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SEQUENCE CHARACTERISATION OF β–1, 3-GLUCANASE GENE FROM *HEVEA BRASILIENSIS* THROUGH GENOMIC AND cDNA CLONING

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Accumulation of different isoforms of β–1,3-glucanase gene has been reported in various plant species in response to pathogen infection and other forms of stress. Although their major role is in disease resistance, they are also involved in various developmental and physiological processes. The genomic and cDNA sequences encoding β–1,3-glucanase gene in *Hevea brasiliensis* were amplified with gene specific primers, which were designed based on the available cDNA sequence and conserved amino acid domains of different plant β–1,3-glucanases. Under optimal PCR conditions a 968 bp DNA fragment was amplified from genomic DNA. Reverse transcription and amplification of the cDNA also yielded a similar 968 bp fragment. These bands were cloned and sequenced. Both PCR and RT-PCR products were the same and showed homology with the previously reported sequences. No intron was present in the coding region of 316 amino acid final functional protein. Southern hybridisation confirmed the presence of a low copy number gene with no difference among the *Phytopththora* tolerant and susceptible *H. brasiliensis* clones studied. Northern hybridisation and RT-PCR analysis showed higher expression of β–1,3-glucanase in latex than in leaves. The possible roles of β–1,3-glucanase gene in combating the abnormal leaf fall disease in *Hevea* and its likely involvement in somatic embryogenesis are also discussed.

Key words: β–1,3-glucanase, cDNA cloning, Gene amplification, Gene expression, *Hevea brasiliensis*.

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INTRODUCTION

The β–1,3-glucanases (PR-2 proteins) have been well characterized in different plant species (Castresana *et al.*, 1990; Beerhues and Kombrink, 1994; Pneumans *et al.*, 2000). They exist as multiple structural isoforms that differ in size, isoelectric point, primary structure, cellular localization and pattern of regulation (Meins *et al.*, 1992) and are subdivided into different classes. The Class I and Class II enzymes are pathogen inducible basic proteins, the former accumulating predominantly in the vacuoles (Keefe *et al.*, 1990) and the latter in the extra cellular compartments (Ward *et al.*, 1991). An acidic extra cellular PR-2d protein induced during infection is classified as Class III, as it differs at least by 43% from Class I and Class II enzymes (Payne *et al.*, 1991). The Class IV isoforms of β–1,3-glucanase are non-responsive to pathogen infection (Van Eldick *et al.*, 1996).

Of the several important roles suggested for β–1,3-glucanases in plants, their possible role in defense against invading pathogens evokes major attention. Accumulation of different isoforms of β–1,3-glucanase has been reported in various plant species in response to pathogen infection, or by other forms of stress, such as treatment with elicitors, ethylene, hormones, chemicals or heavy metals as well as by wounding, low temperature, ozone and UV light (Thalmair *et al.*, 1996; Hincha *et al.*, 1997; Brederode *et al.*, 1991). Plants have developed a variety of constitutive and inducible mechanisms to resist the colonization of a potential pathogen. Induction and accumulation of PR-proteins including β–1,3-glucanases in the infected tissue was often observed in incompatible plant-pathogen interactions (Egea *et al.*, 1999; Philip *et al.*, 2001; Jebakumar *et al.*, 2001). β–1,3-Glucanase catalyzes the

endotype hydrolytic cleavage of β–1,3-glycosidic linkages in β–1,3-glucans, the major cell wall component in many pathogenic fungi. *In vitro* studies have shown that the purified protein can effectively promote the lysis of hyphal tips and thereby inhibit the fungal growth (Sela-Buurlage *et al.*, 1993). β–1,3-Glucanase can also act indirectly by releasing certain oligosaccharides from cell wall of pathogens, which can act as elicitors of defense reactions (Boller, 1995). Transgenic plants over-expressing β–1,3-glucanase have shown higher resistance against pathogens (Yoshikawa *et al.*, 1993; Jogendijk *et al.*, 1993).

The β–1,3-glucanases also have important roles in various physiological and developmental processes in healthy plants. These include cell division (Fulcher *et al.*, 1976), microsporogenesis (Worrall *et al.*, 1992), germination (Casacuberta *et al.*, 1992), senescence (Hanfrey *et al.*, 1996) and flowering (Van Eldik *et al.*, 1996). In *in vitro* plant cell culture, β–1,3-glucanases have associations with callus emergence. Auxin induced elongation of cell wall during callus formation involves the degradation of cell wall β–1,3-glucans by glucanase (Yoshida, 1995). High levels of β–1,3-glucanase activity was reported in embryogenic cultures, compared with the non embryogenic cultures of *Cichorium* (Helleboid *et al.*, 2000). During somatic embryogenesis, the callose deposition on the cell walls of embryogenic cells gradually disappears once the cells start to divide and develop into somatic embryos. Since callose (a mixture of β–1,3-glucans) is a potential substrate of β–1,3-glucanase, it was hypothesised that the enzyme plays an important role in somatic embryogenesis (Helleboid *et al.*, 2000; Dong and Dunstan, 1997).

The rubber tree, *Hevea brasiliensis* is the major source of natural rubber, the most important industrial raw material of plant origin. The abnormal leaf fall caused by *Phytophthora* spp. is the most destructive fungal disease of rubber in India. Molecular mechanisms involved in disease tolerance and somatic embryogenesis mediated plant regeneration are two among the thrust areas of research for crop improvement in *H. brasiliensis*. β–1,3-Glucanase play a crucial role in both these aspects.

A cDNA clone encoding a β–1,3-glucanase has previously been reported in *Hevea* (Chye and Cheung, 1995). In this study, an attempt has been made to isolate and characterise the genomic DNA and cDNA sequences of β–1,3-glucanase, which is a preliminary step for investigating the role of this protein in disease tolerance and somatic embryogenesis in *H. brasiliensis*.

MATERIALS AND METHODS

Isolation of nucleic acid

DNA was isolated from the leaves of *H. brasiliensis* clone RRII 105 by a modified CTAB method (Doyle *et al.*, 1990). About one gram leaf tissue was powdered in liquid nitrogen and suspended in 10 ml of isolation buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA and 100 mM Tris-HCl pH– 8.0) and the mixture was incubated at 55°C for 30 min. After removing the debris by centrifugation, the supernatant was extracted with phenol-chloroform-isoamyl alcohol (24:24:1). The aqueous phase was saved and the RNA was removed by 5 ml DNase free RNase (10 mg/ml). After extracting twice with chloroform, the DNA was precipitated with 0.6 volume isopropanol. The pellet was washed in 70% alcohol, dried and re-dissolved in TE buffer (pH- 8.0).

Leaf RNA was extracted as described by Venkatachalam *et al.* (1999). Latex RNA was isolated by a modified protocol of Kush *et al.* (1990). The latex was collected directly into the tube containing equal volume of extraction buffer (Tris-HCl 50 mM pH 9, LiCl 150 mM, EDTA 5 mM, SDS 2% and β-mercaptoethanol 0.1%). The tube was

immediately frozen in liquid nitrogen. After thawing, centrifugation was carried out at 14,000 rpm for 30 min at 4°C twice. Rubber particles were removed and the remaining solution was extracted with extraction buffer saturated phenol. After centrifugation, the aqueous phase was extracted twice with chloroform. The RNA was precipitated overnight by adding 1/3 volume of 8M LiCl. The RNA pellet was washed first with 2M LiCl and then with 70% ethanol. The pellet was dissolved in DEPC treated H_2O. For further purification, RNA was re-precipitated with 2.5 volumes ethanol in presence of 0.3 M sodium acetate and the pellet was re-dissolved in DEPC treated H_2O.

Amplification of β–1,3-glucanase gene through PCR

For the gene amplification, the primers Gl- 5′ TGCCCAGGTAGGTGTTTGC 3′ and G2- 5′ CCCAGTTCTTTTCTGCACC 3′ were used. These primers correspond to the conserved amino acid domains at N and C terminals of different plant glucanases. PCR was performed in 20 µl reaction containing 20 ng template DNA, 250 nM primers, 1X reaction buffer containing 1.5 mM Mg Cl_2, 100 µM each dNTPs and 0.5 U *Taq* DNA polymerase (Bangalore Genei, India). Reaction was performed in a Perkin Elmer 480 thermal cycler. Initial denaturation was performed at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min 30 sec and extension at 72°C for 2 min. A final extension was performed at 72°C for 7 min.

β–1,3-Glucanase cDNA amplification by reverse transcription (RT)-PCR

Total RNA from the latex and leaves were used as template for the synthesis of cDNA using a one step RT-PCR kit (Super Script, Invitrogen Life Technologies, USA). The primers used for β–1,3-glucanase gene amplification from the genomic DNA were used for the amplification of cDNA also. Both the primers (0.2 µM of each) with 1 µg of total RNA was used in a 50 µl reaction according to the manufacturer's protocol. cDNA was synthesized by incubation at 40°C for 30 min. The cDNA was amplified through PCR as described above.

Cloning and sequencing

Cloning of the PCR product has been carried out at the *Sma* I restriction site of plasmid vector pUC 18 through blunt end ligation (Sambrook *et al.*, 1989). The dephosphorylated vector was incubated overnight with end filled, phosphorylated PCR product at 16°C with 10 U of T4 DNA ligase. After transformation, the recombinants were selected by blue/white screening in X-gal medium. The sequencing of the cloned insert was done at the DBT facility for DNA sequencing, Indian Institute of Science, Bangalore, India, in an automated sequencer using M 13 forward and reverse primers. Sequence comparison was done through BLAST analysis (Altschul *et al.*, 1990) at the NCBI (National Centre for Biotechnology Information, US) site.

Southern hybridisation

Genomic DNA (10 µg) from two clones, RRII 105 and RRIM 600 was digested with 20 U each of *Bam*H I, *Sau*3A I and *Xba* I at 37°C overnight. The fragments were size fractionated in 1% agarose gels and blotted on to nylon membranes (Amersham). The β–1,3-glucanase probe was labeled with α-^{32}P labeled dCTP using 'Multiprime DNA Labeling System' (Amersham Pharmacia, UK). Hybridisation was performed for 16 hours at 65°C in a buffer containing 6X SSC, 5X Denhardt's solution and 0.5% SDS. The membrane was washed with 0.1X SSC, 0.1% SDS for 30 min at 65°C and exposed to X-ray film (Kodak).

Northern hybridisation

Total RNA from latex and leaves (20 µg) were separated in 1% formaldehyde denaturing agarose gel and transferred to nylon membranes. Hybridisation was done using the same probe at 42°C for 16 hours in a buffer containing 50% formamide, 5X SSC, 5X Denhardt's solution and 1% SDS. Filter was washed in 0.5X SSC, 0.1% SDS at 42°C for 15 min and exposed to X-ray film at -80°C with an intensifying screen.

RESULTS AND DISCUSSION

The β–1,3-glucanases which belongs to a large gene family, has been well characterised in different plant species and divided into four subgroups based on isoelectric point, expression pattern and sequence similarity (Van Eldik *et al.*, 1996). This enzyme activity has been implicated in plant responses to environmental stress, pathogen attack and in various developmental processes. In *H. brasiliensis*, an increased level of β–1,3-glucanase enzyme activity was reported in the leaves of tolerant clones in response to *Corynespora cassiicola* infection (Philip *et al.*, 2001). This enzyme has previously been isolated by screening of a latex cDNA library with a heterologous probe from *Nicotiana plumbaginifolia* (Chye and Cheung, 1995). In the present study both genomic and cDNA sequences coding for β–1,3-glucanase gene were amplified through gene specific primers.

Good quality DNA and RNA could be obtained from the leaves and latex by the methods described. The gene specific primers designed based on the cDNA sequence (Chye and Cheung, 1995) and conserved amino acid sequences of plant β–1,3-glucanases could, under optimal PCR conditions, amplify a 968 bp single product when genomic DNA was used as the template (Fig. 1). Reverse transcription and amplification of the cDNA also yielded a 968 bp band. The amplification was more when

Fig. 1. Gene amplified from the genomic DNA: a: Marker (lambda *EcoRI, Hind* III double digest), b: amplified product

latex RNA was used as the template (Fig. 2). This could be attributed to the abundance of β–1,3-glucanase mRNAs in the latex when compared with that of leaf. This was corroborated by an intense signal generated in northern hybridisation analysis (Fig. 3) with latex RNA. Chye and Cheung (1995) have also reported higher expression of β–1,3-glucanase gene in the laticifers than in leaves of *H. brasiliensis*. Constitutive expression of defense related genes like chitinase and lysozymes has also been reported in rubber tree latex (Martin, 1991). The transcription levels of plant defense or stress induced genes were 10-50 fold higher in laticifers compared to leaves (Kush *et al.*, 1990). Similarly, various enzymes stored in the laticifers of *Carica papaya* provide an important contribution to the plant defense mechanism by sanitizing and sealing the wounded areas on the tree (El Moussaoui *et al.*, 2001).

The PCR product could be cloned in plasmid vector pUC 18 through blunt end ligation at the *Sma* I site (Fig. 4). The cloned

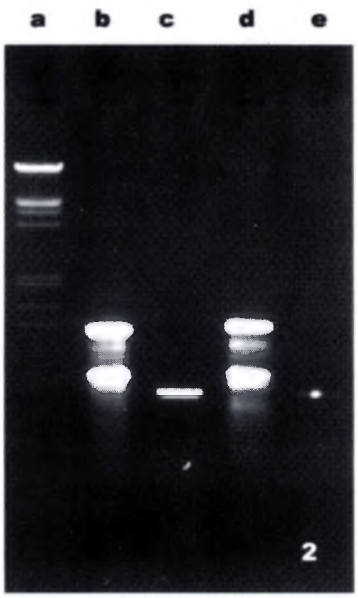

Fig. 2. cDNA amplified from latex and leaf RNA: a: Marker, b: RNA from latex, c: cDNA from latex RNA, d: RNA from leaf, e: cDNA from leaf

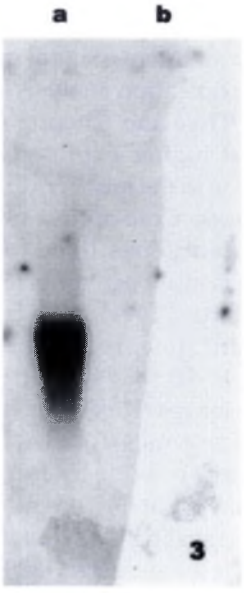

Fig. 3. Northern hybridisation showing high expression in the latex: a: RNA from latex, b: RNA from leaf

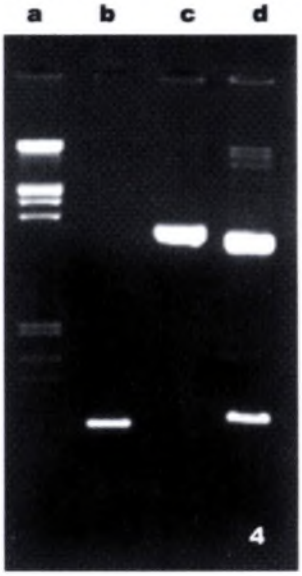

Fig. 4. Cloning of the gene in pUC 18: a: Marker, b: purified PCR product, c: plasmid with insert, d: insert released through restriction digestion

fragment could be successfully excised out using two restriction enzymes on either side of the cloning site. Sequence data (Fig. 5) for both the genomic and cDNA were found to be identical when subjected to online BLAST analysis. The present sequence shows high degree of homology to β–1,3-glucanase sequences reported earlier from rubber (Chye and Cheung, 1995; Yeang and Chow, 2000) (Fig. 6). Moreover, this sequence shows 30 to 60% similarity to the β–1,3-glucanase genes reported from different plants like *Phaseolus vulgaris* (Edington *et al.*, 1991). *Nicotiana tabacum* (Ohme-Takagi and Shishi, 1990), *N. plumbaginifoia* (Gheysen *et al.*, 1990), *Solanum tuberosum* (Beerhues and Kombrink, 1994) and *Pisum sativum* (Chang *et al.*, 1993) (data not shown). In rubber, β–1,3-glucanase is synthesized as a preprotein of 374 amino acids with N and C terminal extensions. These extensions are cleaved during or after transport to the vacuole (Chye and Cheung, 1995). The amplified fragment contains the

complete reading frame coding for the 316 amino acid final functional protein. By comparing the sequence of the cDNA and genomic DNA, it is found that no intron was present in the coding region of the mature protein. In most of the other cases introns are reported as in the case of *N. plumbaginifolia* β–1,3-glucanase, where two introns were observed in the coding region of the gene (Castresana *et al.*, 1990). Single exonic genes are very rare in the case of eukaryotes. Absence of mRNA splicing may facilitate the translational process for protein synthesis.

The abnormal leaf fall disease of *H. brasiliensis* is caused by the oomycete fungi, *Phytophthora* spp. This is a unique class of fungus as their cell wall is devoid of chitin, while most other fungi have chitin as the major component of cell wall (Wessels and Sietsma, 1981). In oomycetes, β–1,3-glucans comprise around 80 to 90% of the dry weight of cell wall. Therefore, β–1,3-glucanase may have a crucial role in combating the disease caused by *Phytophthora* spp. in *Hevea*. In the genomic Southern performed with DNA isolated from abnormal leaf fall tolerant (RRII 105) and susceptible (RRIM 600) *H. brasiliensis* clones, about 3-4 bands were obtained with the enzymes tested, indicating the presence of a low copy number gene (Fig. 7). As no difference was observed between tolerant and susceptible clones, the difference in tolerance to disease may not be due to difference in the copy number of the gene. However, timing and magnitude of induction might be different as observed in the case of pepper infected with *P. capsici* (Egea *et al.*, 1999). Any difference in the number and distribution of laticiferous vessels on leaves and petioles of various clones can also contribute to the clonal difference existing in susceptibility to the disease, as these are the main targets of *Phytophthora* infection.

It is also reported that the activity of β–1,3-glucanase enzyme will be considerably

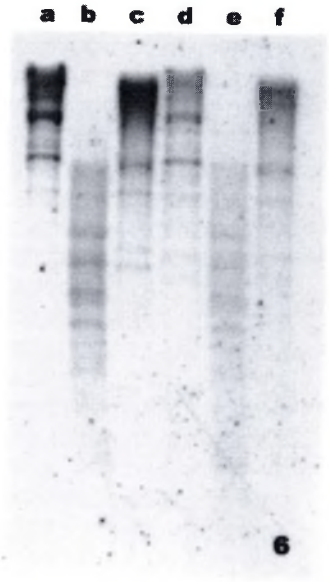

Fig. 7. Southern hybridisation with genomic DNA Lane a-c: Genomic DNA from clone RRII 105 digested with *BamH* I, *Sau* 3 and *Xba* I, d - f: DNA from clone RRIM 600 digested with the same enzymes

higher in the embryogenic cultures than in the non-embryogenic cultures (Helleboid *et al.*, 2000). Therefore, this gene can also be used as a marker for the early detection of embryogenic competence of emerging calli. A study on the regulation of enzyme activity during different stages of somatic embryogenesis will provide sufficient insight into the molecular mechanisms involved, which would further enhance the process of somatic embryogenesis and subsequent plant regeneration in *Hevea*.

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1     TGCCCAGGTAGGTGTTTGCTATGGAATGCAAGGCAACAACCTTCCACCTG  50
51    TTTCAGAGGTCATAGCTCTCTATAAAAAATCTAACATCACGAGAATGAGA 100
101   ATTTATGATCCAAATCGAGCAGTATTGGAAGCCCTTAGAGGCTCAAACAT 150
151   TGAACTCATACTAGGTGTTCCAAACTCAGATCTCCAAAGCCTTACCAATC 200
201   CTTCCAATGCAAAATCATGGGTACAAAAAAATGTTCGTGGCTTCTGGTCA 250
251   AGTGTCCTGTTCAGATATATAGCAGTTGGCAACGAAATTAGTCCTGTCAA 300
301   TAGAGGCACAGCTTGGTTGGCTCAATTTGTTTTGCCTGCCATGAGAAATA 350
351   TACATGATGCTATAAGATCAGCTGGTCTTCAAGATCAAATCAAGGTCTCT 400
401   ACTGCGATTGACTTGACCCTGGTAGGAAATTCCTACCCTCCTTCTGCAGG 450
451   TGCTTTCAGGGATGATGTTAGATCATATTTGGACCCAATTATTGGATTCC 500
501   TATCCTCTATCAGGTCACCTTTACTTGCCAATATTTATCCTTACTTTACT 550
551   TATGCTGGTAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTCACTTC 600
601   ACCATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTG 650
651   ATGCAACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGT 700
701   TCTCTGGAGGTGGTTGTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATT 750
751   TGCTGCCACATTTGACAATGGGCGTACTTATCTCTCAAATTTGATCCAGC 800
801   ATGTTAAAAGAGGTACTCCTAAGAGGCCTAACAGAGCTATAGAGACTTAC 850
851   TTATTTGTTATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAGAAACA 900
901   CTTTGGACTTTTCTTTCCTGATAAATGGCAAAAATATAATCTCAATTTTG 950
951   GTGCAGAAAAGAACTGGG                                  968
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Fig. 5. Nucleotide sequence of β-1,3-glucanase gene in *Hevea brasiliensis*. Both genomic and cDNA were sequenced. No introns were found within the coding sequences for the final functional protein.

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Hb.  QVGVCYGMQGNNLPPVSEVIALYKKSNITRMRIYDPNRAVLEALRGSNIELILGVPNSDL 60
Y&C  ------------------------------------------------------------
C&C  ------------------------------------------------------------

Hb.  QSLTNPSNAKSWVQKNVRGFWSSVLFRYIAVGNEISPVNRGTAWLAQFVLPAMRNIHDAI 120
Y&C  ------------------------------------------------------------
C&C  ------------------------------------------------------------

Hb.  RSAGLQDQIKVSTAIDLTLVGNSYPPSAGAFRDDVRSYLDPIIGFLSSIRSPLLANIYPY 180
Y&C  ------------------------------------N---R-----------------
C&C  ------------------------------------------------------------

Hb.  FTYAGNPRDISLPYALFTSPSVVVWDGQRGYKNLFDATLDALYSALERASGGSLEVVVSE 240
Y&C  -----------------------------------------V------------------
C&C  ----Y-------------------------------------------------------

Hb.  SGWPSAGAFAATFDNGRTYLSNLIQHVKRGTPKRPNRAIETYLFAMFDENKKQPEVEKHF 300
Y&C  ------------------------------------------------------------Q-
C&C  ----------------------------G-------------------------------

Hb.  GLFFPDKWQKYNLNFG                                             316
Y&C  ----------------
C&C  -------RP-------
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Fig. 6. Amino acid sequence of the mature *Hevea* β-1,3-glucanase protein deduced from genomic DNA and its comparison with the cDNAs reported earlier. Positions of identity are denoted by lines. Hb.: genomic DNA; Y & C: cDNA (Yeang and Chow, 2000); C & C: cDNA (Chye and Cheung, 1995)

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