Induction and differential expression of β-1,3-glucanase mRNAs in tolerant and susceptible *Hevea* clones in response to infection by *Phytophthora meadii*

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**Summary**  Most cultivated rubber tree (*Hevea brasiliensis* Willd. ex A. Juss.) clones in India are susceptible to abnormal leaf fall disease (ALF), which is caused by various *Phytophthora* species and results in yield losses of up to 40%. Because the conventional breeding programs for this perennial tree crop are complex and time consuming, we attempted to find a molecular solution to increase the tolerance of rubber trees to ALF. The expression patterns of the gene coding for the pathogenesis-related β-1,3-glucanase (β-glu) enzyme in a tolerant (RRII 105) and a highly susceptible (RRIM 600) clone of rubber tree were examined, following infection with ALF-causing *Phytophthora meadii* McRae. Infected leaf samples were collected at different times after inoculation, and RNA was extracted and subjected to Northern blot hybridization and reverse transcriptase polymerase chain reaction (RT-PCR). On hybridization with a 1.25 kb β-glu probe, Northern blots showed a marked increase in β-glu transcript levels in both clones 48 h after inoculation. However, compared with the susceptible RRIM 600 clone, the tolerant RRII 105 clone had a higher rate of increase and a more prolonged induction, with β-glu transcript levels remaining high for 4 days after inoculation. In RRIM 600, the mRNA levels decreased significantly 48 h after inoculation. On re-hybridization with an 18S rRNA probe, uniform signals were detected in all the lanes, indicating that an equal amount of total RNA was present in all samples. Similar results were obtained in relative quantitative RT-PCR experiments with the housekeeping actin gene as an internal control. Thus, although induction of the β-glu gene occurred in both tolerant and susceptible clones, the predominant difference between clones was in the intensity and duration of the response. The tolerance of clone RRII 105 may be associated with the prolonged expression of the gene following infection. The antifungal activity of these hydrolyase enzymes makes them rational candidates for overexpression by genetic transformation to produce disease resistant crops.

**Keywords:** disease resistance, *Hevea brasiliensis*.

**Introduction**

*Hevea brasiliensis* Willd. ex A. Juss., the Para rubber tree, accounts for more than 99% of the world’s natural rubber production. It was introduced to India by the British in the 19th century, and many plantations were successfully established by the early 20th century. Bud grafting is the chief method for propagation of *H. brasiliensis*. From the initial germplasm collections of the 19th century, many clones with desirable traits have been developed through conventional breeding programs. However, many of the high-yielding clones are susceptible to abnormal leaf fall disease (ALF), which is caused by various *Phytophthora* species and results in a yield loss of up to 40% in highly susceptible clones, making it the most destructive disease of rubber trees in India (Jacob 2003). Abnormal leaf fall disease occurs during periods of prolonged wet weather, when cool, overcast and humid conditions persist for several days. Green pods are generally the first to be infected by the motile zoospores of the fungus, which in turn provide abundant inocula for infection of leaves. The fungus attacks the petiole and leaf blades, causing leaves to shed. After defoliation, the pathogen often attacks the leaf-bearing twigs, causing extensive dieback.

The millions of hectares of rubber plantations across Asia originate from a small sample of seeds collected from the Amazon River basin by Dr. Henry Wickham in 1876 (Imle 1978). This narrow genetic base, along with a prolonged breeding cycle and juvenile period, and a highly heterozygous nature, have made conventional breeding of *Hevea* complex, labor-intensive and time-consuming. Because classical molecular approaches to tag resistance genes, such as constructing segregating populations or near-isogenic lines, are difficult to employ in rubber trees, there is a need to develop biotechnological methods for improving rubber crops. An understanding of the molecular mechanism of disease-resistance, mediated through pathogen-related (PR) proteins, could provide an alternative approach for developing more sophisticated molecular tools to combat ALF disease in rubber trees.

β-1,3-Glucanases (β-glu) are PR-2 proteins that catalyze...
endotype hydrolytic cleavage of 1,3-β-D glucosidic linkages in β-1,3-glucans present in the cell wall of many pathogenic fungi. These abundant and highly regulated enzymes are widely distributed throughout the plant kingdom and have been intensively investigated at the physiological and molecular levels because of their well-proven role in plant defense response (Simmons 1994, Leubner-Metzger and Meins 1999). It has been proposed that they can act in at least two ways: directly, by degrading the cell walls of the pathogen, or indirectly, by promoting the release of cell wall-derived materials that can elicit an active defense reaction (Boller 1993). There are several reports on the induction of β-gluc in pathogen infection in plants such as tobacco (Vogeli-Lange et al. 1988), potato (Kombrink et al. 1988), tomato (Joosten and De Wit 1989), soybean (Yi and Hwang 1996), bean (Xue et al. 1998), pepper (Egea et al. 1999), wheat (Kemp et al. 1999) and peach (Zemanek et al. 2002). The antifungal activity of these hydrolases makes them rational candidates for overexpression by genetic transformation to produce disease-resistant crops.

Among woody plants, the β-gluc gene was first isolated from Hevea (Chye and Cheung 1995). Different isoforms of β-gluc have been detected in the latex of rubber trees (Churngchow et al. 1995). Earlier work on β-gluc from rubber trees concentrated mainly on its allergenic properties (Subroto et al. 1996, Subroto et al. 2001), focusing on the biochemical and immunological characterization of β-gluc (Hev b2) protein as a latex allergen (Sunderasan et al. 1995, Yagami et al. 1998, 2002). However, there are few reports on the role of β-gluc in disease-resistance of woody species in general, and of rubber trees in particular. In India, the widely cultivated clone RRIM 600 clone, which is also widely cultivated, is highly susceptible to the disease. The objective of the present study was to compare the expression patterns of β-gluc in tolerant and susceptible clones of rubber, following infection with the ALF disease-causing fungus Phytophthora meadii McRae. Northern blot hybridization and relative quantitative RT-PCR were employed to study the differential gene expression. The importance of the timing and magnitude of β-gluc induction in combating a Phytophthora challenge is discussed.

Materials and methods

Plant material

The Phytophthora-tolerant Hevea clone RRIM 105 and the highly susceptible clone RRIM 600 were obtained from the Germplasm collection nursery of the Rubber Research Institute of India (RRII). The bud-grafted plants were raised in polyethylene bags in a greenhouse.

Fungal isolate and inoculation

The fungus used was a highly virulent isolate of P. meadii. Plates containing potato-dextrose agar medium were inoculated with the stock culture and incubated in light at 25 °C for 2 days. For sporulation, white oats broth was inoculated with 2-day-old fresh mycelium and incubated in the dark for 2 days. The mycelia were then washed and exposed to light for 24 h to promote sporangial growth. The motile zoospores were liberated by a mild heat shock and young leaves (about 2 weeks old) were inoculated with the zoospore suspension (10⁶ zoospores ml⁻¹). After inoculation, the plants were covered with transparent polyethylene bags to maintain the required humidity and observed for the development of symptoms. Control plants were sprayed with distilled water and maintained under similar conditions. Leaf samples were taken at 0, 24, 48, 72 and 96 h after inoculation and processed for RNA extraction.

Extraction of RNA from leaf samples

Total RNA was isolated from uninfected control leaves and from necrotic areas of infected leaves by the LiCl precipitation method (Sambrook et al. 1989). Around 500 mg of leaf tissue was ground to a fine powder in liquid nitrogen and 5 ml of extraction buffer (0.2 M NaCl, 0.1 M Tris-HCl, pH 8.5, 0.01 M EDTA, pH 8.0, 1.5% SDS, 0.1% 2-mercaptoethanol and 1% insoluble PVPP) was added. Following extraction with an equal volume of extraction buffer-saturated phenol (centrifugation for 15 min at 10,000 g), the aqueous phase was transferred and re-extracted twice with equal volumes of chloroform. The RNA was precipitated overnight in 1/3 volume of 8 M LiCl at −20 °C. The precipitated RNA was recovered by centrifugation for 10 min at 10,000 g, washed with ice-cold 2 M LiCl and dissolved in 250 µl of sterile distilled water. The RNA was then re-precipitated by adding 0.1 vol of 3 M sodium acetate and 2.5 vol of ethanol at −20 °C. Following centrifugation for 10 min at 10,000 g, the pellet was re-suspended in 100 µl of sterile distilled water.

Preparation of probes for Northern blot analysis

Two probes were used. A 1.25 kb β-gluc fragment was amplified from genomic DNA with gene-specific forward (5’-CTT CTT AAT GCC TAT CTC TTC-3’) and reverse (5’-CTC ACA TAA GTT CTT GCC AG-3’ and 5’-GCG ATC CGA ACA TTT CAC CG-3’). The PCR was performed in 20-µl reactions containing 20 ng template DNA, 1× enzyme buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂), 100 µM each of dNTPs, 10 pmol of each primer and 0.5 U Taq DNA polymerase (AmpliTaq Gold, Applied Biosystems, Foster City, CA). Amplification was carried out in a PerkinElmer 480 thermal cycler (Boston, MA) as follows: 94 °C for 4 min; 30 cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 2 min, with a final extension at 72 °C for 7 min. The PCR products were gel-purified and radiolabeled with α-32P-dCTP using the Multiprime DNA Labeling System (Amersham, Buckinghamshire, U.K.).

Northern blot analysis of total RNA

Ten µg of total RNA was resolved in a 1% formaldehyde-agarose gel. The RNA was then transferred to a nylon mem-
brane (Hybond N+ , Amersham Biosciences, Piscataway, NJ) by blotting overnight in 1× SSC (Sambrook et al. 1989). After UV-cross-linking (Hoefer, San Francisco, CA), the membrane was pre-hybridized for 2 h in pre-hybridization solution (5× SSC, 5× Denhardt’s reagent, 1% SDS and 50% formamide) with constant rotation. The denatured α32P-dCTP labeled probe was subsequently added and the membrane was hybridized overnight at 42 °C. The membrane was first washed with 2× SSC + 0.1% SDS at room temperature and then with 0.2× SSC + 1% SDS at 42 °C. The labeled membrane was exposed to X-ray film (Kodak X-Omat, Rochester, NY) with intensifying screens at –70 °C.

Relative RT-PCR
First strand cDNA was synthesized from total RNA by reverse transcription with oligo-(dT) primer using the ImProm-II Reverse Transcription System (Promega, Madison, WI) according to the manufacturer’s protocol. One µl of the first strand cDNA was used to co-amplify the β-glu and β-actin transcripts in a 20 µl reaction. The primer set selected for the amplification of the housekeeping β-actin included an upstream 5′-TCCATAATAGTGATGT-3′ and a downstream 5′-GGA CCTGACTCGTCATAC-3′ primer (Kobayashi et al. 2000). The RNA samples were checked for genomic DNA contamination using the extracted RNA directly as the PCR template, prior to cDNA synthesis, under the same PCR conditions. The PCR products were resolved in a 1.5% agarose gel, visualized by ethidium bromide staining under UV light and the image was documented using EDAS 290 (Kodak Electrophoresis Documentation and Analysis System, New Haven, CT).

Analysis of autoradiograms and RT-PCR products
The relative abundance of β-glu mRNAs relative to the 18S rRNA gene was determined by measuring the net intensity of the band in Northern blots with Kodak 1D image analysis software. Net intensity is the sum of background-subtracted pixel values in the band rectangle. The RT-PCR gel images were scanned to determine the net intensities of β-glu and actin bands. The net intensity data for β-glu were corrected for housekeeping gene data and normalized to 0 h. Corrected values were calculated by dividing the β-glu value by its corresponding housekeeping gene value and multiplying 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 (Zemanek et al. 2002).

β-1,3-Glucanase enzyme assays
β-1,3-Glucanase activity was assayed spectrophotometrically as described by Pan et al. (1991). The crude enzyme extract (62.5 µl) was added to an equal volume of the substrate, 4% laminarin, and incubated at 40 °C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylate and the sample was kept in a boiling water bath for 10 min. The colored solution was diluted 10 times with distilled water and absorbance at 500 nm was measured. One unit of enzyme activity was defined as the amount of enzyme that produces reducing sugars equivalent to 1 µM of glucose per 10 min under standard assay conditions.

Results
Dense sporangial growth was observed when fresh mycelia were grown for 2 days in white oats medium. A brief heat shock effectively released the motile zoospores and these were used for inoculation (Figure 1). The plants were kept under optimum conditions for infection. Disease symptoms began to appear 2 days after inoculation and were more prevalent in the susceptible clone than in the tolerant clone (Figure 2). The necrotic spots induced by the pathogen were more prevalent in areas where the inoculum density was high.

Total RNA was isolated from uninfected control leaves and the necrotic zones of infected leaves at various times after inoculation. Good quality RNA, without degradation or DNA contamination, was obtained. Samples of RNA were subjected to Northern blot hybridization 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 rehybridized with the 18S rRNA gene probe. The signals generated indicated that the 18S rRNA content did not vary with the treatments or the time course, and therefore, there was no non-specific shift in the relative amounts of mRNA. Thus, the 18S rRNA levels remained almost constant during the 96-h period after inoculation, whereas the β-glu mRNA levels changed considerably (Figure 3). The β-glu data were corrected for 18S rRNA values and then normalized to 0 h (control) for each clone. In both tolerant and susceptible clones, the β-glu probe hybridized to a 1.2 kb mRNA, whereas the 18S rRNA hybridized to a 1.7 kb mRNA.

The accumulation patterns of β-glu mRNA demonstrate that expression levels differed between the clones. The quantities of β-glu mRNAs present in the uninfected control samples were below the limits of detection by Northern blots in both tolerant and susceptible clones. Control plants sprayed with distilled water and maintained in similar conditions as infected plants did not exhibit induction of β-glu mRNAs until 96 h. In contrast, in infected leaves of both clones, the transcript levels of β-glu showed a marked increase 48 h after inoculation. The patterns of expression indicated a higher rate of increase in β-glu mRNA in the tolerant clone than in the susceptible clone, as indicated by the more intense signal observed 48 h after inoculation, and the difference in the magnitude of induction between clones was even more evident at 96 h. Although β-glu induction occurred in susceptible clones at 48 h after inoculation, it dropped to drastically low levels at 96 h after inoculation (Figure 3).

The β-glu expression patterns quantified by relative RT-PCR confirmed the results of Northern blot analysis. The β-glu gene and a fragment of the housekeeping actin gene, from each sample of total RNA, were co-amplified from the first strand cDNA synthesized, using gene-specific primers. The RNA was quantified to ensure an equal amount of template for first strand cDNA synthesis. The internal control in RT-PCR was actin because the 18S rRNA genes cannot be reverse transcribed with oligo-(dT) primers. Transient levels of the internal control remained almost the same in control and treated samples at all time intervals, and in both tolerant and susceptible clones. However, as observed in the Northern blots (Figure 3), β-glu appeared to be differentially regulated in the tolerant and susceptible clones. Unlike the Northern blots, we observed PCR amplification of β-glu mRNAs, albeit at low frequency, in the control samples of both clones. This discrepancy reflects the fact that tiny amounts of template are sufficient for amplification by PCR, whereas larger quantities of template are required in Northern blots. The net intensities of
bands amplified from the control samples of both clones were similar in the ethidium bromide-stained agarose gels. In the infected samples, the band intensities began to increase 24 h after inoculation, and an exponential increase in band intensity was observed at 48 h (Figure 4). This exponential increase was probably associated with an increase in the quantity of target sequences in the cDNA template available for PCR, because all other constituents remained the same. Therefore, this increase was correlated to increased gene activity.

The β-glu band intensity corrected to that of actin and normalized to 0 h was plotted against time after inoculation (Figure 5). The results were concordant with those of the Northern blots, with higher and more rapid accumulation of β-glu transcripts observed in RRII 105 than in RRIM 600. The β-glu levels reached a peak at 48 h after inoculation, with a 25-fold increase over the basal value in the tolerant clone compared with a 20-fold increase in the susceptible clone. Compared with the susceptible clone, the induction was more prolonged in the tolerant clone, with β-glu levels showing only a marginal decrease from maximum values for 4 days following inoculation. In contrast, in the susceptible clone, the transcript level of β-glu decreased drastically after 48 h and was only four times greater than the basal level at 96 h. There was no amplification when total RNA was used as the template for PCR, which eliminated the possibility of DNA contamination. Genomic DNA used in the control reaction generated 1.25 kb β-glu and 400 bp actin fragments due to the presence of introns, whereas the RT-PCR products were 1.12 and 260 kb, respectively (Figure 4).

Both Northern hybridization and relative RT-PCR provide a measure of the β-glu mRNA population only. An increased mRNA population, although indicative, does not provide unequivocal proof of the presence of the protein. β-1,3-Glucanase activity was thus measured in the control and infected samples. No significant difference in β-glu activity was observed between healthy tissues of RRII 105 and RRIM 600. Enzymatic activity was several-fold higher in all the Phytophthora-infected samples, consistent with the increased transcript levels in response to infection. Maximum activity was recorded at 48 h after inoculation in both clones (Figure 6). There was a marked difference in the rate of increase in enzyme activity between the clones. The tolerant clone had higher β-glu activity than the susceptible clone at all times studied. Enzymatic activity remained high for 4 days following inoculation in RRII 105, whereas it decreased 3 days following inoculation in RRIM 600.
Discussion

Disease resistance in plants can be due to constitutive or induced mechanisms. Accumulation of PR proteins is one of the most common markers for actively induced plant defense. Among them, proteins with β-gluc activity have been well investigated in several plant species (Simmons 1994, Lebner-Metzger and Meins 1999). These enzymes are believed to play a key role in combating fungal pathogens, presumably by inhibiting fungal growth through their hydrolytic capacity (Mauch et al. 1988). The hydrolytic products of the fungal cell wall can also act as elicitors, which may induce other types of defense reactions in plants (Keen and Yoshikawa 1983). β-1,3-Glucanase and other enzymes with β-gluc activity destroy fungi by degrading β-glucan in the fungal cell walls, thus thinning the cell wall at the hyphal tip. This thinning causes swelling that ultimately leads to lysis of the hyphal tip (Arlorio et al. 1992). The fungal hyphal tip is thought to be particularly susceptible to lysis because its cell wall synthesis involves a delicate balance between β-glucan hydrolysis and synthesis, which could be disrupted by the host β-gluc activity. This could be particularly important to Phytophthora diseases like ALF, because the causative organism belongs to the class Oomycetes, the cell walls of which are primarily made up of β-1,3-linked glucans. In contrast, the principal cell wall component in most other pathogenic fungi is chitin.

Earlier, it was reported that β-gluc was constitutively expressed in the latex of rubber trees (Chye and Cheung 1995). Of the 245 expressed sequence tags (ESTs) from a latex cDNA library, defense-related genes were found to be the second most-frequent transcripts, next to rubber biosynthesis-related genes (Han et al. 2000). Because tapping to collect latex is a process of controlled wounding, various enzymes stored in laticifers may make an important contribution to the plant defense mechanism by sanitizing and sealing wounded areas on the tree as reported for Carica papaya L. (El Moussaoue 2001). In a related study, we observed that constitutive expression of β-gluc was particularly evident in the latex of tolerant clones, especially in FX 516, a hybrid of H. brasiliensis and H. benthamiana Muell. Arg.; however, no detectable level of gene expression was observed in healthy leaves, which are the major sites of Phytophthora attack in rubber trees.

In our study, the time-dependent induction of β-gluc in the leaves of two Hevea clones support the hypothesis that the enzyme is involved in defense against fungal infection. To determine the role of β-gluc in conferring resistance to P. meadii mediated ALF in Hevea, we used a susceptible clone (RRIM 600) and a highly tolerant clone (RRII 105), and analyzed the induction of glucanase following pathogen inoculation, by two experimental approaches: Northern hybridization and relative RT-PCR with suitable internal controls. Although quantification of RNA transcript levels can be performed by Northern blot hybridization analysis, RT-PCR has many advantages such as the ability to detect lower levels of gene expression. However, to be quantitative, RT-PCR must be analyzed in the linear range of amplification for both the gene of interest and the internal control, before reaction components become limiting.

We observed the induction of β-gluc in response to infection by the pathogen in both tolerant and susceptible rubber tree clones. However, the timing and magnitude of induction varied between the clones. Previous studies have reported that the level and onset of β-gluc expression is often positively correlated to the degree of resistance to the pathogen. Muskmelon and tomatoes infected with Fusarium oxysporum f. sp. elaidis exhibited a higher and more rapid expression of β-gluc in resistant varieties than in susceptible varieties (Netzer and Kritzman 1979, Ferraris et al. 1987). Similarly, β-gluc activity was found to increase more rapidly in resistant melon cultivars than in susceptible cultivars following inoculation with cucurbit powdery mildew fungus (Rivera et al. 2002). More glucanase activity has been reported in the Phytophthora-infected tissues of a tolerant variety of black pepper compared with two susceptible varieties (Jebakumar et al. 2002). Our results corroborate these findings: at 48 h after inoculation, there was an exponential increase in β-gluc transcripts, and this induction was higher and more prolonged in the tolerant clone than in the susceptible clone. At 72 h following infection, there was a 2.5-fold increase in β-gluc transcripts in the tolerant clone and a 6-fold increase by the fourth day. Similar results have been reported by Egea et al. (1999) who studied β-gluc expression in resistant and susceptible pepper cultivars infected with Phytophthora capsici L. and found that, although induction occurred in both cultivars 2 days after inoculation, Northern blot hybridization revealed that the β-gluc transcript level was depleted in the susceptible cultivar by Day 3, in the region immediately below the necrotic zone.

The β-gluc enzyme often exists in multiple isoforms; the basic isoforms are usually intracellular, whereas the acidic isoforms are secreted into the intercellular spaces. Although induced proteins are mostly acidic, induction of basic β-gluc has also been reported in many incompatible plant–pathogen interactions. In tobacco, the RNA encoding the basic form of β-gluc was detected within 1 day of inoculation with tobacco.
mosaic virus and reached a peak by Day 2 (Ward et al. 1991). Inoculation of soybean plants with an elicitor from Phytophthora spp. results in increased expression of the basic isoform of β-glucanase gene (Cheong et al. 2000). In disease resistance, basic β-glucanase seems to be particularly important, because overexpression of β-glucanase genes in transgenic plants affords substantial protection against pathogens only when basic glucanase is constitutively expressed (Jongedijk et al. 1995). Intracellular accumulation of basic β-glucanase in the vacuoles of penetrated cells leads to a rapid deployment of β-glucanase at the exact site of penetration. Further, in vitro anti-fungal studies have shown that the vacuolar basic isoform of β-glucanase can inhibit the hyphal growth and spor germination of pathogenic fungi (Yi and Hwang 1996, Anfoka and Buchenauer 1997). A basic β-glucanase isolated from pepper was able to lyse the germinating zoospores and inhibit growth of P. capsici (Kim and Hwang 1997). In our study, a basic isoform of β-glucanase was used as the probe in Northern blot hybridization. The primers used for RT-PCR experiments also corresponded to this basic isoform. However, because the different β-glucanase isoforms have a high degree of homology, the signals generated in Northern blots may not be fully attributed to the expression of the basic isoform alone. Immunocytolocalization studies are necessary, therefore, to determine the subcellular localizations of β-glucanase isoforms specifically induced in tolerant and susceptible rubber clones, in response to Phytophthora infection. At the amino acid level, our isoform (GenBank Accession No. AY325498) is only 97.5% identical with the sequence of Chye and Cheung (1995) (U22147). We suppose that they represent different isoforms of β-glucanase in rubber trees, although both are intracellular basic proteins. In a related study (data not presented here), we constructed a functional cDNA clone of our isoform. The recombinant protein inhibited the growth of Phytophthora meadii, which causes ALF disease in rubber, confirming the role of this β-glucanase isoform in mediating fungal resistance.

In conclusion, the role of β-glucanase in mediating defense against ALF disease in Hevea is supported by evidence that β-glucanase transcription is induced during pathogen attack. The predominant difference between the tolerant clone and susceptible clone was in the intensity of the response, which was directly related to the degree of tolerance. The tolerance of RRII 105 may be associated with the rapid induction and prolonged expression of the gene, resulting in an intensely inhospitable environment for fungal growth, thus enabling the plant to respond to pathogen attack more efficiently. The constitutive expression of β-glucanase in laticifer cells may be another factor determining tolerance to Phytophthora. Our results indicate that it may be possible to enhance the tolerance of rubber plants to invading fungal pathogens through genetic manipulation with the antifungal β-glucanase gene.

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