escherichia coli* JM 109 competent cells. The recombinants were taken by blue white screening in X-gal medium. Colony PCR was carried out in recombinant colonies to confirm the transformation. The sequencing of gene was carried out at Macrogen INC, Korea. The sequence data blasted to get the gene identity.

The mRNA was isolated from the samples of both susceptible (RRII 105) and tolerant clone (GT 1) and cDNA was synthesized and used as template DNA for the relative quantitative PCR analysis

(qRT PCR). ADP ribosylation factor gene was used as reference gene for the relative quantification. Gene expression values of control plants were treated as calibrator and relative quantification of gene expression in treated plants was carried out.

Chitinase specific primers flanked with restriction sites (*Bam HI* in the forward primer and *Not 1* in the reverse primer) based on the chitinase sequence of rubber (Philip *et al.,* 2006). (Chit.F-5'GGATCC ATGGCCAAAGAACCCAAGCC-PCR-3', Chit.R-5'AAGCTT CCGAATA ATTTCTCC CAATTCA-3'), amplification of chitinase gene was carried out with the above primers and PCR product was gel purified. The chitinase gene was cloned in 'pGEM-T Easy' vector system (Promega, USA). The plasmids were sequenced and tested the orientation of restriction sites in the gene. The plasmid was double digested with *Bam H1* and *Not 1* enzymes with multi-core enzyme buffers (Fermentas, Fast digest enzymes) in 20 µL reaction volume. The digestion was performed at 37 °C and the enzyme was heat inactivated. The digested product was purified and used for cloning in expression vector.

The expression system pET 32a+ (Novagen, Germany) were tried for recombinant protein expression. The plasmids were isolated in large quantities and double digested with *Bam H1* and *Not 1* enzymes to create cohesive ends. The 5.9 kb pET vector was digested with the same enzyme and the digestion was confirmed in 0.8 per cent agarose gels. When the digestion was complete, the vector was dephosphorelated by directly adding one unit of calf intestinal alkaline phosphatase to the digestion mix and incubated at 37 ^{0}C for 30 min. The vector was then gel purified to remove the residual nicked and super-coiled plasmid. The fragment with *Bam HI* and *Not 1* cohesive ends was used for unidirectional cloning to vectors cut with *Bam HI* and *Not 1*. The ligation reaction (10 µL) was prepared. The reaction was incubated at 16 ^{0}C overnight. The *E. coli* strain DH5 α was used for transformation and maintenance. Transformants were selected in antibiotic (ampicillin) plate and plasmid was isolated. The cloning was confirmed through PCR and restriction digestion. The sequencing of recombinant plasmid was carried out at M/s. Macrogen Inc. Korea to ensure orientation and correct reading frame.

The strain BL 21 (DE 3) lysis were used for the transformation. The transformed single colony from a freshly streaked plate was inoculated to LB media containing 50 µg mL^{-1} ampicillin and incubated overnight. The 1 mL of the culture was inoculated in 50 mL broth with ampicillin. The cultures were incubated overnight at 37 ^{0}C with shaking. The expression of targeted protein was induced by addition of IPTG to the growing culture where the OD 260 reached 0-6. Control colonies containing the vector without insert were also inoculated. An aliquot of 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 1mM and continued the incubation for 1 to 3 hrs. The cells were harvested at 1.5 h and 3h. The cells were harvested from a 1 ml aliquot of both induced and uninduced cultures by centrifuging at 5000 rpm for 5 minutes at 4 °C. The media drained out and the cells were treated with Bugbuster protein. The control vector without chitinase insert was also analysed along with induced and uninduced samples through SDS-PAGE in 10 per cent polyacrylamide gel. Cells were harvested from the remaining of 50 mL cultures and pellets were frozen at -70 °C for further analysis and purification of target protein.

The Trx (thioredoxin) fusion protein of pET vector were purified from the induced and control cells using the His Bind purification kit (Novagen,USA Cat.no. 70239-3). The purified proteins were tested on 10% SDS PAGE.

The polyclonal antibody was raised commercially using the recombinant chitinase protein in experimental mouse (Chromous Biotech, Banglore). The separated protein from induced and control samples of both tolerant /susceptible clones of *H. brasiliensis* (10% SDS PAGE) were transferred to nitrocellulose membrane. The polyclonal antibody raised with the recombinant chitinase protein was coupled with goat antimouse antibody HRP conjugate and finally the antigen antibody-antibody complexes on nitrocellulose membrane were located with the substrates.

The antifungal activity of purified protein was confirmed using filter paper disc method on PDA plates, inoculated with a growing culture of *C. cassiicola*. Aliquots of the purified recombinant protein 10 µg preparations were added to the filter disc on agar plate after two days of initial vegetative growth of the fungi. The plates were further incubated and observed for the appearance of inhibition zone.

RESULTS AND DISCUSSION

The PCR amplification of the cDNA yielded ~1 kb band (Fig. 1). The nucleotide sequence data of chitinase showed 978 bp and found to be identical with other chitinase gene when subjected to on line blast analysis (NCBI Gen Bank Acession No. DQ873889). The sequence showed 88 percent similarity to the chitinase gene.

The results indicate that chitinase expression is induced till 24th hour in GT 1 (the tolerant clone) and eventually it falls down below in RRII 105 control. In RRII 105,

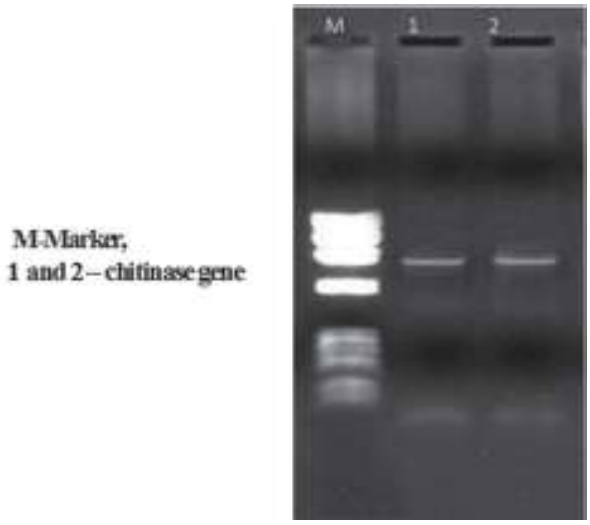


Fig. 1. PCR amplification of chitinase gene

chitinase level was lesser in treated plants than untreated control plants (Fig. 2). The results indicate that level of chitinase increased only in early infection in tolerant clone.

In susceptible clone RRII 105, chitinase activity had been lesser in treated plants than control. As reported in other crops the PR genes expressed quickly and the mRNA produced are translated into PR protein and prevent the further attack of the pathogen. The result of the present study confirmed the results from the other similar studies.

Chitinase gene was cloned in pET32a+ vector and confirmed the presence of insert in expression vector (Fig. 3).

The protein expression levels were tested by SDS –PAGE analysis of cell extracts followed by staining. The target protein was revealed as a unique band in the TCP sample and in the soluble cytoplasmic fraction. It shows that the most of the induced protein are in the soluble form. In pET32a+ vector a very high induction of the fusion protein (Trx+target gene) was detected. A~48 kD band was observed in all induced samples (Fig. 4).

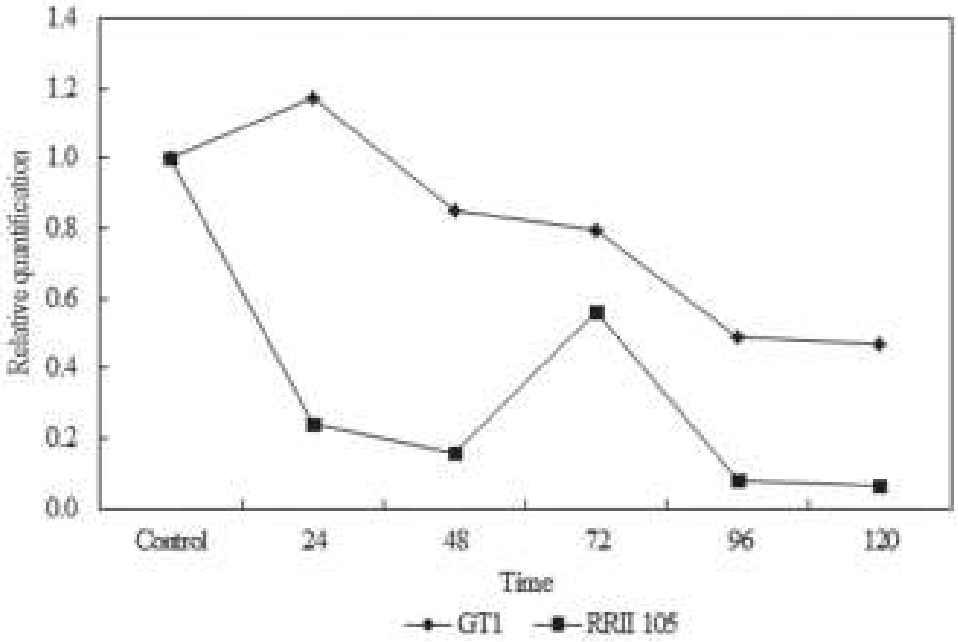
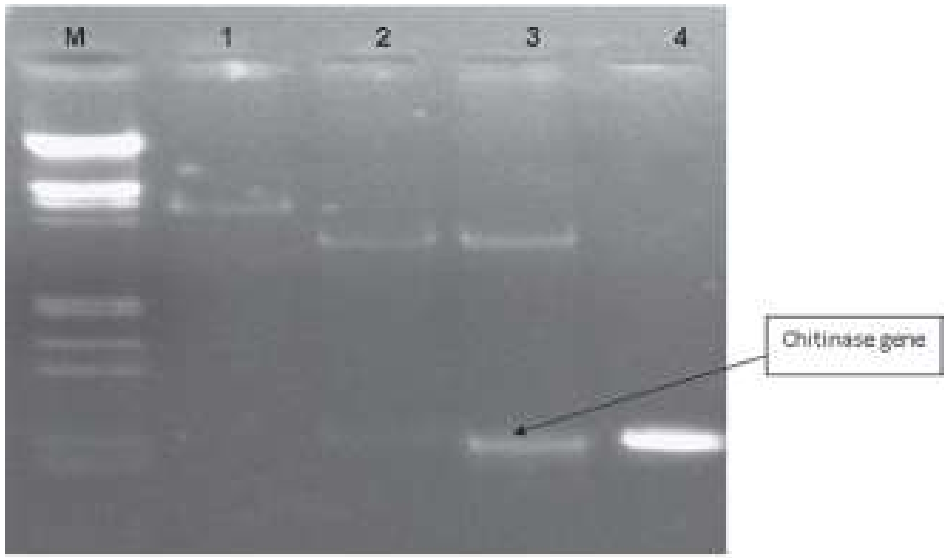


Fig. 2. Relative quantification of chitinase gene in GT1 and RRII 105 clones of *H. brasiliensis*



M-Marker; 1-Single digest; 2-Double digest; 3-Double digest; 4-PCR amplified product

Fig. 3. Chitinase gene insert in pET32a + vector

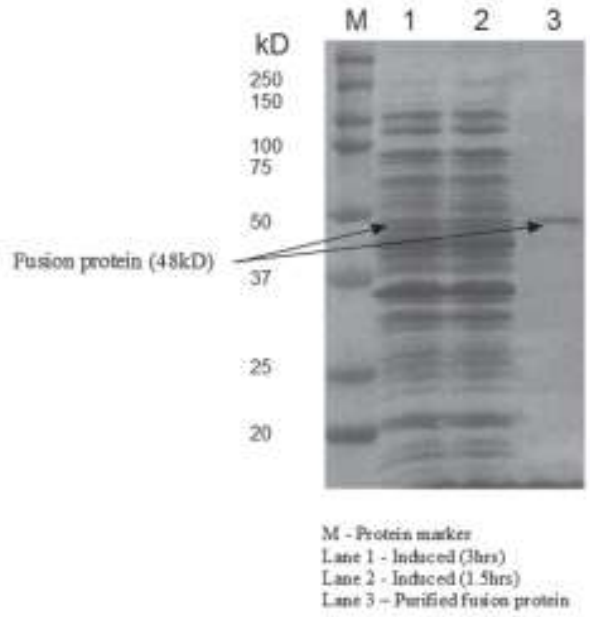


Fig. 4. PAGE analysis of total cell protein from induced samples of pET32a+ vector in BL21/ DE3 host cells

The fusion protein expressed in pET32a+ vector was effectively purified with His Bind purification kit. The system allowed the protein to be purified under native non denaturing conditions.

The tolerant clone GT I showed a prominent band in western blot compared to a minor band and in RRII 105 in induced condition (Fig. 5).

The total protein from induced *E. coli* colonies with recombinant vector were analyzed by SDS –PAGE, the band of expected size (48 kD) of predicted fusion protein was obtained. The predicted pI value of the protein translated from the cDNA sequence of the clone is 8.7. The recombinant fusion protein further purified through His Bind purification kit and polyclonal antibody raised. The recombinant chitinase protein was coupled with goat antimouse antibody HRP conjugate and finally the antigen antibody-antibody complex on nitrocellulose membrane was located with the substrates.

The purified recombinant chitinase showed its antifungal activity against *C. cassiicola*. Clear inhibition zone developed around the filter paper soaked with 10 µg of purified proteins. The results clearly indicated the antifungal activity of purified protein (Fig. 6). In the present study 10 µg of purified protein could effectively inhibit the growth of *C. cassiicola*. *In vitro* assays with purified rice chitinase showed the inhibited growth of rice sheath blight pathogen *Rhizoctonia solani* (Velazhakan, 2000). Tonon *et al*. (2002) reported the complete inhibition of germination of sporangia of *Phytophthora* spp. by 0.3 µg of purified enzyme. from infected tissue. Mauch *et al.,* (1988) reported the combined effect of antifungal activity of β-1,3 glucanase and chitinase in pea.

1 2 3 4

1- GT 1 Control 2 and 3 - GT 1 Induced 4 - RRII I05 induced

Fig. 5. Western blot detection of chitinase in *H. brasiliensis*

Recombinant protein showing inhibition

Control

Fig. 6. Antifungal activity of recombinant chitinase protein against *C. cassiicola* in PDA plates

The role of chitinase in the inhibition of potential pathogen has been reported in many higher plants (Vidhyasekaran, 1998). The major components of the cell wall of many fungi are polysaccharide chitin and β,1-3 glucan and are the substrates for chitinase and glucanase, respectively (Wesselas and Sietsma, 1981). A hypersensitive reaction of plants to pathogenic fungus leads to the induction of chitinase and glucanase activities both locally and systemically (Young and Pegg, 1982). At the same time, the resistance is induced towards a broad range of pathogens including fungi (Broekaert and Peumans, 1988). The *in vitro* antifungal activity exhibited by chitinase and glucanase lead to the speculations regarding a direct antifungal role of these hydrolytic enzymes. It has previously been reported that several plant species expressing chitinase gene showed enhanced resistance to fungal diseases (Nirla *et al.*, 2010). In rice, transgenic indica variety (Pusa Basmati 1) exhibited enhanced resistance to *Rhizoctonia solani*. (Sreedevi *et al*., 2003). In rubber *in vitro* antifungal activity of recombinant β-1,3 glucanase protein against *P. meadii* was reported (Thanseem *et al*., 2005).

Different isoforms of chitinase with different functions are reported in many crop plants (Collinge *et al*., 1993). In the present study *C.cassiicola* induced chitinase was isolated through PCR from infected leaf tissue. The nucleotide sequence data of chitinase showed 978 bp and found to be identical with other chitinase gene when subjected to online blast analysis.

The expression of chitinase gene and purification of chitinase protein may help in the development of suitable molecular diagnostic kit for the early detection of chitinase activity in different clones. Moreover, there is a scope for the development of transgenic *H. brasiliensis* with over expressing chitinase gene which may contribute tolerance against *C. cassiicola.*

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