Isolation and Molecular Characterisation of β-1,3-Glucanase Gene and Its Role in Combating Abnormal Leaf Fall Disease in Rubber Tree (*Hevea brasiliensis* Muell. Arg.)

Thesis

Submitted to

Mahatma Gandhi University
Kottayam

For the Partial Fulfillment of the Requirements for the award of the Degree of

Doctor of Philosophy
In BOTANY Under the Faculty of Science

By

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July 2004

The Rubber Research Institute of India
Rubber Board, Ministry of Commerce and Industry, Govt. of India
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July 05, 2004

Certificate

This is to certify that the thesis entitled "Isolation and Molecular Characterisation of β-1,3-Glucanase Gene and Its Role in Combating Abnormal Leaf Fall Disease in Rubber Tree (*Hevea brasiliensis* Meull. Arg.)" is an authentic record of original research work carried out by Mr. Thanseem Ismail at the Rubber Research Institute of India, under my supervision during the period of November 1999 – May 2004 for the award of the degree of Doctor of Philosophy in the Faculty of Science, Mahatma Gandhi University, Kottayam. It is also certified that the work presented in this thesis has not been submitted to any other degree or diploma earlier.

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Declaration

I, hereby declare that this thesis entitled "Isolation and Molecular ecular Characterisation of β-1,3-Glucanase Gene and Its Role in Combating Abnormal Leaf Fall Disease in Rubber Tree (*Hevea brasiliensis* Muell. Arg.)" is a bona fide record of the research work done by me at the Rubber Research Institute of India, Kottayam and that no part of this work has been presented earlier for any degree or diploma in any other university.

Kottayam
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Acknowledgements

I wish to express my indebtedness and gratitude to Dr. A. Thulaseedharan (Deputy Director, Biotechnology Division, Rubber Research Institute of India, Kottayam), my Ph. D. supervisor, for his encouraging guidance, constant support and valuable suggestions, which have helped a lot in my Ph. D. work, and the final structuring of my thesis.

I owe a special thanks to Dr. N. M. Mathew, Director, RRII, for allowing me to undertake the programme at RRII and for providing the necessary infrastructure support for the successful completion of my Ph. D. program. I also express my gratitude to Dr. K.R. Vijayakumar, Joint Director (Research), RRII.

It is my pleasure to acknowledge the Council for Scientific and Industrial Research (CSIR), New Delhi, for providing the financial support for my Ph. D. program.

Sincere thanks are due to Dr. George Thomas (Inter-field Laboratories, Kochi) and Dr. R. V. Omkumar (Scientist, Rajiv Gandhi Centre for Biotechnology, Trivandrum); lively discussions with them have helped to improve the overall quality of my work.

I record my deep sense of gratitude to Dr. P. Venkatachalam, Dr. S.Sushamakumari, Mrs. P. Kumari Jaysree, Mrs. S. Sobha, Mrs. R.G. Kala, Mrs. K. Rekha and Mrs. R. Jayashree, Scientists, Biotechnology Division, RRII, for their encouragement and advice. I would like to express my warm thanks to Mrs. A. Saleena and Ms. P. Priya, Ph. D. students, Biotechnology Division and Mrs. P. Leda (Scientific Assistant) for their co-operation and help. The services offered by all the supporting staff of the Biotechnology Division are greatly appreciated.

I thank all the RRII Scientific Staff especially, Dr. Kuruvilla Jacob (Dy. Director, Pathology Division), Dr. Annakkutty Joseph, Mr. Sabu P. Idikula, Dr. Shaji Philip (Pathology Div.), Dr. Thakurdas Saha (Genome Analysis), Dr. C.P. Reghu (Germplasm Div) and Mr. M.B. Mohammed Sathik (Physiology Div), for their help extended during my Ph. D. work. I would also like to thank Mr. Thomson Abraham and Mr. Francis Mathew, Research fellows, RRII for their help during the preparation of the manuscript. I am grateful to Mr. B. Biju, (Asst. Director, Systems, RRII) and Mr. K.P. Paulson (Mechanic, RRII) for their valuable technical services rendered. The support and goodwill of the administrative and library staff of RRII are also remembered and appreciated.

Last, but not the least, I would like to express my sincere gratitude to my parents for their blessings and encouragement.

Contents

1

INTRODUCTION

Plants are means for the human survival and comforts starting from food, home to several other requirements. A wide range of microorganisms including fungi, bacteria and viruses are also exploiting plants as a source of food and shelter, as in the case of all living forms on earth. Some of these can act as pathogens, which cause a variety of diseases that result in significant yield reduction leading to heavy economic loss in many of the agricultural and horticultural species. Among these, fungal diseases are rated as the most prevalent biotic stress contributing to yield loss (Grover and Gauthaman, 2003). According to a recent survey, contribution of fungal diseases towards total yield loss of important crops in India is 18 – 31% (Grover and Pental, 2003). Incidence of plant diseases can be controlled conventionally by application of agro-chemicals or by breeding new varieties that contain new resistance conferring genes. The uncontrolled use of many agro-chemicals is environmentally hazardous and it also increases the input cost of farmers. Breeding for resistance is time consuming and has to be a continuous process since new races of pathogens evolve and crops become susceptible. Therefore, novel alternative strategies that would circumvent these problems are required to produce fungus resistant crop varieties. An understanding of the molecular mechanisms involved in disease resistance would be necessary for the development of any such alternative molecular tools for crop improvement. Molecular mechanism of disease resistance involves the structure, organization, expression and regulation of genes that govern plant-pathogen interactions.

Nature has not provided any other industrial raw material of plant origin as flexible as natural rubber. Rubber has number of applications and there is hardly any segment of life, which does not make use of rubber-based material. About 80%

of the natural rubber is used in the motor industry for the manufacture of tires followed by electrical industry. Natural rubber cannot be completely substituted by synthetic rubber because of its unique mechanical properties. Majority of the rubber producing plants belong to the family Moraceae, Euphorbiaceae and Apocyanaceae. Although 50 species are available as sources, only a few have been commercially important. *Hevea brasiliensis*, which belongs to the family, Euphorbiaceae accounts for 99% of the world's natural rubber production (Wycherley, 1992). At present, more than 9.5 million ha in about 40 countries are devoted to rubber tree growth, producing 6.5 million tons of dry rubber each year worldwide (Chen *et al.*, 2003). The British introduced the rubber tree in India and by first half of 20th century many plantations were successfully established in the country (Thomas and Panikkar, 2000). Today, India is the fourth highest producer of natural rubber in the world having a total of 5.7 lakh ha under rubber cultivation (Rubber Statistical Bulletin, 2004) with a total annual production of 6.5 lakh tons (Rubber Statistical News, 2004). More than 98% of the rubber production in India is from traditional rubber growing tracts of Kerala and parts of Tamil Nadu and Karnataka.

Various diseases, most of them are fungal in origin, that strikes through the rubber plantations are responsible for considerable loss in terms of latex yield in every year. Abnormal leaf fall caused by *Phytophthora*, powdery mildew by *Oidium hevea*, and leaf spot by *Cornyspora cassicola* are major fungal diseases affecting the rubber tree. Abnormal leaf fall (ALF) is the most economically significant fungal disease in India. Initial field trails indicated that this disease could cause yield loss up to 38 to 56%, when left unsprayed for one disease season (Ramakrishnan, 1960). Ongoing studies indicate up to 48% yield loss in susceptible clones due to ALF disease (Jacob, 2003).

Although, many promising clones with a fair degree of tolerance to ALF disease were evolved through conventional breeding programmes from the initial germplasm collections of the 19th century, a fully resistant clone to *Phytophthora* with high yield has so far not been developed. Such conventional breeding programmes are elaborate, labour intensive and time consuming particularly in the case of perennial tree crops like *Hevea*. Millions of rubber plantations across Asia

and Africa had actually originated from a minuscule of seeds collected from three specific localities of Amazon River basin in Brazil by Sir. Henry Wickam in 1876 (Imle, 1978). This narrow genetic base along with its long breeding cycle and high juvenility period, which extends up to seven years, makes the conventional crop improvement programmes rather difficult in rubber. Further, the time taken for identification of parents with desired characters, their highly heterozygous nature and difficulties in raising F_2 progenies has slowed down the genetic improvement programmes in the last few decades. All these emphasize the need for biotechnological interventions in crop improvement programmes in *Hevea*. However, classical molecular approaches to tag *R* genes like construction of segregating populations or near isogenic lines will be difficult in a tree crop. Molecular studies revealed that none of the *R* genes isolated so far code for any toxic chemicals with direct fungicidal actions. This job is being done by another group of genes called defense genes. An understanding of the role of such an established defense-related gene like β-1,3-glucanase could be a viable alternative approach for the development of more sophisticated molecular tools to combat ALF disease in *Hevea*.

Several classes of proteins, called pathogenesis related (PR) proteins, are induced in response to the infection of plants with microbial pathogens. β-1,3- Glucanases are included in the family of PR-2 proteins, which are abundant highly regulated enzymes widely distributed throughout the plant kingdom. They are able to catalyse the endo-type hydrolytic cleavage of the 1,3-β-D- glucosidic linkage in β-1,3-glucans. The cell wall of *Phytophthora*, causative organism of ALF disease, is made principally of β-1,3-linked glucans, which constitutes 80 – 90 % of the cell wall dry weight. Therefore, host β-1,3-glucanase enzyme might have a pivotal role in combating the ALF disease in *Hevea*.

Although, the major interest in β-1,3-glucanase is because of their possible role against the invading pathogens, there is strong evidence that these enzymes are also implicated in diverse physiological and developmental processes in uninfected plants including microsporogenesis, fertilization, seed germination, cell division, flowering, fruit ripening and somatic embryogenesis. Over the past 12 years, considerable progress has been made in understanding the structure and regulation

of plant β-1,3-glucanases. It is now recognized that species of higher plants produce a broad range of β-1,3-glucanases differing in primary structure, cellular localization and catalytic activity. Many of them were proved as effective anti-fungal agents both *in vitro* and when over-expressed in transgenic plants. In the present study, an attempt has been made for the isolation and characterisation of β-1,3-glucanase gene in *Hevea* and to find out its role in the defense mechanism of the plant against *Phytophthora* attack. The results could be utilised to explore the possibility of a transgenic approach to control the fungal pathogens. β-1,3-Glucanases have been reported as a part of plant's general or horizontal resistance, which is more durable, and hence commercially more attractive, compared to the race-specific resistance mediated by the *avr-R* gene interaction that will breakdown rapidly whenever a new race of virulent pathogen evolves.

Therefore, the present study has been undertaken with following major objectives.

Objectives of the Study

1. Isolation, cloning and characterisation of genomic and cDNA sequences coding for β-1,3-glucanase gene in *Hevea*.

2. Investigation of the role of β-1,3-glucanase in combating ALF disease. This involves studying the expression patterns of the gene through northern hybridisation and RT- PCR analysis in the infected tissues of tolerant and susceptible clones, after challenge with the pathogen.

3. Isolation of the promoter elements of the gene and to characterise the regulatory sequences in the promoter.

4. Construction of a functional cDNA clone for the bacterial expression of *Hevea* β-1,3-glucanase and purification of the recombinant protein.

5. Study of the *in vitro* anti-fungal properties of *Hevea* β-1,3-glucanase.

2

REVIEW OF LITERATURE

In contrast to animals, plants are unable to move their locations and they lack a circulating adaptive immune system to protect themselves against pathogens. Yet, plants are not passive hosts to the constant onslaught of microorganisms with which they interact in their environment. Like most other eukaryotic organisms, plants defend themselves against an array of pathogenic weapons with an innate arsenal of defense mechanisms. The understanding of how plants 'sense' or 'identify' a foreign microbe as a pathogen and how they interact with it, is beneficial not only to our basic understanding of how these processes work, but also has practical applications for crop improvement.

2. 1. Defense Mechanisms in Plants – An Overview

2. 1. 1. Pre-existing defense structures

Pathogens have developed sophisticated weapons to attack plants and plants have developed diversified defense strategies to ward off pathogens. The resistance can be pre-formed or induced. Pre-formed resistance involves the factors, which are present in plants prior to the contact of pathogen. This includes surface features of plants such as thickness of the cuticle, configuration of cuticular wax, topography of leaf surface, degree of stomatal opening etc. A thick cuticle may increase resistance to infections in which the pathogen enters its host only through direct penetration. Waxes on leaf and fruit surfaces form a water repellent surface, thereby preventing the formation of a thin layer of water on which, pathogen spores might be deposited and germinate. Thick, tough outer walls of epidermis make direct penetration by fungal pathogens difficult or impossible. All these structural features cause delay in pathogen penetration, thereby contributing significantly to plant defense by allowing time for the induction of more sophisticated defense mechanisms.

In addition to these structural barriers, some plants exude a variety of chemical substances even before infection of a pathogen through the surface of their above ground parts as well as through the surface of their roots. Many of these compounds are shown to have inhibitory action against certain pathogens. Fungi-toxic exudates on the leaves of some plants like tomato and sugar beet has been found to inhibit the germination of spores of the fungi *Botrytis* and *Cercospora* respectively. Similarly, in the case of onion smudge caused by the fungus *Colletotrichum circinans*, resistant varieties were generally found to have red scales that contain the phenolic compounds, protocatechuic acid and catechol. These fungitoxic substances could inhibit the growth of conidia (Nicholson and Hammerschmidt, 1992). The fungitoxic exudates were missing in white-scaled susceptible onion varieties. Several phenolic compounds, tannins and some fatty acid-like compounds such as dienes, which are present in high concentrations in the cells of young fruits, leaves or seeds are potent inhibitors of several hydrolytic enzymes, including proteolytic macerating enzymes of plant pathogens. Several other types of pre-formed compounds such as avenacin, a saponin from oats, exclude fungal pathogens from infecting host, which lack enzymes that breakdown saponin (Osbourn, 1996).

2. 1. 2. Induced biochemical defense

Plants have induced cellular defenses as well that prevent further colonization of the tissues, once the structural barriers of the host have been breached by the invading pathogen. These induced defenses are described as active defense mechanisms because they are a response to invading pathogen and require host metabolism to function. Induced or active resistance involves substances either absent, or present in very low levels before infection, and are produced, or activated during infection. Infection of resistant plants by a pathogen triggers a complex series of biochemical and cellular events at or near the site of infection, often referred to as hypersensitive reaction (HR) (Agrois, 1988). The resistance involving HR is characterised by localized cell and tissue death at the site of infection (Van Loon, 1997). As a result, the pathogen remains confined to necrotic lesions near the site of infection. A ring of cells surrounding the necrotic lesions become fully refractory to subsequent infection, referred to as localized acquired resistance (Baker *et al*, 1997; Fritig *et al.*, 1998). These local responses often

trigger non-specific resistance throughout the plant. Thus, the whole plant becomes resistant to the challenge of infection by broad range of pathogens after a previous and local interaction with a pathogen. This long lasting non-specific defense is known as systemic acquired resistance (SAR) (Ryals *et al.*, 1996).

2. 1. 3. Recognition of the pathogen

Early recognition of the pathogen by the plants is very essential if the plant is to mobilize the induced defense to protect itself from the pathogen. This specific recognition process triggers activation of inducible defenses (Fig. 1). Fungal spores adhere to the host cell surface when they come in contact with it. In most cases, the fungal spores produce a mucilage composed primarily of a mixture of glycoproteins as an adhesive material (Ramadoss *et al.*, 1985). The plant apparently begins to receive signal molecules that indicate the presence of a pathogen, as soon as the pathogen establishes physical contact with the host cell surface. These signal molecules are known as elicitors, recognition of which by the host plant results in the activation of defense mechanisms. These elicitors include a variety of substances like glycoproteins (*Phytophthora megasperma* in soybean hypocotyls; Keen, 1971), carbohydrates like β-1,3 glucan or chitin (*Fusarium moniliforme* in rice; Ren and West, 1992), fatty acids like arachidonic acid (*Phytophthora infestans* in potato; Bostock *et al.*, 1981) or peptides (Ricci *et al.*, 1992). Several members of *Phytophthora* spp. produce a novel family of protein elicitors called elicitins. Cryptogein, an elicitin from *P. cryptogea*, elicits the production of ethylene and phytoalexin in tobacco cell suspension cultures (Blein *et al.*, 1991). It was also seen that several kind of elicitors were produced by a single pathogen. *P. infestans*, the potato late blight pathogen. It induces the production of some fatty acids, β-1,3 glucan and a peptide called infestin, as elicitors (Bostock *et al.*, 1986).

2. 1. 4. The 'gene-for-gene' hypothesis

Pathogen elicitors usually have receptor molecules at the host cell surface. When a particular receptor molecule in plant binds with the pathogen elicitor, it is assumed that the plant recognize the pathogen. The newly emerged tools of molecular biology refines this model and leads to the hypothesis that a ligand

produced by the pathogen interacts, presumably directly, with a corresponding plant receptor, which then triggers activation of defense response. The genetics of host-pathogen interactions was first studied in detail by Flor (1956) using flax (*Linum usifatissimum*) and flax rust (*Melampsora lini*) as a model system. The conclusion from this study was, "for each gene that confers virulence to the pathogen there is a corresponding gene in the host that confers resistance to the host and vice versa". This hypothesis is popularly known as 'gene-for-gene' hypothesis. Generally, in the host, the genes for resistance (*R* genes) are dominant over genes for susceptibility. In the pathogen, on the other hand, it is the genes for avirulence (*avr* genes) are usually dominant, while the genes for virulence are recessive. Incompatible plant-pathogen interaction involves the recognition of the pathogen *avr* gene product (elicitor) by the host *R* gene product (receptor molecule). Mutation of an existing *avr* gene of a pathogen avoids gene-for-gene recognition by the host *R* genes and the resistance of the host breaks down. However, through survival pressure and selection a new *R* gene will soon evolve that encodes a new or modified receptor that can recognize the modified elicitor molecule produced by the mutated *avr* gene. In this way numerous diverse *R* genes have evolved in a plant host to counteract corresponding virulence genes in the various races of the pathogen. The gene-for-gene interaction has occurred in a step -wise fashion over time, and continues till date. Plant breeders apply the gene-for-gene concept every time they incorporate a new resistance gene into a desirable variety that is susceptible to a new strain of pathogen.

The first *R* gene isolated was maize *Hm 1* gene (Johal and Briggs,1992), that makes certain varieties of corn plants resistant to race 1 of *Cochliobolus carbonum* by inactivating the HC toxin produced by the fungus. During the last decade, more than 30 *R* genes, which confer resistance against a wide range of pathogens including viruses, bacteria, fungi and nematodes have been isolated and cloned, from both monocots and dicots (Takken and Joosten, 2000; Hulbert *et al.*, 2001). Various plant *R* genes have several structural similarities and can be sub-divided into five classes based on the predicted protein structures. Despite the differences between the various classes of *R* genes characterised to date, majority of them encode signal transduction proteins suggesting that different plants utilise similar strategies in the prevention of diseases. All the R proteins contain a common motif of leucine rich repeat (LRR) with the only exception of R protein of

tomato Pto. Besides the LRR domain, some R proteins contain nucleotide binding sites (NBS) and/or leucine zipper (LZ) (Grover and Gowthaman, 2003). LRRs are believed to mediate protein-protein interactions or determine specific recognition of ligands by the receptor molecules (Kobe and Deisenhofer, 1994). One group of LRR containing resistance gene include the *Cf* class from tomato, comprising *Cf2, Cf4, Cf5,* and *Cf9* (Parniske *et al.*, 1997). They provide resistance to the fungal pathogen *Cladosporium fulvum.*

The gene-for-gene model was well demonstrated at least in three plant-pathogen interactions. These are the *Pto-avrPTO* system in tomato-*Pseudomonas* interaction (Tang *et al.*, 1996), the *Pita-avrPITA* in rice-*Magnaporthe* interaction (Jia *et al.*, 2000) and the *TIP-TCV* coat protein in *Arabidopsis-Turnipcrinkle* virus interaction (Ren *et al.*, 2000). Efforts are on to establish the functional roles of numerous *R* genes. All the receptors show high degree of specificity for pathogen strains and individual *R* genes have only narrow recognition capabilities (Odjakova and Hadjiivanova, 2001). Thus, specific recognition of an aggressor by a plant requires the presence of matching *avr* and *R* genes respectively in them. Despite significant insights into the *R* gene structure and function in the last decade, much remains to be elucidated about the molecular mechanisms by which, R proteins recognize and transduce information in plants.

2. 1. 5. Signal transduction and the hyper sensitive response

The hypersensitive response is the culmination of plant defense responses initiated by the receptor-mediated recognition of the pathogen elicitor at the site of infection. Activation of the multi-component defense responses at local and systemic levels result in the rapid establishment of local resistance and delayed development of systemic acquired resistance (Scheel, 1998).

The immediate effect of invasion of a pathogen into the plant is reported to be an oxidative burst that results in cytotoxicity leading to HR, which prevents further advance of pathogen (Keppler *et al.*, 1989). Changes in the permeability of plasma membrane lead to calcium and proton influx and potassium and chloride efflux (McDowell and Dangl, 2000). Ion fluxes subsequently induce extra-cellular production of cytotoxic reactive oxygen intermediates such as superoxide (O_2^-),

hydrogen peroxide (H_2O_2) and hydroxyl free radical (OH^-) within the infected cell (Somssich and Hahlbrock, 1998). Normal cells appear to have a balance between them to escape from cytotoxicity. Several scavengers like catalase and peroxidase that removes OH^- and H_2O_2 and superoxide dismutase that removes O_2^- are present in the plant cells to keep this balance. It was reported that in susceptible varieties, these scavengers might be highly active, preventing the oxidative burst and the resulting fungal toxicity (Gonner and Schlosser, 1993). The burst of H_2O_2 production at the plant cell surface drives rapid peroxidase mediated oxidative cross-linking of structural proteins in the cell wall, thereby reinforcing this physical barrier against pathogen ingress (Scheel, 1998). Lipoxigenase activity was also shown to be increased by pathogen attack (Koch *et al.*, 1992). Several lipid-breakdown products are shown to be fungi toxic (Namai *et al.*, 1991).

The changes in the ion flux and oxidative burst are crucial for further signal transduction events resulting in a complex, highly integrated signaling network. The localized production of reactive oxygen intermediates and nitric oxide can act as second messengers for HR induction and defense gene expression (Piffanelli *et al.*, 1999). Another components of the signaling network is the specifically induced phospholipases, which act on lipid bound unsaturated fatty acids within the membrane, resulting in the release of linolenic acid, which in turn serve as a substrate for the production of jasmonate, methyl jasmonate and related molecules via a series of enzymatic steps. Most of the inducible defense-related genes are regulated by signal pathways involving one or more of the three regulators jasmonate, ethylene or salicylic acid (Delaney *et al.*, 1994; Reymond and Farmer, 1998). During SAR, salicylic acid levels rise throughout the plant. Defense genes such as pathogenesis related (PR) genes are expressed and plant becomes more resistant to pathogen attack (Fig. 1). Plants that cannot accumulate salicylic acid due to the presence of a transgene that encodes salicylic acid degrading enzyme do not exhibit systemic expression of defense genes (Glazebrook, 1999).

The activation of signal transduction network after pathogen recognition results in reprogramming of cellular metabolism, involving large changes in the expression of many downstream defense genes for PR proteins, enzymes involved in the generation of phytoalexins, the enzymes of oxidative stress protection, tissue repair, lignification, *etc.* Phytoalexins are low molecular weight toxic antimicrobial

Plant Defense Responses

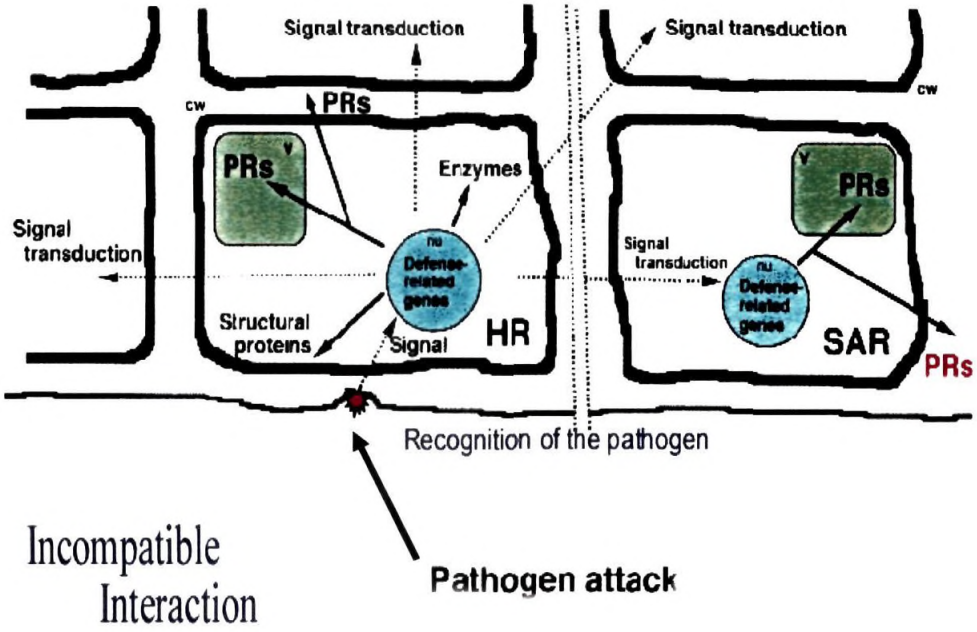


Fig. 1- Development of hypersensitive response and systemic acquired resistance in a resistant plant on infection with a pathogen

secondary metabolites produced by healthy cells adjacent to the localized infected necrotic cells, in response to materials diffusing from the damaged cells. Resistance occurs when one or more phytoalexins reach a concentration sufficient to restrict pathogen development. More than 300 compounds with phytoalexin-like properties have been isolated from plants belonging to more than 30 families. Chemically phytoalexins can be isoflavanoids, terpenoids, coumarins, polyacetylenes, *etc.* (Ebel, 1986). The mechanism of phytoalexin biosynthesis has been well studied in case of the bean phytoalexin, phaseollin. *De novo* synthesis or induced activities of important enzymes involved in the phaseollin biosynthesis like, phenylalanine ammonia-lyase, chalcone synthase, chalcone isomerase, *etc.* has been detected in response to fungal infection (Bell *et al.*, 1984; Robbins and Dixon, 1984; Bianchini *et al.*, 1999). Transgenic plants, which are deficient in phytoalexin biosynthesis, were shown to have increased susceptibility to fungal pathogen (Thomma *et al.*, 1999).

2. 1. 6. Pathogenesis related proteins

In several plant species, upon infection with a pathogen, development of symptoms is accompanied by *de novo* synthesis of one or more new proteins. Such proteins were first identified in tobacco, reacting hypersensitively to tobacco mosaic virus (TMV) (Van Loon and Van Kammen, 1970). Since these proteins are induced under specific pathological conditions, they have been described as pathogenesis related proteins (PR proteins). Later, PR proteins were shown to be induced not only by pathogens, but also by abiotic stresses like wounding, ethylene treatment, UV light, heavy metals, *etc.* The PRs accumulate in the infected tissues during HR and are also induced systemically with the development of SAR, against further infection by the pathogen. Induction of PRs has been found in many plant species belonging to various families (Van Loon and Van Strien, 1999). Based first on serological properties, and later on sequence data, the major tobacco PR proteins have been grouped in to five major classes (PR-1 to PR-5). Members of all the five PR families have been shown to have anti-fungal activity (Linthrost, 1991). Later, as additional pathogen-induced proteins with potential anti-pathogenic action were being described from various plant species, a uniform nomenclature for PRs was proposed based on their grouping into families sharing amino acid sequences, serological relationship and enzymatic or biological activity

(Van Loon *et al.*, 1994). By then, 17 families were recognized and classified for various plant species (Table-1).

The family of PR-1 consists of low molecular weight proteins. Their biological function is not clearly understood; nevertheless, constitutive expression of PR1*A* gene in tobacco enhances resistance of the plant to *Peronospora tabacina* (Alexander, 1993). Three distinct 14 kD PR 1 proteins isolated from tomato exhibited anti-fungal activity against *Phytophthora infestans* (Niderman *et al.*, 1995). The PR-2 groups of proteins show β-1,3 glucanase activity that can hydrolyse β-1,3 glucan, which are components of fungal cell wall (Yoshikawa *et al.*, 1993). Pathogen-induced over-expression of many isoforms of β-1,3 glucanases, both acidic and basic forms have been reported from many plant species including tomato, tobacco, maize, pepper, wheat and melon (Joosten and DeWit, 1989; Ward *et al.*, 1991; Lozovaya *et al.*, 1998; Egea *et al.*, 1999; Kemp *et al.*, 1999; Rivera *et al.*, 2002).

The PR-3, PR-4, PR-8 and PR-11 families of proteins are classified as endochitinases, which differ in their specific activity, catalytic mechanism and primary structure. They were the first pathogen-induced proteins whose function was identified. They hydrolyse the β-1,4 linkage between *N*-acetylglucosamine residues of chitin, a structural polysaccharide of the cell wall of many fungi. The PR-3 family of chitinase has been classified into six major structural classes (Meins *et al.*, 1994). Pathogen- induced over-expression of different classes of chitinases has been reported in various plant species. Hevein, an anti-fungal protein isolated from the lutoid bodies of the latex of rubber tree (Walujono *et al.*, 1975) and wound induced genes (*WIN 1* and *WIN 2*) of potato (Stanford *et al.*, 1989) are included in the PR-4 family.

The PR-5 family includes the thaumatin-like proteins with homology to permatins that permeabilise the fungal membranes (Vigers *et al.*, 1991). Some members of this family have been shown to possess anti-fungal activity, particularly against Oomycetes (Takemoto *et al.*, 1997). PR-6 includes proteinase inhibitors implicated in defense against insects and other herbivores, microbes and nematodes (Koiwa *et al.*, 1997). PR-7 has so far been characterised only in tomato,

Table 1- Recognized families of pathogenesis-related proteins

Family	Properties	Type member	Reference
PR-1	anti-fungal	Tobacco PR-1a	Antoniw *et al.*, 1980
PR-2	β-1,3-glucanase	Tobacco PR-2	Antoniw *et al.*, 1980
PR-3	chitinase type I, II, IV, V, VI, VIII	Tobacco P, Q	Van Loon, 1982
PR-4	chitinase type I, II	Tobacco 'R'	Van Loon, 1982
PR-5	thaumatin-like	Tobacco S	Van Loon, 1982
PR-6	proteinase-inhibitor	Tomato inhibitor I	Green and Ryan, 1972
PR-7	endoproteinase	Tomato P_{69}	Vera and Conejero, 1988
PR-8	chitinase type III	Cucumber chitinase	Metraux *et al.*, 1988
PR-9	peroxidase	Tobacco 'lignin-forming peroxidase'	Lagrimini *et al.*, 1987
PR-10	'ribonuclease like'	Parsley 'PR-1'	Somssich *et al.*, 1986
PR-11	chitinase type I	Tobacco 'class V' chitinase	Melchers *et al.*, 1994
PR-12	defensin	Radish Rs-AFR3	Terras *et al.*, 1992
PR-13	thionin	Barley LTP4	Bohlmann, 1994
PR-14	lipid-transfer protein	Barley OxOa (germin)	Garcia-Olmedo *et al.*, 1995
PR-15	oxalate oxidase	Barley OxOLP	Zhang *et al.*, 1995
PR-16	'oxalate oxidase'-like	Tobacco PRp27	Wei *et al.* 1998
PR-17	lipid-transfer protein	unknown	Okushima *et al.*, 2000

where, it is a major PR and acts as an endoproteinase. As the lysis of the fungal cell wall often requires degradation of cell wall proteins in addition to hydrolysis of chitin and glucan, it was proposed that PR-7 serve as an accessory to antifungal action (Van Loon and Van Strien, 1999). A peroxidase from tobacco that is involved in lignin depositions to strengthen the cell wall in reaction to microbial attack was later classified as PR-9 (Lagrimini *et al.*, 1987). The PR-10 family is structurally related to ribonucleases (Moiseyev *et al.*, 1997) and is supposed to be active against viruses. However, its ability to cleave the viral DNA remains to be demonstrated. The PR-12 type defensins, PR-13 type thionins and PR-14 type lipid transfer proteins all exhibit anti-fungal and anti-bacterial activity (Terras *et al.*, 1992; Bohlmann, 1994; Garcia-Oimedo *et al.*, 1995). Not all families of PRs have been identified in each plant species examined, suggesting that plant species differ in the types of PRs present or at least expressed upon infection.

2. 2. The Abnormal Leaf Fall Disease

2. 2. 1. *Phytophthora* spp.- The Unique Class

Phytophthora is a remarkable genus of plant pathogenic fungi. The name *Phytophthora* is derived from Greek and literally means plant (phyto) destroyer (phthora). It belongs to the Kingdom Chromista, Class Oomycete and Family Phytiaceae. Its unique morphological, genetic and physiologic features combined with the wide range of diseases caused in large number of plant species, make *Phytophthora* one of the most fascinating subjects for investigation. The complicated life cycle with very distinct and strikingly different spore forms ranging from motile zoospores to thick walled oospores, make management of diseases caused by *Phytophthora* difficult and time consuming. Although, they are commonly referred to as fungi, *Phytophthora* and other Oomycetes have number of biological characteristics that are relatively uncommon in other fungi (Griffith *et al.*, 1992). The major part of their life history is primarily diploid, whereas other higher fungi are haploid. The cell walls of *Phytophthora* are composed of cellulose and β-1,3 linked glucan and not chitin, which is the common cell wall component of fungi outside Oomycetes (Bartnicki-Garcia and Wang, 1983). The cell wall is made principally of β-1,3 linked glucose polymers, which comprise about 80 – 90% of the cell wall dry weight. The cytoplasm of *Phytophthora* spp. contains mycolaminarin, a β-1,3 linked glucan, as the characteristic storage polysaccharide instead of glycogen (Wang and Bartnicki-Garcia, 1974). Another unique feature is their ability to produce biflagellate heterokont zoospores. Most of them can produce sexual oospores that are not only capable of surviving for long periods under harsh environmental conditions, but also are sources of genetic variability in the progeny. These resting spores can germinate to produce single or multiple germ tubes, and when the environmental conditions, the most important of which is the availability of water, become favaorable, a rapid production of asexual sporangia and zoospores will occur. This recapitulation of the asexual cycle of the pathogen results in the rapid amplification from an initial infection. All the Oomycetes microorganisms have a short generation time and great reproductive capacity (Dick, 1992). For this reason, *Phytophthora* caused diseases of fruit, foliage, crowns and roots of plants are generally considered to be multi-cyclic that results in severe epidemics.

There are 67 recognized species of *Phytophthora*, that cause disease in a wide range of agricultural, horticultural and ornamental plants as well as causing devastating diseases in native ecosystems, which account collectively for multibillion dollar losses in world cash crops. The most prominent examples are *P. infestans* and *P. palmivora*. The importance of these groups of organisms has historic as well as economic impact. Historically, their importance predate the development of the germ theory of diseases and the science of plant pathology has its origin in the analysis of the major disease epidemic of the 19th century, the Irish potato famine (1845 – 46) caused by *P. infestans* (Erwin and Ribeiro, 1996). *P. palmivora* alone attacks over 80 plant species including plantation crops like cocoa, coconut, pepper, oil palm and rubber. They cause shoot rot, patch canker and black stripe in addition to abnormal leaf fall in rubber in different rubber growing countries.

2. 2. 2. Attacks on *Hevea*

The most destructive disease of rubber in India is the abnormal leaf fall (ALF) caused by different species of *Phytophthora* (Fig. 2). Four species viz. *P. palmivora*, *P. meadii*, *P. nicotianae* var. *parasitica* and *P. botryose* have been isolated from infected specimens. However, the species most common in India is *P. meadii* (Edathil *et* al., 2000). The first instance of this disease in India was reported in 1910 from Palapilly estates in Thrissur dist. Kerala state (McRae, 1918). Later the disease had spread to other rubber growing areas of the country and subsequently to other rubber growing countries like Sri Lanka, Myanmar, Cambodia, Vietnam, Nigeria, Cameroon, Brazil, Costa Rica and Venezuela. Outbreak of the disease was also noticed in major natural rubber producing countries like Malaysia and Thailand (Chee, 1969). Though the disease occurs in several countries, severe incidence necessitating adoption of control measures every year is observed only in the South India.

2. 2. 3. Symptoms of the disease

In South India, the disease occurs annually in the southwest monsoon months of June - August, during which 70% of the annual rainfall is being received. A continuous spell of 250 – 350 mm for 7 – 10 days without intermittent hot sunshine, with a temperature ranging from 22 – 28°C and relative humidity above 90% are the most congenial conditions for the outbreak of the disease.

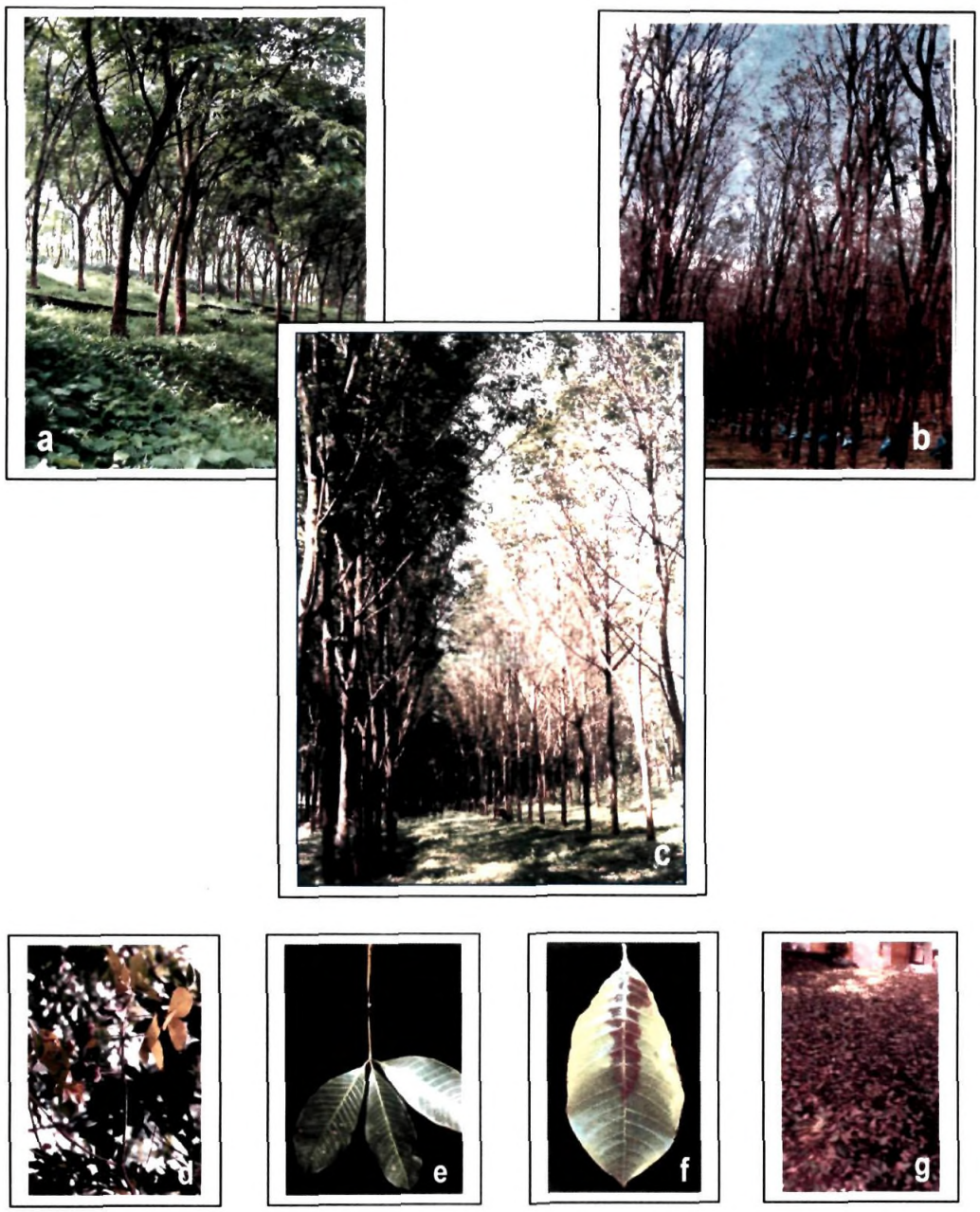


Fig. 2- Occurrence of Abnormal Leaf Fall disease and its symptoms

a. healthy rubber plantation; **b**. an ALF affected plantation; **c**. tolerant and susceptible plants during a disease outbreak; **d**. twig of an affected plant bearing dried pods; **e**. infected petiole with lesion and coagulated latex; **f**. infected lamina; **g**. carpet of fallen leaves

Inoculum development starts with the germination of previous seasons oospores, which serve as resting spores, present in infected dry pods, leaves and twigs deposited in the soil. Green pods are generally the first to be infected, providing abundant inoculum for leaf infections. Green fruit pods in all stages of development are attacked by the fungus, turning them to dull gray colour with water soaked lesions. The fungal mycelia ramify inside the pericarp, and also penetrate into the endosperm of the seed. The pericarp rots very soon, and infected fruits very often do not produce viable seeds. They usually do not absciss, but remain on the tree, blackened, mummified and undehisced. The spread of the disease is assisted by rain splash and wind or by insects. The disease affects mature leaves, where the fungus attacks the petioles causing the leaves to shed while they are still green. The fallen leaves exhibit one or more dark brown lesions with one or two white spots of coagulated latex in the middle of each, usually towards the base of the petiole. Sometimes, the leaf blades are affected with translucent water soaked lesions, which in course of time develop various colours from brown to black. Often the infected leaves turn copper red before falling. After defoliation, the pathogen invades leaf-bearing twigs and causes extensive dieback. On young rubber plants up to three years and in nursery plants, growth is retarded resulting in an extended period of immaturity. In mature plantations, extensive defoliation results in considerable loss of crop. Initial trials conducted four decades ago on three clones have indicated that this disease can cause yield loss of 38 – 56% when left unsprayed for one disease season (Ramakrishnan, 1960). Based on the observations of crop loss due to this disease at different regions over years, the yield loss was predicted to be 30 – 50 % (Pillai *et al.*, 1980). The disease adversely affects growth and bark renewal of the trees. Increase in plugging index, reduction in dry rubber content of latex, and more weed growth are other secondary effects of the disease (Jacob *et al.*, 1989).

2. 2. 4. Clonal susceptibility

In general, all high yielding clones of *Hevea* are susceptible to abnormal leaf fall disease under Indian conditions. However, clones like RRII 105, GT , Gl 1, BD 10 and RRII 33 show certain degree of tolerance to the disease, while clones like RRIM 600, PB 86, Tjir 1, PR 107 and PB 28/59 are highly susceptible. Clones like FX 516 and F 4542, which are hybrids of *H. brasiliensis* and a resistant species of

Hevea called *H. benthamiana*, are resistant to *Phytophthora*, but are not popular due to their low yield.

2. 2. 5. Control measures

Crown budding *ie.* replacement of the crown of a susceptible high yielding clone with that of a resistant variety like FX 516, was tried to control the disease with limited success (Pillay *et al.*, 1986). *Trichoderma* spp. was tried as a biocontrol agent and was shown to inhibit the growth of *P. meadii* (Vanitha *et al.*, 1994). However, chemical control is the most widely used measure to check the spread of the disease. Spraying of 1% Bordeaux mixture as close to monsoon, but before its onset, was found to be very effective for the control of the disease and is being adopted extensively by rubber planters. As an alternative to Bordeaux mixture, copper oxychloride dispersed in agricultural spray oil and sprayed through low volume applicators proved effective for the control of the disease.

2.3. Plant β-1,3-Glucanases and their Importance in Disease Resistance

2. 3. 1. Nomenclature

In the IUBMB nomenclature, all β-glucanases are listed in the enzyme catalog number under EC 3.2.1.# (1992). The first digit places them in class 3, the hydrolases. The second digit (2) indicates that they hydrolyse glycosyl compounds and the third digit (1) indicates that they hydrolyse *O*-glycosidic compounds. Glucan endo-1,3-β-D-glucosidase (EC 3.2.1.39), commonly known as β-1,3-glucanases, are one of the major classes of *O*-glycosidic hydrolyses. The IUBMB enzyme nomenclature of glycosidic hydrolases is based on their substrate specificity and occasionally on their molecular mechanism, and such a classification does not reflect the structural features of these enzymes. A classification of glycosyl hydrolases based on amino acid sequence similarities has been proposed (Henrissat, 1991). In this classification, β-1,3-glucanases constitute Family 17 of glycosidic hydrolases, which comes under CAZy (Carbohydrate Active enZYmes). The CAZy database describes the families of structurally related catalytic and carbohydrate binding modules of enzymes that degrade, modify or

create glycosidic bonds. β-1,3-Glucanases are also classified as PR-2 proteins under pathogenesis-related protein families, which are able to catalyse endo-type hydrolytic cleavage of the 1,3-β-D- glucosidic linkage in β-1,3-glucans. They are abundant highly regulated enzymes widely distributed throughout the plant kingdom (Simmons, 1994; Leubner-Metzger and Meins, 1999).

2. 3. 2. Structural classes

In plants, β-1,3-glucanases (β- glu) exist as multiple structural isoforms that differ in size, isolelectric point, primary structure, cellular localization and pattern of regulation. These isoforms are encoded by multi-gene families of considerable complexity in higher plants (Jin *et al.*, 1999). Such multiplicity could provide more flexibility for spatial and temporal regulation of diverse functions of the β- glu such as induced or tissue specific expression of pathogenesis related isoforms. Multiplicity of β- glu functions might confer advantages to plants by providing several lines of defense against invading microorganisms. Also, the diversity of β- glu, as well as their organ specificity and developmental and differential expression patterns indicate that this enzyme has additional, as yet unidentified, biological functions in plant growth and development. The most detailed sequence information of β- glu isoforms is available from cDNA and genomic DNA clones of tobacco. The various isoforms of the gene in *Nicotiana* have been classified in to 4 distinct structural classes. Similar structural isoforms have been reported from potato, tomato and other plant species also (Beerhues and Kombrink, 1994; Domingo *et al.*, 1994; Thimmapuram *et al.*, 2001). Class I β- glu, which are induced either by pathogen infection or ethylene treatment are the first characterised isoforms. They are also thought to be involved in the normal development of healthy plants during seed germination. The class I genes encode proteins with basic isoelectric points and accumulate primarily in vacuoles. The class I enzymes are usually produced as a pre-protein with an N-terminal hydrophobic signal peptide which is co-translationally removed and a C-terminal extension, *N*-glycosylated at a single site. Glycosylation during processing is thought to facilitate targeting them to the vacuole. The vacuolar class I β- glu were shown to be secreted into the medium in cultured tobacco cells via a novel pathway (Melchers *et al.*, 1993). The class I β-glu gene encoded protein Gn2 of *N. plumbaginifolia* shares 98% amino acid identity with the intracellular vacuolar

isoforms of β- glu isolated from *N. tabacum* earlier (Castresana, 1990). The class I basic isoforms isolated from potato are predominantly localized intracellularly, but are also found in considerable amounts in the extracelluar spaces of infected potato leaves (Schroder *et al.*, 1992). The potato class I basic β-glu also show structural features similar to tobacco class I β-glu. It contains a hydrophobic signal peptide of 25 amino acids and a putative C-terminal extension of 23 amino acids, including a potential glycosylation site. N and C-terminal processing results in a mature protein of 315 amino acids (Beerhues and Kombrink, 1994). The β-glu isolated from monocots (*Pisum sativum*) contains a long amino acid extension at the C-terminal end compared to other isoforms with a single glycosylation site (Buchner *et al.*, 2002). It is expressed in young flowers and in the seed coat, and is weekly expressed in the vegetative tissues during seedling development. The two basic vacuolar isoforms in *Arabidopsis*, BG1 and BG3, show significant structural homology to tobacco class I proteins except for the absence of C-terminal extension (Uknes *et al.*, 1992).

In contrast to class I, the class II isoforms are acidic and are secreted into the extracellular space. The tobacco class II isoforms PR-2a, PR-2b and PR-2c are pathogen- induced acidic proteins without C-terminal extension with an apparent size ranging from 34 – 36 kDa (Linthrost *et al.*, 1990). The class II tobacco isoforms are at least 82% identical in amino acid sequence and differ from the class I enzymes at a minimum of 48.8% of the positions. Class II also includes two acidic 41 kDa stylar β- glu isoforms, Sp41a and Sp41b, which are exclusively accumulated to higher levels in the transmitting tract of tobacco flowers (Ori *et al.*, 1990). They do not appear to be pathogen-inducible, and hence are referred to as 'PR like' proteins.

A pathogen-inductive acidic 35 kDa PR-2d (PR-Q') has been classified as class III β- glu as it differs at least 43% in amino acid sequence from the class I and class II enzymes (Payne *et al.*, 1990). They are also secreted proteins localized in the extracellular spaces. Two highly homologous cDNA clones for class III glucanase have been isolated from tomato plants infected with a viroid. Based on deduced amino acid sequences, TomPR-Q'a was found to be an acidic isoform, 86.7% identical to tobacco PR-Q', and TomPR-Q'b was found to be a basic isoforms, which shows 78.7% identity (Domingo, *et al.*, 1994). The fourth class is

an acidic secreted β-glu that is not pathogen-induced; but is expressed in the anther tapetum, and is involved in the tetrad callose wall dissolution (Bucciaglia and Smith, 1994). As this 'Tag 1' is structurally divergent from other classes it is included as a novel class of tobacco β-glu. The mature Tag 1 is a 35 kDa protein with an N-terminal signal peptide, but without C-terminal extension, and it shares absolutely conserved sequences found in all class of tobacco β-glu. It is 37 – 38% identical to class I, class II and class III tobacco β-glu. The classification, nomenclature and salient features of different plant β-glu enzymes are summarized in Table-2.

Table 2- Classification of ß-1,3-glucanases of tobacco and other *Nicotiana* species

Class	Member name	Trivial name	Origin	MW (kDa)	pI	Localization
I	PR-2e	Glb	*N. tabacum*	33	Basic	Vacuole
I	PR-2e	Gla	"	33	Basic	Vacuole
I	PR-2e	Gglb50	"		Basic	Vacuole
I	PR-2e	Gln2	"		Basic	Vacuole
I		Gn2	*N. plumbaginifolia*		Basic	Vacuole
I		Gnl	"	34	Basic	Vacuole
II	PR-2a	PR-2	*N. tabacum*	35	Acidic	Secreted
II	PR-2b	PR-N	"	35	Acidic	Secreted
II	PR-2c	PR-O	"	35	Acidic	Secreted
II		PR-2d	"		Acidic	Secreted
II	stylar ßGLU	Sp41a	"	41	Acidic	Secreted
II	stylar ßGLU	Sp41b	"	41	Acidic	Secreted
III	PR-2d	PR-Q'	"	35	Acidic	Secreted
IV	anther ßGLU	Tagl	"	35	Acidic	Secreted

In addition to these four classes, two flower specific *Arabidopsis* genes encoding putative new isoforms (BG4 and BG5) of β-glu have also been identified (Delp and Palva, 1999). The deduced amino acid sequences were highly homologous to each other (89%), but only moderately related (32 – 41% amino acid identity) to the different categories of previously characterised β- glu; suggests that BG4 and BG5 may represent a novel class of β- glu in plants. BG4 and BG5 are not responsive to pathogens and are not induced developmentally or hormonally in the vegetative parts of the plant. The specific enzymatic activities

and substrate specificities of the various classes of β-glu vary considerably. The tobacco class I and class II PR-2c appear to be 50 – 250 times more active in degrading its substrate laminarin, than class II PR-2a, PR-2b and class III enzymes (Linthorst, 1991).

2. 3. 3. Biological functions of β-1,3-glucanases: Role in developmental processes

β-Glu are involved in diverse physiological and developmental processes in the uninfected plant including microsporogenesis, pollen germination and tube growth, fertilization, cell division, embryogenesis, fruit ripening, seed germination *etc.* In higher plants, the tetrads of haploid microspores, formed after the meiosis of pollen mother cells, are surrounded by a thick callose wall composed of β-1,3 glucan. At a critical stage of the microspore development, there is a large increase in the β-glu activity that results in the degradation of callose wall, which contributes to the release of microspore into the anther locule, where they develop into mature pollen grains (Steiglitz, 1977). More evidence for the role of β-glu in callose wall dissolution and microsporogenesis has come from transformation experiments. Transgenic tobacco plants over-expressing a vacuolar β-glu in the tapetum tissues exhibited premature degradation of callose, production of abnormal microspores and partial to complete male sterility (Worrall *et al.*, 1992). The result suggests that formation of the callose wall and its proper developmental degradation by β-glu are critical for microsporogenesis (Leubner-Metzger and Meins Jr., 1999).

The interactions between the transmitting tissue of the style and the growing pollen tube are important for guiding pollen tube towards the ovules for successful fertilization. A 41 kDa tobacco β-glu called sp41 was found principally in the transmitting tract in the style (Ori *et al.*, 1990). The specific high-level expression of sp41 at transmitting tract strongly suggests that it plays a significant role in facilitating or regulating pollen tube growth. Another possible role may be in the form of constitutive defense against pathogen attack, as the flower is often visited by insects and various microorganisms that could communicate diseases. More over, the pistil, especially via transmitting tract, is open and vulnerable to pathogens that, like the incoming pollen, may exploit this nutrient rich channel to access the plant.

The process of germination culminates in the rupture of the covering layers of the seed and emergence of the radicle. In many plant species, the seed envelope imposes a physical constraint to radicle protrusion, which has to be overcome by the growth potential of the embryo. Endosperm rupture is the main germination-limiting factor in plants like tomato, tobacco and pepper, and endosperm weakening, a decline in the mechanical resistance of the micropylar endosperm, seems to be necessary for germination to be completed (Leubner-Metzger, 2003). First evidence that suggests the involvement of β-glu in the regulation of germination came from the observation that β-glu is induced during germination (Leubner-Metzger *et al.*, 1995). In tobacco seeds, β-glu is induced after testa rupture and just prior to endosperm rupture. This induction is localized exclusively in the micropylar endosperm at the site where the radicle will emerge. Class I β-glu mRNA and enzyme activity were also observed in the micropylar endosperm of tomato seeds prior to radicle protrusion (Wu *et al.*, 2000). This close correlation between β-glu induction and onset of endosperm rupture under a variety of physiological conditions support the hypothesis that β-glu contributes to endosperm rupture and thus seed germination.

2. 3. 4. Defense-related functions of β-1,3-glucanases

The wide spread role of β-glu in plant defense response have been well investigated in several plant species. β-Glu were proposed to be involved in plant defense as early as 1971 (Abeles *et al.*, 1971). Around the same time the PR proteins were first described as a novel set of abundant proteins accumulated in leaves of resistant tobacco cultivars reacting with HR to TMV infection (Van Loon and Van Kammen, 1970). Later, β-glu were included as PR-2 family of proteins. There is now compelling evidence that β-glu, acting alone or in combination with chitinase, can help to defend against pathogen attack, especially against fungal pathogens. This idea stems principally from the following observations:

1) β-glu expression is induced upon pathogen attack
2) β-glu has anti-fungal activity *in vitro* and
3) β-1,3 linked glucan is found at relatively low concentrations in both monocots and dicots, but is a common component of the fungal cell walls.

It has been proposed that they can act at least in two different ways: directly by degrading the cell walls of the pathogen, and indirectly by promoting the release of cell wall derived materials that can act as elicitors of defense reaction (Boller, 1993)

2. 3. 4..1. Over-expression in response to pathogen attack

The level and onset of β-glu expression is often positively correlated to the level of resistance to the pathogen. There are several reports on the induction of β-glu expression during pathogen infection. Muskmelon (*Cucumis melo*) and tomatoes infected with *Fusarium oxysporum* indicated a higher and more rapid expression of β-glu in resistant than susceptible varieties (Netzer and Kritzman, 1979; Ferraris *et al.*, 1987). Tomato plants resistant to fungal pathogen *Cladosporium fulvum* produced β-glu earlier than susceptible varieties (Joosten *et al.*, 1989). Maize β-glu expression was induced by the fungus *Exserohilum turcicum*, and the level of expression was correlated positively to the various resistance levels of near-isogenic lines (Jondle *et al.*, 1989). Infection of several rice cultivars with the sheath blight pathogen *Rhizoctonia solani* resulted in the induction of β-glu and chitinase (Anuratha *et al.*, 1996). Even though this induction occurred in all cultivars, some cultivars that are moderately resistant appeared to have higher levels of specific isozymes. Infection of ground nut leaves with the early leaf spot pathogen *Cercospora arachidicola* leads to a marked increase in extracellular glucanase activity, limited to the infected tissue (Roulin and Buchala, 1995). Three isoforms with extreme pI values were induced, which when acting together, were capable of degrading pathogen cell wall *in vitro*.

Western blot analysis revealed the strong induction of glucanase in all barley lines infected with a powdery mildew fungus, but the induction was more pronounced in two resistant lines (Jutidamrongphan *et al.*, 1991). The expression of β-glu mRNAs in response to infection by TMV was studied by Ward *et al.*, (1991). The acidic glucanase mRNAs increased to detectable levels within 2 days after inoculation, and peaked at 4–6 days. RNA encoding the basic form of glucanase increased within one day of inoculation, reaching a peak approximately 10-fold greater than its basal level by day 2. The basic β-glu mRNAs remained significantly elevated until day 12. In green house experiments with maize

genotypes Tex 6 and B7 3, the resistant and susceptible inbreds respectively, an elevated glucanase activity in the kernels was found to be associated with lower *Aspergillus flavus* infection in the resistant genotype (Lozovaza *et al.*, 1998). One of the PR proteins known to accumulate in *Cucumis sativus* reacting hyper sensitively to TMV was shown to have β-glu activity through isozyme staining (Repka *et al.*, 1997). This TMV induced acidic β-glu activity was increased six fold after infection. Defense response to stem rust causing fungus in wheat mediated by *R* genes *Sr5* and *Sr24* was studied by Munch-Garthoff *et al.*, (1997). Complete resistance mediated by the *Sr5* gene in cultivar Pre-Sr5 closely correlated with the HR of penetrated cells at early stage of the interaction, when the first haustorium is formed. The HR included a rapid increase in β-glu activity between 24 and 48 h after inoculation.

The induced proteins are mostly acidic and are secreted into the intercellular spaces. Basic isoforms, which are mostly present in the vacuoles at low levels, are also induced upon infection (Van Kan *et al.*, 1992). In disease resistance, basic glucanase seems to be particularly important since over-expression of β-glu genes in transgenic plants afforded substantial protection against pathogens, only when basic glucanases were constitutively expressed (Jongdijk *et al.*, 1995). Inoculation of soybean plants with the bacterium *Pseudomonas syringae* pv. *glycinae* or a fungal elicitor from *Phytophthora* spp. has been found to increase expression of a basic class III isoform of β-glu (Cheong *et al.*, 2000). Infection of pepper (*Capsicum annum*) with *Phytophthora capcici* led to the accumulation of β-glu in the stem tissues soon after inoculation (Egea *et al.*, 1999). After appearance of the symptoms on the pepper stems, β-glu accumulation becomes much more pronounced in the resistant, than susceptible cultivar. β-Glu activity was also detected in the control-uninfected stems, but only in the resistant cultivar. The direct detection of β-glu on polyacrylamide gels revealed a basic isoform in the intracellular fraction, which may be associated with expression of resistance to *P. capcici,* since its activity was detected only in the resistant cultivar. A basic β-glu cDNA clone, isolated from a cDNA library constructed from hypersensitive response lesions of pepper leaves infected with an avirulent strain of *Xanthomonas campestris*, encodes a protein with C-terminal extension, which is the characteristic of class I isoforms (Jung and Hwang, 2000 a). Transcripts of this

gene were more induced in incompatible interactions than in compatible interaction, when inoculated with *X. campestris*. Accumulation was also strongly induced in pepper leaves by both ethephone and methyl jasmonate (Jung and Hwang, 2000 b). This indicates that the basic β-glu may be induced during abiotic stresses also.

An increase in β-glu mRNA levels was reported from shoot cultures of three peach (*Prunus persica*) genotypes treated with culture filtrates of two bacterial and a fungal pathogen (Zemanek *et al.*, 2002). Analysis of accumulation of β-glu mRNAs demonstrated that activation trends were different among the three peach genotypes. The differences between responses in bacterial and fungal treatments support a specific response that may involve different or multiple production pathways of β-glu (Krishnaveni *et al.*, 1999). The β-glu activity was increased more rapidly in resistant melon cultivars than in susceptible ones upon inoculation with the cucurbit powdery mildew fungus *Sphaerotheca fusca* (Rivera *et al.*, 2002). However, strong accumulation of the enzyme was reported in the susceptible cultivar also; but only at advanced stages of infection. Hence, it was concluded that in the susceptible cultivar delayed induction of β-glu does not afford protection and that the rapid accumulation of β-glu at the time of penetration in the resistant cultivar could play an important role in plant defense against powdery mildew.

A β-glu, isolated from pea pods, was detected within 4 - 8 h after challenge with *fusarium solani* (Chang *et al.*, 1992). Accumulation of these β-glu transcripts in pea pods remained high for 48 h in the incompatible reaction, whereas it rapidly decreased in the compatible reaction. The varying resistance levels of different cotton cultivars to the crude toxin of *Verticillium dahliae* (VD) was found to be correlated with the activities of β-glu and chitinase in callus cells. Their activities in the callus cells treated with VD toxin increased to higher level at an earlier time point in resistant cultivars, than in susceptible cultivars (Ying-Zhang *et al*, 2003). Exogenous salicylic acid also induced the accumulation of chitinase and β-glu, which resulted in the resistance to VD toxin. Induction of β-glu and chitinase were reported in the leaves of both resistant and susceptible varieties of sorghum infected with the fungal pathogen *Fusarium moniliforme* (Krishnaveni *et al.*,

1999). It was suggested that β-glu is expressed constitutively at low levels and is secreted into the cell wall and intercellular spaces, where it encounters the invading fungus. cDNA coding for an acidic β-glu isoform has been isolated from wheat spikes infected by *F. graminearum* (Li *et al.*, 2001). Northern hybridisation showed that the expression of this gene along with chitinase is induced upon infection with the fungi. The accumulation of transcripts of these PR proteins was more rapid in the resistant variety 'Sumai 3' than its susceptible mutant during the first 24 h.

The β-glu activity in both leaf and root tissues of one tolerant and two susceptible varieties of black pepper (*Piper nigrum*) infected with *P.capcici* was studied by Jebakumar *et al.*, (2002). Compared with the healthy control, the enzyme activity was found to be more in infected tissues and among the three varieties studied, *Phytophthora*-tolerant p24 expressed higher rate of this defense-related enzyme. Differential expression of a 39 kDa β-glu was observed between two potato cultivars with different degree of field resistance to *P. infestans* (Tonon *et al.*, 2002). In the resistant cultivar, induction (four fold with respect to healthy tubers) occurred 14 h after inoculation, and remained over the basal levels up to 38 h after inoculation. By contrast, in the susceptible cultivar, β-glu was induced at lower levels than those observed in resistant cultivar.

All these results indicate that elevated β-glu expression during pathogen infection contributes to increased resistance, or that it is a limiting factor in host resistance.

2. 3. 4. 2. *In vitro* Studies

Although β-glu and chitinase (chn) are induced in pathogen-infected tissues, they are usually constitutively expressed in roots and lower stems as these plant parts are always in the vicinity of soil- born pathogens. Their ability to co-operatively degrade and kill fungi *in vitro*, indicates their role in destroying the pathogenic fungi, whose cell wall is often made up of chitin and β-glucan. These enzymes can destroy the fungi by thinning their cell wall at the hyphal tip by degrading chitin and β-glucan. This thinning causes swelling and ultimately leads to the bursting and death of the hyphal tip (Arlorio *et al.*, 1992). Although in some

cases, treatment with β-glu and chn alone can inhibit fungal growth *in vitro*, more often combinations of the two enzymes are required for anti-fungal activity. However, in the case of Oomycetes like *Phytophthora* β-glu alone may cause hyphal lysis as their cell wall is devoid of chitin.

Several studies have been made in which, different β-glu and chn isoforms are tested for *in vitro* anti-fungal activity. Only class I basic vacuolar isoforms of tobacco β-glu and chn were effective in promoting the lysis of hyphal tips and inhibiting the growth of *Fusarium solani* (Sela-Buurlage *et al.*, 1993). These effects were greatly enhanced by using combinations of β-glu and class I chn. In contrast, the class II β-glu, PR-2a, PR-2b and PR-2c did not exhibit anti-fungal activity either alone or in combinations tested. Similar studies with tomato β-glu have shown that the vacuolar class I isoforms inhibit growth of *Alternaria solani*, *Trichoderma viride* and *P. infestans* (Anfoka and Buchenauer, 1997). Dual culture experiments of the aflatoxin forming fungus *Aspergillus flavus* with maize callus on the same plate demonstrated that the growth of the *A. falvus* was inhibited by presence of calli (Lozovaya *et al.*, 1998). The fungal growth inhibition was more pronounced in the presence of calli of the resistant genotype TxM, while co-cultivation of the fungus with susceptible Pa91 callus resulted in less inhibition. The inhibition of fungal growth was correlated with the activity levels of β-glu in the callus and in the culture medium. A basic β-glu of 34 kDa was purified from soybean hypocotyls infected by an incompatible race of *P. sojae*. The purified protein inhibited spore germination and hyphal growth of the chitin negative fungus *P. sojae*, but did not show any anti-fungal activity against chitin containing fungi *Alternaria mali* and *Colletotrichum gloeosporioides* (Yi and Hwang, 1997). Genes encoding trichosanthin, tobacco class I chn and tobacco class I β-glu has been expressed in *E.coli* (Hu *et al.*, 1999). All these recombinant proteins showed anti-fungal activity. When two of them were combined, the activity was enhanced greatly, and further stronger anti-fungal activity was observed when all of them are combined. A 39 kDa β-glu purified from resistant potato cultivar produced a direct inhibitory effect on the germination of sporangia of *P. infestans* (Tonon *et al.*, 2002). However, when purified basic β-glu was applied to the hyphae of the arbuscular mycorrhizal fungus *Glomus mosseae* grown *in vitro*, no effect was observed (Vierheilig *et al.*, 2001). It has been demonstrated that some phytopathogenic fungi, whose growth is inhibited by chn and β-glu *in vitro* are

able to overcome the effects of these enzymes over a period of several hours, indicating the ability of those fungi to adapt to these enzymes (Ham *et al.*, 1997). They isolated a glucanase inhibitor protein (GIP) from the culture fluid of fungal pathogen *P. sojae* that can inhibit only specific isoforms of β-glu, but not all. Some reports regarding the inconsistency of endoglucanases as inhibitors of fungal growth *in vitro* and in transgenic plants, might be attributed to this ability of certain phytopathogens to secrete inhibitors of plant endoglucanases (Rose *et al.*, 2002).

Further, it has been suggested that β-glu and chn may act as key enzymes in the lysis of phytopathogenic fungal cell walls during the antagonistic action of bio-control agents like *Trichoderma* (Ait-Lahsen *et al.*, 2001). A novel 78 kDa β-glu isolated from *T. harzianum* showed hydrolysis of yeast and fungal cell walls *in vitro* (de la Cruz *et al.*, 1995). An exo-α-1,3-glucanase isolated from *T. harzianum* could effectively inhibit spore germination as well as hyphal growth of phytopathogens like *A. niger, Botrytys cinerea* and *Penicillium aurantiogriseum* (Ait-Lahsen *et al.*, 2001). However, the enzyme showed no activity against fungi like *Rhizoctonia solani* and *P. syringae*. The difference in sensitivity of the various fungi to the enzyme might be due to the different molecular architecture of spore and hyphal cell walls and / or the presence of specific inhibitors of enzymes. A 43 kDa chn and a 74 kDa β-glu isolated from *T. harzianum* inhibited growth of the phytopathogen *Sclerotium rolfsii* in an additive manner showing promisive ED 50 (50 % effective dose) value of 2.7 μg/ml (El-katatny *et al.*, 2001). A mutant of *T. harzianum*, which produce about three times more chn and β-glu performed better than the wild type during *in vitro* experiments by destroying the pathogen faster, and thereby giving better protection for grapes against *B. cinerea* (Rey *et al.*, 2001)

2. 3. 4. 3. Release of fungal elicitors

In addition to induction by pathogens, β-glu and other PR or stress related proteins are also expressed in response to numerous varied agents called elicitors, which may be either biotic or abiotic in origin. Molecules derived from pathogens that can induce stress related responses in plants are called biotic elicitors. They often are simply crude extracts from pathogens. For example, pea pod β-glu expression was induced by the autoclaved spore extracts of the pathogenic fungus

F. solani (Mauch *et al.*, 1984). Parsley β-glu is expressed in response to elicitors from phytopathogenic fungi (Kombrink and Hahlbrock, 1986). Two β-glu were induced in potato upon treatment with a fungal elicitor (Kombrink *et al.*, 1988). In barley leaves, there was an enhanced expression of β-glu in response to elicitor derived from *R. secalis*, the pathogen responsible for barley leaf scald (Mazars *et al.*, 1990). A specific factor in the crude elicitor preparation, such as proteins, lipids or carbohydrates is actually responsible for inducing the PR gene expression. An indirect defensive role for β-glu was suggested by the observation that β-1,3/ 1,6–glucan oligosaccharides termed oligosaccharide elicitors or oligosaccharins, which are released from pathogen walls by the action of host glucanases can induce wide range of plant defense responses (Ebel and Cosio, 1994). This was well documented for interactions between soybean and the β-glucan elicitor from the pathogenic Oomycete, *P. megasperma*. Yoshikawa *et al.* (1981) found that soybean tissues contain a factor capable of releasing soluble and highly active carbohydrate elicitors from mycelial walls of *P. megasperma* .sp.*glycinea*. This factor was shown to be β-1,3-endoglucanase, which is constitutively present at low levels in soybean tissues (Takeuchi *et al.*, 1990). β-1,6 linked glucans of various chain lengths with frequent β-1,3 linked glucan side chains are constituents of the fungal cell wall. Upon contact of the fungal cells with host tissues during the infection process, the β-1,3-linked glucan side chains are presumed to be attacked by the host β-glu resulting in the release of elicitors. The smallest β-glucan released with elicitor activity was a β-1,3-β-1,6-heptaglucoside and structural requirements for the elicitor activity of these oligosccharides have been investigated (Okinaka *et al.*, 1995). Proteins, which bind this oligosaccharide elicitor have been purified from soybean membranes (Mithofer *et al.*, 1996). Umemoto *et al.* (1997) isolated the cDNA for a β-glucan elicitor binding protein (GEBP), which is localized in the plasma membrane of soybean root cells. Expression of soybean GEBP gene has been shown to confer β-glucan-binding activity to *E.coli* and to tobacco cells cultured in suspension, suggesting that GEBP might be an elicitor receptor. Soybean β-glu that are able to release active β-glucan elicitors from fungal cell walls has been purified (Ham *et al.*, 1995).

Further evidence for the release of elicitors by the host β-glu came from the experiments of Klarzynski *et al.*, (2000). The elicitor activity of laminarin, a linear β-1,3-glucan and a substrate for β-glu, was tested in tobacco cell suspension cultures. They found that treatment with laminarin triggered broad spectrum of defense responses. In accordance with its strong stimulating effect in tobacco cells, infiltration of laminarin into tobacco leaves triggered accumulation of the four major families of anti-microbial PR proteins including β-glu within 48 h. Challenge of laminarin-infiltrated leaves five days after treatment with the soft rot pathogen *Erwinia carotovora*, resulted in a strong reduction of the infection when compared with water-treated leaves. The results showed that linear β-1,3-glucans could elicit defense responses in tobacco and that host β-glu is involved in the release of this elicitor molecule. Rose *et al.*, (2002), studied in detail the mode of action of GIPs from *P. sojae* on soybean. They found that GIPs form complexes with host endoglucanases both *in vitro* and *in vivo* during pathogenesis and as a consequence, the release of glucan elicitor from the *P. sojae* cell walls is inhibited.

2. 3. 4..4. Enhanced disease resistance resulting from transgene expression

The feasibility of transgenic approaches is being tried in several advanced laboratories all over the world as alternative approach to conventional crop improvement programmes. The estimated global area of transgenic or genetically modified crops in the year 2001 was 526 million ha (James, 2003). There is strong evidence that expression of anti-fungal β-glu transgenes alone or in combination with chn transgenes could reduce the susceptibility of plants to infection by certain fungi. Defense involving these genes are particularly interesting because unlike many other secondary metabolites, which are complex in type and expression, and often toxic to pathogens and humans alike, enzymes such as β-glu are not thought to be toxic and are produced by relatively direct gene-to-enzyme pathways. Thus, prospects seems promising for bio-engineering plants towards improved resistance through manipulation of these enzyme expression systems, since these active anti-microbial agents are individually encoded by single genes. The first report on the success of such an approach in developing fungus resistant transgenics came in 1991. Broglie *et al.* (1991) constitutively expressed bean chitinase gene in tobacco and *Brassica napus*; and the plants showed enhanced resistance to *Rhizoctonia*

solani. Since then, there have been a number of reports on transgenics developed for the constitutive expression of many such PR genes including β-glu (Table –3).

Table 3- β-1,3-Glucanases and other PR-genes used for making fungus resistant transgenic plants

Resistance conferring genes	Transgenic plant species	Fungus tested	Reference
Bean chitinase	Tobacco	*Rhizoctonia solani*	Broglie *et al.*, (1991)
Tobacco chitinase	*N. sylvestris*	*Cercospora nocotianae*	Neuhaus *et al.*, (1991)
Soybean glucanase	Tobacco	*P. infestans*	Yoshikawa *et al.*, (1993)
Tobacco PR-1 gene	Tobacco	*Peronospora tabacina*	Alexander *et al.*, (1993)
Glucanase (alfalfa) and chitinase (rice)	Tobacco	*C. nicotianae*	Zhu *et al.*, (1994)
Rice chitinase	Rice	*R. solani*	Lin *et al.*, (1995)
Tobacco glucanase and chitinase	Tomato	*Fusarium oxysporium*	Jongedijk *et al.*, (1995)
Barley glucanase and chitinase	Tobacco	*R. solani*	Jach *et al.*, (1995)
Soybean glucanase	Egg plant	*P. capcici*	Ito *et al.*, (1995)
Tomato chitinase	*Brassica napus*	*Cylindrosporium concentricum*	Grisson *et al.*, (1996)
Chitinase (petunia, tobacco and bean)	Cucumber Carrot	Different fungal pathogens	Punja and Raharjo (1996)
Alfalfa glucanase	Alfalfa	*P. megasperma*	Masoud *et al.*, (1996)
Rice chitinase	Cucumber	*Botrytis cinerea*	Tabei *et al.*, (1998)
Soybean glucanase	Potato	*P. infestans*	Borkowska *et al.*, (1998)
Soybean glucanase	Kiwifruit	*B. cinerea*	Nakamura *et al.*, (1999)
Barley chitinase	Wheat	*Erysiphe graminis*	Bliffeld *et al.*, (1999)
Tomato chitinase	Tomato	*Verticillium dahliae*	Tabaeizadeh *et al.*, (1999)
Rice chitinase	Grapevine	*Elisinoe ampelina*	Yamamoto *et al.*, (2000)
Tobacco glucanase	Brassica napus	*Sclerotinia sclerotiorium*	Lan *et al.*, (2000)
Rice glucanase	Rice	*Magnaporthe grisea*	Nishizawa *et al.*, (2003)
Wheat glucanase, chitinase and PR-5	Wheat	*F. graminearum*	Anand *et al.*, (2003)

Yoshikawa *et al.* (1993) produced transgenic tobacco plants over-expressing the soybean β-1,3-glucan elicitor releasing β-glu under the control of a strong CaMV 35S promoter. The transgenic plants showed reduced symptoms when infected with *A. alternata* or Oomycetes *Phytophthora parasitica* and *Peronospora tabacina*. In many cases, a pronounced synergic effect was obtained when β-glu and chn trangenes were expressed in combination. Sela-Buurlarge

et al. (1993) transformed tobacco plants with transgenes encoding modified class I tobacco β-glu and chn. They found that the extracellular wash fluids from the leaves of plants expressing β-glu and chn showed strong anti-fungal activity against *Fusarium solani*, whereas this effect was less for plants expressing either transgene alone. Zhu *et al*, (1994) made transgenic tobacco plants with genes encoding a basic chitinase from rice and an acidic glucanase from alfalfa. Hybrid plants were generated, by crossing transgenic parental lines exhibiting strong constitutive expression of the transgenes. Evaluation of disease development in these hybrids, heterozygous for each transgene, showed that combination of the two transgenes gave substantially greater protection against the fungal pathogen *Cercospora nicotianae*, than either transgene alone. However, some protection was also observed in the parental lines strongly expressing either transgene alone, when compared with wild type plants. Such results were also reported by Jach *et al.* (1995). They observed that transgenic tobacco plants individually over-expressing barley chn, class II β-glu and type I ribosome inactivating protein resulted in increased protection against *R. solani*. The co-expression of all the transgenes together significantly enhanced protection against fungal attack. Tomato plants exhibiting tobacco class I β-glu and chn transgenes showed reduced susceptibility to infection by *F. oxysporium* (Jongdijk *et al.*, 1995). Transgenic plants showing constitutive over-expression of an inducible acidic β-glu reduced disease severity caused by Oomycete fungi *P. megasperma* (Masoud *et al*, 1996). But no reduction in disease symptoms was observed with several chitin containing fungal pathogens. Ito *et al.* (1995) transformed egg plants with soybean β-glu and found that the transgenic plants showed a statistically significant degree of disease resistance to *P. capcici*. Potato plants transformed with soybean β-glu were found to have increased resistance against *P. infestans* (Borkowska *et al.*, 1998). Soybean β-glu was also used to transform kiwifruit, and some of the transformants showed over six-fold increase in enzyme activity compared with the control (Nakamura *et al.*, 1999). When leaves from control and transformants were inoculated with *B. cinerea*, which cause gray mold disease, the disease lesions on transformants were smaller than on control plants. *Brassica napus* plants transformed with β-glu and chn genes showed increased resistance to *Sclerotinia sclerotiorium* (Lan *et al.*, 2000). The *Gns I* gene of rice, which encodes 1,3;1,4-β-glucanase was over-

expressed in transgenic rice under the control of CaMV 35S promoter (Nishizawa *et al.*, 2003). When these transgenic plants were inoculated with virulent blast fungus (*Magnaporthe grisea*), they developed many resistant type lesions on the inoculated leaf accompanied by an earlier activation of defense-related genes. Genes encoding β-glu and chn, isolated from a cDNA library of *F. gramineorum*-infected wheat spikes of scab resistant cultivar 'Sumai 3', were transformed in to susceptible spring wheat (Anand *et al.*, 2003). One transgenic wheat line, which showed stable inheritance and co-expression of β-glu and chn, showed a delay in the spread of the infection under green house conditions.

The pattern of induction of β-glu in response to pathogen infection is more or less similar for viral, bacterial and fungal pathogens. However, Beffa *et al.* (1996) proposed that the induction of class I β-glu in viral pathogenesis could be a counter defensive mechanism employed by viral pathogens by studying the response of a mutant tobacco plant deficient for class I β-glu through antisense transformation to TMV infection. Transformants showed greatly reduced levels of the enzyme and quite unexpectedly the β-glu deficient mutants showed markedly reduced mosaic disease symptoms indicating another role of these enzymes in viral pathogenesis.

Callose, a substrate for β-glu, can act as a physical barrier for the spread of the virus. Callose deposition in and surrounding TMV-induced lesions was found to be higher in the β-glu deficient hosts, suggesting that the decreased susceptibility to virus resulted from increased callose deposition. Later, it was observed that the movement of plant viruses like TMV is hindered in β-glu deficient mutants of tobacco as the intercellular trafficking via plasmodesmata is delayed (Iglesias and Meins, 2000). They found that this delay was due to the reduction in plasmodesmatal size exclusion limit (SEL) in mutant tobacco because callose turn over is thought to be regulate the plasmodesmatal SEL. Further, the tobacco class I β-glu was inserted into TMV for the over-expression in infected cells (Bucher *et al.*, 2001). Plants infected with this virus showed increased symptoms of the disease; supporting the view that local induction of β-glu during infection helps to promote cell-to-cell movement of viruses by hydrolysing callose. Another intriguing possibility is that viruses can use a defensive response of the

host against fungal infection –production of β-glu – to promote their own replication and speed. These findings are of particular interest since it suggests a novel means based on antisense transformation with host genes, for protecting plants against viral infection.

2. 4. Molecular approaches in *Hevea*

Although the past three decades witnessed tremendous advancements towards understanding of the molecular mechanisms of plant growth and development, most of these studies were carried out in annuals and very few in the tree species. The advanced state of cell wall development, their long generation time and presence of high amount of phenolics, terpenoids, tannin *etc.*, which can react with proteins and nucleic acids have made molecular studies in *Hevea* rather difficult (Thulaseedharan *et al.*, 2000). In spite of such constraints, attempts have been made at different laboratories, mainly in the areas including isolation and characterisation of latex allergens, laticifer-specific gene expression and rubber biosynthesis. Another important area in which research is currently focused is regarding the isolation and characterisation of laticifer specific promoters and on the possible use of *Hevea* laticifer system for producing recombinant proteins of therapeutic and nutritional value (Yeang, 2002). However, only limited studies have been reported so far to understand the molecular mechanism of disease resistance in *Hevea*.

Many of the defense and rubber biosynthesis related enzymes found in the latex are described as latex allergens. To date, 12 proteins of natural rubber (NR) latex have been classified as allergens. β-Glu is also listed among them as a major latex allergen (Hev b2). The other major allergens isolated and characterised include Hevein (Hev b6), rubber elongation factor (REF- Hev b1) and lipid transfer protein (Hev b12) (Wagner and Breiteneder, 2001). A cDNA clone encoding hevein has been isolated from *Hevea* latex cDNA library (Broeakaert *et al.*, 1990). Hevein is believed to be involved in the plugging of wound during latex collection and thereby, plays a major role in the protection of wounded sites from fungal attack. The genes coding for important enzymes involved in the rubber biosynthesis pathway, which are specifically expressed in the laticifers, namely

3-hydroxy-3-methyl glutaryl co-enzymeA reductase (HMGR), REF and farnesyl diphosphate synthase (FDP) have been isolated. Chye *et al.* (1991) isolated a cDNA clone for HMGR using a heterologous probe. Three members of a gene family viz. *hmg 1, hmg 2* and *hmg 3* encode transcripts of HMGR. In a study to over-express *hmg 1* in transgenic *Hevea* to increase the latex biosynthesis, transformed embryoids, which showed 250 – 300% more HMGR activity were obtained (Arokiaraj *et al.*, 1996). However, they were unable to regenerate the transgenic plantlets. Attanayaka *et al.* (1991) screened a *Hevea* latex cDNA library and isolated a cDNA insert of 681 bp, which encodes the entire coding region of the mature REF. Using degenerate oligonucleotide primers for FDP synthase protein, Adiwilaga and Kush (1996) could isolate a full-length cDNA clone which encodes a 47 kDa protein. Isolation and functional analysis of a cDNA clone encoding small rubber particle protein (SRPP) showed that it plays a positive role in rubber biosynthesis (Oh *et al.*, 1999). Recently, a functional cDNA clone encoding geranylgeranyl diphosphate synthase was isolated, and its role in NR biosynthesis was examined (Takaya *et al.*, 2003).

Genomic and cDNA clones of the free radical scavenging enzyme, superoxide dismutase, which has important roles in diseased and stressed conditions, was isolated from *Hevea* (Miao and Gaynor, 1993). Transgenic plants over-expressing SOD were produced in an attempt to give rise to plants tolerant to tapping panel dryness (TPD) (Sobha *et al.*, 2003). To investigate the molecular mechanism of TPD, isolation and characterisation of genes, which change their expression pattern during development of TPD were attempted. Decreased expression levels of a Myb transcription factor was observed in the barks of TPD affected trees (Chen *et al.*, 2003).

The lutoid bodies present in the latex play an important role in the protection of rubber trees against fungal infection. It has been shown that upon wounding, the lutoid bodies burst and release anti-fungal proteins like hevein, hevamine and β-glu. Hevein is shown to bind chitin to inhibit the growth of several chitin-containing fungi (Van Parijs *et al.*, 1990). Hevamine, an enzyme with chitinase/lysozyme activity, which is important for plant defense against pathogenic fungi and bacteria, was purified from lutiod bodies (Jekel *et al.*, 1991).

Recently, the cDNA sequences of hevamine was isolated (Bokma *et al.*, 2001) and the active sites of the enzyme were determined (Bokma *et al*,. 2002). β-Glu activity was observed in the lutoid extracts of *Hevea* latex. Five isoforms consisting of three basic and two acidic isozymes were detected in native IEF gels after activity staining (Breton *et al.*, 1995). A cDNA clone encoding class I β-glu was isolated by screening a *Hevea* latex cDNA library with a heterologous probe from *N. plubaginifolia* (Chye and Cheung, 1995). Expression of *Hevea* glucanase in different tissues was examined through northern hybridisation, and it was observed that the gene is constitutively expressed in the laticifers. *In situ* hybridisation studies were carried out to examine the distribution of β-glu mRNAs in the petiole sections of *Hevea*, and it was found that anti-fungal β-glu is expressed exclusively in the laticifer cells of the petiole.

Further, it was reported by Subroto *et al.* (1996) that the lutoid body fraction from three rubber tree clones differed in their content of β-glu and hevamine. Later the focus on β-glu shifted from its role in defense response to its importance as latex allergen. Although much work has been done on the biochemical and immunological characterisation of *Hevea* β-glu (Hev b2) as a latex allergen (Sunderasan *et al.*, 1995; Yagamai *et al.*, 1998; Wagner and Breiteneder, 2001), significant progress has not been made to study its efficacy in countering the invading fungal pathogens in *Hevea,* except a preliminary work by Philip *et al.* (2001). They found that the tolerant clones of *Hevea* exhibit more β-glu enzyme activity compared with the susceptible clones in the leaf tissues infected with the fungal pathogen *Corynespora cassicola.*

3

MATERIALS AND METHODS

3.1. Isolation, Cloning and Characterisation of Genomic and cDNA Sequences Coding for β-Glu Gene

3.1.1. Genomic DNA isolation

Young, uninfected leaves were selected from *Hevea* clones maintained in the Germplasm collection nursery of Rubber Research Institute of India. The samples were washed thoroughly in tap water and rinsed with sterile water. DNA extraction was done with a modified protocol of Doyle *et al.*, (1990). This modified CTAB procedure consists of the following steps:

i) Two g leaf tissue was ground to a very fine powder in Liquid Nitrogen using a mortar and pestle.

ii) The ground tissue was homogenized with 20 ml 2X CTAB extraction buffer.

> **2X CTAB buffer**: 2% CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl (pH-8.0), 1% Polyvenylpolypyrrolidone and 0.1% β-mercaptoethanol

iii) The sample was then kept at 60°C for 30 min in a 50 ml centrifuge tube.

iv) Centrifuged at 8000 rpm for 10 min, pellet was discarded and the supernatant transferred to a new tube.

v) Equal vol of Tris-saturated phenol-chloroform-isoamyl alcohol (25:24:1) was added and mixed gently.

vi) The sample was then spun at 10,000 rpm for 10 min. and the aqueous phase was transferred to a new tube. The organic phase containing the denatured proteins was discarded.

vii) RNA in the sample was degraded by incubating at 37°C for 1 h after the addition of 5 µl of DNAase free RNAase (10 mg/ml-Sigma).

viii) Proteinase K (20 mg/ml- Bangalore Genei) was added to inactivate the RNAase and other residual proteins. The incubation was continued for another 1 h.

ix) Equal vol of chloroform:isoamyl alcohol was added to the sample, mixed gently and centrifuged at 10,000 rpm for 10 min.

x) The aqueous phase was transferred to a fresh tube and the organic phase contains the lipids and carbohydrates was discarded.

xi) To the sample equal vol of chloroform:isoamyl alcohol was added, mixed gently and centrifuged at 10,000 rpm for 10 min.

xii) Aqueous phase was transferred to a fresh tube and the organic phase was discarded.

xiii) To the sample 0.6 vol ice-cold isopropyl alcohol was added to precipitate the DNA.

xiv) The tube was then kept in ice for 20 min and the precipitated DNA was pelleted by centrifuging at 8000 rpm for 10 min at 4°C.

xv) The DNA was washed twice in 70 % ethanol and once in absolute ethanol.

xvi) The pellet was air-dried and suspended in TE buffer.

xvii) The DNA samples were stored at –20°C.

3.1.1.a. DNA quantification

The quality and quantity of genomic DNA was checked in a UV spectrophotometer (Beckman, USA). The quality was checked by measuring the ratio of absorbance at 260 nm and 280 nm (260/280) respectively. A ratio between 1.7 – 1.8 indicates good quality DNA without protein contamination. DNA quantification was done according to the following formula.

1 O.D. at 260 nm = 50 ng of DNA

The O.D. of each DNA sample at 260 nm was measured and quantified accordingly.

3.1.2. Isolation of RNA from latex

RNA from the latex of rubber tree was extracted through a modified protocol of Kush *et al.*, (1990). All the reagents required were prepared in DEPC (an RNAase inhibitor) treated water. The protocol involved the following steps.

i) Equal vol of latex was collected directly in to a centrifuge tube under ice-cold conditions which contains 10 ml extraction buffer.

> **Latex RNA extraction buffer:** 50 mM Tris-HCl (pH-8.5), 150 mM LiCl, 5 mM EDTA and 2 % SDS

ii) The sample was centrifuged at 12,000 rpm for 30 min at 4°C.

iii) The upper white creamy layer, which consists of rubber particles were removed and the latex serum was transferred to a fresh tube.

iv) It was then treated with an equal vol of extraction buffer saturated phenol, mixed gently and centrifuged at 10,000 rpm for 10 min.

v) The aqueous layer was recovered to fresh tube and phenol extraction was done once more.

vi) The aqueous layer was then treated with an equal volume of choloform:isoamyl alcohol to remove carbohydrates, lipids and traces of phenol; centrifuged and the organic layer was discarded.

vii) RNA in the aqueous layer was precipitated by adding 1/3 vol of 8 M LiCl. The precipitation continued overnight in –20°C.

viii) RNA was pelleted by centrifugation at 10,000 rpm for 20 min. at 4°C.

ix) The pellet was washed with 2 M LiCl, air dried and suspended in 500 µl of DEPC treated H_2O.

x) RNA was further purified and concentrated by precipitation with 2.5 vol ethanol in presence of 0.1 vol, 3 M sodium acetate (pH- 5.2).

xi) The RNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C.

xii) The pellet was washed twice in 70% alcohol, air dried and re-suspended in sterile H_2O.

xiii) The quantity of RNA was checked using UV sphectrophotometer (Section- 3.1.1.a) and its quality and DNA contamination, if any, was checked in 1% agarose gels.

xiv) The isolated RNA samples were stored in 3 vol of 100% ethanol at –70°C.

The agarose gel electrophoresis was carried out according to standard protocols (Sambrook *et al.*, 1989).

3.1.3. First strand cDNA synthesis

First strand cDNA was synthesised from the isolated latex RNA by reverse transcription reaction with oligo-(dT) primers using the 'Improm-II™ Reverse Transcription System' (Promega, USA) as follows:

i) Primer annealing to the target RNA and denaturation was performed in a 0.5 ml reaction tube in ice. 1 µl of total latex RNA (1 µg) was combined with 1 µl oligo-(dT) primers (0.5 µg). The reaction was made up to 5 µl by the addition of nuclease free water.

ii) The tube was incubated for 5 min at 70°C in a pre-heated block and immediately chilled in ice for 5 min.

iii) The tube was then spin down for 10 sec in a micro-centrifuge to collect the condensate and maintain the original volume. This RNA-primer combination was kept on ice until the reverse transcription reaction mix gets ready.

iv) The RT-reaction mix was combined in a 1.5 ml tube on ice. 4 µl of reaction buffer supplied by the manufacturer along with 1.5 µl $MgCl_2$ (1.8 mM), 1 µl dNTP mix (0.5 mM of each dNTP) and 1 µl reverse transcriptase was made up to 15 µl with nuclease free water.

v) The RNA-primer mix (5 µl) was added to the reaction mix to form the final volume of 20 µl.

vi) Annealing was done by incubating the reaction at 25°C for 5 min.

vii) Primer extension was carried out at 42°C for 1 h in a heated block.

viii) The reaction was stopped by inactivating the reverse transcriptase by keeping the tube at 70°C for 15 min.

ix) The synthesised first strand cDNA was stored at –20°C for subsequent PCR amplification.

3.1.4. Design of gene specific primers

Based on a previously published cDNA sequence of β-1,3- glucanase gene (Chye and Cheung, 1995), two gene specific oligonucleotide primers were designed with the help of 'PrimerSelect' programme of 'Lasergene' software (DNASTAR, USA). The primer sequences and their Tm value are shown below

Forward primer- 5' CTT CTT AAT GGC TAT CTC CTC 3' Tm- 55.9
Reverse primer- 5' CTC ACA TAT CAC TCT TAA GG 3' Tm- 53.2

The synthesised primers (Metabion, Deutschland) were dissolved in sterile double distilled water to get a concentration of 100 pmols/μl. The primer stock solutions were stored in –20°C.

3.1.5. PCR amplification of the gene from genomic DNA and cDNA

PCR amplification was performed with 10 ng of genomic DNA or one μl of cDNA from the step **3.1.3.** as templates in separate reactions. Amplifications were carried out in 20 μl reactions, which contains the following constituents:

	Amount	Final concentration
Template	1.0 μl	10 ng
Reaction buffer (Tris-HCl, pH- 9 – 10 mM, KCl- 50 mM, $MgCl_2$- 15 mM)	2.0 μl	1 X
dNTP mix	2.0 μl	100 μM of each dNTPs
Forward primer	1.0 μl	10 pmol
Reverse primer	1.0 μl	10 pmol
Taq DNA polymerase (Amli Taq® from Roche, USA)	0.15 μl	0.75 U
Sterile dist. Water	12.85 μl	
	20 μl	

The reaction mix was overlaid with a drop of mineral oil and amplification was carried out in a Perkin-Elmer 480 DNA thermal cycler. The PCR conditions were as follows:

Step I-	Initial denaturation-	4 min-	94°C
Step II-	Denaturation-	30 sec.-	94°C
	Annealing-	1 min.-	50°C
	Extension-	2 min.-	72 °C
Step III-	Repeat step II-	35 times	
Step IV-	Final elongation-	10 min.-	72°C
Step V-	Hold		4°C

The PCR products were analysed in 1.5 % agarose gels. Gel images were captured using 'EDAS 290' (Electrophoresis Documentation and Analysis System-Kodak, USA). Molecular weight of the amplified products was determined using Kodak I D Image Analysis software.

3.1.6. Cloning of the PCR products

3.1.6.a. Elution of amplified products from agarose gels

The samples were run in 0.7 % low melting point agarose gels and the DNA bands were cut out from the lane after viewing the gel over long wavelength UV light quickly so as to avoid nicks. The gel slices were taken in a 1.5 ml micro-centrifuge tube and kept at 65°C for 10 min or till the agarose melt completely. To the melted agarose 1/10 vol, 5 M NaCl was added, mixed well and again kept at 65°C for 10 min. Equal vol of Tris-saturated phenol was added, mixed gently and the tube was centrifuged at 8000 rpm for 10 min. The aqueous layer was recovered and treated twice with chloroform. The DNA was precipitated by adding 1/10 vol, 3 M sodium acetate and 2.0 vol ethanol. Precipitation was continued for 30 min at –20°C and was pelleted by spinning at 8000 rpm for 10 min at 4°C. The DNA was washed in 70% alcohol, air-dried and re-suspended in an appropriate quantity of TE buffer.

Elution of DNA bands from agarose gels had also been performed using 'Clean Genei kit' (Bangalore Genei) according to the manufacturers instructions.

3.1.6.b. Ligation

'pGEM-T Easy' vector system (Promega, USA) was used for the cloning of purified PCR products. A vector to insert ratio of 1:3 was used for ligation. The amount of insert required for ligation with 50 ng of the vector was calculated using the following formula:

$$\text{Amount of insert} = \frac{\text{ng of vector x kb size of the insert}}{\text{kb size of the vector}} \text{ X molar ratio of insert: vector}$$

The ligation mix was prepared as follows.

2X rapid ligation buffer (supplied by the manufacturer)	-	5.0 µl
pGEM-T Vector	-	1.0 µl (50 ng)
PCR product	-	1.0 µl
T4 DNA ligase	-	1.0 µl
H_2O	-	2.0 µl
		10 µl

The ligation was performed overnight at 4°C or at 16°C for 4 h. The ligation mix was frozen till the time of transformation.

3.1.6.c. Transformation protocols for *E coli*

The *E.coli* strain *JM 109* was used for the transformation purpose. Competent cell preparation and transformation was done as follows:

i) From a glycerol stock, the *E.coli* strain was streak plated to LB.

ii) Single colonies from the plate was picked and cultured in 3 ml LB overnight at 37°C in an environmental shaker (New Brunswick Scientific, USA).

iii) Next day, 2 % of the overnight grown cultures (100 µl) were inoculated to 5 ml LB and grown for 3- 4 h (till the OD reaches 0.3 – 0.5).

iv) The cultures were then kept in crushed ice and distributed 1 ml each to 1.5 ml micro-centrifuge tubes.

v) The cells were harvested by spinning at 5000 rpm for 3 min at 4°C.

vi) After discarding the supernatant, the tubes were kept in ice and 200 µl 0.1 M freshly prepared $CaCl_2$ was added with a pre-cooled pipette tip. The cells were kept suspended in 0.1 M $CaCl_2$ for 20 min in ice.

vii) The tubes were then spun at 5000 rpm for 3 min at 4°C and the supernatant was discarded.

viii) The cells were re-suspended in 200 µl 0.1 M ice-cold $CaCl_2$ and either quickly frozen to –70°C for storage or kept in ice for immediate use.

ix) For transformation, 4 µl of the ligated mix was added to the competent cells and incubated in ice for 30 min.

x) Then a heat shock was given at 42°C for 1 min.

xi) After the heat shock, the tube was immediately transferred to ice and allowed to chill for 1 – 2 min.

xii) Then 800 µl LB medium was added and the culture was incubated at 37°C for 90 min with shaking.

xiii) The transformed competent cells (100 µl) were spread over an LB plate (90 mm) containing 50 µg/ml ampicillin coated with 40 µl X-gal (20 mg/ml) and 4 µl IPTG (200mg/ml).

xiv) The plates were incubated for 12 – 16 h at 37°C.

xv) Transformants containing the insert were selected by blue/white screening.

3.1.7. Confirmation of cloning

3.1.7.a. Through PCR

Both blue and white colonies were cultured overnight in 3 ml LB containing the appropriate antibiotic and were pelleted down quickly by spinning down at maximum speed for 30 sec in a table-top micro-centrifuge. The pellet was suspended in 50 µl sterile double distilled water and boiled for 10 min. The suspension was spun down and the supernatant was used as the template for PCR reactions.

PCR reactions were performed as mentioned in section **3.1.5.** Only 30 PCR cycles were performed with 2 µl of the template plasmid DNA. The PCR products from blue and white colonies were analysed in 1.5 % agarose gels.

3.1.7.b. Alkaline lysis procedure for isolation of plasmids in large scale

Plasmid extraction from recombinant bacteria was done according to the alkaline lysis procedure of Birnboim and Doly (1979).

i) An overnight grown 3 ml culture was inoculated in to 100 ml of LB broth containing the respective antibiotic and kept under constant shaking for 12 – 16 h at 37°C.

ii) The cells were harvested by centrifugation at 6000 rpm for 5 min at 4°C and the supernatant was drained completely.

iii) The bacterial pellet was washed with 10 ml ice-cold STE buffer

STE Buffer 0.1 M NaCl, 10 mM Tris-Cl (pH-8), 1 mM EDTA (pH-8)

iv) The washed pellet was re-suspended in 3 ml of solution I.

Solution I 50 mM Glucose, 25 mM Tris-Cl (pH-8), 10 mM EDTA (pH-8)

v) Cells were lysed by adding freshly prepared solution II (6 ml), mixed thoroughly and kept at room temperature for 5 – 10 min.

Solution II 0.2 N NaOH, 1 % SDS

vi) Finally 4.5 ml of ice-cold solution III was added to the lysate, mixed thoroughly and kept in ice for 10 min.

Solution III 5 M Potassium acetate- 60 ml, Glacial acetic acid- 11.5 ml H_2O- 28.5 ml

vii) The precipitated mix which contains chromosomal DNA and high mol.wt. RNA was removed by centrifuging at 6000 rpm for 15 min at 4°C.

viii) The supernatant was treated with 5 µl of RNAase (10 mg/ml) and incubated at 37°C for 1 h.

ix) Supernatant was then washed twice with an equal vol of chloroform:isoamylalcohol and centrifuged at 10,000 rpm for 10 min.

x) Plasmid DNA from the supernatant was precipitated by the addition of 0.6 vol of isopropanol and incubated at room temperature for 10 min.

xi) The plasmid DNA was recovered by centrifugation at 10,000 rpm for 10 min.

xii) Pellet was washed in 70% ethanol, air-dried and dissolved in 100 µl of TE buffer and stored at –20°C.

xiii) The plasmids isolated from recombinant colonies were checked in 0.8% agarose gels and compared with control plasmids.

3.1.7.c. Restriction analysis of the plasmid DNA

To confirm the cloning event, the recombinant plasmids were subjected to restriction digestion. To release the insert, 1.0 U of *Eco*R I enzyme (Bangalore Genei) was added along with the enzyme buffer supplied by the manufacturer to around 1 µg of the isolated plasmid DNA. The pGEM-T vector is having *Eco*R I restriction sites on either side of the inserted DNA fragment. The reaction mix was incubated for 3 h at 37°C. Self ligated, non-recombinant plasmids were also analysed as a control. The digested plasmids along with undigested controls were analysed in 0.8% agarose gels.

3.1.8. Sequencing and sequence analysis

3.1.8.a. PEG purification of plasmid DNA

The plasmid DNA isolated through alkaline lysis procedure was purified through PEG precipitation for sequencing purpose.

i) To 32 µl of plasmid DNA, 8 µl 4 M NaCl was added, followed by 40 µl of 13% PEG_{8000}.

ii) After thorough mixing, the sample was incubated on ice for 20 min.

iii) The precipitated plasmid DNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C.

iv) The supernatant was discarded and the pellet was rinsed with 70% ethanol.

v) Pellet was air-dried and re-suspended in 20 µl of sterile double distilled H_2O and stored at –20°C.

3.1.8.b. Sequencing

The sequencing was done at DBT sponsored DNA sequencing facility, Department of Biochemistry, Indian Institute of Science, Bangalore in an automated sequencer (ABI PRISM). Sequence similarity search was done with 'BLASTN' programme of NCBI (National Center for Biological Information), USA (Altschul *et al.,* 1990). The deduced amino acid sequence analysis was performed using the various protein handling tools available on the web at the ExPASy site (Expert Protein Analysis System), which is the proteomics server of the Swiss Institute of Bioinformatics (Gasteiger *et al.*, 2003). Computation of the various physical and chemical parameters of the predicted protein like molecular weight, theoretical pH, amino acid composition, estimated half-life, instability index *etc.* were carried out using the ProtParam tool. Post translational modification prediction like presence of signal peptides and its cleavage sites were found out with SignalP (Nielsen *et al.*, 1997). Putative glycosylation site in the protein was determined by NetNGlyc protein analysis tool (Gupta *et al.*, 2004).

3.1.9. Southern hybridisation

3.1.9.a. Restriction digestion of genomic DNA

Good quality genomic DNA with sufficient quantity was isolated from a *Phytophthora* tolerant (RRII 105) and susceptible (RRIM 600) clone of rubber tree and also from another 12 different genotypes. Around 10 µg of genomic DNA was digested with four restriction enzymes namely *Bam*H I, *Sau3*A I, *Xba* I and *Hind* III in separate reactions. The reaction mix (30 µl) was prepared as,

DNA	-	15 µl (10 µg)
Enzyme buffer	-	3.0 µl
Restriction enzyme	-	2.0 µl (20 U)
ddH_2O	-	10 µl

The digestion was continued overnight at 37°C. the fragments were size fractionated in 1% agarose gels.

3.1.9.b. Blotting

The method was based on the standard procedure developed by Southern (1975). The gel was properly documented before the blotting, and processed after electrophoresis as follows:

i) DNA in the gel was depurinated by soaking in a solution of 0.25 N HCl for 12 – 15 min. Then the gel was rinsed twice with dist.H_2O briefly.

ii) Denaturation of the DNA was carried out by treating the gel in denaturation solution for 25 min with gentle shaking. Then it was rinsed with water

Denaturation Solution 0.2 M NaOH, 1.5 M NaCl

iii) Gel was neutralized by soaking in neutralization buffer for 30 min.

Neutralization Solution 1 M Tris-HCl (pH-8), 1.5 M NaCl

Care was taken to see that gel is completely immersed in all solutions while treatment.

DNA was transferred from the treated gel to nylon membrane (Hybond N^+, Amersham, UK) through capillary blotting method (Sambrook *et al.*, 1989).

i) The gel after neutralization was briefly washed in 10X SSC and kept ready.

20 X SSC 3 M NaCl, 0.3 M Sodium citrate pH adjusted to 7

ii) A tray was filled to a height of 5 cm with 10X SSC. A suitable platform with dimensions bigger than the gel was placed in the tray.

iii) The surface of the platform was covered with Whatman No. 3 filter paper presoaked in 10X SSC, in such way that the ends of the paper are immersed in the SSC. Then 3 sheets of Whatman No.1 filter paper was placed after trimming to the same dimensions of the gel and presoaked in

10X SSC on top of the platform. Any air bubbles was removed by rolling the surface with a tube.

iv) The gel was placed carefully on top of this and then a Hybond N+ nylon membrane, presoaked in 10X SSC was placed on top of the gel. Any air bubbles were removed by gently rolling a glass rod on the surface.

v) Two sheets of the Whatman No.1 presoaked filter papers were placed on top of this assembly. Three more sheets of clean dry filter paper was then stacked on it over which ordinary filter papers cut to the gel dimensions were stacked to a height of 10 cm. Over this, a suitable weight of around 200-300 g was placed. The weight should not crush the gel but should be sufficient to keep the papers tight..

vi) The transfer was allowed to proceed overnight for a period of 12-16 h.

vii) After transfer, the assembly was disassembled and the nylon membrane was briefly washed in 5X SSC and air-dried.

viii) The membrane was fixed using a UV cross linker (Hoefer, USA) at 12000 J/cm^2. The membrane was wrapped in Saran and stored between ordinary filter papers in the refrigerator till use.

3.1.9.c. Preparation of labeled probes

The β-1,3-glucanase gene probe was radiolabeled using 'Multiprime DNA labeling system' from Amersham, (UK) following manufacturers instructions. It utilizes random hexanucleotides to prime DNA synthesis on denatured template DNA at numerous sites along its length. The procedure was as follows:

i) About 50 ng of template DNA was diluted to 5 µl with dist. H_2O and boiled for 5 min to denature.

ii) Chilled immediately on ice for 5 min and centrifuged briefly.

iii) Added 2.5 µl of buffer, which contains all the dNTPs except dCTP.

iv) Then 2.5 µl of random primer solution was added.

v) To this 2.5 µl of α-^{32}P labeled dCTP (sp. activity ~ 3000 Ci/mMol or 10 µ Ci/µl) was added.

vi) Then 11.5 µl of nuclease free autoclaved water was added.

vii) Finally 1 µl of the enzyme (Klenow fragment of DNA polymerase I) was added and mixed gently by pipetting up and down.

viii) Spun for few seconds and incubated at 37°C for 30 min.

ix) The reaction was stopped by adding 0.5 µl of 0.5 M EDTA and the probe was diluted to 100 µl with distilled water.

The labeled probe was purified by passing through a Sephadex G-50 column as follows:

i) Sephadex G-50 was added to dist. water to form a slurry (10 g of dry powder yields around 160 ml of slurry).

ii) Glass wool was placed at the bottom of a 1 ml column and 1 ml of the slurry was added without trapping of air bubbles.

iii) The column was spun at 3000 rpm for 3 min in a swinging bucket rotor.

iv) More slurry was added until the Sephadex tightly packed up to 1 ml level.

v) The column was equilibrated first with STE buffer and then with dist. water.

vi) The labeled probe was then passed through the column and purified.

vii) The column purified probe was denatured by boiling at 100°C for 3 min and immediately chilled in ice. It was stored in the freezer till use.

Such purification helps to remove all the small nucleotides and other unincorporated nucleotides to avoid background signals in the blot.

3.1.9.d. Hybridisation

Hybridisation of the labeled probe to the nylon membrane was performed according to Sambrook *et al.*, (1989).

i) The blotted membrane was placed in a hybridisation tube and appropriate amount of pre-hybridisation solution (0.2 ml/cm^2 of the blot – 25 ml for the 13x10 cm membrane) was added.

Pre-hybridisation solution 6 X SSC, 5 X Denhardt's reagent, 0.5 % SDS

ii) The pre-hybridisation was carried out at 65°C for 1 h in a hybridisation oven (Amersham, UK) with rotary movement at very low speed.

iii) The pre-hybridisation solution was poured out and hybridisation solution (pre-hybridisation solution containing denatured probe DNA labeled with α-^{32}P) was poured into the tube and then incubated with slow rotation for 12 – 16 h at 65°C.

3.1.9.e. Washing of the blot and autoradiography

After hybridisation, the membrane was washed twice at room temperature for 5 and 15 min respectively with solution I

Washing solution I 2 X SSC, 0.1 % SDS

Then the blot was subjected to two high stringent washes at 65°C for 30 min each with solution II

Washing Solution II 0.1 X SSC, 0.5 % SDS

The membrane was then floated briefly in 0.1 X SSC at room temperature, air-dried and subjected to autoradiography. The membrane was wrapped in a cling film and placed inside the X-ray cassette. An X-ray sheet was placed over it after marking the orientation. An intensifying screen was placed over this assembly and the cassette was closed tightly and placed in -70°C for 1-2 days.

After this, the X-ray sheet was removed and developed in the developer solution under safe red light. As soon as the spots develop, the X-ray sheet was cleaned with water and quickly placed in the fixer for a few minutes. The sheet was extensively washed in water and air-dried.

3.1.10. Isolation of the promoter elements of the gene

Random Amplification of Genomic DNA Ends (RAGE) technique was utilised to amplify the 5' regulatory sequences of the gene (Siebert *at al.*, 1995). At first, the genomic DNA was digested with blunt end cutting enzymes. The

enzymes used in the present study are *Eco*R V, *Stu* I, *Ssp* I, *Dra* I and *Hae* III. Around 3- 5 µg of DNA were digested overnight at 37°C with 10 U of each enzyme separately in 30 µl reactions. The digestion was checked in agarose gels. Then the DNA fragments were purified by phenol:chloroform:isoamyl alcohol extraction followed by a chloroform extraction and precipitated in 75% ethanol in presence of sodium acetate. The fragments were washed with 80% ethanol and air-dried. Finally, the blunt ended fragments were dissolved in 10 µl TE. Now an adapter sequence was ligated to the DNA fragments.

The adapter consists of a long arm and a short arm, which is amino-linked at 3' end to prevent any erroneous extension during subsequent PCR.

Adapter Sequences

Adaptor long arm
5'- CTA ATA CGA CTA ACT ATA GGG CTC GAG CGG CCG CCC GGG CAG GT- 3'
3'- H_2N- CCC GTC CA- 5'
Adaptor short arm

The two arms were synthesised separately and the adapter was prepared by mixing 100 µM each of the arms. The mix was kept at 96°C for 2 min and then cooled slowly to room temperature for annealing. The adapter was then ligated to DNA fragments of the 5 restriction enzymes separately as follows:

DNA	-	10 µl
Ligase buffer	-	2.0 µl
Adapter (100 µM)	-	1.0µl (5 µM final concentration)
T4 DNA ligase	-	3.33 µl (10 U)
Dist. water	-	to 20µl

The reaction was performed overnight at 16°C. Then the ligation mix was diluted 1:10 and used as the PCR template with adapter specific forward and gene specific reverse primers.

Adapter specific primer (ASP I)
5'- ATA CGA CTC ACT ATA GGG- 3'

Gene specific primer (GSP I)
5'- TAG AGA GCT ATG ACC TCT G-3'

To ensure more specificity, a nested PCR reaction was performed with the products of the first PCR as template.

Primers for the nested PCR

ASP II- 5'-ATA GGG CTC GAG CGG C-3'

GSP II -5'-CAG GTG GAA GGT TGT TGC C-3'

The binding sites of the second pair of adapter specific and gene specific primers are within the first PCR product. The amplified products of the nested PCR reaction was cloned in pGEM vector and sequenced.

3.2. Gene Expression Studies through Northern Hybridisation and RT-PCR Analysis

3.2.1. Plant materials used

All the plant materials used in this study were obtained from the Germplasm collection and nursery of Rubber Research Institute of India. The *Phytophthora* tolerant clone RRII 105 and a highly susceptible clone RRIM 600 were selected for challenge with the pathogen and subsequent northern hybridisation and RT-PCR analysis to study the induction pattern of β-glu gene. Budded stumps of the selected *Hevea* clones were grown in polybags in a glass house, in which the temperature is maintained around 25°C with a high relative humidity.

3.2.2. Fungal culture and inoculation

The fungus used was a highly virulent isolate of *P.meadii*, which was maintained in PDA medium, obtained from Plant Pathology Division of RRII. PDA plates were inoculated with the stock culture and were incubated at 25°C for two days in light. White oats medium was used for sporulation (Rajalakshmy and Joseph, 1986). The oats broth was inoculated with two-day old fresh fungal mycelium from the PDA plates and kept for two days under darkness. The mycelia were then collected in a mesh and washed four times with sterile dist.H_2O in petridishes. All the media components were removed and the mycelia were dispersed in water using forceps and it is then kept in light for 24 h for sporangial growth. The mycelia were observed under microscope to check the sporangial

growth. The plate containing the sporangia were kept in refrigerator for 10 min and then kept in room temperature. This heat shock will liberate the motile zoopspores. Light green leaves of 10 –12 days old were inoculated with this zoospore suspension (10^6 zoospores/ml). After inoculation, the plants were covered with transparent polythene bags to maintain the required humidity and observed for the development of the symptoms. Control plants were also maintained under similar conditions. Leaf samples were taken at different time intervals (24, 48, 72 and 96 hrs after inoculation) and processed for the RNA isolation.

3.2.3. Isolation of RNA from leaf samples

RNA was isolated from necrotic and near infected zones as well as from control leaves according to the procedure of Venkatachalam *et al.* (1999) with suitable modifications. All the reagents required are prepared in DEPC treated H_2O. The protocol involved the following steps:

i) One g of leaf tissue was rinsed with DEPC treated H_2O and ground to a fine powder in Liquid Nitrogen.

ii) Then 10 ml of extraction buffer was added and the homogenate was transferred to a centrifuge tube.

iii) Equal vol of extraction buffer saturated phenol was also added, mixed gently and centrifuged at 10,000 rpm for 15min.

Extraction buffer 0.2 M NaCl, 0.1 M Tris-HCl (pH-8.5), 0.01 M EDTA, 1.5% SDS, 0.1% β-mercaptoethanol (added immediately before use) and insoluble PVPP (added to the homogenate).

iv) The upper aqueous phase was transferred to a new tube and re-extracted with equal vol of chloroform.

v) Centrifuged at 10,000 rpm for 10 min and the aqueous phase was recovered.

vi) Then 1/3 vol of 8 M LiCl was added and the precipitation was continued overnight in ice.

vii) The RNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4 °C.

viii) The pellet was washed first with 2 M LiCl followed by 100% ethanol, air-dried and dissolved in 1 ml DEPC treated sterile H_2O.

ix) For further purity, the RNA was re-precipitated with 0.1 vol 3 M sodium acetate (pH- 5.2) and 2.5 vol of ethanol.

x) The precipitated RNA was pelleted at 10,000 rpm for 10 min at 4°C and washed twice with 70% ethanol.

xi) Pellet was air-dried, re-suspended in 200 µl of sterile H_2O.

xii) The quantity of RNA was estimated spectrophotometrically and its quality and contamination, if any, with DNA was assessed in 1% agarose gel.

xiii) The isolated RNA was used immediately for subsequent analysis or stored in 3 vol of ethanol at –70°C.

3.2.4. Amplification of sequences for using as internal controls

3.2.4.a. Amplification of 18S ribosomal RNA gene

The 18S ribosomal RNA sequence was amplified from *Hevea* genomic DNA using gene specific sense, 5'- TAC CTG GTT GAT CCT GCC AG-3' and antisense, 5'- GCG ATC CGA ACA TTT CAC CG-3' primers. Amplifications were carried out in 20 µl reactions, which contain the constituents as mentioned in Sec. **3.1.5**. The PCR conditions were also the same. The amplified fragment was gel purified, cloned in pGEM vector and sequenced.

3.2.4.b. Amplification of β-actin gene

The partial sequence coding for β-actin in *Hevea* was amplified using gene specific sense, 5'- TCC ATA ATG AAG TGT GAT GT-3' and antisense, 5'- GGA CCT GAC TCG TCA TAC TC-3' primers (Kobayashi *et al.*, 2000). The reaction constituents and PCR conditions were as mentioned in the previous section. The amplified product was cloned and sequenced.

3.2.5. Northern hybridisation protocols

3.2.5.a. Electrophoresis of RNA

Agarose (1%) was melted in 31 ml of H_2O and cooled to 60°C. In a fume hood, 10 ml of 5X formaldehyde gel running buffer (FGRB) and 9 ml of

formamide was added to give a final concentration of 1X and 2.2 M respectively. The gel was allowed to set for at least 30 min at room temperature.

5X Formaldehyde gel-running buffer 0.1 M MOPS (pH- 7.0), 40 mM sodium acetate, 5 mM EDTA (pH- 8.0)

Around 20 µg of RNA (9 µl) was incubated for 15 min at 65°C along with 4 µl FGRB, 7 µl formaldehyde and 20 µl formamide. After a brief spin, 4 µl of formaldehyde gel loading buffer was added to the sample. The six samples analysed include, RNA isolated from uninfected control leaves, infected leaves after 48 and 96 h from the clones RRII 105 and RRIM 600.

3.2.5.b. RNA blotting

Before transfer to the membrane, the gel was washed thrice in DEPC treated H_2O to remove the formaldehyde. The nylon membrane (Hybond N^+, Amersham, UK) was cut in to the size of the gel and was presoaked in 10 X SSC. Blotting was carried out as described in the Southern protocol (Sec. **3.1.9**). After transfer, the membrane was air-dried and UV cross-linked by keeping the RNA side down.

3.2.5.c. Hybridisation and washing

The membrane was kept inside the hybridisation bottle, pre-hybridisation buffer was added and incubated at 42°C for at least 2 h.

Pre-hybridisation solution 5 X SSC, 5 X Denhardt's, 1 % SDS and 50 % formamide

The probe preparation and purification was as mentioned in the Southern protocol. The α-^{32}P dCTP labeled β-1,3 glucanase gene probe was added and incubated overnight at 42°C. The membrane was washed first with solution I (2X SSC + 0.1 % SDS) for 5 min at room temperature. Then two low stringent washings were given with Solution II for 5 min each at room temperature. This was followed by a stringent washing in pre-warmed solution II at 42°C for 15 min. The membrane was rinsed with 2X SSC and excess liquid was removed with blotting paper. It was then wrapped in a UV transparent plastic wrap (Saran) and

exposed to X-ray film with intensifying screens. The X-ray cassettes were placed at –70° for 2 days and the film was washed and fixed.

3.2.5.d. Stripping of the blot

For re-probing with 18S RNA the β-glu probe was stripped off from the membrane. Prior to re-probing, membranes may be stored in refrigerator (2-8°C), wrapped in Saran. When ready to commence re-probing, membrane was rinsed in 5X SSC for 1-2 minutes. Then a boiling solution of 0.1% SDS was added to membrane and placed on a bench-top shaker for 10 min. The operation was repeated twice, using freshly boiling SDS each time. Finally the membrane was rinsed in 5X SSC, pre-hybridised, probed and detected as described earlier.

3.2.6. RT-PCR assay for differential expression

RNA was isolated from infected zones on the leaves of tolerant clone RRII 105 and susceptible clone RRIM 600 at different time intervals such as 0, 24, 48, 72, and 96 h as well as from uninfected controls as described previously. First strand cDNA was synthesised from total RNA by reverse transcription with oligo-(dT) primers using the 'Improm II reverse transcription system' (Promega, USA) according to manufactures protocols. One µl of the first strand cDNA was used to co-amplify the β-glu and β-actin transcripts in a 20 µl reaction. RNA samples were tested for the presence of genomic DNA contamination by using extracted RNA directly as a PCR template, prior to cDNA synthesis, under the same PCR conditions. The PCR reaction products were separated on a 1.5% agarose gel, visualized with Et Br staining under UV and the image was captured using EDAS 290.

3.2.7. Analysis of autoradiograms and RT-PCR products

The relative abundance of β-glu mRNAs with respect to 18S RNA gene was determined by measuring the net intensity of band in northern blots using Kodak 1D image analysis software. Net intensity is the sum of background-subtracted pixel values in the band rectangle. The RT-PCR gel images also were scanned to determine the net intensities of β-glu and actin bands. The net intensity

data for β-glu were corrected for house keeping gene data and then normalized to 0 h. Corrected values were calculated by dividing the β-glu value by its corresponding house keeping gene value and multiplied by the highest house keeping 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).

3.2.8. β-1,3-Glucanase enzyme assays

Enzyme assay was done spectrophotometrically according to Pan *et al.*, (1991). The crude enzyme extract (62.5 µl) was added to equal volume of substrate, laminarin (4%) and incubated at 40°C for 10 min. The reaction was stopped by adding 375 µl dinitrosalicylate and the sample was kept in a boiling water bath for 10 min. The coloured solution was diluted 10 times with dist. water and absorbance at 500 nm was measured. One unit of enzyme activity was defined as the amount of enzyme that produce, reducing sugars equivalent to one µM of glucose equivalent per 10 min under above conditions.

3.2.9. Tissue specific expression

RNA was isolated from leaf and latex of an uninfected plant and northern hybridisation was performed as described earlier with β-glu probe to study the tissue specific expression. As constitutive expression was observed in the latex, in order to find out any difference in the expression pattern in different clones, RNA was isolated from the latex of three tolerant (RRII 105, RRII 33, Fx 516) and three susceptible clones (RRIM 600, PB 86 and PR 107) and northern hybridisation was performed with β-glu gene probe.

3.3. Construction of a Functional cDNA Clone and Purification of Recombinant Protein

3.3.1. Design of primers with restriction sites and PCR amplification

Four primers that will amplify the sequences coding for mature functional protein were designed with appropriate restriction enzymes. One set of forward

and reverse primers has an *Eco*R I site at their 5' end, while the second set possesses a *Bam*H I site.

The sequences of primers were as follows,

1. 5'- GGAATTCCAGGTAGGTGTTTGCTATGG-3'
2. 5'- GGAATTCCCAGTTCTTTTCTGCACC-3'
3. 5'- CGGGATCCCAGGTAGGTGTTTGCTATGG-3'
4. 5'- CGGGATCCCCAGTTCTTTTCTGCACC-3'

Primer No.1 and 3 are sense primers, while No.2 and 4 are antisense primers. The first two primers contain an *Eco*R I site and a *Bam*H I site present in the last couple. Additional nucleotides were added to the 5' end of the restriction sites, since certain endonucleases require additional bases flanking their recognition sequences. This will facilitate the cleavage close to the end of the DNA fragments. The primers were synthesised with Metabion (Deutschland).

PCR amplifications were tried with four different combinations of primers. These were primer No. 1 & 2, No.1 & 4, No. 3 & 2 and primer No. 3 & 4. The constituents of the reactions and PCR conditions were as mentioned earlier.

3.3.2. Cloning in expression vectors

The 963 bp glucanase gene fragment amplified with primer combinations 3 & 2 (BE) and 1 & 2 (Eco) were gel purified. The BE fragment is having a *Bam*H I site at 5' end and an *Eco*R I site at 3' end. The 'Eco' fragment has *Eco*R I sites at both ends. The purified BE fragment was double digested with *Eco*R I and *Bam* H I enzymes with multi-core enzyme buffer (Promega, USA) in 20 µl reaction volume. The digestion was performed overnight at 37°C and then the enzyme was heat inactivated by incubating the reaction at 75°C for 10 min. After a chloroform wash, the digested fragments were precipitated with double vol ethanol and 0.1 vol sodium acetate (3 M), washed in 70 % ethanol, air-dried and dissolved in 10 µl H_2O. The fragment is now ready for cloning with sticky *Bam*H I and *Eco*R I sites at 5' and 3' ends respectively. The 'Eco' fragment is digested with *Eco*R I and purified as described.

Two expression systems namely pGEX-2T (Amersham, UK) and pET 32a+ (Novagen, USA) were tried for recombinant protein expression. The plasmids were isolated in large quantities and double digested with *Eco*R I and *Bam*H I enzymes and also with *Eco*R I alone in separate reactions, to create cohesive ends. The 4.9 kb pGEX and 5.9 kb pET vectors were checked for digestion in 0.8% agarose gels. When the digestion is complete, the vector was dephosphorylated by directly adding one U of calf intestinal alkaline phosphatase to the digestion mix and incubation at 37°C for 30 min. The vector was then gel purified to remove the residual nicked and super-coiled plasmid. The BE fragment with *Eco*R I and *Bam*H I cohesive ends was used for unidirectional cloning to vectors cut with *Eco*R I and *Bam*H I. The 'Eco' fragment was cloned to vectors opened with *Eco*R I. The ligation reaction (10 µl) was setup as follows:

linearised, dephosphorylated vector	-	1.0 µl (50 ng)
DNA fragment	-	2.0 µl
ligase buffer	-	1.0 µl
10 mM ATP	-	1.0 µl
H_2O	-	4.0 µl
T4 DNA ligase	-	1.0 µl (3 U)

The reaction was incubated at 16°C overnight. The *E.coli* strain *JM 109* was used for transformation and maintenance. Transformants were selected in antibiotic (amp) plate and plasmid was isolated. The cloning was confirmed through PCR and restriction digestion. To determine the insert orientation and size one vector specific forward and insert specific reverse primers were used in PCR reaction.

3.3.3. Sequencing

Sequencing primers for pGEX vectors were brought from Bangalore Genei Ltd. The pET sequencing primers, S-Tag and T7 terminator primers, were synthesised (Metabion). Sequencing of the PEG purified plasmids was carried out to ensure orientation and correct reading frame.

3.3.4. Expression of the target gene

The *E.coli* strain *DH5 α* was selected as the expression host for pGEX vectors and strain *BL 21 (DE 3)* was used in the case of pET. These strains were

transferred with their respective plasmids and transformed single colonies were streaked to LB plates. A single colony from a freshly streaked plate was inoculated to 3 ml LB media containing 50 μg/ml ampicillin. The culture was grown overnight and in the following morning 1% of this culture was inoculated to 50 ml LB containing the appropriate antibiotic in a 250 ml flask. Control colonies containing the vector without insert was also inoculated. The cultures were incubated with shaking at 37°C until the OD_{600} reaches 0.6 (around 2- 3 h). Then the expression of the target protein was induced by the addition of IPTG to the growing culture. An aliquot of the 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 1 mM and continued the incubation for 2 – 3 h. Just prior to harvest, 1 ml of culture from both induced and uninduced samples was removed and the OD_{600} was measured after suitable dilution.

Total protein of the samples were analysed for checking the expression. The cells were harvested from a 1 ml aliquot of both induced and uninduced cultures by centrifuging at 5000 rpm for 5 min at 4°C. The media was completely drained out and 100μl phosphate buffered saline (PBS) was added to yield a concentration factor of 10X (100 μl vs. starting vol of 1 ml culture). Then the cells were lysed with 100μl 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 analysed through SDS-PAGE. Host alone and control vector without insert samples were also analysed along with induced and uninduced samples. Equal amount of samples were added to 10% acrylamide gel after measuring the OD_{600} and concentration factors into consideration. Cells were harvested from the remaining of the 50 ml cultures and the pellets were frozen at –70°C for further analysis and purification of the target protein.

3.3.5. Purification of the target protein

The frozen bacterial pellet of 50 ml culture was thawed on ice. For the purification of fusion protein cloned in pGEX vector, GST-fusion protein purification kit from Bangalore Genie was utilised. After thawing, the pellets were suspended in 10 ml PBS and sonicated in a tube on ice until the sample is no longer viscous. Sonication was performed 3 times at 40 amplitude for 30 sec with

30 sec interval. Then 0.5 ml wash solution (provided by the manufacturer) was added and centrifuged at 10,000 rpm for 20 min at 4°C. The equilibrated glutathion column was loaded with the supernatant. Then the column was washed with the equilibration buffer till the OD_{280} reaches 0.05. Finally column was eluted with 10 ml of elution buffer and 1 ml fractions were collected. The eluted fractions were assayed by SDS-PAGE with appropriate controls. The fractions containing the maximum amount of protein were desalted by dialysis with PBS

The Trx (thioredoxin)-fusion proteins of pET vector were purified using the His.Bind purification kit (Novagen, USA). The induced bacterial pellets of 50 ml culture 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 and the soluble proteins were collected by centrifuging at 10,000 rpm for 20 min. Column was prepared with 5 ml of His.Bind slurry to yield a final bed volume of 2.5 ml of settled resin. After washing with 3 vol of sterile deionised 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 then 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). Finally the bound protein was 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 analysed as a control.

3.3.6. Confirmation of the induced target proteins

3.3.6.a. Western blotting

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 to nitrocellulose membranes (Sigma, USA) as per standard protocols (Sambrook *et al.*, 1989) using a western blot apparatus (Broviga). The membrane was then washed twice for 10 min each in 15 ml TBS buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5). Then incubated for 1 h in 15 ml blocking solution (3% BSA in TBS). Then the membrane was

washed twice for 10 min each with 20 ml of TBSTT buffer (0.5 M NaCl, 20 mM Tris-HCl , 0.2% v/v Triton X-100 , 0.05% v/v Tween -20, pH- 7.5). The primary antibody (His.Tag monoclonal antibody) was then added, diluted in the blocking solution, and incubated for 1 h. Unbound antibodies were removed by washing twice with 20 ml TBSTT followed by a single wash in 15 ml of TBS buffer. Now the secondary antibody (Goat anti-mouse IgG AP conjugate) was added and incubated for another 1 h. Membrane was washed thoroughly for 5 times with 20 ml each of TBSTT. Finally the membrane was developed with BCIP and NBT and incubated in room temperature until the colour develops. When the signals appear, the reaction was stopped by washing the blot thoroughly with deionized water and air-dried.

3.3.6.b. Thrombin Cleavage

A thrombin cleavage site is available on the pET vector that will allow the enzymatic removal of the N- terminal fusion tags. Around 20 µg of the purified recombinant protein from the pET vector was used in the reaction, which contains 1 U of thrombin and 1 X 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°C and the products were analysed in 12% SDS-PAGE gel.

3.3.7. Anti-fungal assay of purified recombinant proteins

All manipulations were carried out under sterile conditions. PDA plates (90 mm) were prepared and a single growing mycelial plug of 4 mm diameter of the Oomycete fungi *Phytophthora meadii* was inoculated at the center. To allow initial vegetative growth, the plates were incubated at 25 ± 2°C for 24 h. At this time, sterile filter paper discs (5 mm and 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 which contains around 10 µg of recombinant protein were used for each disc. The plates were further incubated and observed for the appearance of inhibition zones and photographed.

4

RESULTS

4.1. Isolation, Cloning and Characterisation of Genomic and cDNA Sequences Coding for β-Glu

4.1.1. Isolation of nucleic acids

The genomic DNA was isolated in good quality and concentration (Fig. 3). The DNA isolated from 2 g leaf tissues were dissolved in 1 ml TE buffer. A 100 µl aliquot of this DNA sample was diluted 10 times with dist. water and the absorbance at 260 and 280 nm were measured. OD_{260} was 1.2 and OD_{280} was 0.68. The amount of DNA obtained was calculated as 300 µg/g of the leaf tissue used. The ratio of A_{260} / A_{280} was 1.76, indicating that the isolated DNA is of good quality without protein contamination and the preparation was free from phenol.

RNA was isolated from the latex as well as from leaves without degradation and DNA contamination (Fig. 4). The concentration of RNA isolated was approximately 70 µg/ 100 mg of leaf tissue or 700 µg/ 10 ml of latex.

4.1.2. PCR amplification of β-1,3-glucanase gene

First strand cDNA was synthesised using oligo-(dT) primers from the isolated latex RNA. Genomic DNA and first strand cDNA was used as templates for PCR amplification with gene specific primers. Under optimal PCR conditions, that include concentrations of template DNA, dNTPs, *Taq* DNA polymerase and Mg^{2+}, a 1.25 kb single product was amplified when 10 ng of genomic DNA was used as the template. A 1.12 kb fragment was amplified when one µl of first strand cDNA was used as the template (Fig. 5). The isolated RNA as such, used in one reaction, did not produce any amplicons, confirming the absence any DNA contamination in RNA preparations (Fig.6).

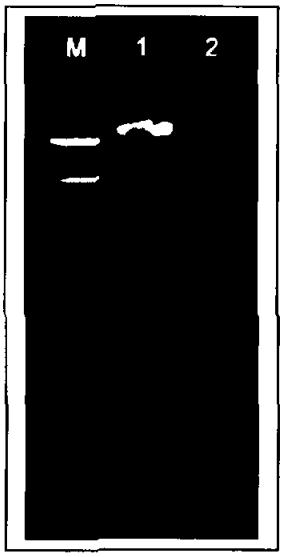


Fig. 3- DNA isolated from leaf tissues

M- mol.wt. marker (λ DNA- *Eco*R I & *Hind* III double digest); 1- DNA isolated from 2 g leaf tissue dissolved in 1 ml TE buffer; 2- diluted DNA sample

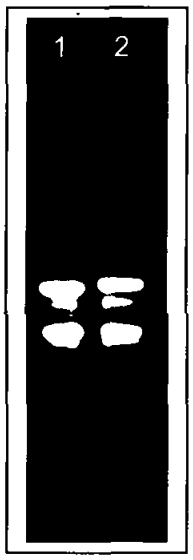


Fig. 4- RNA isolated from latex and leaf tissues

lane 1- from latex; 2- from leaves.

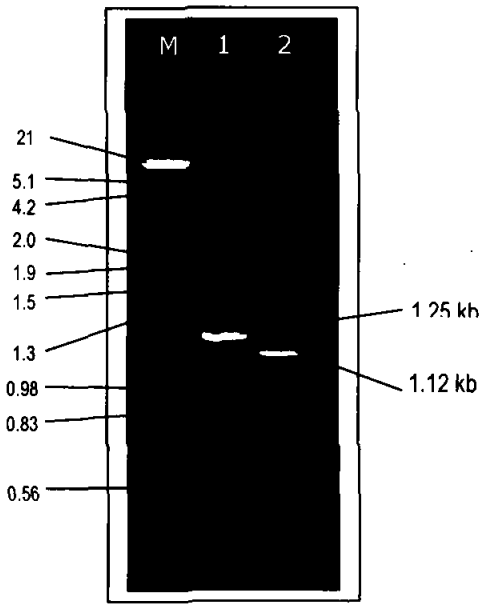


Fig. 5- PCR amplification of the genomic and cDNA sequences coding for β-glu with gene specific primers

M- marker; lane 1- 1.25 kb genomic DNA fragment; 2- 1.12 kb cDNA fragment

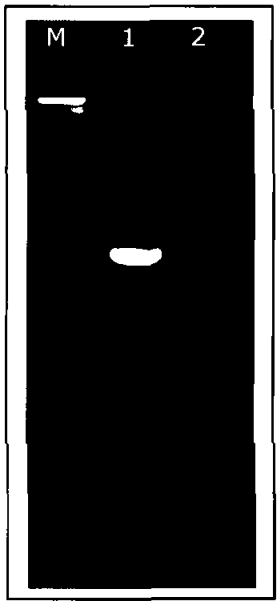


Fig. 6- Amplification of the first strand cDNA

M- marker; Lane 1- cDNA amplified; 2- no amplification when RNA was used as template in PCR

4.1.3. Cloning of the PCR products

The pGEM-T vector system used for the cloning of PCR products utilises a simple, yet efficient method for cloning known as TA cloning. It exploits the inherent property of several *Taq* DNA polymerases to preferentially add a template-independent single adenosine residue to the 3' ends of double stranded PCR products. Fragments carrying such 3'-A overhangs can efficiently be cloned using linearised T- vectors with 3'-T overhangs on both ends (Fig. 7). The T-vector was prepared by adding a 3' terminal thymidine residue to both ends of the vector that was opened with a blunt end cutting enzyme. These single 3'- T overhangs at the insertion site will prevent the recircularization of the vector during ligation reactions and greatly improve the efficiency of ligation of the PCR product.

A vector:insert ratio of 1:3 was found to be optimum as approximately 200 colonies were formed per plate with 50 μg/ml ampicillin when the ligated mix was transformed to *E.coli* strain *JM 109*. A blue/white ratio of around 50% was observed in presence of X-gal and IPTG. Plasmid mini-preparation from 15 white and two blue colonies were performed through alkaline lysis and a PCR was performed with insert specific primers. Fragment of predicted size was amplified from most of the white colonies, confirming the presence of the insert (Fig.8).

Plasmids were isolated in large quantities from three positive colonies and restriction analysis was performed. Both control and recombinant plasmids were linearised by digestion with *Pst* I, which is having only a single restriction site on the vector (Fig.9). On agarose gels, the mol. size of the linearised recombinant plasmid was observed to be around 4.2 kb, while control vector is 3 kb in size. Further, digestion with *Eco*R I, which is having recognition sites on either side of the cloning site, could release the cloned fragment of predicted size (Fig. 10). These results confirmed the cloning event. Fragments amplified from both genomic DNA and cDNA were cloned. The recombinant plasmids were isolated in large quantities and was PEG purified for sequencing purpose.

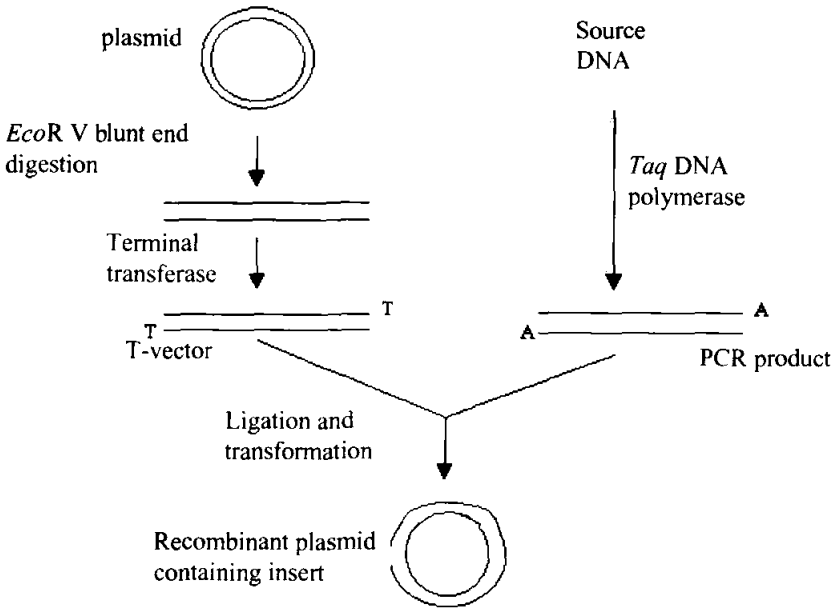


Fig. 7- Schematic diagram of cloning of PCR products in T-vectors

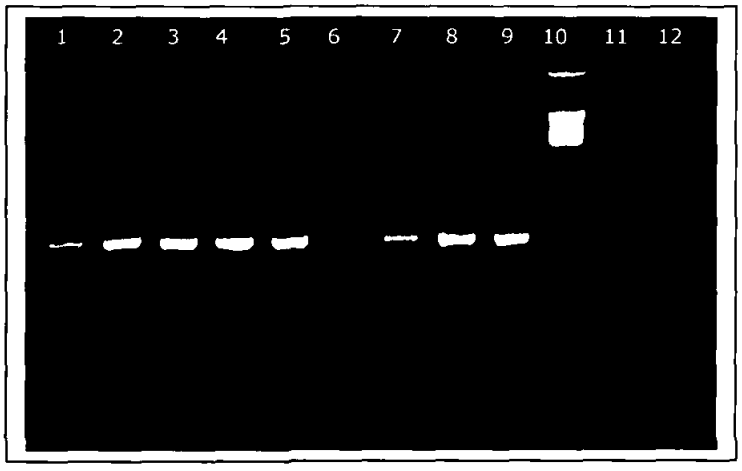


Fig. 8- PCR analysis of the white/blue colonies to confirm the presence of the insert

lanes 1-9- white colonies; 10- marker; 11 – 12- blue colonies

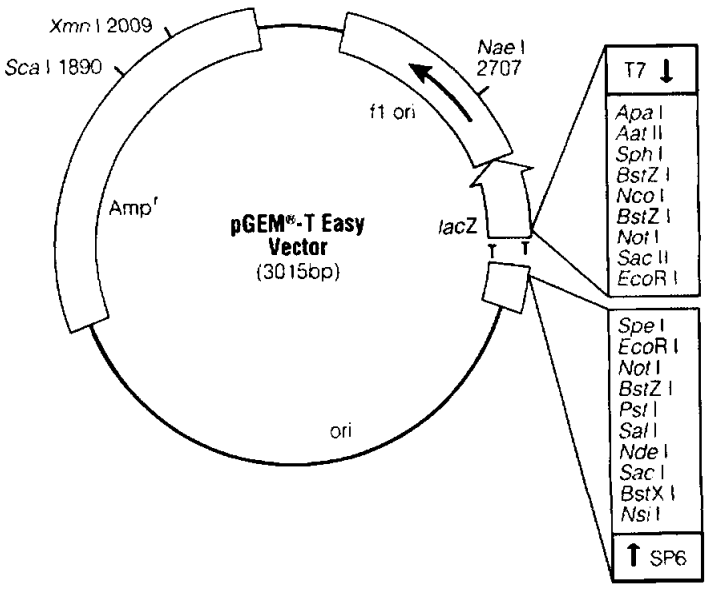


Fig. 9- The pGEM vector used for the cloning of PCR products

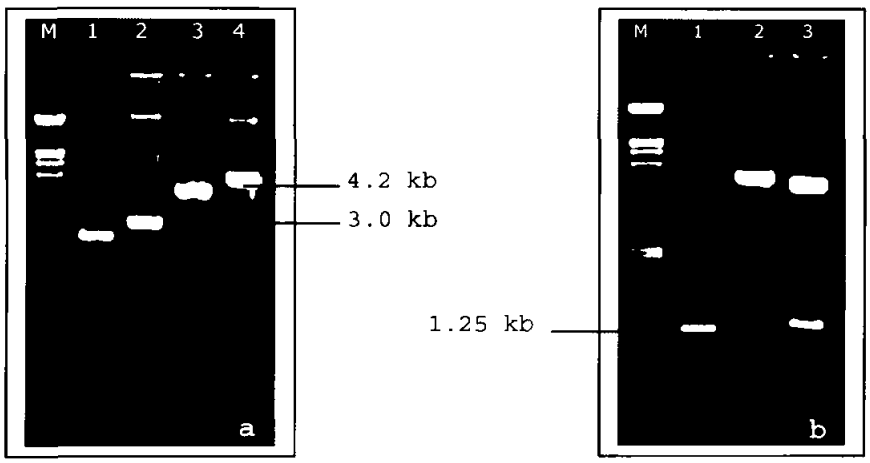


Fig.10- Confirmation of cloning through restriction analysis

a. Linearising plasmid DNA with *pst* I .
M- marker; lane 1- control plasmid uncut; 2- control plasmid digested; 3- plasmid with insert uncut; 4- plasmid with insert digested

b. Release of the cloned fragment by digestion with *Eco*R I
M- marker; 1- gel purified PCR product; 2- plasmid with insert uncut; 3- insert released

4.1.4. Nucleotide sequencing and sequence analysis

The PEG purified recombinant plasmids were sequenced with T7 and SP6 sequencing primers. Both 1.25 kb genomic DNA and 1.12 kb cDNA fragments were sequenced. Both the sequences were similar except for the presence of a 132 bp intervening sequence in the genomic fragment. The sequence of the genomic DNA fragment was presented in Fig 11. The complete *Hevea* genomic sequences coding for β-1,3-glucanase gene isolated in the present study was submitted to database (GenBank Accession No.AY 325498).

The cDNA sequence obtained in this study was aligned with three earlier reported sequences. Maximum similarity of 99% was observed with the *Hevea* β-1,3- glucanase sequence reported by Chye and Cheung, (1995) from the clone RRIM 600. The cDNA codes for a protein consist of 374 amino acids in length. The predicted protein is found to be basic as the pI was theoritically measured as 8.96. The molecular weight was determined as 41202.88 Da with tools in Expasy Molecular Biology server (Switzerland). The deduced protein sequence is shown in Fig. 12.

The amino acid sequence shows differences at certain positions when compared with the three earlier reported sequences coding for the mature protein (Table- 4 and Fig. 13). It was identical to the basic isoform of glucanase reported by Chye and Cheung (1995) at 99.1% of positions. It also shows similarities with pathogen inducible β-glu isolated from grapevine (GenBank Accession No. AAF 44667- 77% similarity), tobacco (JQ 0982- 69%), bean (CAA37289- 66%), chickpea (CAA 10287- 65%), arabidopsis (AAL 36038-64%), potato (AAC 19114- 63%), garden pea (T 06552- 63%), tomato (S26241- 62%), alfalfa (T09401- 62% similarity) and many other acidic and basic isoforms of β-glu isolated from various plant species.

The 374 amino acid pre-protein consists of N-terminal and C-terminal extensions, which are cleaved during the posttranslational modifications (Shinshi *et al.*, 1988). A 36 amino acid length signal peptide at the N-terminal was detected with the SignalP prediction programme (Center for Biological Sequence analysis) (Fig. 14).

```
1     atg*gctatctcctcttcaac ttcaggaact agtagttccc
41    tgccctcaag aactactgtc atgcttcttc tgattttctt
81    tacagcaagc cttggtataa caggtctctc tctctctctc
121   tctctctctc tctctctctc tccatctatc tactctcatg
161   ttaaagttga cgatgctctt tttttctctc cttgttctaa
201   aaggtttcaa ctaatacctg tatttaggaa ttcagatgcc
241   caggtaggtg tttgctatgg aatgcaaggc aacaaccttc
281   cacctgtttc agaggtcata gctctctata aacaatctaa
321   catcaagaga atgagaattt atgatccaaa tcgagcagta
361   ttggaagccc ttagaggctc aaacattgaa ctcatactag
401   gtgttccaaa ctcagatctc caaagcctta ccaatccttc
441   caatgcaaaa tcatgggtac aaaaaaatgt tcgtggcttc
481   tggtcaagtg tcctgttcag atatatagca gttggcaacg
521   aaattagtcc tgtcaataga ggcacagctt ggttggctca
561   atttgttttg cctgccatga gaaatataca tgatgctata
601   agatcagctg gtcttcaaga tcaaatcaag gtctccactg
641   caattgactt gaccctggta ggaaattcct accctccttc
681   tgcaggtgct ttcagggatg atgttagatc atacttggac
721   ccaattattg gatttctatc ctctatcagg tcacctttac
761   ttgccaatat ttatccttac tttacttatg ctggtaatcc
801   aagggatatt tcccttccct atgctttgtt cacttcacca
841   tcagttgttg tgtgggatgg tcagcgaggt tataagaacc
881   tttttgatgc aacgttggat gcattgtact ctgctcttga
921   gagggctagt ggtggttctc tggaggtggt tgtttcggaa
961   agtggctggc cgtctgccgg agcatttgct gccacatttg
1001  acaatgggcg tacttatctc tcaaatttga tccagcatgt
1041  taaaggaggt actcctaaga ggcctaacag agctatagag
1081  acttacttat ttgccatgtt tgatgaaaat aagaagcaac
1121  cagaggttga gaaacacttt ggacttttct ttcctgataa
1161  acggccaaaa tataatctca attttggtgc agaaaagaac
1201  tgggatattt ctactgaaca caatgcaaca atacttttcc
1241  ttaagagtga tatgtga*    1257
```

Fig. 11- Genomic sequences coding for Hevea β-1,3- glucanase.

The intron sequence was indicated in red. The splice donor (GT) and splice acceptor (AG) consensus sequences are underlined. The start (ATG) and end (TGA) codons are denoted by asterisks(*).

MAISSSTSGTSSSLPSRTTVMLLLIFFTASLGITDAQVGVCYGMQGNNL
PPVSEVIALYKQSNIKRMRIYDPNRAVLEALRGSNIELILGVPNSDLQSL
TNPSNAKSWVQKNVRGFWSSVLFRYIAVGNEISPVNRGTAWLAQFVL
PAMRNIHDAIRSAGLQDQIKVSTAIDLTLVGNSYPPSAGAFRDDVRSY
LDPIIGFLSSIRSPLLANIYPYFTYAGNPRDISLPYALFTSPSVVVWDGQ
RGYKNLFDATLDALYSALERASGGSLEVVVSESGWPSAGAFAATFDN
GRTYLSNLIQHVKGGTPKRPNRAIETYLFAMFDENKKQPEVEKHFGLF
FPDKRPKYNLNFGAEKNWDISTEHNATILFLKSDM

Fig. 12-The amino acid sequence deduced from the cDNA sequence of Hevea β-1,3-glucanase.

The N and C-terminal extensions, which are absent in the mature protein, were shown in red

Percent identity							
Divergence		1	2	3	4		
	1	-	97.2	99.1	97.2	1	Present sequence
	2	2.9	-		100	2	Sponer *et al.*, 1999
	3	1.0	2.6	-	97.5	3	Chey and Cheung, 1995
	4	2.9	0	2.6	-	4	Yeang and Chow, 2000
		1	2	3	4		

Table- 4- Sequence similarity and distances between the reported Hevea β-glu proteins as determined through MegAlign programme

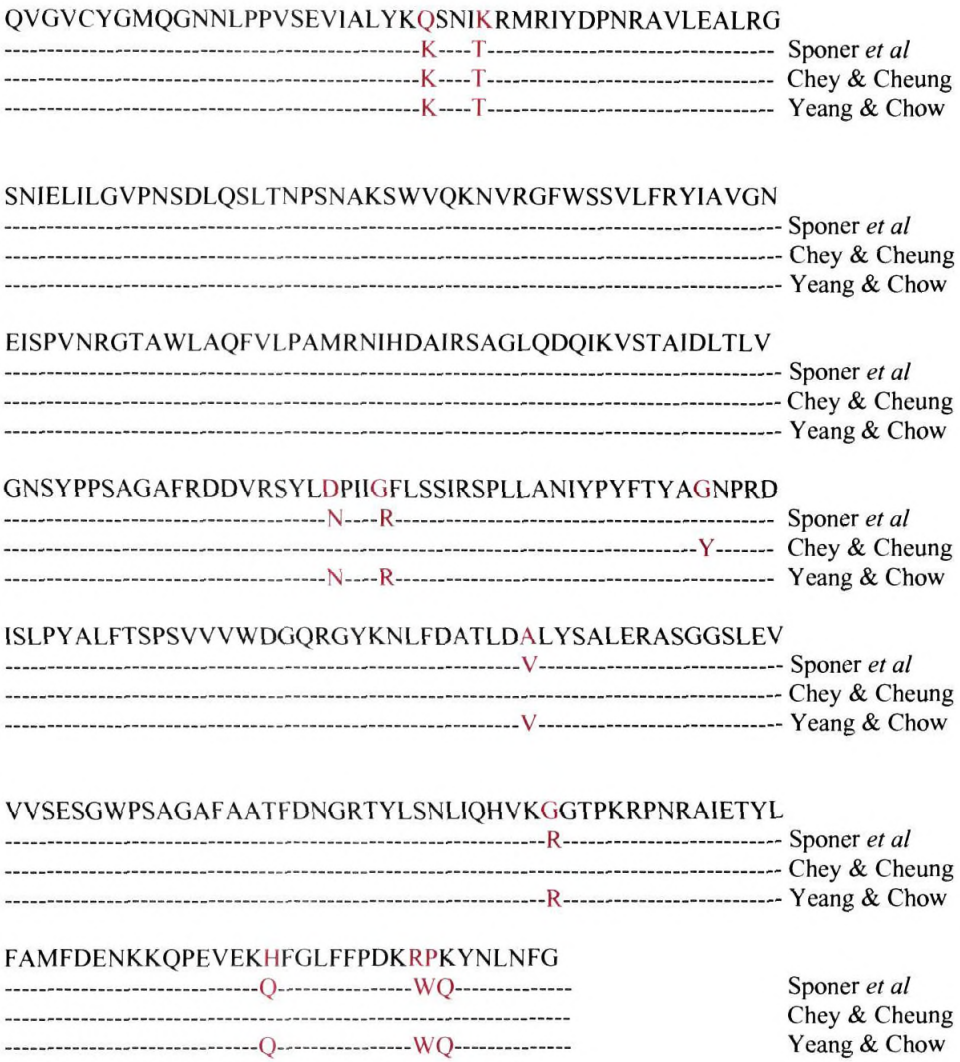
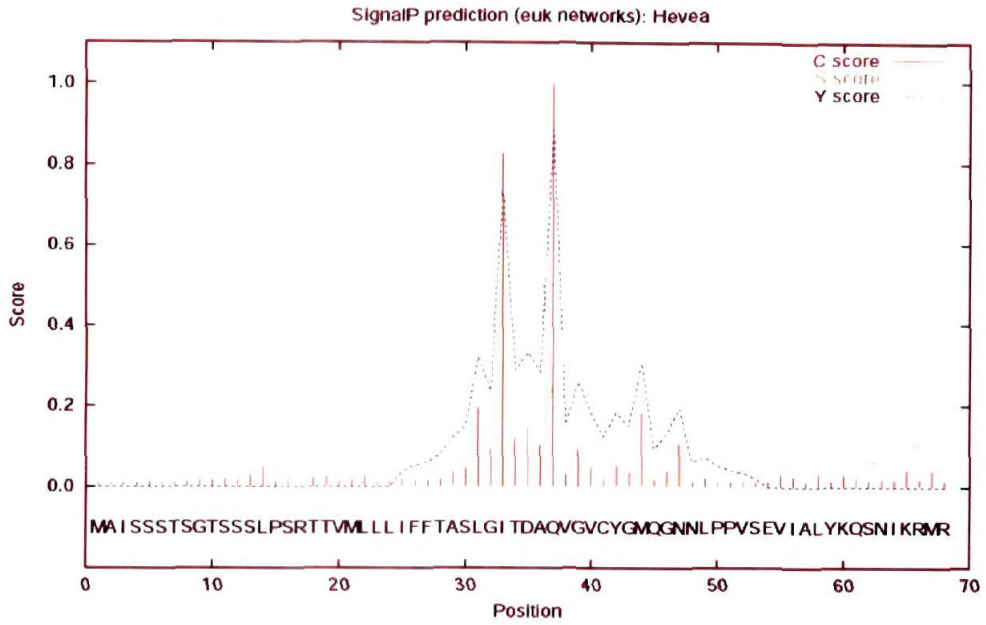


Fig. 13- Amino acid sequence comparison of Hevea mature β-glu with three earlier reported sequences

The places of identity are denoted by dots and differences were shown in red



Is the sequence a signal peptide?

Measure	Position	Value	Cutoff	Conclusion
max. C	37	0.998	0.37	YES
max. Y	37	0.893	0.34	YES
max. S	30	0.961	0.88	YES
mean S	1-36	0.674	0.48	YES

Most likely cleavage site between pos. 36 and 37: TDA-QV

C-score - raw cleavage site score
S-score - signal peptide score
Y-score - combined cleavage site score

Fig. 14- Prediction of the N-terminal 36 amino acids as the signal peptide using SignalP protein analysis software.

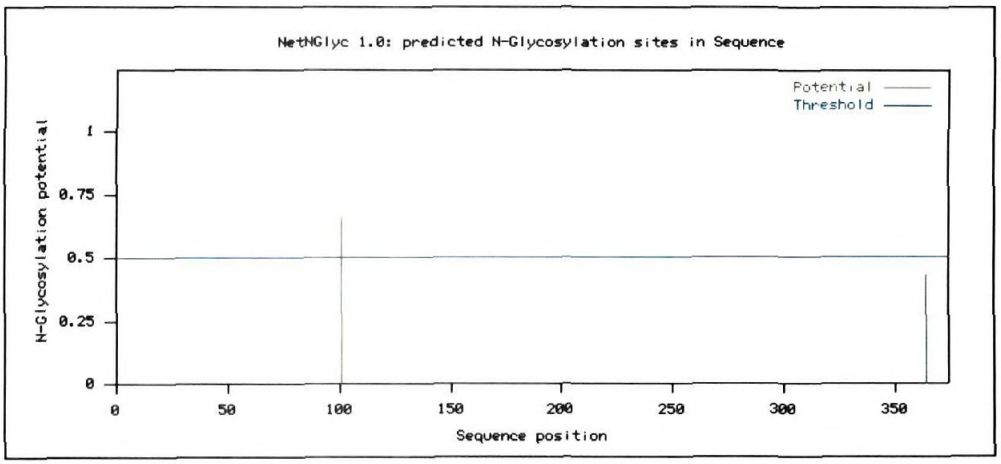


Fig. 15- Putative glycosylation site in the protein as determined by NetNGlyc protein analysis tool.

The potential glycosylation site is the asparagine residue at position 101 (NPSN)

Many proteins are modified after translation by the attachment of carbohydrates; they are said to by glycosylated. In plants, many proteins are glycosylated by *N*-linked oligosaccharides. This *N*-glycosylation of proteins has a great impact both on their physio-chemical properties and on their biological functions (Rayon *et al.*, 1998). The amino acid asparagine serves as the attachment site in many cases and is characterised by the three amino acid motif: Asn-X-Ser/Thr [Asparagine (Asn), any of the 20 amino acids (X), and Serine (Ser) or Threonine (Thr)]. All such sites need not be glycosylated as it depends on many other factors also. NetNGlyc software can predict the putative glycosylation sites in a given sequence after considering all such factors. One potential glycosylation site was found in the present sequence (Fig. 15).

4.1.5. Southern hybridisation

To confirm the presence of the gene, genomic Southern was performed.The genomic DNA of the tolerant and susceptible clone was digested with three restriction enzymes namely *Bam*H I, *Sau3*A I and *Xba* I (Fig.16). The fragments blotted on nylon membranes were probed with PCR amplified β-glu gene fragment. Hybridising bands were detected in both clones. Presence of 3 - 4 hybridising bands indicates the presence of a multi-copy number gene (Fig. 17). Different copies may correspond to the various isoforms of glucanase. More number of bands obtained with *Sau* 3AI is due to the internal restriction sites for the enzyme as shown by the sequence data. However, both tolerant and susceptible clones generated same pattern of signals with the enzymes tested, indicating that there is no difference in the copy number of this defense-related gene in both the clones. This was further confirmed by testing the presence of the gene in 14 different genotypes of *Hevea*, in which 5 are tolerant to abnormal leaf fall disease, 5 are highly susceptible and the remaining four are wild plants which are resistant to most of the fungal diseases. *Hind* III digested fragments generated same pattern of signals in Southern with β-glu gene probe (Fig. 18).

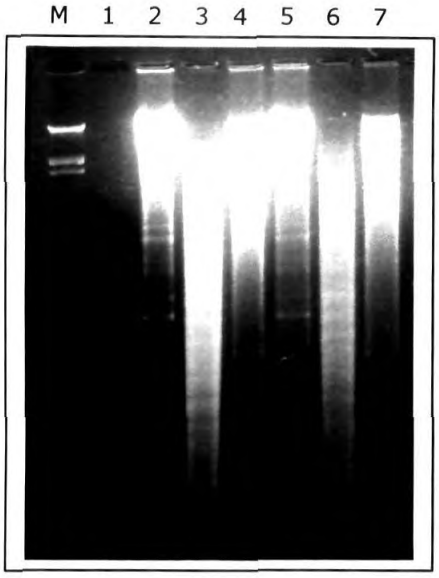


Fig. 16- Genomic DNA digested with restriction enzymes prior to blotting

M-marker, lane 1- positive control; 2 – 4: DNA from clone RRII 105 digested with *Bam*H I, *Sau*3A I and *Xba* I; lanes 5 – 7: DNA from clone RRIM 600 digested with the respective enzymes

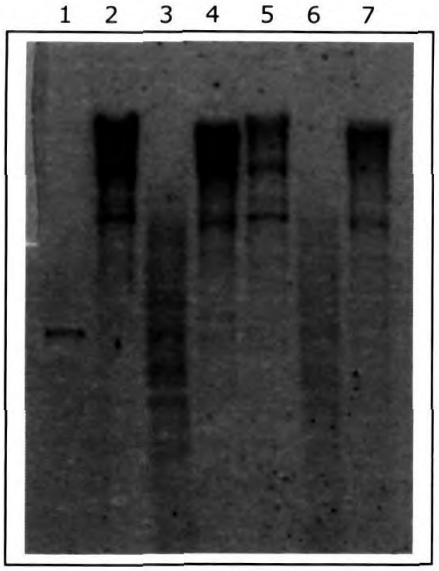


Fig. 17- Southern hybridisation c genomic DNA of tolerant and susceptibl clones with β- glu probe

lane 1- positive control; 2 – 4: DNA from clone RR 105 digested with *Bam*H I, *Sau*3A I and *Xba* I; lane 5 – 7: DNA from clone RRIM 600 digested with th respective enzymes

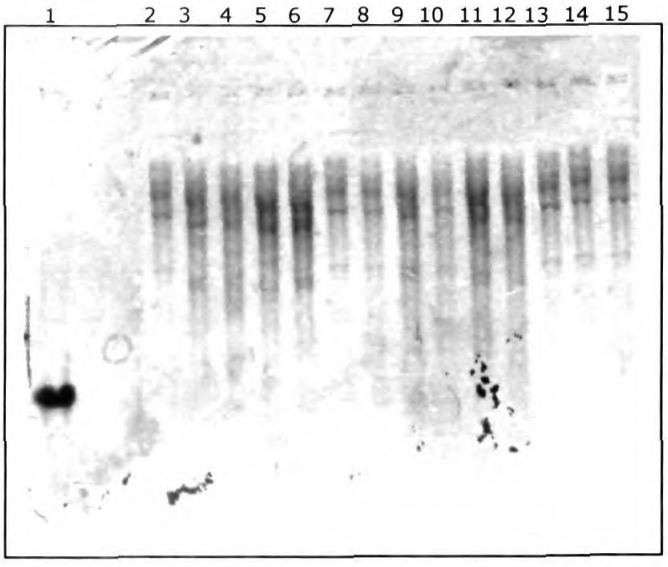


Fig. 18- Southern hybridisation of Hind III digested genomic DNA fragments of 14 genotypes of Hevea with β-glu probe

Lane 1- positive control; lanes 2 – 6: RRII 105, GI 1, BD 10, GT 1, RRII 33 (tolerant clones); lanes 7 – 10: FX 516, F 4542, two seedling plants (wild); lanes 11 – 15: RRIM 600, Tjir 1, PB 86, RRIM 701, PR 107 (susceptible clones)

4.1.6. Isolation of the promoter elements of β-glu gene through RAGE

An attempt has been made to isolate the 5' regulatory elements of the gene of interest using RAGE technique. The technique involves the PCR amplification of the region of interest using a reverse primer based on a known DNA sequence and a forward primer based on a specially designed adapter (Fig. 19).

Genomic DNA was digested to generate blunt ended fragments. Many blunt end-cutting enzymes were tried and those, which provide good digestion, were selected. These include *Eco*R V, *Stu* I, *Ssp* I, *Dra* I and *Hae* III. The digested fragments were ligated with the adapter. PCR was performed with adapter specific and β glu gene specific primers, immediately after stopping the ligation reaction. A gradient PCR was tried with different annealing temperatures. Only *Stu* I, *Ssp* I and *Dra* I enzymes produced some amplicons in the first PCR reaction (Fig. 20). The digestion of genomic DNA with *Eco*R V was comparatively less and it was over-digested with the four-cutter *Hae* III. These factors affected the ligation and the subsequent PCR.

The diluted first PCR products were re-amplified in a nested PCR with a second pair of adapter and gene specific primers for confirmation. Here only *Ssp* I and *Dra* I digested-samples produced amplicons, which are of around 500 bp long (Fig.21). Two PCR products from *Ssp* I and *Dra* I digested DNA samples were cloned in pGEM vectors (Fig. 22). Recombinant plasmids were isolated in large quantities and PEG purified for sequencing. The sequence data was presented in Fig. 23.

A minimal promoter with CAAT and TATA boxes was present in the PCR amplified fragments. The 513 bp long PCR fragment contains only 198 bp upstream elements. The CAAT box was located at the position –139 and the TATA box was seen at –82 from the ATG start codon. This is the first report on the isolation of promoter elements of β-glu gene in *Hevea* and the sequence data was submitted to the GenBank. (Accession No. AY 325498).

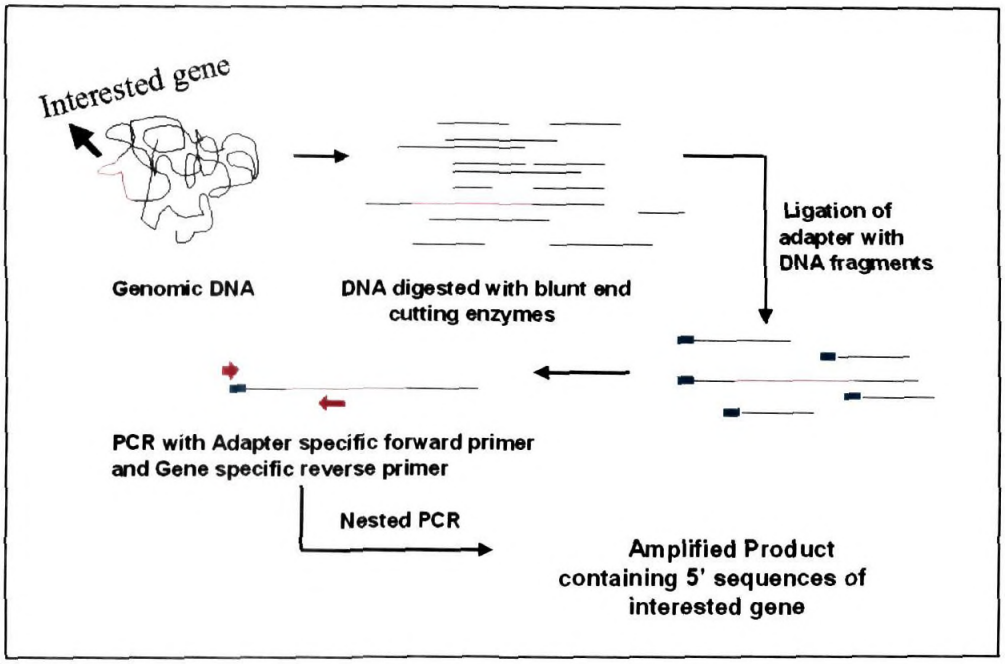


Fig.19- Diagrammatic representation of RAGE technique for the isolation of 5' upstream elements of the gene

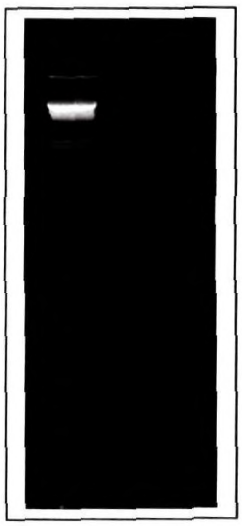


Fig. 20- Ssp I digested adapter ligated genomic DNA fragments amplified with adapter specific and gene specific primers

M- marker; lane 1- amplicons

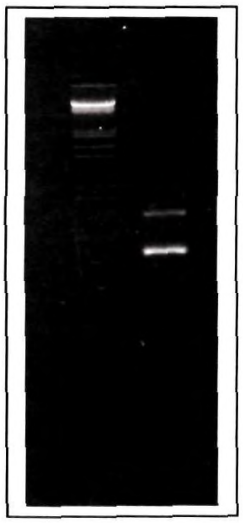


Fig. 21- Nested PCR with the products of the first PCR as template

M- marker; lane 1- amplification from the *Ssp* I digested samples

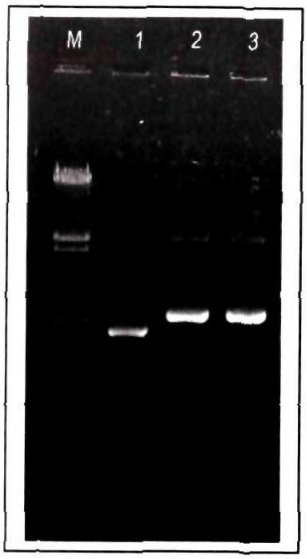


Fig. 22- Cloning of the RAGE products in pGEM vector

M- marker; lane 1- control plasmid without insert; 2 – 3: plasmid containing the inserts

ataggg ctcgagcggc cgcccgggca ggtatttctt ccattaaata
aatatattag ttttaatttg gcgatagttt aaattcaaac cccaattgac
attaaataaa taaataagct ccagaaatag tatgctaagg agtccctata
taaagcatag acttggtagt aatgtaatgc atatgcatgc tccaaattaa
gctgctctct tcttaggttc catccttctt aatggctatc tcctcttcaa
cttcaggaac tagtagttcc ctgccctcaa gaactactgt catgcttctt
ctgattttct ttacagcaag ccttggtata acaggtctct ctctctctct
ctctctctct ctctctctct ctccatctat ctactctcat gttaaagttg
acgatgctct ttttttctct ccttgttcta aaaggtttca actaatacct
gtatttagga attcagatgc ccaggtaggt gtttgctatg gaatgcaagg
caacaacctt ccacctg

Fig. 23- The promoter sequence of rubber β-glu obtained through RAGE protocol

The PCR primers were underlined. The ATG start codon, CAAT box and TATA box are shown in red.

4.2. Gene expression studies through Northern hybridisation and RT-PCR analysis

4.2.1. Fungal infection to the plant material

Fresh mycelia of the pathogen were inoculated to white oats medium and grown for 2 days in darkness. Then the mycelial mat was washed with sterile water and exposed to light for sporulation. It was observed that the fungal mat in water produced sporangia profusely after 24 h. The complete mat was studded with sporangia (Fig. 24). A brief heat shock effectively released the motile zoospores. This medium free zoospore suspension was used for inoculation (Fig. 25). After inoculation, the plants were covered with transparent polythene bags to maintain the required humidity and observed for the development of the symptoms (Fig. 26). The disease symptoms begin to appear 2 days after inoculation and were more prevalent in the susceptible clone than the tolerant one. The necrotic spots developed by the hyper sensitive responses induced by the pathogen were more in the areas where the inoculum density was higher.

4.2.2. Isolation of RNA from infected leaves

RNA was isolated in good quality and concentration from the infected zones of the leaves (Fig. 27). RNA was also isolated from uninfected control samples. No DNA contamination was confirmed, as it may interfere with the expression analysis.

4.2.3. Amplification of sequences for using as internal controls

A part of the sequences coding for 18S RNA gene and β-actin gene were PCR amplified from *Hevea* for using as internal controls in northern and RT-PCR experiments. The 1.7 kb 18S RNA gene fragment and 260 bp actin gene fragments were cloned in pGEM vectors (Fig. 28 and 29) and sequenced to confirm their identity with the published sequences. The sequence data is presented in Figs. 30 and 31. Both fragments were shown nearly 100% similarity with the published sequences in other species in the databank. These gene fragments are reported for the first time from rubber tree in the present study.

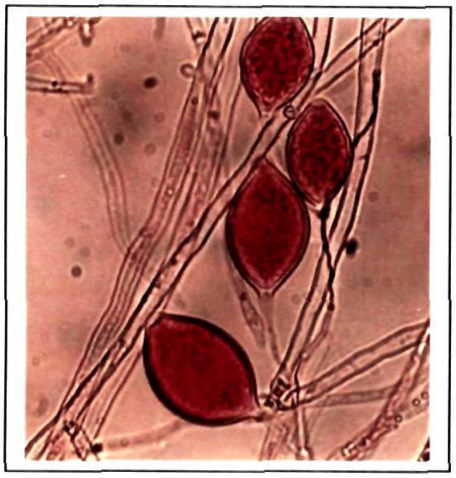


Fig. 24- Sporangial development after 2 days of culture in white oats medium

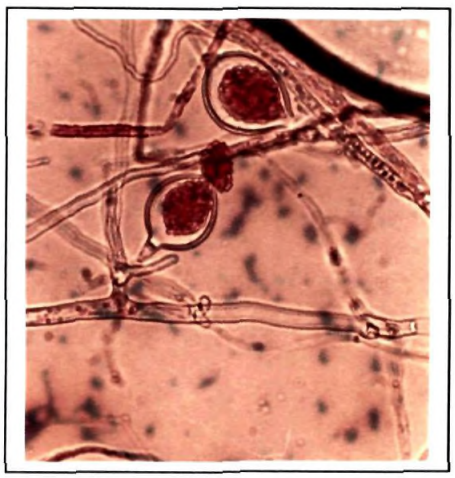


Fig. 25- Release of zoospores through heat shock prior to inoculation

Fig. 26- Tolerant and susceptible plants inoculated with Phytophthora and maintained under favourable conditions for disease development

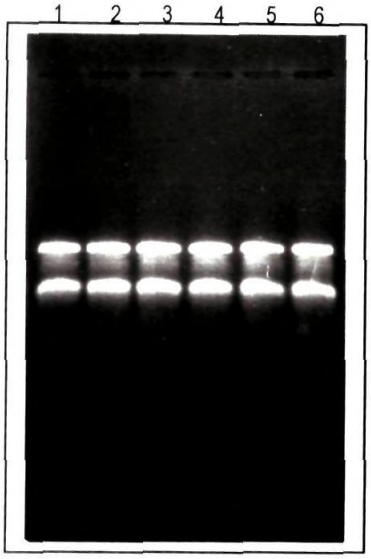


Fig. 27- RNA isolated from infected leaf samples of tolerant and susceptible clones at different time intervals

lanes 1 – 3: RNA isolated from tolerant clone RRII 105 at 0, 48 and 96 h after inoculation. Lanes 4 – 6: RNA isolated from susceptible clone RRIM 600.

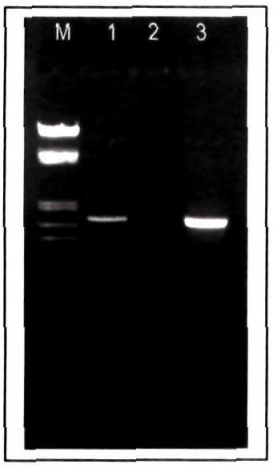


Fig. 28- Amplification of a part of the 18S RNA gene and cloning

M- marker; lane 1- 1.7 kb amplified fragment, 2- PCR using control vector as template; 3- confirmation of cloning by PCR using vector with insert as template

Fig. 29- Amplification of a part of the actin gene

M- marker; lane 1- PCR amplified 260 bp actin gene fragment from cDNA

tacctggttg atcctgccag tagtcatatg cttgtctcaa agattaagcc
atgcatgtgt aagtatgaac taattcagac tgtgaaactg cgaatggctc
attaaatcag ttatagtttg tttgatggta tctgctactc ggataaccgt
agtaattcta gagctaatac gtgcaacaaa ccccgacttc tggaaggaat
gcatttatta gataaaaggt cgacgcgggc tctgcccgtt gctctgatga
ttcatgataa ctcgacggat cgcacggcca tcgtgccggc gacgcatcat
tcaaatttct gccctatcaa ctttcgatgg taggatagtg gcctactatg
gtggtgacgg gtgacggaga attagggttc gattccggag agggagcctg
agaaacggct accacatcca aggaaggcag caggcgcgca aattacccaa
tcctgacacg gggaggtagt gacaataaat aacaataccg ggcgctttag
tgtctggtaa ttggaatgag tacaatctaa atcccttaac gaggatccat
tggagggcaa gtctggtgcc agcagccgcg gtaattccag ctccaatagc
gtatatttaa gttgttgcag ttaaaaagct cgtagttgga ccttgggcgc
ggccgggccg gtccgcctca cggcaggcac cgacctgctc gacccttctg
ccggcgatgc gctcctggcc ttaactggcc gggttcgtgc ctccggcgcc
gttactttga agaaattaga gtgctcaaag caagccatcg ctctggatac
attagcatgg gataacatca taggattccg gtcctattgt gttggccttc
gggatcggag taatgattaa tagggacagt cgggggcatt cgtatttcat
agtcagaggt gaaattcttg gatttatgaa agacgaacaa ctgcgaaagc
atttgccaag gatgttttca ttaatcaaga acgaaagttg ggggctcgaa
gacgatcaga taccgtccta gtctcaacca taaacgatgc cgaccaggga
tcggcggatg ttgcttatag gactccgccg gcaccttatg agaaatcaaa
gtctttgggt tccgggggga gtatggtcgc aaggctgaaa cttaaaggaa
ttgacggaag ggcaccacca ggcgtggggc ctgcggctta atttgactca
acacggggaa acttaccagg tccagacata gcaaggattg acagactgag
agctctttct tgattctatg ggtggtggtg catggccgtt cttagttggt
ggagcgattt gtctggttaa ttccgttaac gaacgagacc tcagcctgct
aactagctat gcggaggtga ccctccgcgg ccagcttctt agagggacta
tggcctttta ggccaaggaa gtttgaggca ataacaggtc tgtgatgccc
ttaggtgttc tgggccgcac gcgcgctaca ctgatgtatt caacgagtct
atagccttgg ccgacaggcc cgggtaatct ttgaaatttc atcgtgatgg
ggatagatca ttgcaattgt tggtcttcaa cgaggaattc ctagtaagcg
cgagtcatca gctcgcgttg actacgtccc tgccctttgt acacaccgcc
cgtcgctcct accgattgaa tggtccggtg aagtgttcgg atcgc

Fig. 30- Sequence of 18S RNA gene in *Hevea* (GenBank Accession No. AY 435212) The PCR primer sequences are underlined

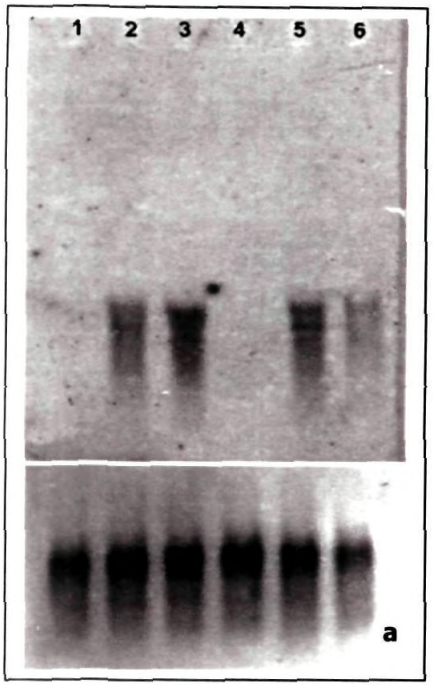
tccataatga agtgtgatgt ggatatcagg aaggatctct atggtaacat
tgtgctgagt ggtggttcca ctatgttccc cggcattgca gatcgaatga
gcaaggagat cactgctctt gctccaagca gcatgaagat caaggtggtt
gctccaccgg agagaaagta cagtgtctgg attggaggat ctatcttggc
atcccttagc accttccagc agatgtggat ttccaagggt gagtatgacg
agtcaggtcc

Fig. 31- Partial sequence of β-actin gene in *Hevea*. The PCR primer sequences are underlined

4.2.4. Northern hybridisation

Accumulation of anti-fungal proteins like β-1,3-glucanases is a common phenomenon associated with HR. RNA isolated from necrotic zones of infected tissues and control uninfected leaves at different time intervals after inoculation were subjected to northern blot hybridisation 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 rehybrdised with the 18S gene probe. The signals generated indicate that the 18S RNA content did not vary with the treatments and time course. This uniform expression of rRNA indicates that there was no non-specific shift in the relative amounts of mRNA. In this study β- glu mRNA levels change dramatically, while the 18S RNA levels remained almost the same till 96 hrs after inoculation (Fig. 32 a). As mentioned in the materials and method section, the β- glu data were corrected for 18S RNA values and then normalized to 0 h (control) for each clone (Fig. 32 b). In both tolerant and susceptible clones the glucanase probe hybridised to 1.2 kb mRNA while the 18S RNA hybridised to a 1.7 kb mRNA.

Analysis of the accumulation of mRNAs encoding β- glu demonstrated that activation trends are different between the two clones. The quantity of glucanase mRNAs present in the uninfected control samples were not up to detectable levels in northern blots in both tolerant and susceptible clones. Control plants sprayed with dist. H_2O and maintained in similar conditions as infected ones, also did not exhibit the induction of glucanase mRNAs till 96 h as no signal were detected. However, the transcript levels of β- glu showed a marked increase, 48 h after inoculation in both clones. From the pattern of expression a faster rate of increase appears to be occurred in tolerant clone as more intense signal was generated. This difference in the magnitude of induction was even more evident at 96 h. Although the level of β- glu transcripts remains almost the same in tolerant clone, the hybridisation signals observed for susceptible clones dropped to drastically low levels at 96 h (Fig. 32).



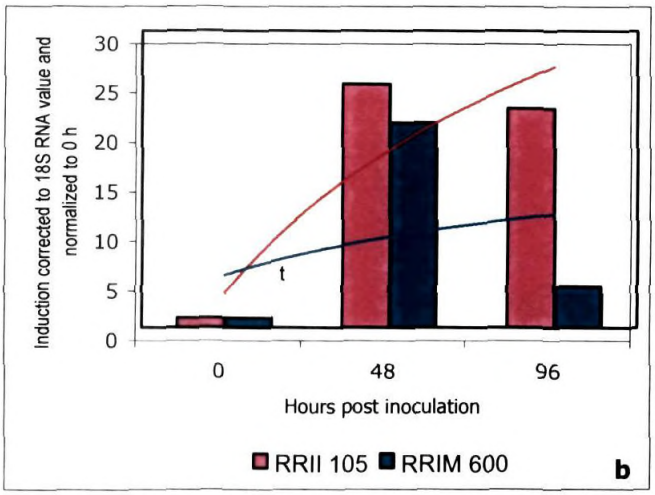


Fig. 32- Northern blot hybridisation analysis and corresponding graphics plot showing differential expression of β-1,3- glucanase gene

a. lanes 1- 3 – RNA isolated from tolerant clone RRII 105 at 0, 48 and 96 h after inoculation. lanes 4- 6- RNA isolated from susceptible clone RRIM 600. The bottom panel of the same blot was re-hybridised with the 18 S RNA probes, which is showing uniform expression.

b. Graphical representation of the northern blot by measuring the net intensity of hybridising bands with the Kodak image analysis software. The net intensity data for β-glu were corrected for 18S RNA gene data and then normalized to 0 h. Trend lines also were shown

The β- glu expression patterns quantified by relative RT-PCR also indicates similar results. Using gene specific primers, the β- glu gene and a part of house keeping actin gene were co-amplified from the first strand cDNA, synthesised from the total RNA isolated from different samples. RNA was quantified to ensure equal amount of template for first strand cDNA synthesis using oligo-(dT) primers. We used actin as the internal control in RT-PCR as the 18S RNA genes cannot be reverse transcribed with oligo-(dT) primers. Transient levels of the internal control remains almost the same in control as well as treated samples, at all time intervals in both tolerant and susceptible clones. But as observed in the northern blots, β-glu appear to be differentially regulated. However, in contrast to northern blots, we got amplification of β- glu mRNAs with low intensity in control uninfected samples also. This may be due to the reason that tiny amount of template is enough for the amplification of bands in PCR, while more quantities of template is required in northern blots (Fig. 33).

The net intensities of β-glu bands amplified from control samples of both clones were more or less same in the ethidium bromide stained agarose gels. In the infected samples, the band intensities begin to increase 24 h after inoculation and an exponential increase in band intensity was observed at 48 h (Fig. 33). This increase could be due to the quantity of the target sequences in the cDNA template available in PCR, as all other constituents remains the same and thus can be correlated with the increased gene activity.

The results are in accordance with that of northern blots as higher and more rapid accumulation of β- glu transcripts observed in the case of tolerant clone RRII 105. The β- glu levels reached a peak at 48 h after inoculation as almost 25-fold increase than its basal level was observed in tolerant clones. At the peak level, only 15-fold increase was seen in susceptible clone. The induction was observed to be more prolonged in tolerant clone, as the induction level remains higher than basal levels 4 days after inoculation, but slightly lower than the peak value. While in susceptible clones, the transcript levels of β- glu decreased drastically and it was only 4-fold higher than the basal levels at 96 h (Fig. 34).

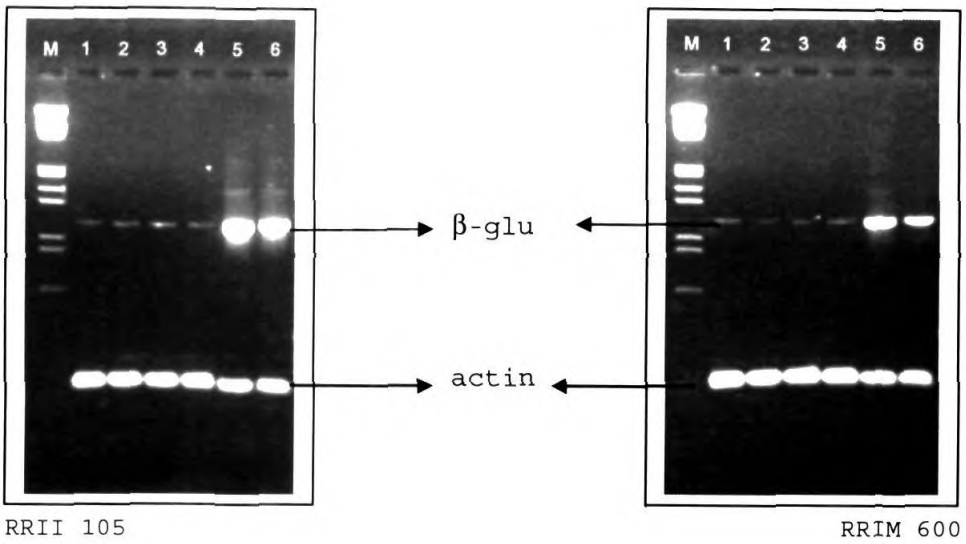


Fig. 33- Relative RT-PCR analysis of control and infected leaf tissues

M- marker; Lane 1 – 3: control uninfected leaf tissues at 0, 48 and 96 h after inoculation; Lanes 4 – 6: infected samples at 0, 48, and 96 h after inoculation

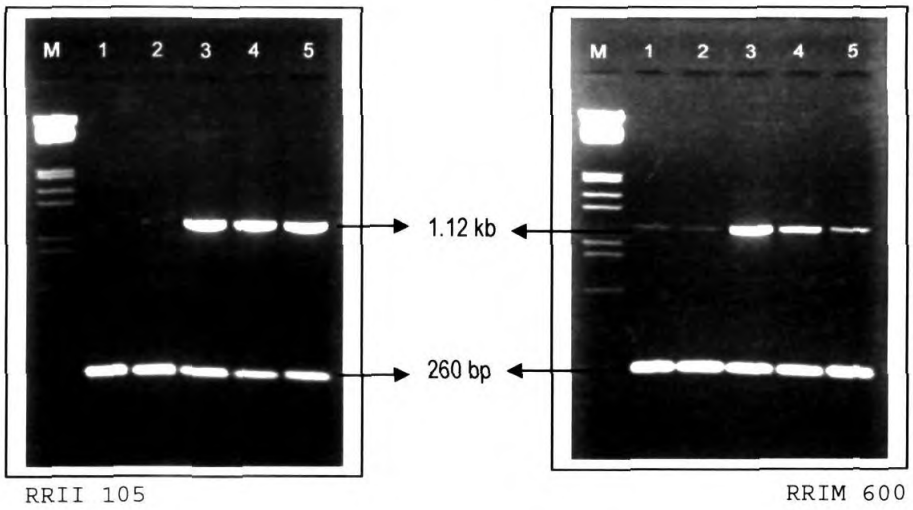


Fig. 34- Induction of β- glu transcripts in the infected leaf tissues at different time intervals

M- marker; Lane 1 – 0 hr after inoculation; 2- 24 h; 3- 48 h; 4- 72 h; 5- 96 h after inoculation.

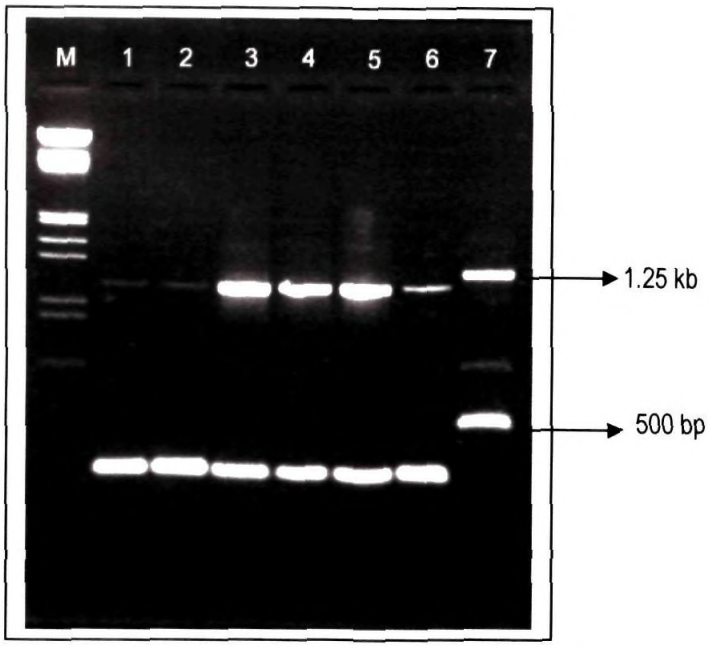


Fig. 35- Comparison of the induction pattern of β-glu in tolerant (RRII 105) and susceptible (RRIM 600) clones of Hevea in response to infection with Phytophthora.

M- marker; lane 1- 2: RRII 105 and RRIM 600, 0 h after inoculation; Lane 3 – 4: 48 h after inoculation respectively; 5 – 6: 96 h after inoculation, 7- 1.25 kb β-glu and 500 bp actin gene fragments amplified from the genomic DNA

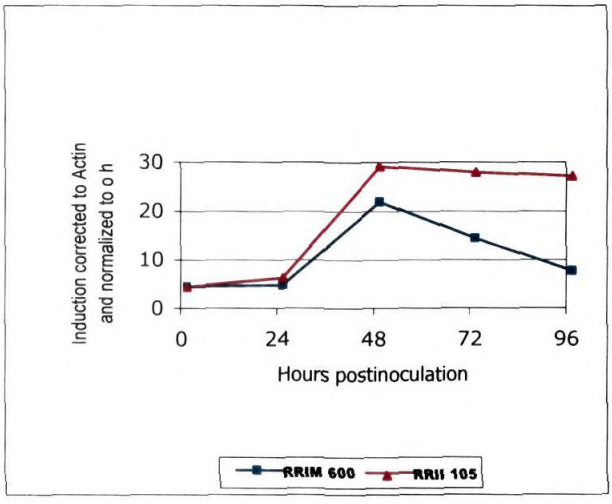


Fig. 36- Graphic representation of the comparison of β-glu induction in tolerant and susceptible clones

The faster and more prolonged induction observed in the case of tolerant clone as revealed by relative RT-PCR analysis

Total RNA used in a PCR reaction did not produce any amplicons and thus eliminates the chance of any DNA contamination. Genomic DNA used in a control reaction generated a 1.25 kb β- glu and 500 bp actin fragments due to the presence of introns, while the RT-PCR products were 1.12 kb and 260 bp respectively (Fig.35).

The β- glu band intensity corrected to that of actin and normalized to 0 h was plotted against 'hours post inoculation'. Activation trends were more evident in these plots. (Fig. 36 – 38). The more rapid and prolonged induction of β-glu in tolerant clone was clearly indicated in the graphical plots. Although induction occurs in susceptible clones also, mRNA level starts to deplete drastically 72 h after inoculation and it was coming down to the basal levels at 96 h after inoculation, while in tolerant clone the induction level recorded only slight decline.

The detection of the differential expression of β-glu mRNA transcripts through RT-PCR by measuring the band intensity and correcting the value for that of a house keeping gene actin, gave almost same results of northern blot hybridisation with house keeping 18S RNA gene as internal control (Fig. 39). This validates the use of relative quantitative RT-PCR in gene expression studies.

4.2.6. Measurement of enzyme activity

Both northern hybridisation and relative RT-PCR will give a measure of the β-glu mRNA population only. An increased mRNA population always need not confirm the presence of the end product. Therefore, β-glu enzyme activity was also measured in the control and infected samples spectrophotometrically through laminarin-dinitrosalicylic acid method. In agreement with the increased transcript levels, the enzyme activity was also several-fold higher in all the infected samples. No significant difference was observed in the healthy tissues of both RRII 105 and RRIM 600. The activity was stimulated by the *Phytophthora* infection and maximum activity was recorded at 48 and 72 h after inoculation in both clones (Fig. 40). There was a marked difference in the increase in enzyme activity between the two clones. Tolerant clone reordered higher activity than the susceptible one at all the time intervals. The activity levels remains high even four days after inoculation in RRII 105, while in RRIM 600 enzyme activity level has come down 3 days after inoculation.

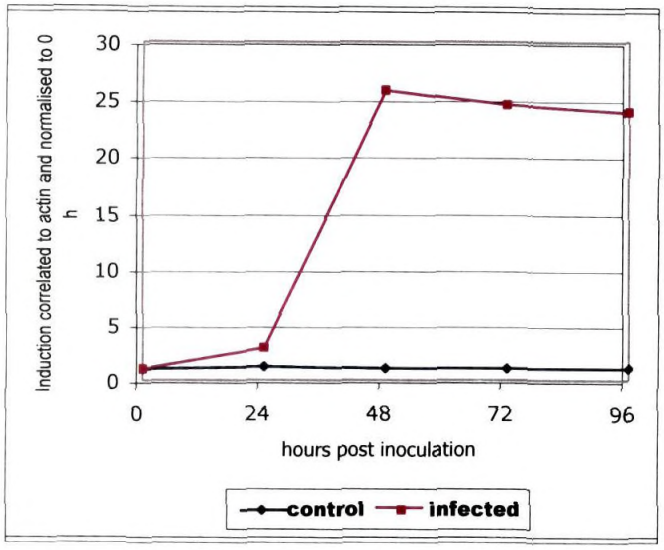


Fig. 37- Graphic plot on the induction of β-glu expression relative to that of actin in control and infected leaves of tolerant clone RRII 105.

Almost 25-fold increase was observed than its basal levels 48 hrs post inoculation

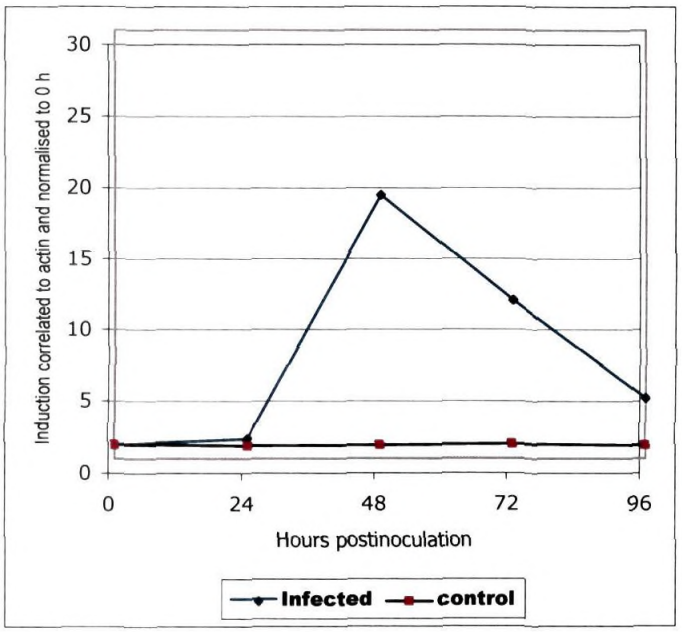


Fig. 38- Graphic plot on the induction of β-glu expression relative to that of actin in control and infected leaves of susceptible clone RRIM 600

Transcript levels depleted drastically after reaching a peak 48 hrs post inoculation

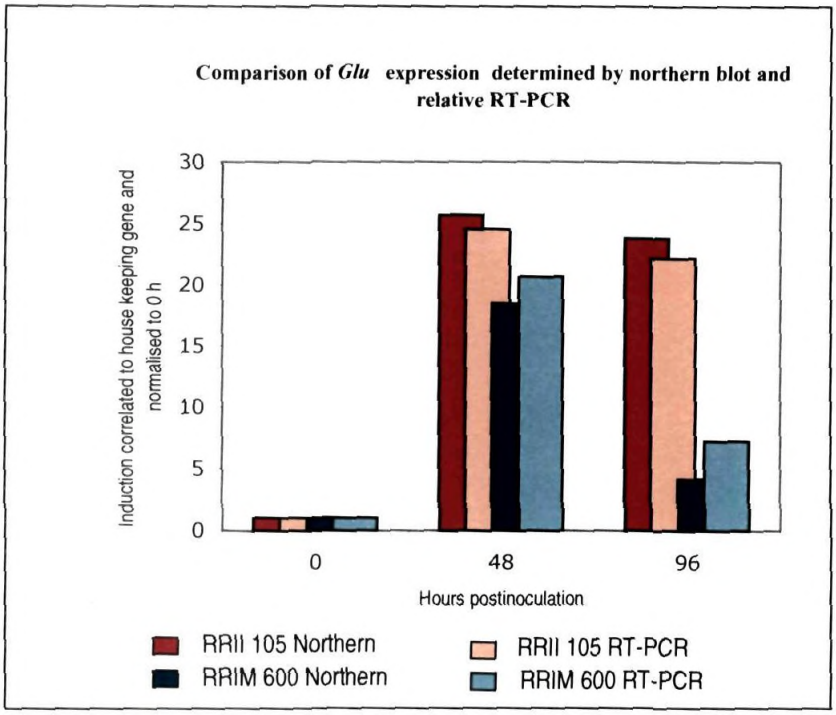


Fig. 39- Northern hybridisation and relative RT-PCR shows almost similar results on the pattern of glucanase induction in tolerant and susceptible clones

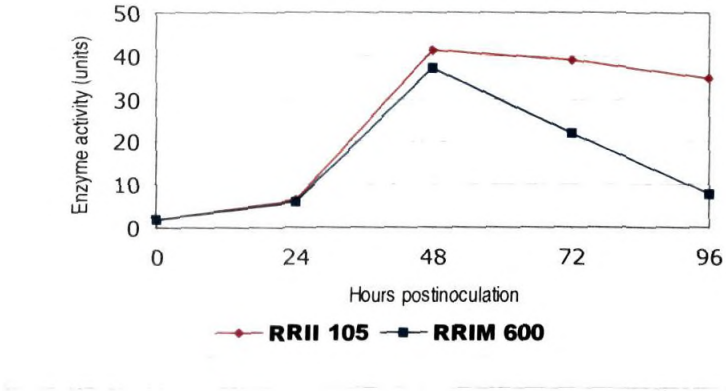


Fig. 40- β-Glu activity in the infected tissues of tolerant and susceptible clones measured by dinitrosalicylic acid method using laminarin as the substrate

In accordance with the induced transcript levels of β-glu, presence of the end product was also detected

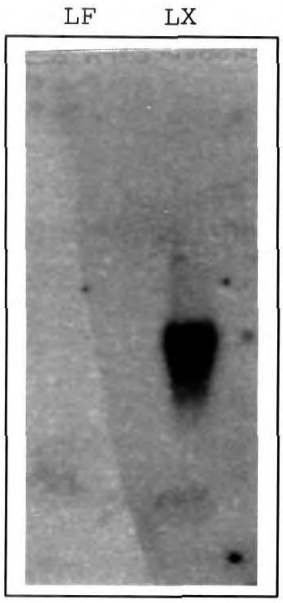


Fig. 41- Constitutive expression of β-glu in the latex

LF- RNA from leaf; LX- RNA from latex

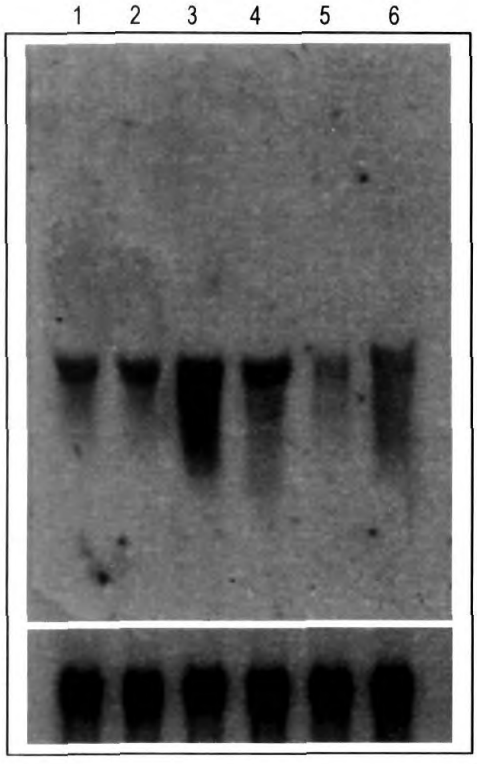


Fig. 42- Expression levels of β-glu in the latex of different clones of Hevea

1- RRII 105; 2- RRII 33; 3- FX 516; 4- RRIM 600; 5- PB 86; PR-107. Highest expression was observe with clone FX 516. Bottom panel of the same membrane was hybridised with 18 S probe

4.2.7. Tissue specific expression

The β-glu gene was found to be constitutively expressing in the latex of rubber tree, but in leaves it is to be induced. Signal with high intensity was also obtained when latex of a newly opened tree was used for northern hybridisation (Fig. 41). To see any correlation between the level of this constitutive expression and tolerance, equal amount of RNA from three tolerant and three susceptible plants were analysed for the presence of β-glu mRNAs. It was found that the level of expression was comparatively more in tolerant clones, with strongest signals obtained from clone FX 516. (Fig. 42). However, susceptible clone RRIM 600 also recorded higher gene expression.

4.3. Construction of functional cDNA clone and purification of recombinant proteins

4.3.1. PCR amplification with restriction site attached primers

Two primer pairs were designed with restriction sites to amplify the region coding for the mature final protein. All the primer combinations successfully amplified the band of expected size (963 bp) under optimal PCR conditions (Fig. 43). The fragments obtained from primer combinations 3 & 2 and 1 & 2 were gel purified for cloning purpose. The first one (BE) is having a *Bam*H I site at 5' end and an *Eco*R I site at 3' end, while the second fragment (Eco) possess *Eco*R I site at both ends. The presence of two different sites on either ends ensured unidirectional cloning. The gel-purified fragments were digested with the respective enzymes to create the cohesive ends.

4.3.2. Cloning in expression vectors

Two bacterial expression systems namely, pGEX and pET systems (Fig. 44 and 45) were tried in the study for the bacterial expression of rubber β-1,3-glucanase gene. The expression vectors were isolated in large quantities. Concentration and purity of the plasmid preparations were checked in agarose gels (Fig. 44). The 4.9 Kb pGEX and 5.9 Kb pET vectors were digested with the appropriate restriction enzymes and digestion was ensured on agarose gels (Fig. 46).

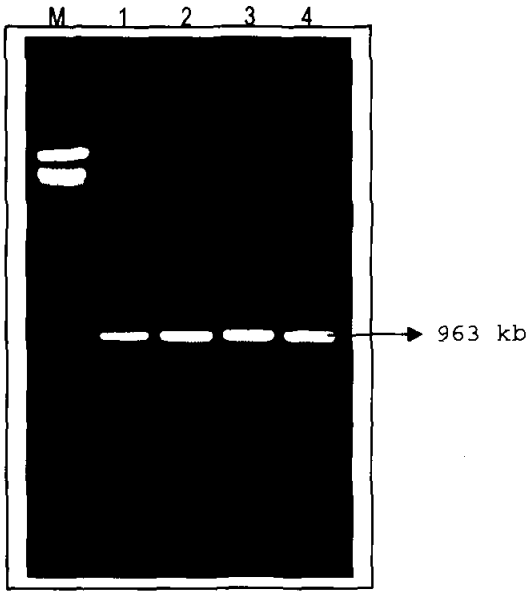


Fig. 43- PCR amplification of the sequences coding for the mature β-1,3-glucanase using four pairs of primers with restriction sites.

M- mol. wt. marker; 1- amplification with primer pairs 1 & 2; 2- with 1 & 4; 3- with 3 & 2 and 4- amplification with 3 & 4

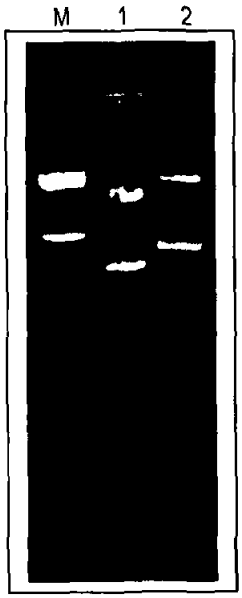


Fig. 44- The 4.9 kb pGEX and 5.9 kb pET vectors isolated for cloning of the gene.

M- marker; 1- pGEX and 2- pET

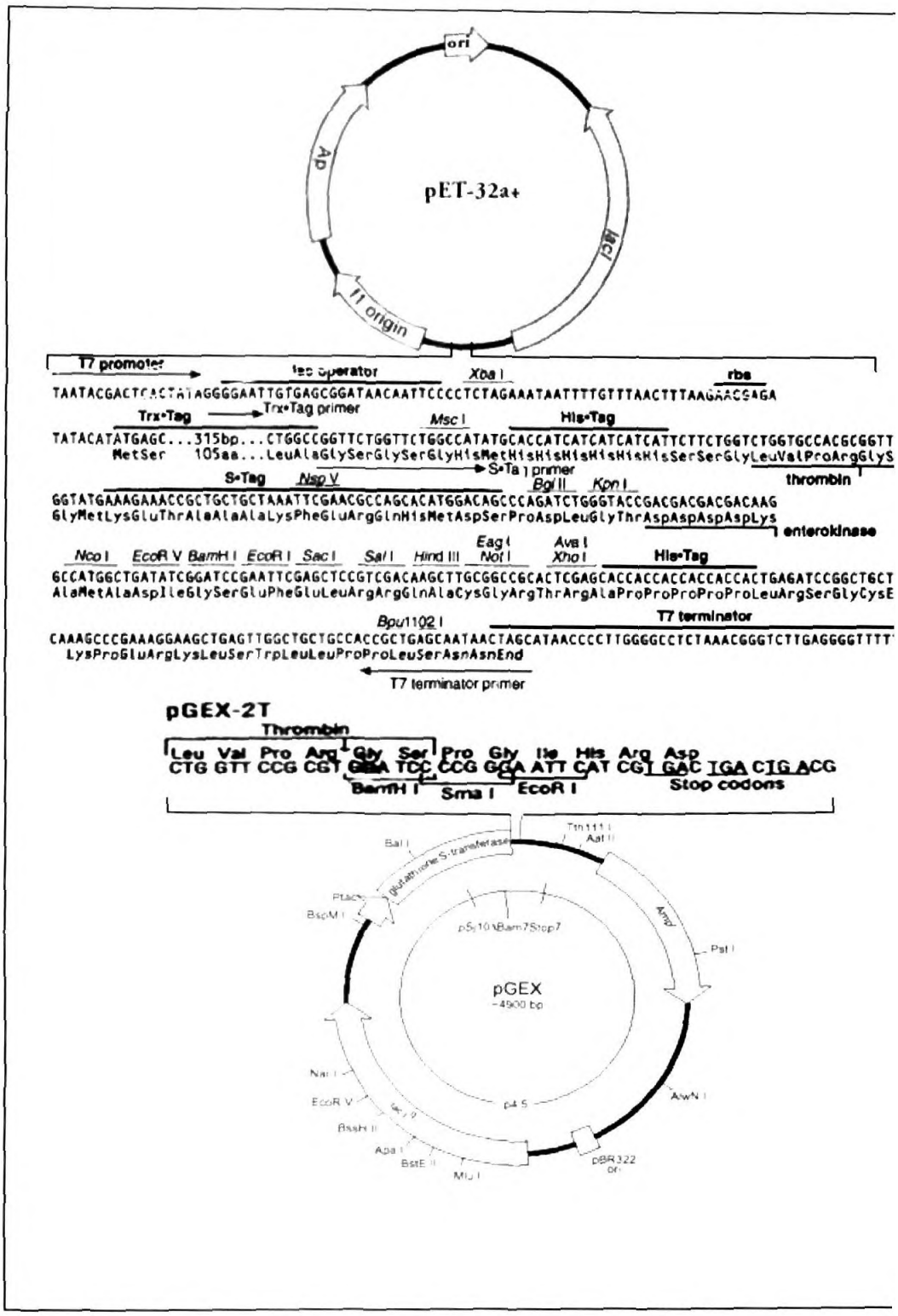


Fig. 45- The pET 32a+ and pGEX-2T expression vectors. Multiple cloning sites and the fusion tags were shown

After ligation and transformation, the cells were spread on amp$^+$ LB plates. Many hundred colonies were obtained with all ligated mix. For analysis of recombinants, plasmid miniprep was carried out from a few colonies and the presence of the insert was ensured by three methods. Difference was observed in the migration of bands, when potential recombinant plasmids were run along with negative controls (plasmid without insert) (Fig. 47). Those plasmids, which shows size difference with control was used for PCR analysis. Band of expected size was amplified from potential recombinant plasmids when insert specific primers were used in the PCR, while no amplification was observed when control plasmid was used as the template (Fig. 48). For further confirmation, plasmids that gave positive results in PCR were used in the restriction digestion analysis. On linearising with a single restriction enzyme (*Bam*H I), the vector size on agarose gel was found to be 5.86 and 6.86 kb for pGEX and pET respectively. This confirms the presence of the insert as the control vector size is only 4.9 and 5.9 kb respectively for pGEX and pET. The 963 bp inserted band could successfully released by double digesting the plasmid with *Eco*R I and *Bam*H I, which are the restriction sites on either side of the insert (Fig. 49 and 50). Finally the correct orientation of the insert was ensured by PCR with vector specific forward and insert specific reverse primers, which yield fragment of expected size.

Recombinant pGEX and pET vectors, which were tested positive in all confirmations, were isolated in large quantities from 100 ml cultures, PEG purified and sequenced to ensure that there is no shift in the translational reading frame. The sequencing data along with the reading frame is presented in Fig. 51 and 52. The sequence coding for the final mature protein only was cloned, excepting the N and C-terminal extensions of pre-proprotein. However, five amino acids of the C-terminal extension were retained for recombinant expression, as it was reported that in some cases these amino acids were not completely removed in the final functional protein (Subroto *et al.*, (2001).

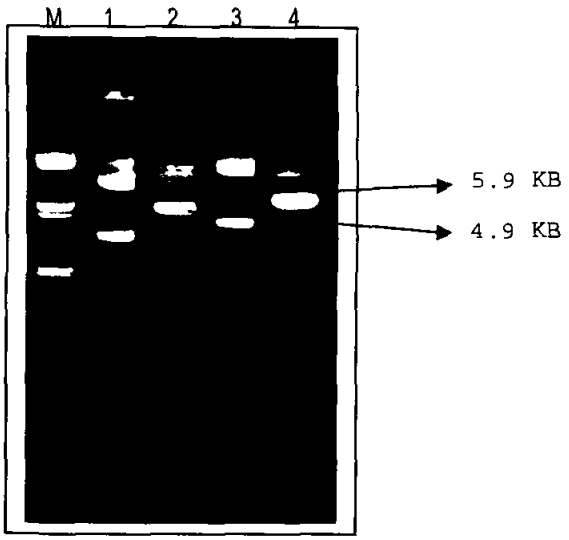


Fig. 46- pGEX and pET vectors double digested with EcoR I and BamH I.

M- marker; 1- pGEX uncut; 2- pGEX digested; 3-pET uncut and 4- pET digested

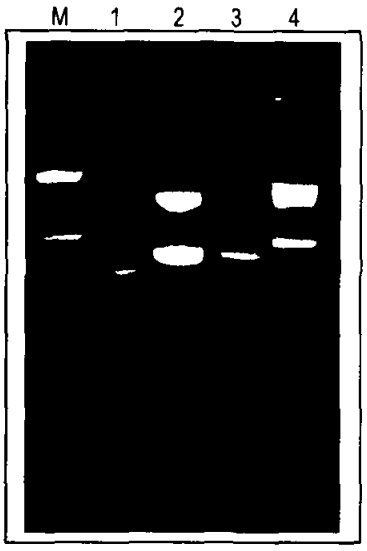


Fig. 47- Cloning in pGEX and pET vectors

M- marker; 1- pGEX control; 2- pGEX with insert; 3-pET control and 4- pET with insert

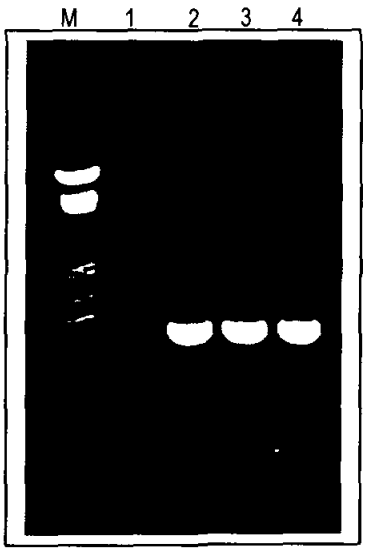


Fig. 48-PCR analysis of the potential recombinant colonies showing amplification of the cloned fragment

M- marker; 1- vector without insert; 2-4: recombinant plasmids as PCR templates

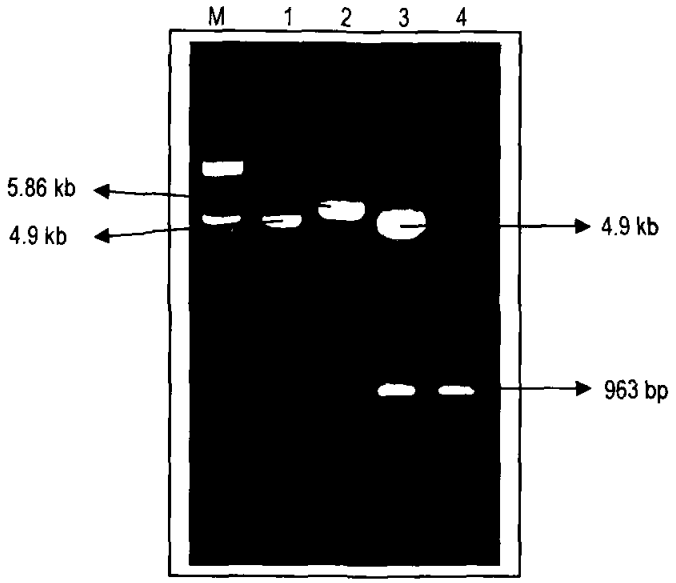


Fig. 49- Confirmation of cloning in pGEX vector through restriction analysis

M- marker; 1- linearised control vector; 2- linearised vector with insert; 3- insert released through double digestion with *Eco*R I and *Bam*H I; 4- PCR amplification of the inserted 963 bp fragment

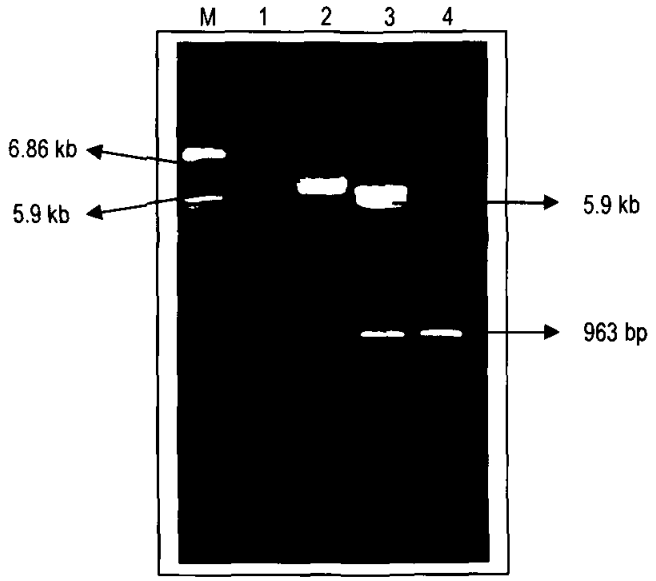


Fig. 50- Confirmation of cloning in pET vector through restriction analysis

M- marker; 1- linearised control vector; 2- linearised vector with insert; 3- insert released through double digestion with *Eco*R I and *Bam*H I; 4- PCR amplification of the inserted 963 bp fragment

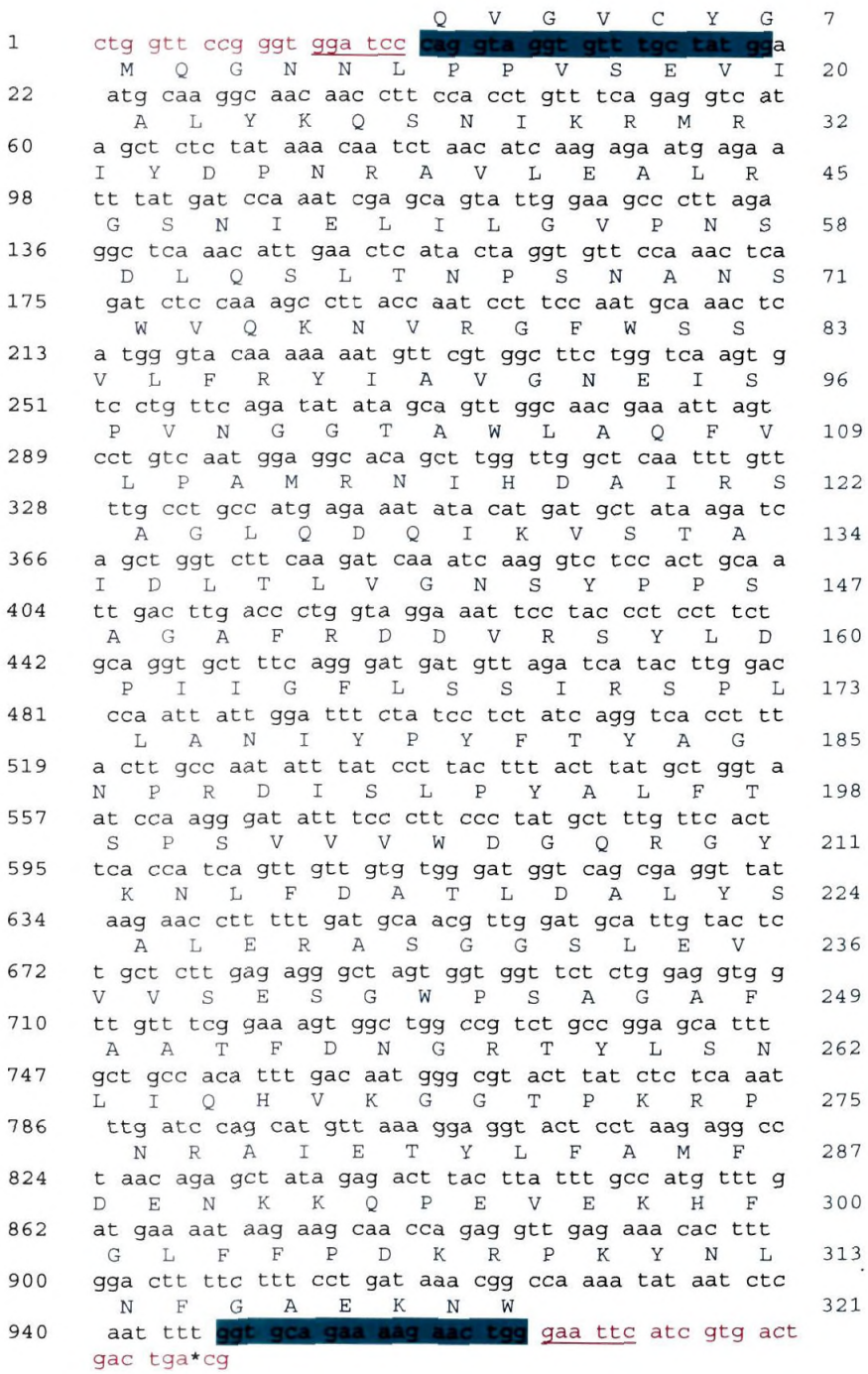


Fig 51- Sequence of pGEX vector inserted with Hevea β-1,3-glucanase gene, showing the reading frame.

The vector sequences are in red and the restriction sites underlined. The primers used for PCR amplification are highlighted

```
gac agc cca gat ctg ggt acc gac gac gac gac aag gcc
                                Q   V   G   V   C   Y   G
atg gct gat atc gga tccCAG GTA GGT GTT TGC TAT GGa
 M   Q   G   N     298 aa         K   Y   N   L
atg caa ggc aac ...894 bp..... aaa tat aat ctc
 N   F   G   A   E   K   N   W
aat ttt GGT GCA GAA AAG AAC TGG gaa ttc gag ctc cgt
cga caa gct
```

Fig 52- Sequence of pET vector inserted with Hevea β-1,3-glucanase gene, showing the reading frame.

The vector sequences are in red and the restriction sites underlined. The primers used for PCR amplification are highlighted

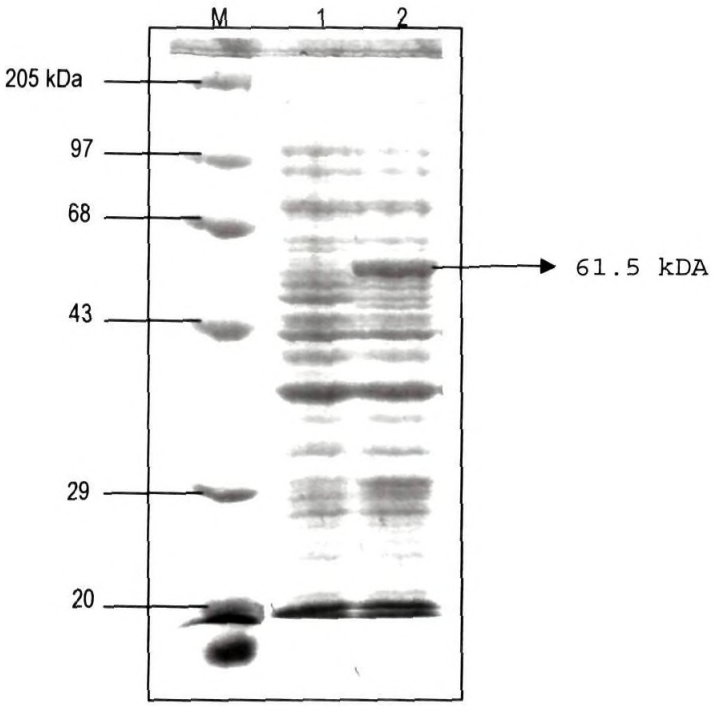


Fig. 53- Recombinant protein expression in pGEX vector (10% SDS-PAGE gel)

M- protein mol.wt. marker; 1- TCP from uninduced colonies; 2- TCP from colonies induced with 1 mM IPTG showing the induction of 61.5 kDa GST-fusion protein

4.3.3. Expression of the target gene

After the establishment of the recombinant plasmid in expression hosts (*E. coli* strains *DH 5α* for pGEX and *BL 21 (DE3)* for pET), the expression of the target DNA was induced by the addition of IPTG to the growing culture, when the OD_{260} reaches 0.6. Concentrations varying from 0.2 – 2.0 mM IPTG were tried for the induction of target proteins. A final concentration of 1 mM IPTG was found to be optimum for the maximum induction in the case of pGEX vector and for pET the optimum level was 0.4 mM. The protein expression levels were rapidly tested by SDS-PAGE analysis of the cell extracts followed by staining with Coomassie blue. Four fractions were analysed in parallel for the detection of target proteins after induction. These are the 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. It shows that most of the induced proteins are in the soluble form; which is very important for its purification in native form. However, some protein was detected in the inclusion bodies also.

DH5α host cells successfully expressed the cloned gene in pGEX vector (Fig. 53). The fusion protein (GST + target gene) of expected size (61.5 kDa) was detected as a prominent band in the induced samples, while it was absent in the uninduced controls. However, a prominent GST band was not induced as expected, when a vector alone control was used. With pET vector also, a very high induction of the fusion protein (Trx + target gene) was detected (Fig. 54). A 55.6 kDA band was observed in all induced samples. Here a very high induction of the control Trx protein was also observed, when the vector alone colonies were induced with IPTG.

4.3.4. Purification of the target protein

The GST-fusion protein expressed in pGEX vector was purified by passing through a glutathione column. Before column purification, release of the target proteins in the soluble fraction after sonication was ensured by SDS-PAGE. The bound proteins were finally eluted with 10 ml of elution buffer and the 1 ml

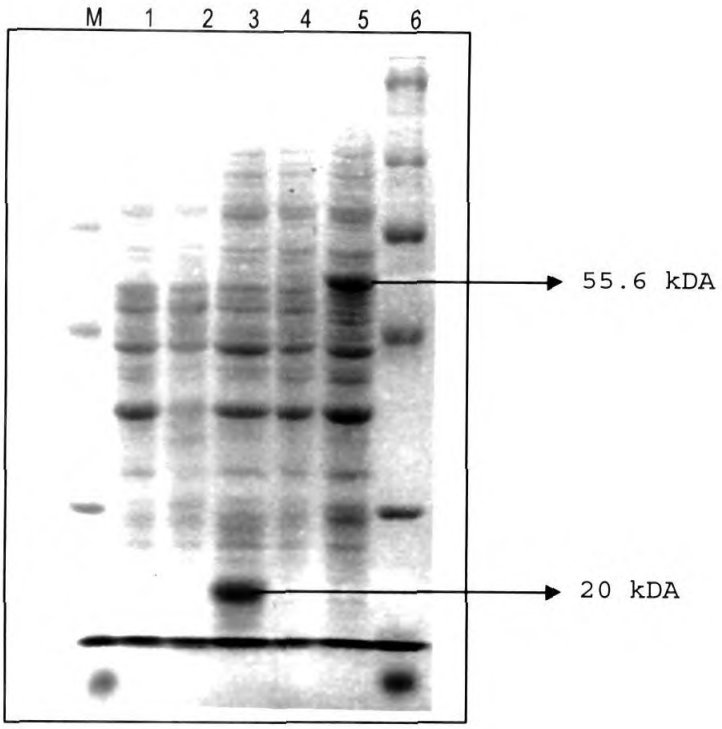


Fig. 54- PAGE analysis of TCP from different samples showing the recombinant protein expression with pET vector in BL21 DE3 host cells

M- protein mol.wt. marker; 1- host cells alone; 2- uninduced vector alone control; 3- induced vector alone control showing the 20 kDA Trx + His tag control protein; 4- uninduced recombinant colonies; 5- induced recombinant colonies showing the fusion protein of expected size (55.6 kDA); 6- protein mol.wt. marker.

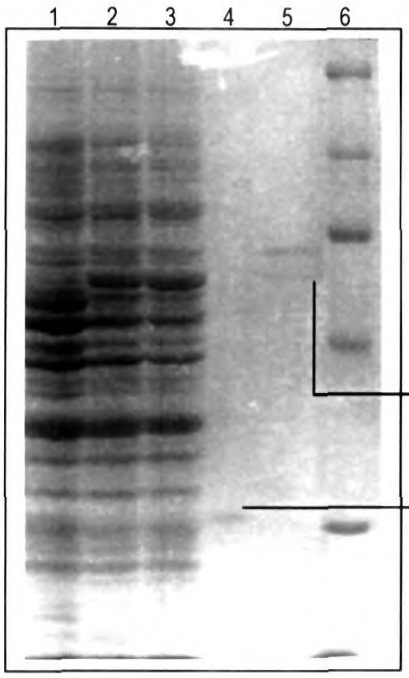


Fig.55-Column purification of the GST-fusion protein expressed by the pGEX vector

1- uninduced control; 2- TCP from induced colonies; 3- total soluble fraction from the sonicated samples; 4- GST control protein purified; 5- GST-fusion protein (61.5 kDa) purified; 6- protein mol.wt. marker

fractions were analysed. It was observed that most of the eluted proteins were present in 2nd, 3rd and 4th fractions. But, in addition to the induced protein of expected size, one additional protein band of higher mol.wt. was also detected in the eluted fractions (Fig. 55).

The Trx-fusion protein expressed in pET vector was effectively purified in mg quantities with the His.Bind purification kit. The fusion tag contains six histidine residues that will bind with Ni^{2+} cations, which are 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. The system allowed the proteins to be purified under native non-denaturing conditions. Most of the eluted protein was present in the first four 1 ml fractions (Fig. 56). No contaminating proteins were seen in purified fractions as obtained in the case of pGEX vector. In order to optimise the protocols, the thioredoxin protein expressed by the vector without insert was also purified (Fig. 57). The eluted fractions were stored in –70°C for further analyses.

4.3.5. Confirmation of the induced target proteins

The induced fusion and control proteins were transferred to nitrocellulose membranes. The identity of the induced protein in pET vector was confirmed through western blotting with the vector encoded fusion partner of the target protein. His tag monoclonal antibody, which is having an epitope consists of 5 consecutive histidine residues, was used as the primary antibody. This will bind with the His.tag sequences encoded by the pET vector. 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 located separately on the western blot; confirming that the induced proteins are His tagged (Fig. 58).

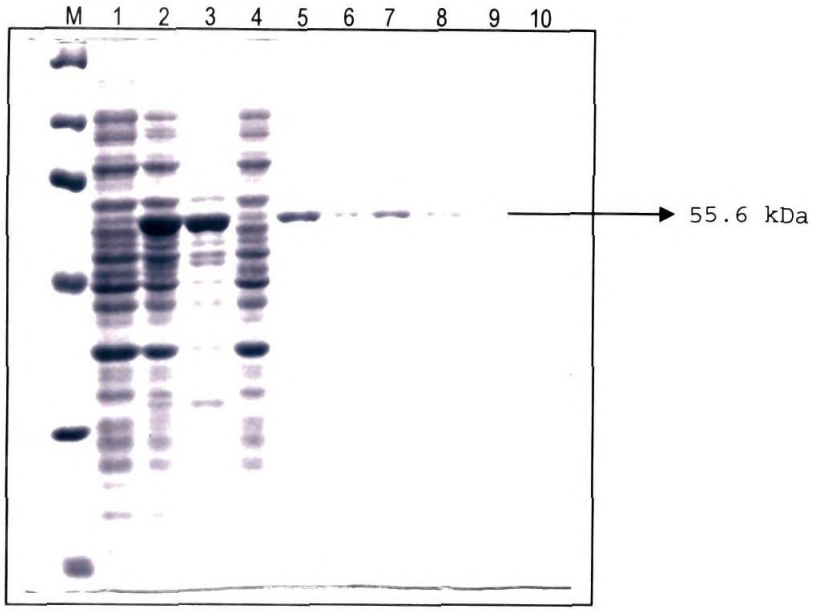


Fig. 56- Purification of pET recombinant protein by passing through the His bind Ni^{2+} column.

M- marker; 1- TCP from uninduced colonies; 2- TCP from induced cells; 3- total soluble proteins after sonication; 4- unbound proteins removed from the column with wash buffer; 5-10: different fractions of the eluted protein

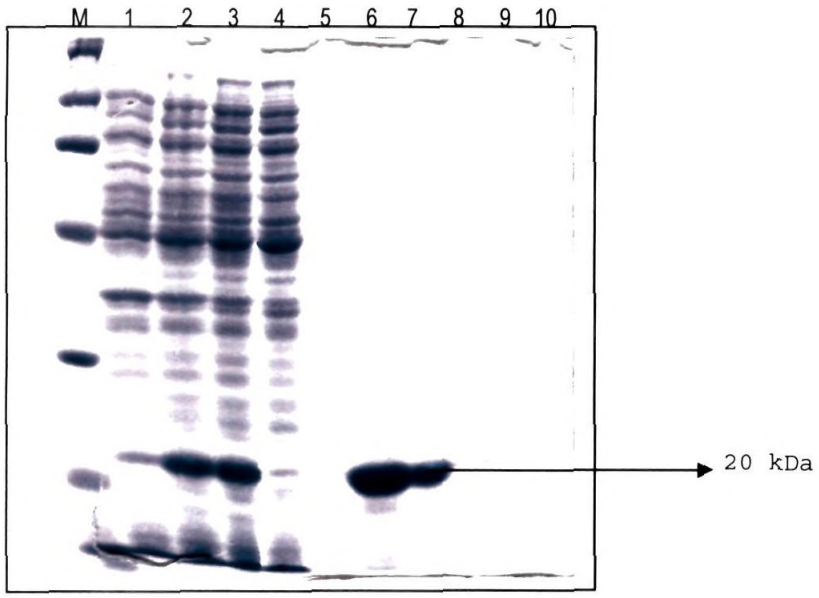


Fig. 57- Purification of pET control thioredoxin protein by passing through the His bind column.

M- marker; 1- TCP from uninduced colonies; 2- TCP from induced cells; 3- total soluble proteins after sonication; 4- unbound proteins removed from the column with wash buffer; 5-10: different fractions of the eluted protein

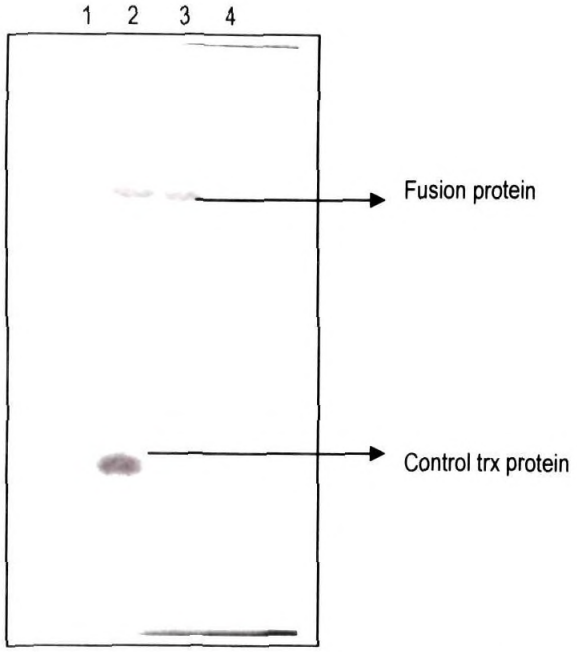


Fig.58- Detection of target proteins through western blot with His. Tag monoclonal antibody

lane 1- uninduced control; 2-TCP from both control and recombinant induced samples loaded in the same well showing 55.6 kDa fusion protein and 20 kDa control thioredoxin ; 3- TCP from induced recombinant colonies; 4- from induced control cells

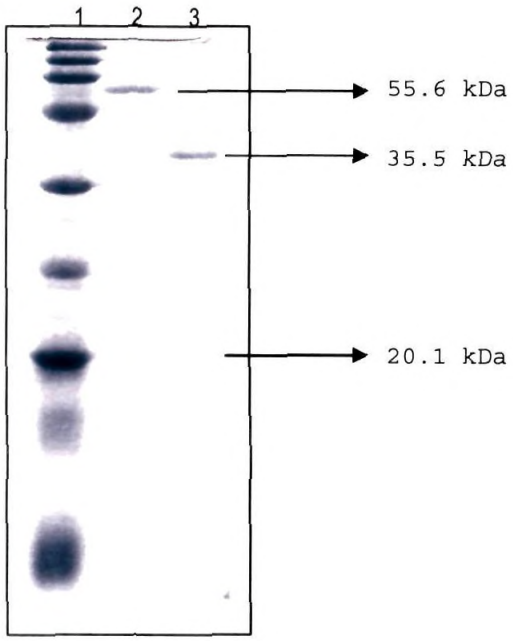


Fig. 59- Cleavage of N-terminal fusion tag of the purified recombinant protein with thrombin

lane 1- protein mol.wt. marker; 2- column purified fusion protein of 55.6 kDa; 3- cleavage with thrombin showing protein bands of 35.5 kDa and 20.1 kDa

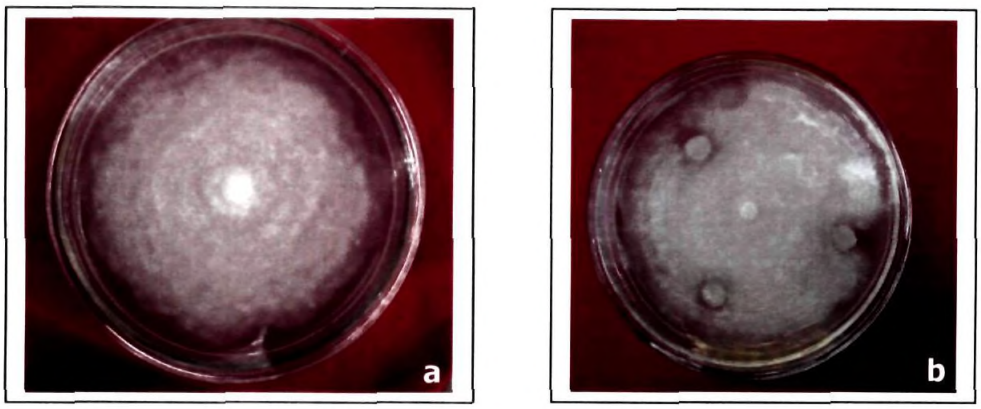


Fig. 60- Anti-fungal activity of purified Hevea recombinant β-1,3-glucanase against Phytophthora meadii.

a. control; **b.** filter discs of 5 mm diameter with 10 μg of purified protein. Clear inhibition zones were observed around filters

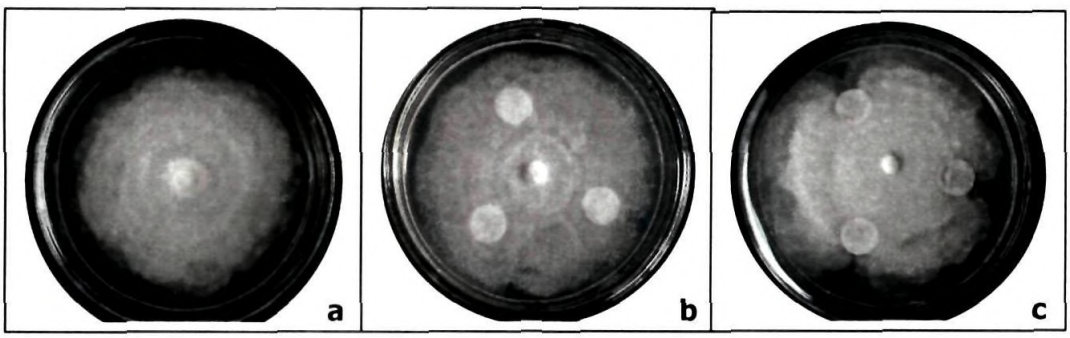


Fig. 61- Inhibition of fungal growth by purified enzyme

a. control; **b.** control plates with filter discs of 9 mm diameter soaked with sterile water; **c.** filter discs soaked with 10 μg of purified recombinant protein. The inhibitory zones were slowly over-grown by the fungus one week after inoculation

The identity of the induced protein was also confirmed by cleavage of the purified fusion protein encoded by the pET vector with restriction grade thrombin. A thrombin cleavage site is present in the vector that will allow the proteolytic removal of the N-terminal fusion tags from the expressed target protein. One unit of thrombin effectively cleaved about 20 μg of target protein when incubated at 23°C overnight. The 20 kDa fusion tag protein along with His tag and 35.5 kDa recombinant *Hevea* β-1-3- glucanase was detected in 12 % PAGE (Fig. 59).

4.3.6. Anti-fungal assay of recombinant proteins

The anti-fungal property of purified *Hevea* β-glu was assayed on PDA plates, which is inoculated with a growing culture of *P.meadii* using filter paper disc method. Aliquots of the purified recombinant protein preparations were added to the filer discs on agar plate after two days of initial vegetative growth of the fungi. In the following two days distinct inhibition zones were developed around the filters soaked with 10 μg of the purified enzyme solution (Fig. 60 and 61). These inhibitory zones remained visible for at least one week and then the fungus starts to overgrow the initial inhibition zones.

5

DISCUSSION

In nature, plants are under constant threat from a variety of microorganisms; most of them are fungal in origin, which often attack them, drastically affecting their yield or even causing death of the affected plants. However, plants are not al all silent towards the constant onslaught of microbes. They have a variety of defense strategies to ward off the pathogenic microorganisms. Through crop improvement programmes involving conventional methods like breeding of disease resistant varieties by hybridisation and selection, man also contributed significantly to the ability of plants to overcome the attack of pathogens. As an alternative to such conventional breeding programmes, which often take decades to complete, agricultural scientists are now using the tools of molecular biology and genetic engineering to develop transgenic plants with built-in resistance to the pathogens. Enormous progress has been made in the past decade in our understanding of the highly complex molecular events that occur in plant-pathogen interactions. This knowledge has in turn provided a number of options and strategies, which can be utilised to make transgenic plants. All these informations, starting from the first step of mutual recognition of the host and pathogen, moving on to immediate response of the plant in terms of hypersensitive response, followed by local resistance response in terms of production of PR proteins and then, to the final step of systemic acquired resistance, have been or are being used to produce pathogen-resistant transgenic plants in different crop species.

Selection of a candidate gene for transgenic expression for crop improvement is very important. Thorough molecular studies have to be performed to establish the role of a particular gene in plant defense. Generally two forms of

genetic resistance have been described in plants: race-specific (vertical resistance) or race non-specific (horizontal). Race-specific resistance is characterised by the interaction between products of dominant *R* gene in the host and corresponding *avr* gene in the pathogen. When a new virulent race of pathogen evolve, the *R* genes become completely ineffective. But, in contrast, race non-specific resistance is assumed to be multi-gene based and hence, it is durable and commercially more attractive than race specific resistance. β-1,3- Glucanases are considered to be one of the major components of this broad generalized defense mechanism against pathogen attack and are classified as PR-2 proteins. They are abundant, highly regulated hydrolytic enzymes widely distributed in plant kingdom. They have been studied in detail in different plant species and their role in combating the invading pathogen during local and systemic acquired resistance has been well documented in many incompatible plant-pathogen interactions. Induction of β- glu has been demonstrated in many plant-fungal pathogen interactions and they are supposed to play a major role in plant defense. Primarily they can degrade the cell wall of many fungal pathogens or disrupts its deposition by hydrolysing β-1,3- glucans, contributing to pathogen death (Mauch *et al.*, 1988). They can also release fungal cell wall fragments that in turn can act as elicitors of active host defense response (Yoshikawa *et al.*, 1993).

The objective of the present study was isolation and characterisation of β-glu gene from *Hevea* and a study on its role in the defense of plants against *Phytophthora* attack. Among the natural rubber producing countries, India occupies the topmost position with an annual productivity of 1592 kg/ha during 2002–'03. Yet, we have not achieved even 2/3 of the potential productivity on our plantations mainly because of the occurrence of diseases (Jacob, 2003). Ever since its first incidence, reported in 1918 (McRae, 1918), ALF has continued to be the major fungal disease affecting the rubber trees in India. Its incidence is particularly severe in the traditional rubber growing tracts of the country, like Kerala, where annual rain fall is around 3000 mm, 60% of which is falling on the monsoon months of June – August that results in cold wet weather with high relative humidity, which are congenial for the outbreak. This study is initiated based on the assumption that, a chitinless fungi like *Phytophthora*, which possess more than 90% β-1,3 linked glucans as their cell wall material, could be effectively dealt

through the host β-1,3-glucanases, as reported in the case of several other plant species.

5.1. Characterisation of β-1,3-glucanase gene in rubber tree

The β-glu gene was amplified by PCR from genomic DNA and cDNA using primers based on a reported sequence (Chye and Cheung, 1995). Nucleic acids were isolated according to published protocols with certain modifications. Genomic DNA was isolated with sufficient purity and concentration that ensure good PCR amplification and restriction digestion. Since *Hevea* leaves have a high phenolic content and other secondary metabolites, it was necessary to purify the DNA to get good amplification in PCR. In the present study, tissue lysis for DNA isolation was done using CTAB buffer. One phenol-chloroform extraction and two chloroform washes were carried out to eliminate all other contaminating compounds and macromolecules other than deoxyribonucleotides. Around 300 μg of DNA was obtained per g of the leaf tissue used. The A_{260}/A_{280} ratio was found to be 1.76, indicating the purity of the isolated DNA. A value below 1.6 indicates protein contamination and if it is above 1.9, the preparation may be contaminated with phenolics.

Efficient extraction of high quality RNA from different plant tissues is very important, as it is the preliminary step in many molecular biology techniques such as gene expression studies, construction of cDNA libraries etc. In the present study, RNA was extracted from latex using a modified protocol of Kush *et al.*, (1990). Concentration of the constituents of the extraction buffer was reduced and the pH was maintained at 8.5. Concentration of SDS was reduced considerably to 2%, as against the 10% in the original protocol, without affecting the purity and concentration of isolated RNA. The alkaline pH and the presence of SDS in the extraction buffer eliminated the problems of RNase contamination and latex coagulation. The protocol proposed by Venkatachalam *et al.*, (1999) for RNA extraction from bark was suitably modified for RNA extraction from leaf samples. Addition of exogenous RNase inhibitors such as guanidinium thiocyanate and vanadyl ribonucleoside complexes were not required in this protocol as all reagents used were prepared with DEPC treated H_2O. The amount of RNA was found to be

more in leaves than in latex and the RNA obtained per gram of leaf tissue was significantly higher than the amount of DNA obtained.

The amplification products from genomic DNA and cDNA show a 132 bp difference in length. This is due to the presence of an intervening sequence near the 5'end of the gene. As shown in Fig. 10, the gene consists of two exons of 102 bp and 1023 bp length, interrupted by a 132 bp intron. The intron is flanked by the intron specific sequences GT at the 5' end and AG at the 3' end. A comparison between the coding region of the genomic and cDNA sequences reveals that they are 100% identical. The intron sequence is AT-rich (61.3%), which is necessary for the processing of pre-mRNA introns in plants. The genomic DNA sequence of *Hevea* was reported for the first time in this study. Almost all β-glu genes isolated from various plant species contain a single intron, with the exception of a gene isolated from *Nicotiana plumbaginifolia,* which contains two (Castresana *et al.*, 1990). The position of the intron is the same in the genes for acidic and basic glucanases, indicating that the structure of these glucanase genes are highly conserved, and that they may have arisen from a single common ancestor. While more or less similar in their coding regions, these genes differ from each other in their upstream, downstream and intron sequences (Thimmapuram *et al.*, 2001). In the case of *Hevea* also, although the position of the intron is the same as in other glucanases, the length is different. The size of the β-glu genes (considering only exons and introns) in different plant species vary from 1177 bp in tobacco (Shinshi *et al.*, 1988) to 4149 bp in rice (Simmons *et al.*, 1992). The variation in the total length of the glucanase genes is mainly due to the difference in the size of the introns, as almost all β-glu genes encode proteins of about same size. The size of the *Hevea* β-glu gene (1257 bp) also falls within the range of other glucanase genes, but the intron size is much smaller than other dicot species, where it ranges from 341 – 974 bp and in monocots, it is still larger. However, the intron size is only 106 bp in the case of a β-glu isoform isolated from sugar beet (Gottschalk *et al.*, 1998).

As the different isoforms of plant β-glu show complex developmental and pathogen related regulation at the mRNA level, cis and trans-acting regulatory elements of the promoter were investigated by several workers (Leubner-Metzger

and Meins, 1999). Expression studies of the *Gus* coding region fused to a deletion series of 5'-flanking sequences have been used to identify regions of class I β-glu promoters important for transcriptional regulation. These promoter activity studies indicate that these genes are primarily regulated at the level of transcription (Van de Rhee *et al.*, 1993). A 10-bp TCA-motif with a consensus sequence 5'- TCATCTTCTT- 3' has been identified in many stress related gene promoters including β-glucanases (Goldbrough *et al.*, 1993). Another type of enhancer like element, known as AGC-box with consensus sequence of AGCCGCC, has reported in the promoters of many basic class I β-glu (Hart *et al.*, 1993). Trans-acting DNA binding protein, which binds to AGC-box were also identified in tobacco (Ohme-Takagi and Shinshi, 1995). Two copies of this AGC-box was found to be located at –1452 to –1193 in class I tobacco β-glu and was observed to be necessary for high-level organ specific expression in leaves (Vogeli-Lange *et al*, 1994). Present study utilises the 5' RAGE analysis to isolate the promoter elements by PCR amplification of the adaptor ligated genomic DNA fragments. To ensure more specificity, a nested PCR reaction was also employed. It means that 2 pairs of primers were used for a single locus. The binding site of second pair of primers will be within the first PCR product. The logic behind this strategy is that if the wrong locus were amplified by mistake, the possibility is very low that it would also be amplified a second time by a second pair of primers. However, in the present study only 198 bp upstream elements from ATG start codon could be isolated, which contains a putative TATA-box at position –139 and a possible CAAT-site at position –82. In a previous study, analysis of the *N. plumbaginifolia* class I β-glu gene has shown that a short 138 bp promoter is sufficient to confer full activity in transgenic tobacco leaves, and is even more active than –736 and –2000 promoters (Alonso *et al*, 1995). It was found that the region –138 to –98 of this promoter is sufficient for high-level response to *Pseudomonas syringae* infection. A promoter analysis has to be conducted to investigate the activity of the promoter of *Hevea* β-glu, isolated in the present study.

It has been reported that in higher plants, β-glu are encoded by gene families of considerable complexity. Such multiplicity could provide more flexibility for spatial and temporal regulation of diverse functions of β-glu, like

microsporogenesis, pollen tube growth, seed germination *etc*, in addition to disease resistance. For example, Jin *et al.* (1999) reported that at least 12 different classes of β-glu exist in soybean. In potato, more than 10 hybridising bands were observed, when the restriction-digested genomic DNA was hybridised to β-glu probe, indicating that the gene is encoded by complex gene families. This diversity of β-glu may be involved in the regulation of pathogenesis related functions as well, since there may be need of either constitutive or induced expression and there may also be requirements for varied responses in different tissues. Such multiplicity of β-glu functions might confer advantages to plants by providing several lines of defense against the invading microorganisms. However, Southern blots of total genomic DNA digested with *Bam*H I, *Hind* III and *Xba* I, revealed the presence of only 3 – 4 hybridising bands in the case of *Hevea*. Since there are no *Bam*H I, *Hind* III or *Xba* I sites were present in the β-glu gene sequence in *Hevea*, this result suggests that the glucanase in *Hevea* may be encoded by members of small gene family of 3 – 4 genes. The same result was reported in another woody species (peach) also, where the β-glu gene family was found to consist of only 3 – 4 genes (Thimmapuram *et al.*, 2001). Low copy β-glu gene families have been reported in *Cichorium* also (Helleboid *et al.*, 2000). Present study confirmed the presence of glucanase genes in equal copy numbers in tolerant and susceptible plants as well as in some wild accessions through Southern hybridisation.

The deduced protein sequence consists of a pre-proprotein of 374 amino acids with a theoretical pI value of 8.96 and a molecular weight of 41.2 kDa. This is similar to that of basic glucanases from other plants. The structural features include a signal peptide of 36 amino acids, enriched in neutral and hydrophobic residues, typical of eukaryotic signal sequences as suggested by Von Heijne (1983). The location of the cleavage site was estimated to be between amino acids 36 and 37 (sequence TDA – QV), by using SignalP V 1.1 software (Nielsen *et al.*, 1997). The signal peptide of *Hevea* consists of a positively charged region at the N-terminus, enriched in serine and threonine residues, followed by a hydrophobic centerpiece and a C-terminal polar region.

Earlier reports suggest that class I basic glucanases are produced as a pre-proprotein with an N-terminal hydrophobic signal peptide, which is co-translationally removed, and a C-terminal extension, N-glycosylated at one site. After removal of the signal peptide, the proprotein is transported from the endoplasmic reticulum *via* the Golgi compartment to the vacuole, where the C-terminal extension is removed to form the mature functional protein (Leubner-Metzger and Meins, 1999). The isolated β-glu from *Hevea* clone RRII 105 also shows the properties of class I β-glu genes. The pre-proprotein consists of a characteristic 36 amino acid N-terminal signal peptide and a 22 amino acid C-terminal extension for vacuolar targeting. The mature protein consists of 316 amino acids with a theoretical pI of 9.26 and a molecular weight of 35 kDa. The C-terminal extension of class I glucanases have been shown to be essential for vacuolar targeting of the protein. Along with prohevein and hevamine, β-glu is also a major component of the lutoid body fraction of the *Hevea* latex. C-terminal vacuolar targeting sequences have been found to be present in all these lutoid body proteins (Subroto *et al.*, 2001).

The C-terminal extension of isolated *Hevea* glucanase, particularly residues 365 – 370, is rich in hydrophobic amino acids. It has been suggested that a hydrophobic acidic motif structure, rather than a specific amino acid sequence, form a sorting signal in carboxy extension polypeptides (Nakamura and Matsuoka, 1993). A putative glycosylation site was found in the C-terminal extension at Asn-364 in the sequence of Asn-Ala-Thr-Ile. But the glycosylation site that is found in the mature protein of *Hevea* glucanase reported earlier from the clone RRIM 600 (Chye and Cheung, 1995) at position 63 (NITR), seems to be absent in the protein observed in the present study. However, the second potential glycosylation site at position 101 (NPSN) is present in the sequence. This result was confirmed by sequencing of the β-glu cDNAs amplified in three independent PCR reactions. There are reports of considerable variation in β-glu from different clones of *Hevea*. Subroto *et al.*, (2001) reported that, the β-glu from clone GT1 is a glycoprotein, while the enzyme from clone PR 261 is not glycosylated, and the clone RRIM 600 contains a mixture of carbohydrate free and glycosylated glucanases. The β-glu from these clones differ in their specific activities as well.

5.2. Differential expression of *Hevea* β- glu during pathogenesis

Disease resistance in plants is brought about by constitutive and induced mechanisms. Accumulation of PR proteins is one of the most common markers for active, induced plant defense. Among these, the proteins with β- glu activity have been well investigated in several plant species. (Simmons, 1994; Leubner-Metzger and Meins, 1999). There are several reports on the induction of β-glu during pathogen infection in different plant species like 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). They are considered to be part of the defense response mainly against fungal pathogens, presumably by inhibiting their growth through their hydrolytic capacity (Mauch *et al*, 1988). This could be particularly important in the case of *Phytophthora* diseases like ALF, as the causative organism belongs to the class Oomycetes, the cell wall of which is primarily made up of β-1,3 linked glucans, while in most other cases the principal cell wall component is chitin. Furthermore, the hydrolysis products of the fungal cell wall can act as elicitors, which may induce other types of defense reactions in plants (Keen and Yoshikawa, 1983).

β-Glu gene from a woody plant species was firstly isolated from *Hevea* (Chye and Cheung, 1995). However, not much work has been done to understand the role of this gene in disease resistance of woody species in general, and of rubber tree in particular. In the present study, detection of time-dependent induction of β- glu in two *Hevea* clones support the hypothesis that this enzyme may be involved in the reaction against fungal infection. To determine the role of β- glu in *Hevea* resistance to *P. meadii*, a susceptible clone (RRIM 600) and a highly tolerant clone (RRII 105) were analysed for the induction of glucanase upon pathogen inoculation. To study gene expression, two experimental approaches were used: northern hybridisation and relative RT-PCR analysis (relative quantitative RT-PCR or RQ RT-PCR). Although, quantification of RNA transcript levels can be performed conventionally using northern blot hybridisation analysis, RT-PCR has many advantages, such as being able to detect even lower

levels of gene expression. But, even though RT-PCR is more rapid and specific, quantification can be difficult since there may be many sources of variation, such as template concentration and amplification efficiency. One approach to overcome this problem is relative RT-PCR, which co-amplifies the gene of interest with an internal control and quantifies the gene of interest relative to the internal control (Gause and Adamovicz, 1995). Relative RT-PCR requires only small amounts of DNA, takes less time to complete, is less expensive than northern blot analysis and requires only standard PCR techniques. However, to be quantitative, RT-PCR must be analysed in the linear range of amplification for both the gene of interest and internal control, before reaction components become limiting. When target transcripts are abundant, PCR amplification may reach the plateau after only 20 cycles; therefore, relative RT-PCR conditions must be determined to ensure that both bands are still accumulating at the end of each cycle. However, in the present study it was found that the PCR products are still below saturation even at the end of 32 cycles.

Relative RT-PCR has been employed to study the expression of β-glu in *Nicotiana benthamiana* infected with *Colltotrichum destructiuum* (Dean *et al.*, 2002). The results were found to be similar to northern hybridisation data. This competitive RT-PCR technique has been increasingly used in animal and human systems as well (Steagel *et al.*, 2000; Amant *et al.*, 2001). In this study, the results from northern blot and relative RT-PCR analysis, regarding β- glu expression were found to be similar, thereby validating the use of relative RT-PCR with a suitable internal control for measuring the host gene expression during infection. The use of primers to amplify the actin gene fragment in each sample helped to account for variation in template concentration and amplification efficiency. Actin is supposed to be a better internal control than 18S RNA in RT-PCR because, Burleigh (2001) recognized that 18S RNA sequences are highly conserved, therefore, the internal control was amplifying both plant and fungal 18S RNA from the fungal infected tissues, leading to an under estimation of plant gene expression.

Present study reports the induction of β- glu in response to infection by the pathogen, both in tolerant and susceptible clones of *Hevea*. However, the timing and magnitude of induction varies between the clones. The level and onset of

β- glu expression has been found to be positively correlated to the level of resistance to the pathogen. Muskmelon and tomatoes infected with *Fusarium oxysporium* induced a higher and more rapid expression of β- glu in resistant, than in susceptible varieties (Netzer and Kritzman, 1979; Ferraris *et al.*, 1987). The β- glu activity was increased more rapidly in resistant melon cultivars than in susceptible ones upon inoculation with cucurbit powdery mildew fungus (Rivera *et al.*, 2002). More glucanase activity was reported in the *Phytophthora* infected tissues of a tolerant variety of black pepper compared with two susceptible varieties (Jebakumar *et al.*, 2002). The results of the present study is concordant with the above-mentioned findings as at 48 h after inoculation there was an exponential increase in β- glu mRNA transcripts, and this induction was 1.5 fold higher in tolerant clone than the susceptible one. This induction was observed to be more prolonged in tolerant clone, as by 72 h tolerant clone recorded an increase in β- glu transcripts by 2.5 fold than the susceptible clone, and it was as high as 6 fold on the fourth day after inoculation. Presence of the end product was also confirmed by spectrophotometrically measuring the glucanase activity. Similar result was reported by Egea *et al.*, (1999) on studying the β- glu expression in resistant and susceptible pepper cultivars infected with *P. capcici*. Although induction occurred in both cultivars 2 day after inoculation, northern hybridisation revealed that β- glu transcript level depleted in susceptible cultivar by day 3, in the region immediately below the necrotic zone.

β- Glu often exist as multiple isoforms of which, the basic isoforms are usually intra-cellular and acidic isoforms are secreted into the intercellular spaces. Although induced proteins are mostly acidic, induction of basic β- glu has also been reported in many incompatible plant-pathogen interactions. RNA encoding basic form of glucanase within one day after inoculation and reached a peak by day 2 (Ward *et al.*, 1991). Inoculation of soybean plants with an elicitor from *Phytophthora* spp. results in an increased expression of basic isoform of β- glu (Cheong *et al.*, 2000). In disease resistance, basic glucanase seems to be particularly important, since over-expression of β- glu genes in transgenic plants afforded substantial protection against pathogens, only when basic glucanase was constitutively expressed (Jongedijk *et al.*, 1995). Intra-cellular accumulation of

basic glucanase in the vacuoles of the penetrated cells would lead to a rapid deployment of β-glu at the exact site of penetration. Further, *in vitro* anti-fungal studies have shown that the vacuolar basic isoform of glucanase can inhibit the hyphal growth and spore germination of pathogenic fungi (Anfoka *et al*, 1997; Yi and Hwang, 1997). A basic glucanase isolated from pepper could lyse the germinating zoospores and inhibited the growth of *P.capcici* (Kim and Hwang, 1997). In this study, the primers used for the synthesis of probes for northern blots and for the RT-PCR experiments, correspond to basic isoforms of glucanase. However, as the different glucanase isoforms share a high degree of homology, the signals generated in northern may not be fully attributed to the expression of basic isoform alone. Immunocyto-localization studies are, therefore necessary to determine the sub cellular localizations of β-glu isoforms specifically induced in tolerant and susceptible *Hevea* clones in response to *Phytophthora* infection.

It was observed that β-glu was constitutively expressing in the latex of rubber tree. This is in accordance with the result of Chye and Cheung, (1995) where higher expression of β-1,3-glucanase gene was reported in the laticifers than leaves. The constitutive nature of gene expression was further confirmed using the latex collected from a freshly opened tree. Constitutive expression of defense related genes like chitinase and lysozymes has also been reported in the latex of *Hevea* (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). As the tapping to collect latex is a process of controlled wounding, various enzymes stored in the laticifers may be providing an important contribution to the plant defense mechanism by sanitizing and sealing the wounded areas on the tree as reported in the case of *Carica papaya* (El Moussaoue *et al.*, 2001). Constitutive expression of β-glu in the latex of six clones was studied using northern hybridisation. It was observed that the band intensity was more in the case of tolerant clones studied, particularly for FX 516, which is a hybrid of *Hevea brasiliensis* with a wild relative *H. benthamiana*. However, a susceptible clone RRIM 600 also recorded higher levels of gene expression in the latex, comparable to that of tolerant clones.

In conclusion, the role of β-glu in defense against ALF disease in *Hevea* is supported by the evidence that their transcription is induced during pathogen attack. The predominant difference between the tolerant clone and susceptible clone lies in the intensity of response. The intensity of β-glu expression was found directly related to the level of tolerance. The tolerance of clone RRII 105 may be due to the rapid induction and more prolonged expression of the gene that produce an intensely inhospitable environment for fungal growth, thus enabling the plant to respond to the pathogen attack more efficiently. The level of constitutive expression in the laticifer cells may also be another factor that determines the tolerance to *Phytophthora*.

5.3. Recombinant *Hevea* β-glu and its anti-fungal properties

The role of plant glucanases in the inhibition of a potential pathogen has been suggested first based on the observation that, although high activity of this enzyme is found frequently in higher plants, its substrate, callose, is usually present only in small quantities (Abeles *et* al., 1971) while its natural substrate, β-1,3-glucan is the main structural element of many pathogenic fungal cell walls. Later, it was shown that the purified enzyme could effectively degrade the fungal cell walls (Young and Pegg, 1982). The enzyme can destroy the fungi by thinning their cell wall at the hyphal tip that causes swelling and ultimate bursting of the hyphal tip (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.

In the present study, the β-glu isolated from *Hevea* was shown to be over-expressed in the infected zones of leaves, suggesting indirectly their role in combating the disease. In order to find out a direct evidence on inhibition of pathogenic fungal growth by the host β-glu, a functional cDNA clone was

constructed that could express the *Hevea* β-glu in *E.coli*, and the recombinant protein was purified and tested for anti-fungal properties.

The 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*). In this vector, the target gene is cloned under the control of T7 promoter, which is not recognized by *E.coli* RNA polymerase. Therefore, virtually no expression occurs until a source of T7 RNA polymerase is provided. After cloning and establishing in a non-expressing host strain *JM 109*, which lack the gene for T7 RNA polymerase, the recombinant plasmid was transferred to an *E.coli* strain containing a chromosomal copy of the gene for T7 RNA polymerase (*BL 21*) under the control of a *lac* promoter, for protein expression. These hosts are lysogens of bacteriophage *DE 3* and carry a DNA fragment containing the *lacI* gene, the *lacUV5* promoter and the gene for T7 RNA polymerase. Once a recombinant plasmid is established in *DE3* lysogen, the only promoter known to direct transcription of T7 RNA polymerase gene is the *lacUV5* promoter, which is inducible by IPTG. Thus, the addition of IPTG to a growing culture of the lysogen induces T7 RNA polymerase, which in turn transcribes the target DNA in plasmid under the control of T7 promoter. The T7 RNA polymerase is very selective and active and almost all the cell's resources are converted to target gene expression. The target protein comprised more than 50 % of the total cell protein, a few hours after induction.

One mM IPTG was found to be optimum for maximal expression for pGEX vectors and 0.4 mM for pET vectors. However, often the conditions for highest mass of the target protein do not correlate with the conditions for highest activity of the protein. Solubility of the target protein was another major concern while attempting the expression. Most of the induced proteins were in the soluble form, although some quantity accumulated in the inclusion bodies also. LaVallie *et al.*, (1993) 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 the present study, it was observed that growth and prolonged induction at 30°C increases the protein solubility. The

fusion protein expressed by pGEX vector could not be used for subsequent analyses because of the failure in purifying it as a single band.

When total soluble proteins from the induced *E.coli* colonies with recombinant pET vector were analysed by SDS-PAGE, the band of 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 is 9.26. Sequences 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 functionality of the glucanase enzyme. This was demonstrated by the hydrolysis of laminarin that served as the glucanase substrate. Earlier, a functional latex cDNA clone was 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 was not tested for its anti-fungal properties. Recently, a recombinant latex class I chitinase was produced using pMAL expression vectors and its immunoglobulin binding reactivity was studied by immunoblot experiments using sera from latex-allergic patients (Rihs *et* al, 2003). Yu *et al.,* (2003) also report bacterial expression of a barley glucanase, which is functionally active.

The purified recombinant *Hevea* β-glu was assayed for its anti-fungal activity against ALF causing *P. meadii.* Clear inhibition zones were developed around the filters soaked with 10 μg of the purified protein, when fungi were grown on PDA plates. This result clearly indicates the inhibitory action of purified enzyme. The fungi over grew these clear inhibitory zones one week after inoculation.

In the present 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 the purified rice chitinase was shown to have 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 germination of 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, a β-glu purified from pea pods was found to be inhibitory only to *Fusarium solani* among the 8 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 make use of combinations of β-glu and chitinase in order to study the *in vitro* anti-fungal properties. In combination, their inhibitory effect seems to be several-fold higher than when these enzymes are used individually (Mauch *et al.*, 1988). Hu *et al.*, (1999), expressed three anti-fungal proteins; trichosanthin, tobacco class I chitinase and tobacco class I glucanase in *E.coli* and the recombinant proteins were tested for anti-fungal activity. All the three kinds of proteins showed inhibitory activities, but in combinations of two, the activity was found to enhance several folds. When all of them are combined, a very high anti-fungal activity was observed. However, in the present study, only β-glu might be playing a major role, since the pathogen is a chitinless fungus.

Proper selection of glucanase genes is very important for the development of transgenic plants with enhanced disease resistance, as 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 have been isolated, which are having major roles in various physiological and developmental processes, but less important in disease resistance. In several studies, different β-glu isoforms have been tested for *in vitro* antifungal activity (Mauch *et al.*, 1988; Sela-Buurlaga *et al.*, 1993). Only class I basic vacuolar isoforms of β-glu were found to be effective in promoting the lysis of hyphal tips and inhibiting the growth of several fungi (Lawrence *et al.*, 1996; Anfoka and Buchenauer, 1997). The extra cellular class II acidic glucanases with week anti-fungal activity may be involved in the releasing of elicitors from fungal cell wall that activate the host defense reactions. Present study also confirms the role of class I basic vacuolar

isoforms of glucanase in fungal resistance. An exception to this was 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), it was shown that preparations of partially purified glucanase from potato tubers were able to degrade the glucans purified from *P. infestans* cell walls. This result and the direct fungicidal action reported herein, suggest that this hydrolytic enzyme could act directly by inhibiting the growth of the invading fungi.

Prospects of the study

The role of β-1,3-glucanase in defending the fungal diseases, especially abnormal leaf fall in *Hevea*, has been confirmed through present studies. This will enlighten the possibility of a transgenic approach in rubber crop improvement. Such a transgenic tree, which over-expresses this anti-fungal protein, with built-in resistance to many fungal diseases, will be beneficial to the rubber plantation industry all over the world. In addition, since different species of *Phytophthora* are the major causative organisms for many of the plant diseases that adversely affect the economic backbone of the agricultural sector of Kerala, like bud rot of coconut, foot rot affecting pepper, mahli disease of areca nut etc., this kind of an approach has significant relevance. Further, development of a functional cDNA clone in a non-pathogenic microorganism with rubber β-1,3-glucanase may be helpful in the bio-control of many *Phytophthora* mediated diseases in several crop plants. From an industrial point of view, a functional cDNA clone would enable the production of recombinant rubber β-1,3-glucanase in large quantities that can be used in immunoassays for latex allergy diagnostics as this enzyme is also classified as a major latex allergen.

6

SUMMARY AND CONCLUSION

Fungal diseases have been one of the principle causes of crop losses from the very early days of organised agriculture. Chemical control of the disease is costly and deleterious to the environment, and eventually becomes less efficient due to the evolution of the pathogen. Breeding for disease resistance is a time consuming process that often took decades to complete in the case of perennial tree crops. Significant new advances at the molecular level in the field of plant-pathogen interactions form the basis for novel transgenic approaches to crop protection. The cloning of disease resistance and defense-related genes and the dissection of the signal transduction components of the hypersensitive response and systemic acquired resistance pathways has greatly increased the diversity of options available for transgenic disease resistance. Establishment of the role of the candidate gene in defense responses would be essential for engineering effective and durable resistance to pathogens in the field.

In the present study, the pathogenesis related β-1,3-glucanase gene in *Hevea* was investigated for its role in combating the abnormal leaf fall disease caused by *Phytophthora* spp. Induction of β-glu has been well documented as a part of plant's broad generalized defense mechanism against a variety of pathogenic fungi in many plant species. Accumulation of this enzyme has always been associated with the advent of hypersensitive response at the infection site and development of systemic acquired resistance throughout the plant. They are hydrolytic enzymes, able to catalyze the endo-type hydrolytic cleavage of β-1,3-linked glucans, which are the principal cell wall component in many phytopathogenic fungi. Thus they can act directly by degrading the cell wall of the pathogen. They can also perform in an indirect way, by releasing the fungal cell wall components that can act as elicitors of other forms of active host defense

responses. For these reasons, considerable effort has been aimed at isolating and characterising these plant hydrolases to evaluate their potential for improving disease resistance of plants against fungi.

Gene specific primers were used to amplify the β-glu gene from *Hevea* genomic and cDNA. For this purpose DNA and RNA were isolated with good concentration and purity from *Hevea* (clone RRII 105) leaf tissues. RNA was isolated from latex also. First strand cDNA was synthesised from the isolated RNA through reverse transcription. PCR conditions were optimised to amplify the bands from genomic DNA and cDNA. A 1.25 kb and a 1.12 kb fragments were amplified from genomic and cDNA respectively. These PCR fragments were cloned in pGEM vectors and sequenced. Both were found to be identical except for the presence of a 132 bp intron in the genomic sequence. The *Hevea* β-glu gene shows similarities with the other reported β-glu genes from different plant species, but the intron size varies. The position of the intron was found to be same in all reported plant glucanases. The genomic DNA sequence of the β-glu gene in *Hevea* was reported for the first time in this study. Deduced amino acid sequence shows that it encodes a protein of 374 amino acids with 36 amino acid N-terminal signal peptide and 22 amino acid C-terminal for vacuolar targeting, which are removed during final processing. It is basic class I glucanase with a theoretical pI value of 8.96 with a predicted size of 41.2 kDa. Differences were observed at certain positions when compared with three earlier reported sequences of mature β-glu from *Hevea*. A protein glycosylation site reported in β-glu from clone RRIM 600 was absent in the present protein from clone RRII 105. Southern hybridisation confirms the presence of a low copy number gene in 14 different genotypes of rubber tree, which include both tolerant and susceptible varieties.

Most of the high yielding clones of rubber tree are susceptible to abnormal leaf fall, which is the most destructive disease of the rubber plantations in India. However, some clones show certain degree of tolerance to *Phytophthora* infection. To determine the effectiveness of β- glu to resist *Phytophthora* infection in *Hevea*, a susceptible clone (RRIM 600) and a highly tolerant clone (RRII 105) were analysed for induction of glucanase upon pathogen infection. Two experimental approaches, northern hybridisation and relative quantitative RT-PCR, were

performed to study the gene expression. β- Actin and 18S RNA genes were used as internal controls in RT-PCR and northern analysis respectively. Expression levels were calculated by measuring the band intensity. The level and onset of β- glu expression were found to be positively correlated to the level of resistance to the pathogen. Present study reports the induction of β- glu in both the tolerant and susceptible clones of *Hevea*, upon pathogen infection. However, the timing and magnitude of induction varies between the clones. In northern no signals were generated in the uninfected control samples. After inoculation, an exponential increase in β- glu transcript levels observed in both clones and it reached a peak at 48 h after inoculation. But a faster rate of increase was observed in tolerant clone and this was more evident in due course. Four days after inoculation, the transcript levels remains 15-fold higher than its basal levels in tolerant clones, while the β-glu mRNA levels were depleted to drastically low levels at this stage in susceptible clone. Relative RT-PCR analysis with house keeping actin gene as internal control also indicate similar trends, validating its usefulness in studying the differential gene expression. As both northern and RT-PCR give a measure of only the β- glu mRNA transcript levels, to confirm the presence of the end product glucanase enzyme assay was also carried out using laminarin as the substrate. The enzyme activity was found to vary between clones and treatments in accordance with the northern and RT-PCR results. The faster, higher and more prolonged induction of β- glu observed in the case of tolerant clone as reported in the case of many other plant species, was shown to have important implications in combating the *Phytophthora* challenge in *Hevea*. Although lesions were formed on tolerant clones, this enzyme may play a significant role in disease resistance by limiting the extension of fungal hyphae within the necrotic tissue.

As reported earlier, the defense-related β- glu was found to be expressed constitutively in the latex of rubber trees. Tapping to collect latex is a process of controlled wounding and hence the defense-related genes may be expressing in order to sanitize and seal the wounded sites of the plant. In the present study it was observed that the level of this constitutive expression was almost similar in tolerant and susceptible clones. However, the more intense signals obtained with the latex of a wild accession, suggests a role for latex glucanase in fungal resistance.

Induction of β- glu in *Hevea* during pathogen infection indirectly suggests their role in plant defense. In order to find out a more direct evidence for the inhibition of pathogenic fungal growth by host β- glu, a functional cDNA clone was constructed that could express the rubber β- glu in *E.coli*. There are many reports on the *in vitro* anti-fungal properties of plant glucanases. Class I basic vacuolar isoforms are tested positively in most of the anti-fungal assays. The effect seems to be enhanced greatly when combinations of glucanase and chitinase were used. While most of the studies utilises native enzymes purified from infected plant tissues, in the present study a recombinant *Hevea* β- glu expressed in *E.coli* was tried. The sequence coding for mature functional protein, excluding the N and C-terminal extensions of pre-proprotein, was cloned unidirectionally in pET vector and transformed to an expression host of *E.coli*. Sequencing of the vector ensured correct orientation and reading frame of the cloned insert. Conditions were optimised for the induction of the recombinant protein in soluble form. The target gene was under the control of a T7 promoter, which could be activated in host cells that contain a chromosomal copy of the T7 RNA polymerase gene. This T7 RNA polymerase gene is under the control of a *lac* promoter and hence, could be induced with IPTG. Thus, the addition of IPTG to a growing culture induces T7 RNA polymerase, which in turn, transcribes the target DNA in plasmid. Under optimum conditions, a band of expected size (55.6 kDa) was detected in SDS-PAGE of the soluble protein fraction, isolated from the induced colonies. The target protein, formed in fusion with an N-terminal affinity tag, was purified by passing through an affinity column. Western blotting and thrombin cleavage of the N-terminal fusion tag confirmed its identity. Hydrolysis of its substrate, laminarin, ensured that the purified recombinant protein is in an active form.

The purified *Hevea* β-glu was assayed for its anti-fungal activity against the ALF causing *P.meadii* using filter paper disc method. A single mycelial plug was inoculated in the center of a PDA plate and were incubated at 25°C. Sterile filter paper discs of 5 and 9 mm diameter were laid on the agar surface 1 cm from the periphery of the petridish and was soaked with 10 μg of the purified enzyme solution. Clear inhibition zones, observed around the filter discs treated with the enzyme, indicate the anti-fungal property of the purified protein. These inhibitory

zones remained visible for at least one week and then the fungus starts to overgrow the initial inhibition zones. β-Glu can destroy the fungi by thinning their cell wall at the hyphal tips by degrading the β-glucan that results in swelling and ultimate burst of the hyphal tip. The possible role of host β-glu in resisting the *Phytophthora* attack in *Hevea* that has been evident by its over-expression during fungal infection is being confirmed through the direct fungicidal action of the purified glucanase reported herein.

Last 15 years have shown considerable progress in understanding the structure and regulation of plant β-1,3-glucanases. It is now recognized that higher plant species produce a broad range of β-glu differing in primary structure, cellular localization and catalytic activity. Their involvement in disease resistance has been confirmed through their induction during pathogenesis, *in vitro* anti-fungal properties, and finally through the development of transgenic plants with enhanced resistance. Host β-glu will be predominantly important in combating Oomycete pathogens like *Phytophthora* because their cell wall is made principally of β-1,3 linked glucans. The most destructive disease of rubber tree in India is ALF caused by different species of *Phytophthora*. Although many promising clones were evolved in the last few decades, the narrow genetic base of rubber trees along with its long breeding cycle, high juvenility period and highly heterozygous nature remains as the major limitations for evolving fungal resistant plants through conventional breeding programmes. Further, a single genotype, RRII 105, occupies more than 70% of our rubber plantations. The intensive use of a monoculture crop with little genetic diversity may significantly enhance their susceptibility to increasingly aggressive pathogens. Considering the obstacles involved and time taken for conventional techniques, alternative molecular strategies involving modern tools of molecular biology hold great potential for crop protection programmes in *Hevea*. Since the present study also supports the importance of β-glu in plant defense, it can be presumed that the resistance of rubber plants to fungal attack can be greatly enhanced through development of transgenic plants, which over-express this anti-fungal protein. Prospects seems to be promising, as efficient protocols for genetic transformation and plant regeneration, which often are the major constrains in the development of transgenics in many crops, is already available in the case of rubber.

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List of Figures

Abbreviations

β-glu	β-1,3-glucanase / s
ALF	Abnormal leaf fall
amp	Ampicillin
avr (gene)	*Avirulance* gene
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
chn	Chitinase / s
CTAB	Hexadecyltrimethylammonium bromide
DEPC	Diethyl pyrocarbonate
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
EC	Enzyme catalogue
EDTA	Ethylenediaminetetraacetic acid
Et Br	Ethidium bromide
ExPAsy	Expert protein analysis system
GIP	Glucanase inhibitor protein
GST	Glutathion-S-transferase
h	Hour / s
ha	Hectare
His. tag	Histidine tagged
HR	Hypersensitive response
IPTG	Isopropylthio-β-D-galactoside
IUBMB	International Union of Biochemistry and Molecular Biology
LB	Luria-Bertani broth
NBT	Nitroblue tetrazolium chloride
PAGE	Polyacrylamide gel electrophoresis
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PEG	Polyethyleneglycol
PR	Pathogenesis-related
PVPP	Polyvinylpolypyrrolidone
R (gene)	*Resistance* gene
RAGE	Random amplification of genomic DNA ends
RQ	Relative quantitative
RRII	Rubber Research Institute of India
RT	Reverse transcriptase
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride-sodium citrate
TCA	Total cell protein
TE	Tris-EDTA
TMV	Tobacco mosaic virus
TPD	Tapping panel dryness
Trx	Thioredoxin
vol	Volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside