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CLONING AND BACTERIAL EXPRESSION OF A PATHOGEN-INDUCIBLE ISOFORM OF β-1, 3-GLUCANASE GENE FROM *HEVEA BRASILIENSIS* WITH ANTIFUNGAL PROPERTIES

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A pathogen-inducible isoform of β-1, 3-glucanase gene (β-glu) from the Para rubber tree (*Hevea brasiliensis* Muell. Arg.) is reported for the first time. In order to induce the production of β-glu, two-week-old leaves of *H. brasiliensis* (clone RRII 105) were inoculated with abnormal leaf fall-causing pathogen, *Phytophthora meadii*. RNA was isolated from the near necrotic zones of the infected samples by LiCl precipitation. First strand cDNA was synthesized and sequence coding for the final mature β-glu (EC 3.2.1.39) was amplified. A functional cDNA clone was constructed using the pET 32a+ expression system. The major difference of the new basic isoform with a predicted pI of 9.26 is the absence of a glycosylation site at Asn-27, which is present in all the reported cDNA sequences of *Hevea* β-glu. Conditions were optimized for IPTG- induced over-expression of the gene in the *E. coli* strain BL21 (DE3), in soluble form. The column purified recombinant protein retained its functionality as proved by its ability to hydrolyze its natural substrate, laminarin. The purified recombinant *Hevea* β-glu was assayed for its antifungal activity against *Phytophthora*. When the fungus was grown on PDA plates, clear inhibition zones were observed around the filter paper discs soaked with 10 µg of the purified protein, indicating the inhibitory action of the purified enzyme.

Keywords: Antifungal protein, β-1,3-glucanase, *Hevea*, Recombinant protein

INTRODUCTION

Disease resistance in plants is brought about by constitutive and induced mechanisms. Accumulation of pathogenesis-related (PR) proteins is one of the most common markers for active, induced plant defense. β-1, 3-glucanases (β-glu) are considered to be one of the major components of this broad, generalized defense mechanism of plants against pathogen attack and are classified as PR-2 proteins. These are abundant, highly regulated hydrolytic enzymes widely distributed in the plant kingdom. β-glu have been studied in detail in different plant species and their role in combating the invading pathogens has been well documented in many incompatible plant-pathogen interactions (Simmons, 1994;

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Leubner-Metzger and Meins, 1999). They are supposed to play a major role in plant defense, primarily by degrading the cell wall of many fungal pathogens or by disrupting β-1, 3-glucans deposition by hydrolyzing it and thereby contributing to pathogen death (Mauch *et al.*, 1988). They can also act in an indirect way by releasing fungal cell wall fragments that in turn can act as elicitors of active host defense response (Yoshikawa *et al.*, 1993).

The role of plant glucanases in the inhibition of potential pathogens was first suggested based on the frequent observation of high activity of this enzyme in higher plants, even though its substrate in plants, callose, is usually present only in small quantities, while β-1,3-glucan is the main structural element of many pathogenic fungal cell walls (Abeles *et al.*, 1971). Later, it was shown that the purified enzyme could effectively degrade the fungal cell wall (Young and Pegg, 1982). The enzyme can destroy the fungi by thinning their cell wall at the hyphal tip causing swelling and ultimate bursting (Arlorio *et al.*, 1992). The fungal hyphal tip is thought to be particularly susceptible to lysis, since its cell wall synthesis involves a delicate balance between β-glucan hydrolysis and synthesis, which could be disrupted by the plant β-glu activity (Simmons, 1994). After decades of biochemical research on plant-pathogen interactions and successful production of transgenic plants, it has been proved that plant β-glu could be an effective tool in controlling many fungal pathogens (Yamamoto *et al.*, 2000; Nishizawa *et al.*, 2003).

The most destructive disease of rubber in India is the abnormal leaf fall (ALF) caused by different species of *Phytophthora*; the most common species being *P. meadii* (Edathil *et al.*, 2000). Field studies indicated up to 48% yield loss in susceptible clones due to ALF disease (Jacob, 2003). The present study was initiated based on the assumption that a chitinless fungus like *Phytophthora*, which has more than 90% of its cell wall composed of β, 1,3 linked glucans, could effectively be dealt with host β-glu. Induction of host β-glu had already been reported in the infected leaf tissues of rubber tree (Thanseem *et al.*, 2005). Higher, faster and prolonged induction of β-glu has been observed in the case of tolerant clones, than in the susceptible ones. In this study, direct fungicidal action of host β-glu was tested by the construction of a functional cDNA clone, enabling the production of recombinant protein. The purified recombinant protein was tested for its antifungal activity.

MATERIALS AND METHODS

Isolation of pathogen-induced cDNA coding for β-glu

Two-week-old leaves of the *Hevea* clone RRII 105, grown in polybags, were inoculated with a highly virulent isolate of *P. meadii*. The plants were maintained under optimum conditions for disease development. RNA was isolated from the near necrotic zones of the infected samples by LiCl precipitation, according to Thanseem *et al.* (2005). First strand cDNA was synthesised from total RNA by reverse transcription with oligo-(dT) primers using Improm-II Reverse Transcription System (Promega, USA), following the manufacturer's protocol. The sequence coding for the final mature β-glu was amplified from first strand cDNA using the primers 5'-CGG GAT CCC AGG TAG GTG TTT GCT ATG G-3' and 5'-GGA ATT CCC AGT TCT TTT CTG CAC C-3', designed based on a previously reported

sequence (Thanseem *et al.*, 2003). PCR was performed in 20 µl reactions containing 20ng template, 1X enzyme buffer (10 mM Tris-HCl pH 8.3, 50mM KCl, 1.5 mM $MgCl_2$), 100 µM of each dNTP, 10 pmoles of each primer and 0.5 U Taq DNA polymerase (AmpliTaq Gold, Applied Biosystems, USA). Reactions were carried out in a Perkin-Elmer 480 thermal cycler as follows: initial denaturation at 94 ^{0}C for 4 min, 34 cycles of 94 ^{0}C for 30 sec, 55 ^{0}C for 1 min and 72 ^{0}C for 2 min, and a final extension at 72 ^{0}C for 7 min. The PCR products were gel-purified.

Cloning in expression vector

The amplified 963 bp glucanase gene fragment contained a *Bam*HI site at 5′ end and an *Eco*RI site at 3′ end. In order to facilitate unidirectional cloning, the gel-purified fragment was double digested with *Eco*RI and *Bam*HI enzymes, using multi-core enzyme buffer (Promega, USA), to generate cohesive ends. The pET bacterial expression system (pET 32 a+, Novagen, USA) was used for recombinant protein expression. The vector was dephosphorylated using 1 U of calf intestinal alkaline phosphatase (Amersham, UK) and ligation was performed using T4 DNA ligase (Promega, USA). *E. coli* strain JM 109 was used for transformation and maintenance. Cloning was confirmed through PCR and restriction digestion.

Expression of the target gene

E. coli strain BL21 (DE3) was used for the expression of the target gene. Fifty ml cultures (LB medium containing 50 µg/ml ampicillin) were incubated with shaking at 37 ^{0}C, until the OD_{600} reached 0.6 (around 2- 3 h). An aliquot of the growing culture was removed for using as uninduced control. Expression of the target protein was induced by the addition of IPTG to a final concentration of 0.4 mM and the incubation was continued for 2–3 h. Just prior to harvest, 1 ml of culture from both the induced and uninduced samples was removed and OD_{600} was measured. The cells were harvested from 1.0 ml aliquots of both the induced and uninduced cultures by centrifuging at 5000 rpm for 5 min at 4 ^{0}C. The medium was completely drained out and 100 µl phosphate-buffered saline was added to yield a concentration factor of 10X. The cells were then lysed with 100 µl 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 analyzed through SDS-PAGE. Host *per se* and control vector without insert were also analyzed along with the induced and uninduced samples. Equal amounts of samples were loaded on to 10% acrylamide gel, after measuring the OD_{600}. Cells were harvested from the remaining portion of the 50 ml cultures and the pellets were frozen at -70 ^{0}C for further analysis and purification of the target protein.

Purification of the target protein

The Trx (thioredoxin) - fusion recombinant protein was purified using the His.Bind purification kit (Novagen, USA). The bacterial pellets from 50 ml of induced cultures 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 in a tube on ice and the soluble proteins were collected by centrifuging at 10,000 rpm for 20 min. Column was prepared with 5 ml of HisBind slurry to yield a final bed volume of 2.5 ml of settled resin. After washing with 3 vol sterile deionized 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 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). The bound proteins were 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 analyzed as controls.

Confirmation of the induced target proteins

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 on to nitrocellulose membranes (Sigma, USA) according to standard protocols (Sambrook *et al.*, 1989) using a western blot apparatus (Broviga, India). The reactions were performed according to the manufacturer's protocol. When signals appeared, the reaction was stopped by washing the blot thoroughly with deionized water and the blot was then air-dried.

A thrombin cleavage site is available on the pET vector allowing the enzymatic removal of N-terminal fusion tags. Approximately 20 µg of the purified recombinant protein from the pET vector was used in the reaction containing 1U of thrombin and 1X thrombin cleavage buffer (20 mM Tris-HCl, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$). The reaction was incubated overnight at 23 ^{0}C and the products were analyzed in 12% SDS-PAGE gel.

Antifungal assay of purified recombinant proteins

Experiments were carried out under sterile conditions. PDA plates (90 mm) were prepared and a 4 mm diameter single mycelial plug growing from the margin of *P. meadii* was inoculated at the center. To allow initial vegetative growth, the plates were incubated at 25 ± 2 ^{0}C for 24 h. At this time sterile filter paper discs (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 containing ~10 µg of recombinant protein was used for each disc. The plates were further incubated and observed for the appearance of inhibition zones and photographed.

RESULTS AND DISCUSSION

RNA was isolated in good quality and concentration from infected leaf samples (Fig. 1). The β-glu cDNA was amplified from the synthesized first strand cDNA (Fig. 2). No amplification was observed when RNA

Fig. 1

Fig. 2

Fig. 1. RNA isolated from the infected leaf tissues
Fig. 2. PCR amplification from the cDNA
Lane 1- Mol. wt. marker (λ DNA- *Eco*R I & *Hind* III double digest); 2- The 963 bp β-1, 3-glucanase cDNA coding for the mature functional protein

isolated as such and cDNA synthesized from uninfected samples were used in PCR. The pathogen-induced 963 bp band obtained was gel-purified, cloned and sequenced.

For the analysis of recombinants, plasmid miniprep was carried out from a few colonies and the presence of the insert was ensured by PCR and restriction analysis. Using a vector-directed forward primer and an insert-specific reverse primer in PCR, a product of expected size (≈1.0 kb) was obtained, assuring the correct orientation of the insert. Plasmid was isolated in large quantities from 100 ml cultures of a recombinant colony, PEG purified and sequenced to ensure that there was no shift in the translational reading frame. Only the sequence coding for the final mature protein was cloned without the N- and C-terminal extensions of the proprotein. However, five amino acids of the C-terminal extension were retained for recombinant expression, since these amino acids could not be completely removed in the final functional protein in some cases (Subroto *et al.*, 2001).

After the establishment of the recombinant plasmid in expression host (*E. coli*), the expression of the target DNA was induced by the addition of IPTG to the growing culture when OD_{260} reached 0.6. Concentrations varying from 0.2 – 2.0 mM IPTG were tried for the induction of target proteins. A final concentration of 0.4 mM IPTG was found to be optimum for maximum induction. Four fractions were analyzed in parallel for the detection of target proteins after induction. These included total cell protein (TCP), media sample, soluble cytoplasmic fraction and insoluble cytoplasmic fraction (inclusion bodies). The target protein was revealed as a unique band in the TCP sample and in the soluble cytoplasmic fraction. This shows that most of the induced proteins are in the soluble form which is very important for their purification in native form. However, some protein was detected in the inclusion bodies also. A very high induction of the fusion protein (Trx + target gene) of the expected size (55.6 kDa) was detected in all the induced samples compared to the uninduced controls (Fig. 3). A very high induction of the control Trx protein was also observed when the vector-alone colonies were induced with IPTG.

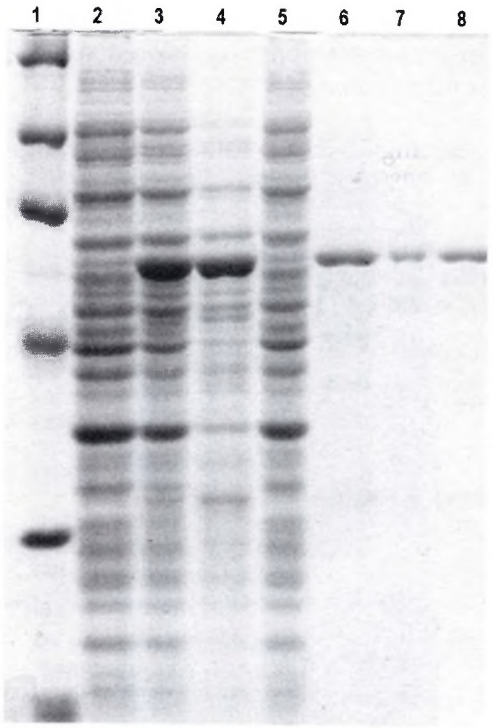

Fig. 3. Purified recombinant protein expressed in pET vector
Lane 1- Protein mol. wt. marker; 2- TCP from uninduced cells; 3- TCP from induced cells; 4- Total soluble proteins after sonication; 5- Unbound proteins removed from the column; 6-8: Different fractions of the eluted protein

The Trx-fusion protein expressed in pET vector was effectively purified with the His.Bind purification kit. The fusion tag contained six histidine residues that bound with Ni^{2+} cations which had been immobilized on the column resin using a charge buffer containing 50 mM $NiSO_4$. After washing away the unbound proteins, the target protein was recovered by elution with imidazole. This system allowed the proteins to be purified under native non-denaturing conditions. Most of the eluted protein was present in the first four of one ml each fractions (Fig. 3). No contaminating proteins were observed in purified fractions. The eluted fractions were stored at –70 ^{0}C for further analyses.

The identity of the induced protein in pET vector was confirmed through western hybridization. The His-tag sequence in the vector encoded fusion partner of the target protein was detected by the His-tag monoclonal antibody. The enzyme-coupled anti-mouse IgG AP conjugate was used as the secondary reagent and finally the antigen-antibody-antibody complexes on nitrocellulose filter were located with the chromogenic substrates NBT and BCIP. The 55.6 kDa fusion protein and 20 kDa Trx control protein were observed separately on the western blot, confirming the identity of the induced proteins (Fig. 4).

The identity of the induced protein was also confirmed by cleavage of the purified

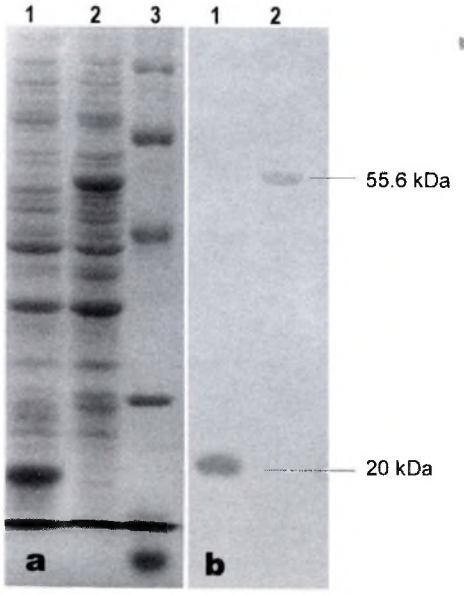

Fig. 4. Detection of target proteins through western blot with His-Tag monoclonal antibody
a. PAGE gel b. Western blot
Lane 1- TCP from induced control cells showing the 20 kDa control thioredoxin protein; 2- TCP from induced recombinant cells showing 55.6 kDa fusion protein; 3-Protein mol. wt. marker

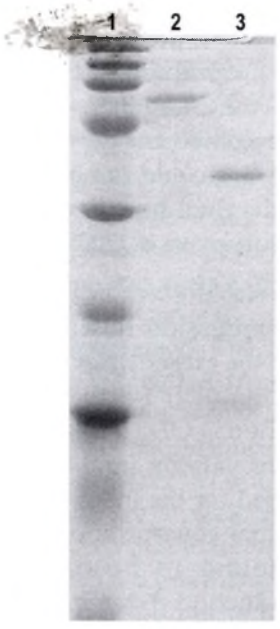

Fig. 5. Cleavage of N-terminal fusion tag of the purified recombinant protein with thrombin
Lane 1- Protein mol. wt. marker; Lane 2- Column purified fusion protein of 55.6 kDa; Lane 3- Cleavage with thrombin showing protein bands of 35.5 kDa (β-1,3-glucanase) and 20.1 kDa (thioredoxin).

fusion protein encoded by the pET vector, with restriction grade thrombin. One unit of thrombin effectively cleaved about 20 µg of the target protein when incubated at 23 °C overnight. The 20 kDa fusion-tag protein along with His-tag and the 35.5 kDa recombinant *Hevea* β-glu were detected in 12% PAGE (Fig. 5). In the antifungal assay, distinct inhibition zones of *P. meadii*.were developed within two days around the filters soaked with 10 µg of the purified enzyme solution (Fig. 6). These inhibitory zones remained visible for one week and then the fungus started to overgrow the inhibition zones.

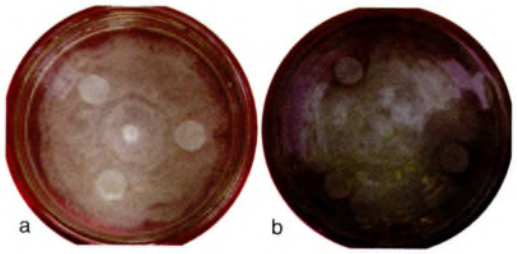

Fig. 6. Antifungal activity of purified *Hevea* recombinant β-1,3-glucanase against *P. meadii*. a. Control; b. Filter discs with 10 µg of purified protein

Many isoforms of plant glucanases with varying functions have been reported. In this study, a pathogen-inducible Class I basic β-glu was isolated through PCR from infected leaf tissues. This is the first report

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DQ989337    QVGVCYGMQG NNLPPVSEVI ALYKQSNIKR MRIYDPNRAV LEALRGSNIE LILGVPNSDL 60
AJ133470    QVGVCYGMQG NNLPPVSEVI ALYKKSNITR MRIYDPNRAV LEALRGSNIE LILGVPNSDL
AF311749    QVGVCYGMQG NNLPPVSEVI ALYKKSNITR MRIYDPNRAV LEALRGSNIE LILGVPNSDL
U22147      QVGVCYGMQG NNLPPVSEVI ALYKKSNITR MRIYDPNRAV LEALRGSNIE LILGVPNSDL

DQ989337    QSLTNPSNAN SWVQKNVRGF WSSVLFRYIA VGNEISPVNG GTAWLAQFVL PAMRNIHDAI 120
AJ133470    QSLTNPSNAK SWVQKNVRGF WSSVLFRYIA VGNEISPVNR GTAWLAQFVL PAMRNIHDAI
AF311749    QSLTNPSNAK SWVQKNVRGF WSSVLFRYIA VGNEISPVNR GTAWLAQFVL PAMRNIHDAI
U22147      QSLTNPSNAK SWVQKNVRGF WSSVLFRYIA VGNEISPVNR GTAWLAQFVL PAMRNIHDAI

DQ989337    RSAGLQDQIK VSTAIDLTLV GNSYPPSAGA FRDDVRSYLD PIIGFLSSIR SPLLANIYPY 180
AJ133470    RSAGLQDQIK VSTAIDLTLV GNSYPPSAGA FRDDVRSYLN PIIRFLSSIR SPLLANIYPY
AF311749    RSAGLQDQIK VSTAIDLTLV GNSYPPSAGA FRDDVRSYLN PIIRFLSSIR SPLLANIYPY
U22147      RSAGLQDQIK VSTAIDLTLV GNSYPPSAGA FRDDVRSYLD PIIGFLSSIR SPLLANIYPY

DQ989337    FTYAGNPRDI SLPYALFTSP SVVVWDGQRG YKNLFDATLD ALYSALERAS GGSLEVVVSE 240
AJ133470    FTYAGNPRDI SLPYALFTSP SVVVWDGQRG YKNLFDATLD VLYSALERAS GGSLEVVVSE
AF311749    FTYAGNPRDI SLPYALFTSP SVVVWDGQRG YKNLFDATLD VLYSALERAS GGSLEVVVSE
U22147      FTYAYNPRDI SLPYALFTSP SVVVWDGQRG YKNLFDATLD ALYSALERAS GGSLEVVVSE

DQ989337    SGWPSAGAFA ATFDNGRTYL SNLIQHVKGG TPKRPNRAIE TYLFAMFDEN KKQPEVEKHF 300
AJ133470    SGWPSAGAFA ATFDNGRTYL SNLIQHVKRG TPKRPNRAIE TYLFAMFDEN KKQPEVEKQF
AF311749    SGWPSAGAFA ATFDNGRTYL SNLIQHVKRG TPKRPNRAIE TYLFAMFDEN KKQPEVEKQF
U22147      SGWPSAGAFA ATFDNGRTYL SNLIQHVKGG TPKRPNRAIE TYLFAMFDEN KKQPEVEKHF

DQ989337    GLFFPDKRFK YNLNFG  316
AJ133470    GLFFPDKWQK YNLNFG
AF311749    GLFFPDKWQK YNLNFG
U22147      GLFFPDKRFK YNLNFG
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Fig. 7. Alignment of deduced amino acid sequence of β-1, 3-glucanases reported from *H. brasiliensis*. Sequence coding for the final mature protein excluding the N-terminal signal peptide and C-terminal extension are only shown. Variant amino acids are shown in boxes

on the isolation of a pathogen-inducible isoform from rubber. The major difference observed in the amino acid sequence of the protein encoded by the cDNA was the absence of a glycosylation site at Asn-27 which is present in all the reported cDNA sequences of *Hevea* β-glu (Fig. 7). Through protein analysis, Subroto *et al.* (2001) had reported differences in the glycosylation properties of β-glu isolated from the lutoid body fractions of different clones. They also reported a non-glycosylated isoform of glucanase from clone PB 261 and found that the specific activity of this isoform was 3–5 times higher than the glycosylated isoform of glucanase from clone GT 1. It was concluded that the different specific activities of β-glu from *Hevea* clones were not exceptional and may reflect the fact that these proteins were evolved through rapid co-evolutionary interactions with pathogens. The β-glu isoform isolated from *Hevea* was shown to be over-expressed in the infected zones of leaves, indirectly suggesting their role in combating the disease (Thanseem *et al.*, 2005). A constitutive expression of another basic isoform of β-glu was reported previously in the latex of *H. brasiliensis* (Chye and Cheung, 1995). This constitutive expression of β- 1, 3 - glucanase gene could be one among the many factors which prevents the fungal infection on the tapping panel. The earlier sequence obtained from latex (Thanseem *et al.*, 2003) was also similar to the pathogen induced isoform from leaves reported herein. It is yet to be verified whether the number and distribution of latex vessels rings or the density of latex vessels within a ring in the leaves and petiole of different *H. brasiliensis* clones is factor that determines the clonal susceptibility to fungal pathogens.

In order to obtain direct evidence on inhibition of pathogenic fungal growth by the host β-glu, a functional cDNA clone was constructed, which could express the *Hevea* β-glu in *E. coli* and the recombinant protein was purified and tested for anti-fungal properties. pET 32a$^+$ vector system (Novagen, USA) was used for the cloning and high level expression of the peptide sequences fused with a 109-amino acid thioredoxin protein (*TrxA*). The target protein comprised more than 50% of the TCP a few hours after induction. Solubility of the target protein was a major concern while attempting the expression. Most of the induced proteins were found to be in the soluble form, although some quantity accumulated in the inclusion bodies also. LaVallie *et al.* (1993) have reported that a number of mammalian cytokines and growth factors, when expressed as C-terminal *trxA* fusion proteins, stayed remarkably soluble in the *E. coli* cytoplasm under certain conditions. In this study, growth and prolonged induction at 30 ^{0}C was found to increase the protein solubility.

When total soluble proteins from the induced *E. coli* colonies with recombinant pET vector were analyzed by SDS-PAGE, a band of the expected size (55.6 kDa) of the predicted fusion protein (trx-*Hevea* glucanase) was obtained. The predicted pI of the protein translated from the DNA sequences of the clone was 9.26. Sequence coding for only the mature functional protein was cloned, excluding N- and C-terminal extensions, which are characteristic of Class I glucanases. The recombinant fusion protein could be purified as a single band by passing through an affinity column for the fusion tag at the N-terminal. The recombinant fusion protein retained the functionality of the glucanase enzyme. This was demonstrated by the hydrolysis of the glucanase substrate, laminarin. Earlier, a functional latex cDNA clone had been constructed from *Hevea* by

Yeang and Chow (2001). A basic isoform of *Hevea* glucanase was produced from the clone with a predicted pI value of 9.83. The protein produced was shown to be Hevb 2, a basic glucanase classified as a major latex allergen. However, this had not been tested for its antifungal properties. Recently, a recombinant latex Class I chitinase has been produced using pMAL expression vectors and its immunoglobulin binding reactivity has been studied by immunoblot experiments using the sera from latex-allergic patients (Rihs *et al.*, 2003). Yu *et al.* (2003) reported bacterial expression of a functionally active barley glucanase.

In our study, 10 µg of purified protein could effectively inhibit the growth of *P. meadii*. However, the potential concentration needed for complete inhibition was not estimated. Similarly, 10 µg of purified rice chitinase has been shown to inhibit the growth of the rice sheath blight pathogen *Rhizoctonia solani*, when applied on sterile filter paper discs (Velazhahan *et al.*, 2000). Tonon *et al.* (2002) reported complete inhibition of the germination of the sporangia of *P. infestans* with 0.3 µg/ml of purified enzymes. They had used enzyme purified from the infected plant tissues and not a recombinant protein. A basic β-glu induced in pepper (*Capsicum annum*) had antifungal activity against *P. capsici* at 100 µg/ml (Kim and Hwang, 1997). When applied individually, β-glu purified from pea pods was found to be inhibitory only to *Fusarium solani* among the eight fungi tested, including three representatives of the Oomycetes. This inhibitory activity was observed at a concentration of 65 µg/ml (Mauch *et al.*, 1988). Most of the reports used combinations of β-glu and chitinase in order to study the *in vitro* antifungal properties. In combination, their inhibitory effect seems to be severalfold higher than when they are used individually (Mauch *et al.*, 1988). Hu *et al.* (1999) expressed three antifungal proteins - trichosanthin, tobacco class I chitinase and tobacco class I glucanase in *E. coli* and the recombinant proteins were tested for antifungal activity. All the three kinds of proteins showed inhibitory activities, but in combinations of two, the activity was found to enhance severalfolds. When all of them were combined, a very high antifungal activity was observed. However, in our study, only β-glu might be playing a major role since the pathogen is a chitin-less fungus.

Proper selection of glucanase genes is very important for the development of transgenic plants with enhanced disease tolerance since β-glu can exist in many structural isoforms that differ in size, primary structure, isoelectric point, cellular localization and pattern of regulation. Several glucanase isoforms with major roles in various physiological and developmental processes, but less important in disease resistance, have been isolated. In several studies, different β-glu isoforms have been tested for *in vitro* antifungal activity (Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993). Only Class I basic vacuolar isoforms of β-glu have been found to be effective in promoting the lysis of hyphal tips and in inhibiting the growth of several fungi (Lawrence *et al.*, 1996; Anfoka and Buchenauer, 1997). The extracellular Class II acidic glucanases with weak antifungal activity may be involved in the release of elicitors from fungal cell wall that activates host defense reactions. This study also confirmed the role of Class I basic vacuolar isoforms of glucanase in fungal resistance. An exception to this has been reported by Tonon *et al.* (2002), where they could isolate and purify an acidic isoform of

glucanase with *in vitro* antifungal properties. In a previous study, Andreu *et al.* (1998) have demonstrated the ability of partially purified glucanase from potato tubers in degrading the glucans from *P. infestans* cell walls. This result and the fungicidal action reported herein suggest that this hydrolytic enzyme could act directly by inhibiting the growth of the invading fungi. Recently, co-expression of a chitinase from *Brassica juncea* (BjCHI1) and a glucanase isoform from *H. brasiliensis* (HbGLU) in transgenic potato was shown to be effective in controlling the growth of the soil-borne phytopathogen, *Rhizoctonia solani* (Chye *et al.*, 2005). The newly isolated isoform of glucanase from rubber can be potentially useful in controlling fungal pathogens, particularly chitin-less fungi like *Phytophthora* spp. in a wide variety of crop plants.

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