Molecular cloning of the partial promoter of β-1,3-glucanase gene isoform-5 (Gln5) from *Hevea brasiliensis*, clone RRIM-600

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Submitted by

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CERTIFICATE

This is to certify that the dissertation entitled "Molecular cloning of the partial promoter of β-1, 3-glucanase gene isoform-5 (Gln5) from *Hevea brasiliensis*, clone RRIM-600" submitted by Ms. Jyothilekshmi. V., for the degree of Master of Science in Biotechnology, Mahatma Gandhi University, Kottayam is an authentic record of the research work carried out under my supervision at the Biotechnology Division, Rubber Research Institute of India, Kottayam. It is also certified that this work has not been presented for any other degree or diploma earlier.

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CERTIFICATE

This is to certify that the dissertation entitled "**Molecular cloning of the partial promoter of β-1,3-glucanase gene isoform-5 (Gln5) from *Hevea brasiliensis*, clone RRIM-600"** is an authentic record of the project work done by Ms. Jyothilekshmi. V Mahatma Gandhi University, Kottayam at Rubber research Institute of India, under the guidance of Dr. A. Thulaseedharan, Deputy Director (Biotechnology), RRII, Kottayam, in partial fulfilment of the requirement for the award of the Degree of Master of science in Biotechnology at the School of Bioscience, Mahatma Gandhi University, Kottayam and the dissertation has not formed the basis for the award of any other degree or diploma earlier.

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DECLARATION

I do hereby declare that the dissertation entitled "**Molecular cloning of the partial promoter of β-1,3-glucanase gene isoform-5 (Gln5) from *Hevea brasiliensis*, clone RRIM-600**" submitted to Mahatma Gandhi University in partial fulfillment for the award of degree of Master of Science in Biotechnology is a record of original research work done by me under the supervision and guidance of Dr. A. Thulaseedharan, Deputy Director (Biotechnology), Rubber Research Institute of India, Rubber Board, Kottayam and it has not formed the basis for the award of any Degree/ Diploma/ Associateship/ Fellowship to any candidate of any University.

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ABBREVIATIONS

β- glu	:	*β-1,3-glucanase*
ALF	:	*Abnormal leaf fall disease*
CAZy	:	*Carbohydrate Active enzymes*
CTAB	:	*Cetyl trimethyl ammonium bromide*
DNA	:	*Deoxyribonucleic acid*
EDTA	:	*Ethylene-diamine-tetraacetic acid*
GUS	:	*Glucouronidase*
HR	:	*Hypersensitive Reaction*
IUBMB	:	*International Union of Biochemistry and Molecular biology*
NCBI	:	*National Center for Biotechnological Information*
PCR	:	*Polymerase Chain Reaction*
PR proteins	:	*Pathogenesis Related proteins*
RNA	:	*Ribonucleic acid*
TMV	:	*Tobacco Mosaic Virus*
UV	:	*Ultra violet*

INTRODUCTION

The Para rubber tree, *Hevea brasiliensis* is an economically important crop. It accounts for more than 99% of the world's natural rubber production. The tree was introduced to the tropical regions of Asia from its primary centre of origin, Brazil, in the nineteenth century. The commercial plantations are now distributed in countries like Malaysia, Indonesia, Thailand, India, Sri Lanka, China, Philippines, Vietnam, Cambodia, Myanmar, Bangladesh, Nigeria, Cameroon, Brazil and Mexico. In India, commercial cultivation of natural rubber was introduced by the British planters, although the experimental efforts to grow rubber on a commercial scale in India were initiated as early as 1873 at the Botanical Gardens, Calcutta. India's natural rubber consumption stood at 978 thousand tons per year, with the production at 893 thousand tons (IRRDB, 2006).

The tree – *Hevea brasiliensis*

H. brasiliensis belongs to the family Euphorbiaceae. The plants of Euphorbiaceae family are mostly monoecious herbs, shrubs and trees, sometimes succulent and cactus-like and comprise one of the largest families of plants with about 300 genera and 7500 species that are further characterized by the frequent occurrence of milky sap. The generic name is derived from a local word in the Amazon,"heve" meaning rubber. There are ten species found to occur in this genus. They are *H. benthamiana, H. camargoana, H. camporum, H. guianensis, H. microphylla, H. nitida, H. pauciflora, H. rigidifolia,* and *H. spruciana* (Clement-Demange *et al, 1997).* Among the ten species *H. brasiliensis* is the only commercially exploited species for natural rubber.

H. brasiliensis is a deciduous perennial crop. The economic life period of rubber trees in plantations is around 32 years – up to 7 years of immature phase and about 25 years of productive phase. The tree comes into flowering after 3-4 years after planting and profuse regular flowering occur in trees of more than 6-7 years old. Flowering takes place annually during February – March, after the annual leaf fall coinciding with the winter period, often called as 'wintering'. Off-season flowering is also reported to occur in India, during August – September period (K.K.Vinod, 2002). The soil requirement of

the plant is generally well-drained weathered soil. The climatic conditions for optimum growth found in the traditional belt of rubber trees consist of:

- Rainfall of around 250 cm evenly distributed without any marked dry season and with at least 100 rainy days per year.
- Temperature range of about 20°C to 34°C with a monthly mean of 25°C to 28°C.
- High atmospheric humidity of around 80%.
- Bright sunshine amounting to about 2000 hours per year at the rate of 6 hours per day throughout the year.
- Absence of strong winds.

Fig.1: Healthy rubber plantation with a clear view of tapping panel in the inset

The trunk is cylindrical, but frequently swollen towards the base and the bark is pale to dark brown with a smooth surface and the inner bark pale brown with abundant white or cream colored latex (Fig.1). The leaves are in spirals and with three leaflets (Duke, 1978). The flowers are small with no petals, bright or creamy-yellow in color and extremely pungent. There are male and female flowers but both are found in the same inflorescence. The female flowers are bold and borne on the tip of the inflorescence, and male flowers are borne in numerous numbers below it. The fruit is an exploding 3-lobed capsule. The milky latex of *Hevea brasiliensis*, produced by a specialized secretary system in the phloem, is the raw material for natural rubber. The latex is a renewable resource that can be sustainably tapped without harming the tree. Latex is a natural polymer of isoprene with a molecular weight of 10,000 to 1,000,000. A small percentage of other material such as proteins, fatty acids and inorganic materials are found in natural rubber. Rubber is water-resistant, does not conduct electricity, is durable and most importantly is highly elastic. These useful properties are due to the large and complex molecular structure of rubber.

Natural rubber

Rubber plantations supply raw materials for the production of many industrial goods required for automobiles, aircrafts, railways, textile industries, sports, and engineering goods and even for building roads. On account of the multifarious uses to which rubber can be put to, the consumption of rubber in the world as well as India has been increasing steadily. Significant uses of rubber are door and window profiles, hoses, belts, malting, flooring, gloves, toy balloons, rubber bands and pencil erasers. Additionally rubber produced as a fiber sometimes called elastic has significant value for use in the textile industry because of its excellent elongation and recovery properties. Seeds are source of rubber seed oil, recommended for manufacture of soap. Oil is also used in paints, varnishes and is effective against houseflies and lice. Press cake or extracted meal is used as fertilizer or feed for stock. The wood from this tree is used in manufacture of furniture. It is valued for its dense grain, minimal shrinkage, attractive color and acceptance of different finishes. It is also prized as an environmentally friendly

wood, as it makes use of trees that have been cut at the end of their latex producing cycle (IRRDB, 2006).

Major diseases in *Hevea*

The consequences of frequent tapping cause infection at the raw surface tapping site. This problem is common in the southernmost part of Thailand where the humidity is high and suitable for pathogen growth, especially fungus. *Phytophthora palmivora* is the causative agent of "leaf fall" and "black stripe" in rubber plants. It attacks the petioles, causing mature leaves to fall prematurely and attacks the tapping surface resulting in poor latex production. Therefore, the resistant cultivar of *H. brasiliensis* should be selected for planting. This is a more cost effective way to prevent low-yielding rubber tree. The commonly used strain of rubber for planting is RRIM600, it gives high-yield of latex but susceptible to *Phytophthora spp.*

Abnormal leaf fall (*Phytophthora spp.*) severe in India, causing the abscission of mature leaves during the monsoon rains; treatment necessitates the application of a pre-monsoon prophylactic copper spray. Secondary leaf fall, caused both by *Oidium heveae* and *Colletotrichum gloeosporioides* which infect the flushes of new leaves produced after the annual leaf change (wintering), varies greatly in severity according to local weather and cultivar (Ramakrishnan, 1960). It can be controlled by repeated rounds of an appropriate prophylactic fungicide or by artificially hastening the onset of wintering by removing the old leaves with a contact herbicide. By far the most important leaf disease is the South American leaf blight (*Microcyclus ulei*); it is largely responsible for the lack of a vigorous rubber industry in South America, the home of the rubber tree. It can only be effectively countered by the use of resistant cultivars.

Many of the high yielding clones of *Hevea brasiliensis* are susceptible to abnormal leaf fall disease which is caused by various *Phytophthora* species and results in a yield loss of upto 40% in highly susceptible clones, making it the most destructive disease of rubber trees in India (Jacob 2003). *Phytophthora* ("phyton" in Greek means plant and "phthora" means destruction) is a genus of plant damaging Oomycetes (water

molds) whose member species are capable of causing enormous losses on crops worldwide as well as environmental damage to the natural ecosystem. The Oomycetes are the largest group of heterotrophic *Stramenopiles*. They are found all over the world in fresh and salt water habitats. Some of the terrestrial Oomycetes are among the most important plant pathogenic organisms that are facultatively or obligately parasitic. The cell walls of the group consist mostly of β -1,3- and β -1,6-glucans with a small amount of cellulose. A few members also have chitin deposits. *Phytophthora* are mostly pathogens of dicotyledons and are relatively host-specific parasites. These include *P. infestans, P. sojae, P. alni, P. meadii, P. cinnamomi, P. kernoviae, P. palmivora, P. ramorum,* etc.

Abnormal leaf fall disease occurs during periods of prolonged wet weather, when cool, overcast and humid conditions persist for several days. Green pods are generally the first to be infected by the motile zoospores of the fungus, which in turn provide abundant inocula for infection of leaves. The fungus attacks the petiole and leaf blades, causing leaves to shed. After defoliation, the pathogen often attacks the leaf bearing twigs, causing extensive dieback. An understanding of the molecular mechanism of disease-resistance, mediated through pathogen-related (PR) proteins, could provide an alternative approach for developing more sophisticated molecular tools to combat ALF disease in rubber trees.

It is known that plants have been infected by many pathogens including bacteria, virus, fungi and nematodes and it can be infected with several pathogens at the same time. Each plant has its own defense mechanism to protect itself from the pathogen. Structural defense mechanisms which prevent an infection are histological and cellular defense structures formed as a result of hypersensitive reaction in the plant, for example, cork layers formation, abscission region formation, formation of tyloses and gum. Cork layers can prevent spreading of pathogen and its toxin by impeding water and nutrient flow to infected tissues resulting in localization of non-viable infected tissue. Abscission regions are formed by tissue disruption creating intercellular space surrounding infected area; therefore it prevents normal, non-infected tissue from further infection. Tyloses formation commonly develops during the invasion of pathogen into the xylem by the in

growth of the protoplasm of the adjacent paranchymal cells of the xylem making it obstructed. Tyloses can be enormously and rapidly formed in the resistant plant whereas it is formed more slowly on the susceptible plant usually after infection was spread. The accumulation of gum intra- or intercellularly surrounding the infected area is also helpful in this regard. The rate of gum accumulation differs between different kinds of plants.

Hypersensitive reaction is one of the one of the most important defense mechanism in plants. It causes the infected and its surrounding tissue of the resistant plant turn brown, having the characteristics of burn–like lesion and hypersensitive cell death. It makes the pathogen inside the dead tissue deprived of nutrients. The rate of hypersensitive reaction is much slower or does not exist in the susceptible plant making it unable to defense itself. In addition to this, ultraviolet rays and certain metal ions can also stimulate plant defense mechanism.

Rubber is an economically important crop and it has wide range of applications. Bud grafting is the chief method for propagation of *Hevea brasiliensis*. From the initial germplasm collections of the 19th centuary, many clones with desirable traits have been developed through conventional breeding programs. Breeding process in *Hevea* has more advantages and disadvantages. The common advantages are: Cross pollination is easy owing to the monoecious nature, seeds are very bold and can be handled easy, and the progenies selected can be multiplied vegetatively by budding. The disadvantages are: crossing is laborious and time consuming, the hybrid recovery is very low, the flowers are susceptible to mechanical manipulation, seed viability is very limited, handling of breeding population require extensive resources like land and labour, genetic base of the breeding stock is very narrow and collection from the wild is very difficult (K.K.Vinod, 2002).

So to improve the yield of rubber, biotechnological methods are introduced. Genetic engineering, on the other hand, employs a very different method to produce improved crops. Instead of relying on sexual recombination, genetic engineering preserves the integrity of the parental genotype, inserting only a small additional piece of information that controls a specific trait. This is done by splicing a well characterized chunk of foreign DNA containing a known gene into a chromosome of the host species

(Richard, 2004). There are many methods are used to transfer a foreign gene to the host such as *Agrobacterium* – mediated gene transfer, particle bombardment, electroporation etc. among these methods *Agrobacterium* – mediated gene transfer is commonly used in *Hevea*.

Regarding abnormal leaf fall disease, β-1,3- glucanase gene plays a major role in *Hevea*. It has been studied in detail in the tolerant clone RRII 105. There are several forms of this gene with different promoters and has been reported in the NCBI by Supriya *et al* and Acc. Nos. are Gln2- GU123623.2, Gln3- GU191335.1, Gln4- JN251022.1, Gln5- JQ650524.1, Gln6- JQ650525.1. It has been reported earlier, that the pathogen inducible β-1,3-glucanse gene is present in tolerant and susceptible clones. But a continuous expression for a long period is observed only in the tolerant clones. Promoters are the controlling elements of gene expression. Elucidation of sequence variation if any between the tolerant and susceptible clones may help in the understanding of the differential gene expression of β-1,3-glucanase in the *Hevea* clones susceptible and tolerant to abnormal leaf fall disease. Therefore, the objective of the present study is to elucidate nucleotide sequence of the promoter region of β-1,3-glucanase gene from the *Hevea* clone RRIM 600 which is susceptible to abnormal leaf fall disease and compare with the already reported sequence from a tolerant clone.

Objectives

- Isolation and molecular characterisation of the promoter of the β-1,3-glucanase gene isoform-5 (Gln 5) from the *Hevea* clone RRIM 600 susceptible to abnormal leaf fall disease.
- Elucidation of nucleotide and *cis*-element variations if any with respect to the same gene reported from a *Hevea* clone RRII 105 tolerant to abnormal leaf fall disease.

REVIEW OF LITERATURE

Hevea brasiliensis accounts for more than 99% of the world's natural production. The majority of diseases affecting *Hevea* rubber tree, considered as economically important, were recorded with the beginning of the 20th century. There has been a changed scene in the maladies of the rubber tree. This is mainly due to the production of clones that can resist the common diseases and the well acceptance of the new genetic material by the growers. Other factors which contributed to the spread of new diseases are the expansion of rubber cultivation to the new localities and the non-adoption of proper cultural practices by the growers. Presently, three diseases, namely the South American leaf blight (SALB), corynespora leaf fall and abnormal leaf fall diseases are the most threatening maladies affecting the rubber tree. However, many of the high yielding clones are susceptible to abnormal leaf fall disease (ALF), which is caused by various *Phytophthora* species and results in a yield loss of up to 40% in highly susceptible clones, making it the most the most destructive disease of rubber tree in India (Jacob 2003). Abnormal leaf fall disease occurs during periods of prolonged wet weather, when cool, overcast and humid conditions persist for several days. Green pods are generally the first to be infected by the motile zoospores of the fungus, which in turn provide abundant inocula for infection of leaves. The fungus attacks the petiole and leaf blades, causing leaves to shed. After defoliation, the pathogen often attacks the leaf bearing twigs, causing extensive dieback.

Bacteria, fungi, viruses and mycoplasma are the microorganisms which causes diseases in plants. Among these only fungi are known to cause disease in rubber trees leaves (Grover and Gauthaman, 2003). The leaf disease is more important because they can lead to fungal diseases affect the different parts of the rubber tree namely root, stem and significant crop loss. In plants, the leaves function as the seat of photosynthesis. Photosynthesis is essential for growth and yield. Leaf diseases cause reduction of leaves and the total photosynthetic area available with the plant. Thus it results in poor growth and yield. The major leaf diseases of rubber occur as epidemic in plantations. They spread very fast in the plantation and lead to quick defoliation. The important leaf diseases of rubber are abnormal leaf fall disease, powdery mildew, corynespora, colletotrichum and bird's eye spot disease.

The stem, shoot, leaf, and pod of the tree are attacked by two *Phytophthora* species, *P. palmivora and P. botryose, P. palmivora* causes black stripe of the tapping panel and patch canker on the untapped bark, pod rot and leaf fall. On the other hand, *P. botryose* is the main cause of leaf fall and pod rot diseases, although it may also cause black stripe under conditions favorable to it. Leaf fall and black stripe are important diseases during the rainy seasons from July to October. Most of the research has been centered on black stripe and leaf fall as they occurred more frequently than other *Phytophthora* diseases (Tan, 1979). *P. palmivora* and *P. botryosa* from rubber were capable of infecting other crops including cocoa, durian, pepper, mango, citrus, and orchid (Chee and Hashim 1971). No *Phytophthora* species has been recorded on rubber roots, although rubber root diseases caused by other groups of fungi are major constraints to the rubber industry.

The Abnormal Leaf Fall Disease

Abnormal leaf fall (ALF) is the most economically significant fungal disease in India this disease is caused by *Phytophthora spp.,* and other major fungal disease affecting the rubber tree are powdery mildew by *Oidium hevea*, and leaf spot by *Corenyspora*. During the south-west monsoon period, wet weather coupled with humid condition favour the diseases. First the fruits rot, later infected leaves fall in large numbers, prematurely, either green or after turning coppery red with a drop of latex, often coagulated in the centre of bark lesion on the petiole. Lesion may develop on the mid rib and leaf blades also. Heavy defoliation may lead on to considerable loss of crop. Initial field trails indicated that this disease could cause yield loss up to 38 to 56%, when left unsprayed for a disease season (Ramakrishnan, 1960). Ongoing studies indicate up to 48% yield loss in susceptible clones due to ALF disease (Jacob, 2003). Clones like PB 86, PB 235, PB 260, PB 311, PB 28/59, RRIM 600, RRIM 628, RRIM 703, RRII 5, PR 255, PR 261 and Tjir 1 are relatively susceptible to the disease. RRII 105, PB 217, GT 1 and GL 1 are clones showing some tolerant reaction to the disease.

Prophylactic spraying of the foliage prior to the onset of south- west monsoon with 1% boardeau mixture or oil based copper oxychloride will check the disease. Two rounds of spraying using 17 to 22 liters of fungicide oil mixture per hectare per round (1:6 proportion) with a gap of 10 to 15 days is necessary.

***Phytophthora* species**

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. There are about 60 species in the genus *Phytophthora*, all of them are plant pathogens. *Phytophthora* diseases have been well studied in the temperate regions of the world. Throughout the wet tropics, agricultural production of a large range of crops is seriously reduced due to the wide range of *Phytophthora* pathogens causing a large number of different diseases.

There are a number of host and pathogen factors which, together with features of their interactions, make *Phytophthora* diseases so troublesome in the wet tropics. One of the important factors to consider is that the genus *Phytophthora* does not belong to the fungal kingdom. It is an Oomycetes, closely related to diatoms, kelps and golden brown algae in the kingdom Stramenopila (Beakes, 1998). These organisms are found commonly in wet tropics. All *Phytophthora* species need high humidity for sporulation and the germination of sporangiospores and zoospores to initiate infections. Frequent or seasonal heavy rainfall, and high levels of humidity, is common throughout the tropical lowlands. Tropical highlands have the added problem of heavy mist and dew during the morning and/or late afternoon, producing free water throughout the night and providing almost daily opportunities for sporangiospores to be formed, transported and start new infections.

Another characteristic of *Phytophthora* species and *P. palmivora* in particular, is their ability to cause multiple diseases on the same host. In addition to causing multiple diseases on the same host, *P. palmivora* can also attack a wide range of different host

species that are widespread and/or cultivated throughout the tropics. 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 (Bartnicki-Garcia and Wang, 1983). The cell wail is made principally of β-1, 3 linked glucose polymers, which comprise about 80 - 90% of the cell wall dry weight. The cytoplasm of *Phytophthara 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, under favorable environmental conditions, the most important of which is the availability of water, a rapid production of asexual sporangia and zoospores will occur. All the Oomycetes, microorganisms have a short generation time and great reproductive capacity (Dick, 1992).

The most destructive disease of rubber in India is the abnormal leaf fall (ALF) caused by different species of *Phyrophthora.* Four species, *P. palmivora, P. meadii, P. nicotianae var. parasitica and P. hotryose* have been isolated from infected specimens. However, the species most common in India is *P. meadii* (Edathil *et al.,* 2000). This disease was first reported in Thrissur district (Mc Rae, 1918) and later this disease had spread to other rubber growing countries like Sri Lanka, Myanmar, Cambodia, Vietnam, Nigeria, Cameroon, Brazil, Costa Rica and Venezuela (Chee, 1969).

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^{0}C and relative humidity of above 90% are the most congenial conditions for the outbreak of the disease.

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 abscise, 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 (Ramakrishnan, 1960).

Pathogenesis Related Proteins

Interactions between plants and pathogens reflect an elaborate co-evolution of recognition, defense and counter defense mechanisms. Infection of resistant strains of plants triggers a complex series of local biochemical and cellular events in the host, known as the hypersensitive reaction (reviewed in Collinge and Slusarenko, 1987). The hypersensitive reaction is a stereotypic response, that is, the nature of the response is similar for viral, bacterial, and fungal pathogens. Some components of the hypersensitive

reaction appears to serve a general defense function independent of the inciting pathogen. Rapid cell death results in necrotic lesions at the site of infection. Antimicrobial compounds such as the phytoalexins are produced. Cell walls are modified by the deposition of lignin and the β-1, 3-glucan callose. This process is thought to isolate the infected area and to help prevent spread of the pathogen (Bell, 1981). Other components of the HR, for example, the induction of antifungal β-1, 3-glucanases and chitinases, appear to be tailored for defense against a particular class of pathogens (Schlumbdum *et al.,*, 1986; Mauch *et al.,*, 1988; Broglie and Broglie, 1993; Sela Buurlage *et al.,*, 1993; Zhu *et al.,*, 1994).

Plant genes induced during the hypersensitive reaction are commonly assumed to be part of the host's defense mechanisms. For example, induction of one class of genes encoding β-1,3-glucanases could be a counter defense mechanism employed by viral pathogens. Higher plants have developed various defense mechanisms against biotic and abiotic stresses, such as pathogen invasions, wounding, exposure to heavy metal, salinity, cold, and ultraviolet rays. These defense mechanisms include: physical strengthening of the cell wall through lignification, suberization, and callose deposition; production of phytoalexins which are secondary metabolites, toxic to bacteria and fungi; and synthesis of pathogenesis-related (PR) proteins such as β 1,3-glucanases, chitinases and thaumatin like proteins (Bowles, 1990).

PR proteins were first observed in tobacco plants infected with tobacco mosaic virus (TMV) (Van Loon and Van Kammen, 1970), and they were subsequently identified in many other plant species. Based on their primary structures, immunologic relationships, and enzymatic properties, PR proteins are currently grouped into seventeen families (PR-1 through 17) (Van Loon, 1999; Görlach *et al.,*, 1996; Okushima *et al.,*, 2000; Christensen *et al.,*, 2002). The PR-1 family consists of proteins with small size (usually 14-17 kD) and antifungal activity. The PR-2 family consists of β-1,3-glucanases, which are able to hydrolyze β-1,3-glucans, a biopolymer found in fungal cell walls. The PR-3, - 4, -8 and -11 families consist of chitinases belonging to various chitinase classes (I – VII). The substrate of chitinases, chitin, is also a major structural component of fungal cell walls. The PR-5 family consists of thaumatin-like proteins and osmotin-like

proteins. Other PR families include proteinase inhibitors, endoproteinases, peroxidases, ribonuclease - like proteins, defensins, thionins, lipid transfer proteins, oxalate oxidases and oxalate oxidase - like proteins.

The β-1,3 glucanases are able to catalyze endo - type hydrolytic cleavage of the 1,3-β-D-glucosidic linkages in β-1,3-glucans. The β-1,3-glucanases are abundant, highly regulated enzymes widely distributed in seed-plant species. Although the major interest in β-1,3 glucanases stems from their possible role in the response of plants to microbial pathogens and these enzymes are also implicated in diverse physiological and developmental process in the uninfected plants including cell division, microsporogenesis, pollen germination and tube growth, fertilization, embryogenesis, fruit ripening, seed germination, mobilization of storage reserves in the endosperm of cereal grains, bud dormancy, response to wounding, cold, ozone and UV etc (Van Loon,1999).

Plant β-1,3,-Glucanase and their Importance In Defense Mechanism

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 hydrolyze glycosyl compounds and the third digit (1) indicates that they hydrolyze- 0-glycosidic compounds. Glucan endo-l,3-β-D-glucosidase (EC 3.2.1.39), commonly known as β- 1,3- glucanases, are one of the major classes of 0-glycosidic hydrolyses. The IUBMB nomenclature of glycosidic hydrolases is based on their substrate specificity and their molecular mechanism, and such a classification does not reflect the structural features of the 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, whlch 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.

Structural classes of β-1,3-Glucanase

β-1,3-Glucanases exist as multiple structural isoforms that differ in size, isoelectric point, primary structure, cellular localization, and pattern of regulation (Meins F, 1992). Among woody plants, the β-glucanase gene was first isolated from *Hevea* (Chye and Cheung, 1995). Different isoforms of β-glucanase have detected in the latex of the rubber tree (Cheung, 1995). The most detailed sequence information for these isoforms is available from cDNA and genomic clones of tobacco β– glucanase, which form a multigene family. Based on the amino acid sequence identity, the various isoforms of the gene in *Nicotiana* have been classified in to 4 distinct structural classes (Meins F Jr *et al.,* 1992; Ward ER *et al.,* 1991; Payne G, 1990). Similar structural isoforms have been reported from potato, tomato and other plant species also (Oh Hy *et al* 1995; Beerhues L, 1994).

The ca. 33 kDa class 1 enzymes of Nicotiana tabacum, which constitute the PR-2e subgroup of tobacco PR-proteins, are basic proteins localized in the cell vacuole (Van den Bulcke, 1989; Keefe, 1990). β- Glucanase 1 is produced as a preproprotein with a N-terminal hydrophobic signal peptide, which is co-translationally removed, and a C-terminal extension N-glycosylated at a single site. The proprotein is transported from the endoplasmic reticulum via the Golgi compartment to the vacuole where the C-terminal extension is removed to give the mature, ca. 33 kDa enzyme, which is not glycosylated (Sticher L, 1992; Skinshi H, 1988). Glycosylation during processing is thought to facilitate targeting them to the vacuole. The vacuolar class I β- glucanase were shown to be secreted into the medium in cultured tobacco cells via a novel pathway (Melchers *et al.,* 1993). Class I β-glucanase, which are induced either by pathogen infection or ethylene treatment are the first characterized isoforms. They are also thought to be involved in the normal development of healthy plants during seed germination.

The known mature β-glucanase 1 of tobacco and Gn2 of *Nicotiana plumbaginifolia* share ca. 98% amino acid identity (Gheysen G *et al.,* 1990). In contrast, with only ca. 76% similarity, the Gn1 of *N. plumbaginifolia* is structurally more distinct (Castresana C *et al.,* 1990). The potato class I β-glucanase is similar to tobacco class I β-glucanase. 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-glucanase gene isolated from monocots *(Pisum sativum)* contains a long amino acid extension at the C-terminal end compared to other isoforms (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 β-glucanase 1, the classI II and class III members of the PR-2 family are secreted into the extracellular space (Stintzi A *et al.,* 1993; Meins F Jr *et al* 1992; Simmons CR, 1994). The tobacco class II β-glucanase PR-2a, PR-2b, PR-2c are acidic proteins without the c – terminal extension with an apparent size from ca. 34 to 36 kDa (Linthrost *et al.,* 1990) . The class II tobacco isoforms are at least 82% identical in amino acid and differ from the class I enzymes at a minimum 48.8% of the positions (Meins F Jr *et al.,* 1992; Simmons CR *et al.,* 1994). Class 2 also includes the two acidic 41kDa stylar β-glucanase isoforms, Sp41a and Sp41b, which are exclusively expressed in the style of tobacco flowers (Ori N *et al.,* 1990). They do not appear to be induced by pathogen infection and, are reffered to as "PR-like proteins" (Van Loon LC *et al.,* 1994).

The acidic ca. 35kDa PR-2d (PR-Q) is the representative of tobacco class III β-glucanase and differs in sequence by at least 43% from the class I and class II enzymes (Payne G *et al.,* 1990). They are also secreted in the extracellular space. Two highly homologous cDNA clones for class 3 β glucanase have been isolated from tomato plants infected with a viroid (Domingo C, 1994). Based on deduced amino acid sequences, TomPR-Q'a is an acidic isoform, 86.7% identical to tobacco PR-Q and TomPR-Q'b is a basic isoforms , which is 78.7% identical to tobacco PR-Q'.

Tag 1 appears to represent a novel class of tobacco β-Glucanase. It is a "PR-like" protein which is expressed specifically in tobacco anthers (Bucciaglia PA *et al.,* 1994). Like the tobacco class 1 β-glucanase, Tag 1 is encoded by a small gene family with at least two members derived from the *N. tomentosiformis* progenitors of tobacco. Based on

deduced amino acid sequence, Tag 1 encodes a polypeptide with an N-terminal signal peptide, but no C-terminal extension. The mature form of Tag 1 is an acidic, 35 kDa protein, which shares absolutely conserved sequences found in all classes of tobacco β-glucanase. It is 37-38% identical in sequence to the mature forms of tobacco class 1 Gla, class 2 PR-2, and class 3 PR-Q (Meins F Jr *et al.,* 1992).

The specific enzymatic activities and substrate specificities of different β- glucanase vary considerably. The β glucanase class 1 and II PR-2c appear to be 50 to 250 times more active in degrading the β-1,3 –glucan substrate laminarian than the class 2 PR-2a and PR-2b and the class 3 PR-2d enzymes (Kauffmann S *et al.,* 1987; Linthorst HJM *et al.,* 1991).

In general, many high yielding clones of *Hevea* are susceptible to abnormal leaf fall disease under Indian conditions. However, clones like RRII 105, GT , Gl I, BD'10and 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. brasiliesis* and a resistant species of *Hevea* called *H. benthamiana,* are resistant to *Phytophthora,* but are not popular due to their low yield.

Role of β-1, 3-Glucanases in plants

β-Glucanases are involved in diverse physiological and developmental processes in the uninfected plants including cell divisin (Waterkeyn L *et al.,* 1967; Fulcher RG *et al.,* 1976), microsporogenesis (Worall D *et al.,* 1992; Bucciaglia PA *et al.,* 1994), pollen germination and tube growth (Roggen HP *et al.,* 1969; Meikle PJ *et al.,*1991), fertilization (Lotan T *et al.,* 1989; Ori N *et al.,* 1990), embryogenesis (Dong JZ *et al.,* 1997; Helleboid S *et al.,* 1998), fruit ripening (Hinton DM *et al.,* 1980), seed germination (Vogeli-Lange R *et al.,* 1994) etc.

In higher plants, the pollen mother cells undergo meiosis to form tetrads of haploid microspores. Each individual microspore of the tetrad is surrounded by a thick callose wall composed of a β-1,3-glucan. At a critical stage of microspore development,

the β-glucanase activity increases resulting in the degradation of callose wall. The microspores are then released into the anther locule where they develop into mature pollen grains (Steiglitz, 1977). Alterations in the timing of β-glucanase expression, or failure to express β-glucanase, leads to abnormal dissolution of the tetrad callose wall, which has been shown to be a primary cause of male sterility (Izhar S *et al.,* 1971; Warmke H E *et al.,* 1972). The evidence for the role of β-glucanase in callose wall dissolution and microsporogenesis has come from transformation experiments. Tobacco plants that express the recombinant extracellular β-glucanase 1 exhibited premature degradation of callose in microspore cell walls, production of abnormal microspores, and partial to complete male sterility (Worall D *et al.,* 1992). The results suggest that formation of callose cell wall and its proper developmental degradation by endo-βglucanase are critical for microsporogenesis (Tsuchiya *et al.,* 1995).

The interactions between the transmitting tissue and the growing pollen tube are important for guiding pollen tube growth towards the ovules and successful fertilization. The class II β-glucanase Sp41 of tobacco is a "PR-like" protein (Lotan T *et al.,* 1980; Ori N *et al.,* 1990). The mature form of *Sp41* is a ca. 41kDa, acidic, glycoprotein, which is secreted into the extracellular matrix. 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.

Germination of seeds is a complex process. Under favorable conditions rapid growth of the embryo culminates in the rupture of the covering layers and emergence of the radicle. In many species, the enclosing tissue acts as a physical barrier which must be overcome to complete its germination (Black M, 1996; Bewley J D, 1997). Rupture of testa and endosperm are the main germination limiting factor in plants like tobacco, tomato and pepper (Leubner-Metzger *et al.,* 1995; Arcila J *et al.,* 1983). The first hint that β-glucanase play a role in tobacco seed germination was came from the observation that β-glucanase 1 is induced during germination (Vogeli *et al.,* 1994; Leubner *et al* 1995). In tobacco plants, β-glucanase 1 is induced after testa rupture in the micropylar region of the

endosperm where the radicle will penetrate, but prior to endosperm rupture. The β-glucanase 1 weakens the endosperm by digestion of cell-wall material and that this promotes radical protrusion to facilitate germination. This close relation between β-glucanase 1 induction and the onset of endosperm rupture under a variety of physiological conditions supports that β-glucanase play a major role in seed germination process. In 1971, Abeles *et al.,* suggested that the glucanohydrolases β-1,3-glucanases and chitinase might function as a defense against fungal pathogens (Abeles *et al.,* 1971). The β-glucanase and chitinase, acting alone and particularly in combination, can help defend plant against fungus infection. It has been proposed that these glucanohydrolases act in at least two different ways: directly, be 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 (Mein *et al.,* 1992; Boller, 1988).

***In vitro* antifungal activity**

β-glucanase and chitinase can hydrolyze β-1,3-glucans and chitin, respectively which are major components of the call walls of many pathogenic fungi (Wessels JGH *et al.,* 1981). In some cases, treatment with β-glucanase and chitinase can inhibit fungal growth *in vitro*, more often combinations of the two enzymes are required for antifungal activity (Boller T. 1988). Only class I vacuolar isoforms of tobacco β-glucanase I and chitinase are very effective in promoting the lysis of hyphal tips and inhibiting the growth of *Fusarium solani* (Sela-Buurlage *et al,* 1993). In contrast, the class II β-glucanase PR-2a, PR-2b, and PR-2c did not exhibit antifungal activity either alone or in any combination. Similar studies with tomato β-glucanase and chitinase have shown that the vacuolar class I isoforms, but not the secreted class II isoforms inhibit the growth of *Alternaria solani, Trichoderma viride* and *Phytophthora infestans* (Lawrence CB, 1996; Joosten MHAJ, 1995). Plant β-glucanase can release oligosaccharides from cell walls of the pathogens, which can then act as elicitors of defense reactions (Boller T *et al.,* 1993, 1995; Bowles JD, 1990).

Enhanced resistance to fungal pathogens resulting from transgene expression

There is strong evidence that the expression of β-glucanase transgenes alone or in combination with chitinase transgenes regulated by CaMV 35S RNA promoter can increase the tolerance of plants to infection by certain fungi. Transgenic tobacco plants expressing a soybean β-1,3-glucan-elicitors releasing β-glucanase or the tobacco class II β-glucanase PR-2b show reduced symptoms when it is infected with *Alternaria alternata* or the oomycetes *Phytophthora parasitica var. nicotianae* and *Peronospora tabacina* (Yoshikawa *et al.,* 1993; Lusso, 1996).

In many cases, an effective synergic action is obtained when β-glucanase and chitinase transgenes are expressed in combination. Tomato plants expressing tomato class I β-glucanase and chitinase transgenes show reduced susceptibility to infection by *Fusarium oxysporum* whereas expression of either gene alone is not effective (Jongedijk *et al.,* 1995; Sela-Buulage *et al.,* 1993). They found that the extracellular wash fluid from the leaves of plants expressing both β-glucanase and chitinase and that have strong antifungal activity against *Fusarium solani,* whereas this effect was less for plants expressing either transgene alone. Tobacco plants expressing a bean class I chitinase gene show decreased susceptibility to the root pathogen *Rhizoctonia solani* (Broglie *et al.,* 1991). Expression of the rice Rch10 basic chitinase gene and alfalfa glucanse I transgenes also increased the protection of tobacco against the chitin-containing fungus *Cercospora nicotiana*, the casual agent of the frogeye disease, relative plants expressing either of the transgenes alone (Zhu *et al.,* 1994).

Regulation of β-1,3-glucanase expression

β-Glucanase show developmental regulation and regulation in response to treatment with hormones or infection with pathogens. In early studies, based on the measurements of enzyme activity (Moore *et al.,* 1972; Felix, 1987). More recently, specific glucanase have been measured immunologically and their mRNAs have been measured semi-quantitatively by RNA-blot hybridization. In certain cases, regulation of transcription has been studied using plants transformed with Gus reporter genes under the

control of promoter region of β-glucanase genes, namely:1) the tobacco class I *N. tomentosiformis* homeologue Glb (Vogeli *et al.*,1994) and the tobacco class I *N.sylvestris* homologues Gglb50 (Van de Rhee *et al.*, 1993), the less-related β-glucanase I gene GnI of *N. plumbaginifolia* (Alonso *et al.*, 1995) and the tobacco class II β-glucanase genes PR-2b and PR-2d (Henning *et al.*, 1993; Shah, 1996).

β-Glucanase accumulates at high concentrations in the roots and in lower leaves of mature, healthy tobacco plants (Shinshi, 1987; Neale *et al.*, 1990). The pattern of expression in leaves and roots of β-glucanase I protein and steady state mRNA is very similar and is correlated with promoter activity of the ca. 1.5 to 1.7 kb 5' flanking region (Shinshi *et al.*, 1987; Ohme, 1990). Therefore, regulation of β-glucanase I in these organs appears to be primarily at the level of transcription. Similar conclusions may be drawn for the less related β-glucanase I gene *Gn1* of *N. plumbaginifolia*, which shows low promoter activity in upper leaves and high promoter activity in lower leaves and roots (Castresana *et al.*, 1990). A rapid form of down regulation is detectable at the mRNA level one hour after suspension-cultured cells are treated with auxin and cytokinin (Sperisen, 1993). Down-regulation by auxin and cytokinin also appears to be at least in part transcriptional since the decrease in steady state RNA is correlated with decreased activity of the *Glb* promoter (Vogeli-Lange, 1993; Rezzonico, 1998).

Many plant species react to treatment with the stress hormone ethylene with induction of β-Glu I activity, protein, and mRNA in leaves (Memelink *et al.*, 1990; Hart CM *et al.*, 1993; Beffa *et al.*, 1995). Ethylene increases the β-glucanase I content of epidermal cells slightly; its inductive effect is highly on mesophyll cells of the tobacco leaf (Keefe *et al.*, 1990). Studies with inhibitors of ethylene production and ethylene action have shown that ethylene is required for the induction of β-glucanase I in cultured tobacco cells (Felix and Meins, 1987). Ethylene treatment also increases the promoter activity of the tobacco β-glucanase I gene *Glb* (Vogeli-Lange, 1994). Similarly, ethephon (2-chloroethylphosphonic acid), which releases ethylene, increases the promoter activity of the tobacco β-Glu I genes *Gglb50* (Van de Rhee, 1993).

Regulation of β-glucanase I and class I chitinase are often tightly coordinated (Meins *et al*, 1992; Brederode *et al*, 1991). During the germination of tobacco seeds, β-glucanase I, but not class I chitinase, are transcriptionally induced in the micropylar endosperm (Leubner-Metzger *et al,* 1995). ABA inhibits this seed specific induction (Leubner-Metzger *et al*, 1995) and also down-regulates β-glucanase I, but not class I chitinase at the transcriptional level in tobacco pith-cell suspensions and cultured leaf discs (Rezzonico *et al*, 1998). The class II PR-2a, PR-2b and PR-2c are present in sepals, but not in other floral organs (Henning *et al*, 1993; Cote *et al,* 1991). Reporter gene experiments showed that the 1.7 kb promoter of the tobacco class II beta-glucanase PR-2d gene is active in sepals, in the base of flowers, and in young seedling, but not in leaves, roots or stem of mature tobacco plants (Henning *et al*, 1993). In contrast, the 1.75 kb class II beta-glucanase PR-2b promoter is active in leaves, stem and root of mature tobacco plants, but at levels much lower than that of the tobacco beta-glucanase I Gglb 50 promoter (Van de Rhee *et al,* 1993).

Pathogenesis Related Regulation

In general, β-glucanase and chitinase are induced in plants infected with viral, bacterial, and fungal pathogens. Similarly, elicitors including fungal glucans (Kombrink *et al*, 1988), chitosan (Mauch *et al*, 1984), *N*-acetylchito oligosaccharides (Kaku *et al*, 1997), and glycoprotein can induce the accumulation of the two enzymes (Boller *et al*, 1993). Class I β-glucanase proteins and their mRNAs are induced in TMV-infected leaves of tobacco as part of the local lesion, HR response (Meins *et al,* 1992; Payne *et al,* 1990). *Gus* reporter-gene experiments with the tobacco β-glucanase *Gglb50* and *Glb* promoters have shown that β-glucanase I is transcriptionally induced by up to ca.10-fold in TMV infected leaves showing HR. (Vogeli-Lange, 1994; Van de Rhee *et al*, 1993). Histological studies indicate that the *Glb* promoter is active in the cells around necrotic lesions induced by TMV infection but not in cells adjacent to the lesions or in the lesions themselves Gus activity is also higher in areas with lesions compared to lesion-free areas (Vogeli-Lange *et al,* 1994).

Activity of the ethylene-inducible *Gn1* promoter of *N. plumbaginifolia* is strongly induced to ca. 21-fold, as a part of the HR of tobacco leaves infected with the incompatible bacterium *Pseudomonas syringae* pv *syringae* and is localized around the necrotic lesions Alonso *et al* , 1995). The PR-related class II and III beta-glucanase are induced both locally in TMV-infected leaves and systemically in non-infected leaves of the same plant (Ward *et al*, 1991; Bol *et al*, 1996; Lusso *et al*, 1995). *Gus* reporter gene studies have shown that the promoters of the tobacco class II *PR-2b* and *PR-2d* are induced both locally around necrotic lesions on TMV-infected tobacco and systemically in non-infected leaves (Van de Rhee, 1994; Alonso *et al*, 1995). The close correlation between systemic induction of class II and class III beta-glucanase has led to the use of these genes as markers for systemic acquired resistance (Delaney *et al*, 1997; Ryals *et al*, 1996).

Salicylic acid is also induces the accumulation of mRNAs of PR-related class II and III beta-glcanases and certain other PR proteins; (Ward *et al*, 1991; Niki *et al*, 1998) and, promoter activity of the class II *PR-2b* (Van de Rhee, 1994) and *PR-2d* (Van Loon LC, 1994). In contrast to the tobacco class I *Gglb50* and *Glb* promoters, the promoter of the less related *N. plumbaginifolia* class I *Gn1* promoter is strongly induced to ca. 14 fold in transgenic tobacco plants treated with salicylic acid (Castresana *et al,* 1990).

MATERIALS AND METHODS

Plant Material

Plant material used in this study was obtained from the germplasm collection nursery of Rubber Research Institute of India, Kottayam, Kerala. In the present study the *Hevea brasiliensis* clones RRIM 600 has been selected. Young uninfected leaves were identified and collected in clean polybags. Leaf samples were immediately brought to the laboratory for DNA isolation.

1. Genomic DNA Isolation

DNA Extraction buffer

2 % CTAB, 1.4 M NaCl , 20 mM EDTA, 0.1 M Tris-HCl (pH- 8.0), 1% Poly Vinyl Polypyrrolidone and 0.1% β - mercaptoethanol.

(β - mercaptoethanol was added freshly at the time of isolation).

All glass wares were soaked in detergent and washed in tap water, rinsed in distilled water, allowed to dry in hot air oven and autoclaved at a pressure of 16 lbs and 121°C for 20 minutes. The samples were washed thoroughly in tap water; adhering water particles were removed using blotting sheets. DNA extraction was done following a modified protocol of Doyle *et al.*, 1989.

i. Leaf sample weighing 1gm was frozen with liquid nitrogen and ground to a very fine powder using a mortar & pestle.
ii. 10 ml of 2X CTAB extraction buffer was added and transferred to a 50 ml centrifuge tube.
iii. The tube was incubated at 55 °C for 30 minutes with occasional swirling.
iv. The suspension was mixed gently and centrifuged at 10,000 rpm for 10 minutes to pellet the debris; the supernatant was transferred to a new tube.
v. Equal volume of Tris saturated phenol: chloroform: isoamylalcohol (25:24:1) was added and mixed by gentle inversion.

vi. The sample was spin at 10,000 rpm for 10 minutes & aqueous phase was carefully transferred to a new centrifuge tube.

vii. RNA present in the sample was eliminated by treatment with *DNase* free *RNase* and solution was incubated at 37° C for 1 hour.

viii. *Proteinase* K was added to inactivate the *RNase* and to remove other residual proteins. Incubation continued for another 1 hour.

ix. Equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently and centrifuged at 10,000rpm for 10 minutes.

x. Aqueous phase was carefully transferred to a new centrifuge tube.

xi. Equal volume of chloroform was added and mixed gently and centrifuged at 10,000rpm for 10 minutes.

xii. Aqueous phase was carefully transferred to a new centrifuge tube.

xiii. To the samples 0.6 volume of ice cold isopropyl alcohol was added to precipitate the DNA.

xiv. The tubes were kept in ice for 20 minutes and the DNA was pelleted by centrifuging at 8000 rpm for 10 minutes at 4° C.

xv. DNA was washed twice in 70% ethanol.

xvi. Suspended in sterile distilled water.

xvii. DNA was stored at -20 ° C.

2. Agarose Gel Electrophoresis

The quality of isolated genomic DNA sample was checked by agarose gel electrophoresis. Agarose gel was prepared by suspending dry agarose powder at a concentration of 0.8 % in 0.5 X TBE (pH 8) buffer in a conical flask and mixed by swirling, boiled in a microwave oven until all the agarose was melted to form a clear solution. The melted agarose was cooled to 50°C and ethidium bromide was added to give a final concentration of 5 µg/ml. This was poured into a suitable clean gel casting tray containing a comb of appropriate size to form wells, and allowed to cool to form a rigid gel. After the gel has set, the comb was carefully removed and placed the gel into an electrophoresis tank filled with running buffer. 2 microliter of loading buffer (0.25 %

bromophenol blue and 30% glycerol in TE buffer) was added to 2 µl of DNA. After thorough mixing the samples were loaded into the resultant wells. Gel was subjected to a constant electric field of 50 V and electrophoresis was done until the bromophenol blue dye front has migrated to the bottom of the gel. The molecular marker used was Lamda DNA double digested with EcoRI and HindIII restriction enzymes. The gel was visualized in a UV transilluminator. DNA bands showed up in the gel as fluorescent bands under UV light and photographed using Kodak EDAS 290 gel documentation system.

3. PCR Amplification of β-1, 3-glucanase gene isoform

PCR could rapidly amplify a specific region of a DNA molecule *in vitro* to yield sufficient quantities that can be cloned sequenced or analysed by restriction mapping. Based on the earlier reported sequence of β-1,3-glucanase (NCBI Acc. No: GU191335.1) two gene specific primers already designed in the laboratory has been used.

Forward primer: 5' GCA TTC TTT TAC GTA TTT A 3'
Reverse Primer: 5' CAC ACA TAT CAC TCT TAA GG 3'

PCR Amplification was performed with 20 ng of genomic DNA. PCR is being carried out in 20 µl reaction volume with the following constituents.

Table 1. PCR Reaction components

PCR Reagents	Quantity
Sterile water	11.84 µl
PCR buffer (10X)	2 µl
dNTPs mix (dATPs, dGTPs, dCTPs, dTTPs [100µM each])	2 µl
Taq polymerase (3 units/µl)	0.16 µl
Template DNA (10ng/ µl)	2 µl
Forward primer (250 nM)	1 µl
Reverse primer (250 nM)	1 µl
Total	**20 µl**

PCR mixture was overlaid with a drop of mineral oil and amplification was carried out in Thermal Cycler (M/S Eppendorf, India).

Table 2. The PCR profile

Step 1	Initial denaturation	92°C	3minutes
Step 2	Denaturation Annealing Extension	92°C 50°C 72°C	1minute 1minute 2minute
Step 3	Repeat the step 2	36 cycles	
Step 4	Final elongation	72°C	10 minutes

PCR products were analyzed by agarose gel electrophoresis. 1.2% agarose gel was prepared and PCR products were loaded into the respective wells. Gel was run at 50V until the bromophenol blue front has migrated to the bottom of the gel. The gel image was captured by Kodak EDAS 290 gel documentation system.

4. Purification of amplified bands from agarose gel

Agarose gel electrophoresis showed an amplification of two distinct bands of approximately 1.2 kb respectively. The amplified gene fragment band was excised with a clean scalpel blade from agarose gel containing the DNA band and transferred into a pre weighed 1.5 ml microcentrifuge tube. Weighed the tube containing the agarose gel slice and subtracted the weight of empty tube and determined the actual weight of the gel fragment. PCR product was eluted and purified from the gel using the Illustra GFX™ PCR DNA and Gel band kit from M/S GE Healthcare.

The purification was done according to the manufacturer's instructions, which are as follows:

i. Three volumes of binding buffer (Capture buffer Type 3) was added for one volume of sliced agarose gel in a tube.
ii. Closed the tube and incubated at 60°C for 15-30 minutes until the agarose is completely melted.

iii. Added 800 µl of sample to the spin column in the supplied collection tube.
iv. Centrifuged at 12,000g for 1 minute and the filtrate was discarded.
v. 500 µl of wash buffer was added to the spin column- collection tube assembly and spun at 8,000g for 1 minute.
vi. The filtrate was discarded and centrifugation was repeated at 12000g for 1 minute.
vii. Spin column was placed in a new 2ml collection tube, 40µl of elution buffer was added and centrifuged at 12,000g for 1 minute.
viii. Removed the spin column and eluted sample was stored at -20° C.

5. Cloning of the PCR product

Cloning of the purified PCR product was carried out in pGEM-T cloning kit, following the manufacturer's instructions.

a. Ligation

'pGEM-T Easy' vector system (M/S 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

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

The ligation mix was prepared as follows.

2X rapid ligation buffer (supplied by the manufacturer)	-	5.0 µl
pGEM-T Vector	-	1.0 µl (5ng)
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 for overnight.

b. Preparation of competent cells of *E.coli*

E.coli DH5α cells were streaked aseptically onto an LB Agar plate containing no antibiotics. The plate was then kept overnight in an incubator at 37°C. On the following day, a single colony from this plate was inoculated into 25 ml of autoclaved LB broth in a conical flask. Incubated at 37°C with overnight shaking (220 rpm). After incubation, 2 ml from this culture was inoculated into another conical flask containing autoclaved 50 ml LB broth, incubated at 37°C with shaking at 220 rpm for approximately 2 hours.

The culture after incubation was transferred to a fresh centrifuge tube and incubated in ice for 10 minutes. Then centrifuged at 2000 rpm for 3 minutes at 4°C. The supernatant was poured off carefully and the pellet was suspended in 10 ml ice-cold 0.1 M $CaCl_2$ solution. Again centrifuged in cold (4°C) at 2000 rpm for 3 minutes. Poured off the supernatant, the pellet was again suspended in 2 ml ice-cold 0.1 M $CaCl_2$ solution and incubated in ice for 30 minutes. 100µl each was then dispensed in to fresh 1.5 ml micro centrifuge tubes and kept at -80°C for further use.

c. Transformation protocols for *E. coli*

Materials:

1) IPTG stock solution (0.1M)

1.2 g IPTG
Add water to 50ml final volume.
Filter-sterilize and store at 4°C.

2) X-Gal (2ml)

100 mg 5-bromo-4-chloro-3-indolyl-b-D-galactoside
Dissolve in 2 ml N, N′-dimethyl-formamide.
Cover with aluminum foil and store at –20°C.

3) Preparation of LB Agar Medium

Luria- Bertani (LB) agar medium was prepared for spread plating the bacteria (Sambrook *et al.*, 1989). The composition of the media is 10 g bacto-tryptone, 5 g bacto-yeast extract and 10 g sodium chloride for one litre of media. For the present study, the ready made media powder supplied by M/S Hi-Media Laboratories Pvt. Ltd, Mumbai, India was used. Added 30 g of LB Agar powder to 950 ml of deionized water taken in a conical flask and dissolved. The volume was then made up to 1000 ml, plugged the flask with cotton and autoclaved for 15 minutes at 15 lbs. After this, the autoclaved medium was kept inside a laminar airflow chamber. At ear-bearable temperature, the antibiotic at the desired concentration was added into the medium and mixed slowly avoiding any air bubble formation. Approximately 25 ml medium was poured into petri dishes under sterile conditions.

4) Preparation of LB Broth

Added 20 g of LB powder (M/S Hi-Media Laboratories Pvt Ltd, Mumbai, India) into a conical flask containing 950 ml deionized water, dissolved and then made up to 1000 ml. Dispensed appropriate quantity of the medium into different tubes and conical flasks, plugged and autoclaved at 15 lbs for 15 minutes. Inside the laminar air flow chamber, appropriate quantity of the required antibiotic was added into each of the conical flasks and tubes containing LB Broth and mixed, at the time of use.

5) LB plates with ampicillin

Added 15 g agar to 1 liter of LB medium. Autoclaved and allowed the medium to cool to 50°C before adding ampicillin to a final concentration of 100 µg/ml. Poured 30ml of medium into 90 x 15 mm petri dishes and allowed to harden.

6) LB plates with IPTG/X-Gal

The above antibiotic LB plates were supplemented with 0.5 mM IPTG and 80 µg/ml X-Gal and pour the plates. (Alternatively, 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal may be spread over the surface of an LB-ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use).

7) SOC medium (100ml)

2.0 g Bacto-trypton
0.5 g Bacto-yeast extract
1 ml 1M NaCl
0.25 ml 1M KCl
1 ml 2 M Mg2+ stock, filter sterilized
1 ml 2 M glucose, filter sterilized

Added Bacto-tryptone, Bacto-yeast extract, NaCl and KCl to 97 ml distilled water. Stirred to dissolve. Autoclaved and cooled to room temperature. Added 2 M Mg^{2+} stock and 2 M glucose, each to a final concentration of 20 mM. Made up to 100ml with sterile, distilled water. Filtered the complete medium through a 0.2µm filter unit. The final pH was 7.0.

8) 2M Mg^{2+} stock

20.33 g $MgCl_2 \cdot 6H_2O$
24.65 g $MgSO_4 \cdot 7H_2O$

Added distilled water to 100 ml. Filter sterilized.

d) Transformation of *E.coli*

About 10 ng of vector DNA was added to DH5α competent cells and mixed uniformly. Incubated in ice for 15 minutes. Heat shocked the cells for 2 minutes at 42°C

in a water bath without shaking. Immediately transferred the tubes to ice and incubated for 10 minutes. Added 250 µl of fresh LB medium kept at room temperature. Then capped the tubes tightly and incubated at 37°C with shaking (220 rpm) for 1 hour. Spreaded 50µl from the transformed cells into LB agar plate (90 mm) containing the appropriate antibiotics for selection. Incubated at 37°C overnight for the proper growth of the colony. After the incubation, plates were observed for colonies.

6. Colony PCR

Colony PCR was carried out to identify positive colonies carrying the cloned genomic DNA (transformed colonies) with gene specific forward and reverse primers. PCR has been done in 20 µl reaction volume. The PCR profile is shown in Table: 4

Table: 4. Colony PCR profile

Step 1	Initial denaturation	96°C	10minutes
Step 2	Denaturation Annealing Extension	92°C 50°C 72°C	1minute 1minute 2minute
Step 3	Repeat the Step 2	36cycles	
Step 4	Final elongation	72°C	10minutes

Agarose gel electrophoresis of PCR reaction product has been done to identify positive amplification. AGE was performed using 1.2% agarose.

7. Isolation of Plasmid

Plasmid DNA extraction from recombinant bacteria was done using Illustra™ Plasmidprep MiniSpin kit of M/S GE Healthcare. Plasmid isolation was confirmed by agarose gel electrophoresis (1 %).

8. Sequencing

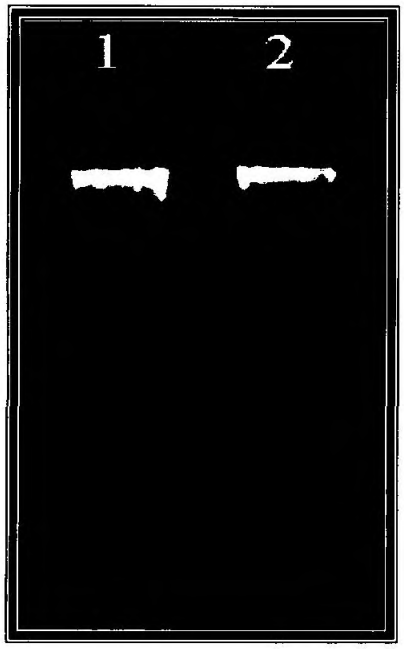
The sequencing of the insert in the isolated recombinant plasmids has been done at M/S Macrogen, South Korea with an automated sequencer. The sequencing was carried out with the M13 forward and reverse primers. The obtained sequence was analyzed with the help of Bioinformatic tools like, BLASTn, ClustalW, and PLACE prediction software.

RESULTS AND DISCUSSION

The *Hevea* clone RRII 105 is a moderately tolerant clone against abnormal leaf fall disease caused by *Phytophthora spp*, while RRIM 600 is a susceptible one. Role of β-1,3-glucanase against ALF was already reported in RRII 105 (Thanseem *et al*, 2005). It was also found that the gene is showing differential expression in the tolerant and susceptible clones upon fungal infection. In susceptible clones the gene expression was found to be inhibited after 48 hrs. What governs this process was not well understood. The expression of a gene is controlled by the promoter region of the gene. So it was assumed that there will be differences in the promoter cis- elements/ binding factors in the respective tolerant and the susceptible clones corresponding to the glucanase gene. Recent studies conducted in RRII 105 revealed that different isoforms of β-1,3- glucanase exist in a single clone of *Hevea* which are regulated by their individual promoters. (Supriya & Thulaseedharan, unpublished data). The present work was undertaken to characterize the promoter region of one of these isoforms of β-1,3-glucanase enzyme, (Gln5) from the *Hevea brasiliensis* clone RRIM 600 to check whether there is any difference in the nucleotide sequence and the promoter region of Gln 5 gene in the tolerant and the susceptible clone. Comparison in the sequence patterns of promoter region of this isoform in both clones could throw light on the anti-fungal functions. In the present study, PCR amplification, cloning and sequencing of the β-1,3-glucanase gene isoforms (Gln 5) along with its promoter from the *Hevea* clone RRIM 600 and comparison with the sequences from the clone RRII105 was carried out.

Genomic DNA isolation

The DNA isolated from young, healthy leaves of *Hevea* clone RRIM 600 by CTAB method was electrophoresed on 0.8% agarose gel. The DNA isolated was of good quality without shearing and RNA or protein contamination (fig 2). When DNA was isolated from 1g leaf sample and dissolved in 50µl sterile distilled water, the concentration was estimated to be about 1 µg/µl. Further the concentration was adjusted to 20 ng/µl with sterile distilled water and used as template in PCR.

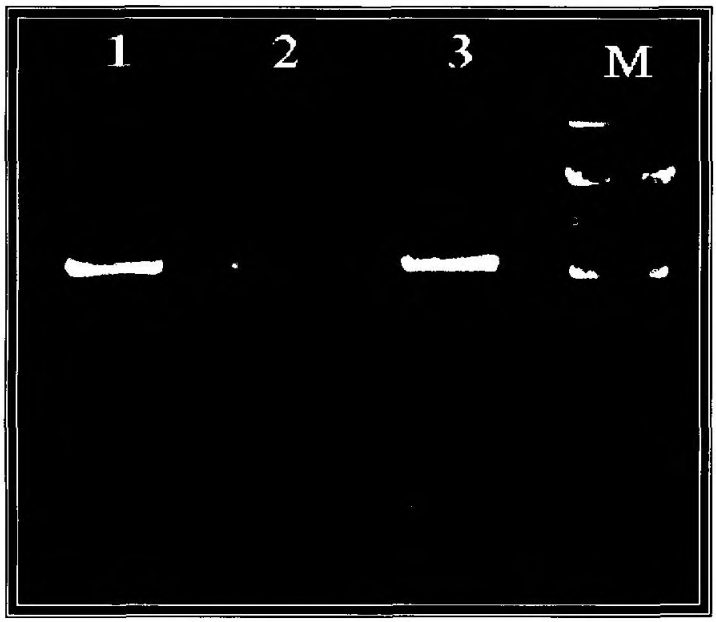


Lanes 1 and 2: DNA samples of clone RRIM 600

Fig 2: DNA isolated from *Hevea* clone RRIM 600

PCR amplification of β-1,3-glucanase gene promoter (Gln 5) and Coding DNA Sequence (CDS)

PCR amplification was done by using specific primers for the β-1,3-glucanase gene and partial promoter, a DNA amplicon of size ~1.4 kb was isolated through PCR. The products were distinct, single bands with no artifacts (fig 3). The above observations were made through agarose gel electrophoresis of the samples.



Lanes 1, 2& 3: Amplified 2 kb fragment

M : Molecular marker (*Eco*R1/*Hind* III lambda DNA double digest marker)

Fig.3: PCR amplification of Gln 5 isoform of β-1,3-glucanase gene

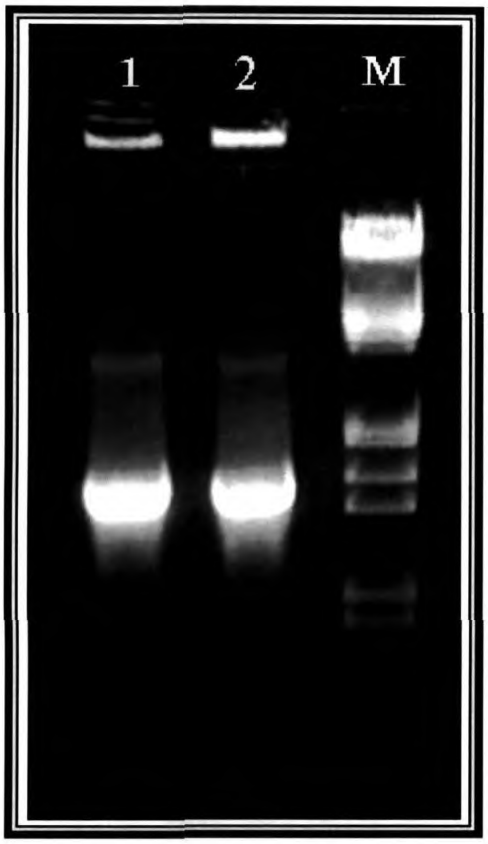
Cloning of β-1, 3-glucanase gene isoform 5 (Gln 5)

The specific DNA bands amplified through PCR was eluted from the gel were ligated in the pGEMT-T Easy vector as per the manufacturer's instructions. After ligation, the ligated mixture was used to transform DH5α competent cells. The transformed colonies were plated on LB agar mediun containing the antibiotic ampicillin. The plates were over layered with x-gal and IPTG for blue-white screening. On overnight incubation, many colonies were developed, most of them are white colonies. It shows that the transformation efficiency was good and it was suitable for plasmid isolation.

Colony PCR

To confirm the presence of insert in white colonies, a few of the colonies were directly used as template in PCR reactions using the insert specific primers. The products where electrophoresed on 1.2 % gel which showed single bands in the exact size as in the

initial PCR (fig 4). The result confirmed with the presence of the insert in the transformed colonies.



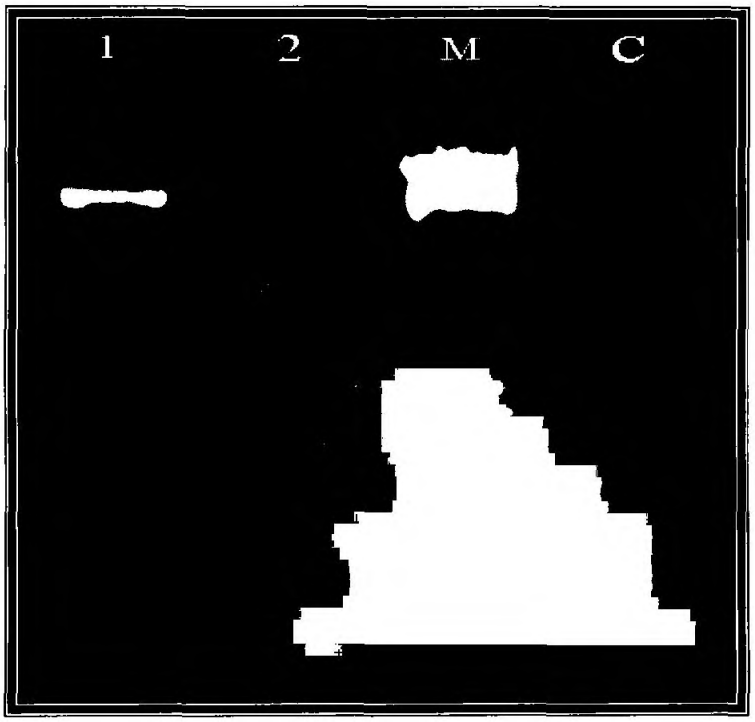
Lanes 1&2 : colony transformed with ligation mixture.

M : Molecular marker (*Eco*RI/*Hind* III lambda double digest marker)

Fig 4: Colony PCR of cells with transformed with cloned vector

Plasmid isolation

Recombinant plasmids isolated from transformed colonies were electrophoresed on 1% agarose gel. Three bands were observed in each lane showing the presence of different conformations of the plasmid (fig 6). The higher size of the recombinant plasmid compared to the insert-less plasmid also confirms the presence of the insert in the recombinant plasmid.



Lanes 1&2 : plasmid with insert

M : molecular marker (*Eco*RI/*Hind*III lambda double digest marker).

C : control without insert

Fig. 6. Recombinant plasmids isolated form the positive colonies

Nucleotide sequencing

The plasmids carrying amplified fragments were sequenced at M/S Macrogen Korea. The nucleotide sequence of Gln 5 isoform of β-1,3-glucanase were analyzed using the online bioinformatics tool- PLACE. The sequence obtained has both promoter and gene sequence with a total of 2057 bp within the given primer sequences (Fig 6).

Fig 6. Nucleotide sequence of Gln 5 isoform of β-1,3-glucanase from RRIM 600

The nucleotide sequence of Gln 5 isoform of β-1,3-glucanase gene and promoter from the *Hevea* clone RRIM 600 contained 2057 nucleotides in the PCR and amplified region. The promoter sequence comprised 847 nucleotides among this 764 to 800 is 5'UTR region. The deduced sequence shows 97% homology with the earlier reported Gln 5 isoform from the *Hevea* clone RRII 105 and more than 95% homology with other isoforms isolated from RRII 105. The sequence showed similarity except for a very few nucleotides. There is an intron sequence with GT-AG splicing junction at 904 to 1035

positions in coding gene portion. The various *cis* elements present in the promoter were analyzed using the online bioinformatics tool- PLACE.

Comparison of sequence with earlier reported Gln 5 sequence from RRII 105

Using ClustalW online software tool, the sequence obtained from the *Hevea* clone RRIM 600 was aligned with that of Gln 5 gene sequence from of RRII 105 (fig 7).

Fig 7. Comparison of nucleotide sequences between RRII 105 and RRIM 600

```
600         ----------------------------------------------------GCATTCTTTTA 11
105         GCTCCCTAGGCTATTATTAAATCATAATTTGTTGGATTAGATTTATATTGCATTCTTTTA 60
                                                               ***********

600         CATATTTATATATTTATATTTTGATTCGTATTTTAATTAGAAACTTTTATTTTAAATTTT 71
105         CATATTTATATATTTATATTTTGATTCGTATTTTAATTAGAAACTTTTATTTTAAATTTT 120
            ************************************************************

600         GTTATCTTATGATAAAATTTTAATAATTATATGATAGAGAATATACATTATCCACTGTAT 131
105         GTTATCTTATGATAAAATTTTAATAATTATATGATAGAGAATATACATTATCCACTGTAT 180
            ************************************************************

600         TAAATAACAATTTTTTTTTAATTTTGGGAAAATAAATCACTGGAAAATCTCAAATTTTTT 191
105         TAAATAACAATTTTTTTT-AATTTTGGGAAAATAAATCACTGGAAAATCTCAAATTTTTT 239
            ****************** ******************************************

600         TTGAAATATCCCTTAATTAATAAAATAACATCCATGTTTAATAGATCCTAAATTTGAATT 251
105         TTGAAATATCCCTTAATTAATAAAATAACATCCATGTTTAATAGATCCTAAATTTGAATT 299
            ************************************************************

600         TAAACGATAAAAATAATTATGTTATAAAGTGACAAATCTTATCCTTTTATCTTTTAAGGA 311
105         TAAACGATAAAAATAATTATGTTATAAAGTGACAAATCTTATCCTTTTATCTTTTAAGGA 359
            ************************************************************

600         GATATGGGGGGGCAGAAAAAAGACTTAAAAGTCTTCAACAGATAGAGACATTCATTTATC 371
105         GATATGGGGGGGCAGAAAAAAGACTTAAAAGTCTTCAACAGATAGAGACATTCATTTATC 419
            ************************************************************

600         CAACGAAAAATTGAAAGATGCCCAAGACATTTCAAACTAAATTAGCATTAATTATCAGGA 431
105         CAACGAAAAATTGAAAGATGCCCAAGACATTTCAAACTAAATTAGCATTAATTATCAGGA 479
            ************************************************************

600         TTATTAATTGGTAAAGAGTGCATTATTTTCAATAAAAGAAGTAAAATAATTTCACCGATT 491
105         TTATTAATTGGTAAAGAGTGCATTATTTTCAATAAAAGAAGTAAAATAATTTCACCGATT 539
            ************************************************************

600         AGTTTTGAGAGATTGTTGTGCATTTTCAAAGCACATGTTTTCTTCCTATCAGACATTGGA 551
105         AGTTTTGAGAGATTGTTGTGCATTTTCAAAGCACATGTTTTCTTCCCATCAGACATTGGA 599
            ********************************************** *************

600         TCCACTTTTTCTTAATTTAGAAAAATAACTATAACTATATATCTGTAAAGAGCACTATAT 611
105         TCCACTTTTTCTTAATTTAGAAAAATAACTATAACTATATATCTGTAAAGAGCACTATAT 659
            ************************************************************

600         TTTAGCGCTCTAAATGCATGGTTGAATTTTTCTTAATTTTAAACCTGATCAGTTGAAATT 671
105         TTTAGTGCTCTAAATGCATGGTTGAATTTTTCTTAATTTTAAACCTGATCAGTTGAAATT 719
            ***** ********************************************************
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600         AAACAAACAAATAAACTCCAGCAACAGTATTCTCAAGAGTCCGCCTATAAAGCTTTGACA 731
105         AAACAAACAAATAAACTCCAGCAACAGTATTCTCAAGAGTCCGCCTATAAAGCTTTGACA 779
            ************************************************************

600         TGGTAGTAACCTAACACATATGCGTGGTCGAAATTATAAGCAACTTTCTTCTAATTTCCC 791
105         TGGTAGTAACCTAACACATATGCGTGGTCGAAATTATAAGCAACTTTCTTCTA-TTTCCC 838
            ********************************************************** ******

600         CCCTTCTTAATGGCTATCTCCTCTTCAACTTCAGGAACTAGTAGTTCCCTGCCCTCAAGA 851
105         CCCTTCTTAATGGCTATCTCCTCTTCAACTTCAGGAACTAGTAGTTCCCTGCCCTCAAGA 898
            ************************************************************

600         ACTACTGTCATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGGTCTCTCT 911
105         ACTACTGTCATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGGTCTCTCT 958
            ************************************************************

600         CTCTCTCTCTCTCTCTCTCTCTCTCTCTCCATCTATCTACTCTCATGTTAAAGTTGAC 971
105         CTCTCTCTCTCTCTCTCTCTCTCTCTCCATCTATCTACTCTCATGTTAAAGTTGAC 1018
            ************************************************************

600         GATGCTTTTTTTTTCTCTCCTTGTTCTAAAAGGTTTCAACTAATACCCCGTATTTGGAAT 1031
105         GATGCTCTTTTTTTCTCTCCTTGTTCTAAAAGGTTTCAACTAATACCT-GTATTTGGAAT 1077
            ****** ****************************************  ***********

600         TTAGATGCCCAGGTAGGCGTTTGCTATGGAATGCAAGGCAACAACCTTTCAATCTGTTTC 1091
105         TTAGATGCCCAGGTAGGCGTTTGCTATGGAATGCAAGGCAACAACCTTCCA-TCTGTTTC 1136
            ********************************************** ** ********

600         AGAGGTCATAGCTCTCTATAAACAATCTAACATCAAGAGAATGAGAATTTATGATCCAAA 1151
105         AGAGGTCATAGCTCTCTATAAACAATCTAACATCAAGAGAATGAGAATTTATGATCCAAA 1196
            ************************************************************

600         TCGAGCAGTATTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAA 1211
105         TCGAGCAGTATTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAA 1256
            ************************************************************

600         CTCAGATCTCCAAAGCCTTACCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGT 1271
105         CTCAGATCTCCAAAGCCTTACCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGT 1316
            ************************************************************

600         TCGTGGCTTCTGGTCAAGTGTCAGGTTCAGATATATAGCAGTTGGCAACGAAATTAGTCC 1331
105         TCGTGGCTTCTGGTCAAGTGTCAGGTTCAGATATATAGCAGTTGGCAACGAAATTAGTCC 1376
            ************************************************************

600         TGTAAATGGAGGCACAGCTTGGTTGGCCCAATTTGTTTTGCCTGCCATGAGAAATATACA 1391
105         TGTAAATGGAGGCACAGCTTGGTTGGCCCAATTTGTTTTGCCTGCCATGAGAAATATACA 1436
            ************************************************************

600         TGATGCTATAAGATCAGCTGGTCTTCAAGATAAAATCAAGGTCTCCACTGCAATTGACTT 1451
105         TGATGCTATAAGATCAGCTGGTCTTCAAGATAAAATCAAGGTCTCCACTGCAATTGACTT 1496
            ************************************************************

600         GACCCTGGTAGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATC 1511
105         GACCCTGGTAGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATC 1556
            ************************************************************

600         ATACTTGGACCCAATTATTGGATTTCTATCCTCTAGCAGGTCACCTTTACTTGCCAATAT 1571
105         ATACTTGGACCCAATTATTGGATTTCTATCCTCTAGCAGGTCACCTTTACTTGCCAATAT 1616
            ************************************************************

600         TTATCTTTACTTTACTTATGCTTATAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTT 1631
105         TTATCCTTACTTTACTTATGCTTATAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTT 1676
            ***** ****************************************************
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600         CACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGC 1691
105         CACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGC 1736
            ************************************************************

600         AACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGT 1751
105         AACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGT 1796
            ************************************************************

600         TGTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCG 1811
105         TGTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCG 1856
            ************************************************************

600         TACTTATCTCTCAAATTTGATCCAACATGTTAAAGGAGGTACTCCTAAGAGGCCTAACAG 1871
105         TACTTATCTCTCAAATTTGATCCAACATGTTAAAGGAGGTACTCCTAAGAGGCCTAACAG 1916
            ************************************************************

600         AGCTATAGAGACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGA 1931
105         AGCTATAGAGACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGA 1976
            ************************************************************

600         GAAACACTTTGGACTTTTCTTTCCTGATAAACGGCCAAAATATAATCTCAATTTTGGTGC 1991
105         GAAACACTTTGGACTTTTCTTTCCTGATAAACGGCCAAAATATAATCTCAATTTTGGTGC 2036
            ************************************************************

600         AGAAAAGAACTGGGATATTTCTACTGAACACAATGCAACAATACTTTTCCTTAAGAGTGA 2051
105         AGAAAAGAACTGGGATATTTCTACTGAACACAATGCAACAATACTTTTCCTTAAGAGTGA 2096
            ************************************************************

600         TATGTG- 2057
105         TATGTGA 2103
            ******
```

From the sequence alignment, sequences show variations in the promoter and gene region for a few nucleotides. The sequence showed nucleotide variations at 150, 538, 617, 785th bases in the promoter region. The other features of the sequences is given in table.4.

Table 4. Features of DNA Gln3 isoform from RRIM 600

Clone	*Characteristics*
RRIM 600	*DNA*
	Total number of bases – 2057 bp *%A = 30.63 [630]* *%T = 34.22 [704]* *%G = 16.53 [340]* *%C = 18.62 [383]* *Base count 630a, 704t, 340g, 383c*

Significance of *cis*-elements present in the promoter region

The *cis*-elements present in the promoter region of the sequence were predicted using the PLACE software (Plant *Cis*-Acting regulatory DNA Elements). The promoter region contained necessary elements usually present in a stress regulated promoter. The result reveals that there is no difference in the presence and number of the *cis*-elements in the promoter region of Gln 5 gene from the *Hevea* clone RRIM 600 and RRII 105. The *cis* elements, their respective positions in the sequence and the sequence of the particular elements are given in table 5.

Table 5. *cis*- elements present in the promoter region of deduced nucleotide sequence

Cis-Element	Site	Sequence
CAATBOX1	381 (+)	CAAT
DOFCOREZM	277,330 (+)	AAAG
MYBCORE	661 (+), 347(-)	CANNTG
MYCCONSAT	523 (-)	CANNTG
EBOX	523, 661(+)	CANNTG
TATABOX	15 (-)	TATATAA
WRKY	281, 727 (+)	TGAC
WBOX	726 (+)	TTGAC

As shown in the table 5, in the present study, the important *cis*-elements present in the Gln 5 gene promoter include Dof CORE, Myc box, E box, W box and WRKY box in addition to the TATA box and CAAT box. TATA box and CAAT box are mandatory elements in eukaryotic promoters that are present in the above sequence. The E- box and Dof CORE elements are related to enhancement of transcription of the downstream region of the gene. They are specifically regions for DNA Binding proteins that help in the initiation and continuation of transcription (Seitz *et al.*, 2010).

Apart from these, there are function-related elements like MYCCONSENSUS, WRKY and W Boxes. *cis* - acting elements like the W boxes (Rushton *et al.*, 1996; Eulgem *et al.*, 2000) have been well studied. The W box [(T) TGAC(C/T)] is the binding site for members of the WRKY family of transcription factors (Rushton *et al.*, 1996). There is increasing evidence that W boxes are a major class of cis- acting elements responsible for pathogen. The importance of W boxes was illustrated recently by studies of the *Arabidopsis* transcriptome during systemic acquired resistance (Maleck *et al.*, 2000; Petersen *et al.*, 2000). In some cases, clustering of W boxes may be associated with inducibility by pathogens. It was shown that the W box–containing promoter element E17 mediates gene expression at pathogen infection sites in transgenic *Arabidopsis* plants. WRKY transcription factors (Hara *et al.*, 2000) have been implicated in gene expression in response to wounding. It was shown recently that wound- and pathogen-induced signaling consists of networks with some shared components (Romeis *et al.*, 1999). It remains an open question, however, whether specific *cis*-acting elements can direct both pathogen- and wound- induced expression in planta or whether these two activities are characteristics of separate elements. Study of pathogen inducible synthetic plant promoters constructed from a range of both well-studied and novel *cis*-acting elements suggests that major differences are seen between many of the elements with regard to their background expression, their induction by different pathogens, and their speed of induction. Additionally, it demonstrates that several pathogen-inducible elements also direct local wound-inducible expression and therefore that components of pathogen and wound-induced signaling are shared.

Abel *et al.*, 2003 reported that Myc box are induced during abscisic acid signaling. There are sufficient evidences supporting this fact. Hence, the MYC CONSENSUS element can be related to stress-related functions as abscisic acid is a stress related hormone. Therefore this promoter may be induced during abiotic stress also. There can be interactions between signaling pathways of biotic and abiotic stress.

Pathogen-inducible plant promoters contain multiple *cis*-acting elements, only some of which may contribute to pathogen inducibility. Recent studies report that defense signaling is largely conserved across species boundaries at the *cis* -acting element level.

Many of these promoters also direct local wound induced expression, and this provides evidence for the convergence of resistance gene, non host, and wound responses at the level of the promoter elements. Synthetic promoters are constructed using these cis-acting elements and it show the effects of varying the number, order, and spacing of such elements. These promoters are valuable additions to the study of signaling and transcriptional activation during plant–pathogen interactions (Rushton *et al.,* 2002)

It has been earlier speculated that the promoter region of β-1,3-glucanase gene form RRII 105 is functionally efficient in attacking the *Phytophthora* pathogen and thus being tolerant to abnormal leaf fall disease. In order to confirm this, a comparative study of the promoter elements in the susceptible clone RRIM 600 is attempted here to find if any variations in the promoter region with respect to the tolerant clone may contribute to its functional variance. The results showed that there are no variations among the nucleotide regions of Gln 5 glucanase gene and promoter between the tolerant clone RRII 105 and the susceptible clone RRIM 600. Therefore, there are no changes in the presence and number of *cis*-elements also in the promoter region of Gln 5 compared with the reported sequence from RRII 105 clone. Though further functional validation is required, presently it can be concluded that the presence of pathogen-inducible elements probably present further upstream to the isolated region may play some role in ALF. There can be other reasons for its susceptibility also.

SUMMARY AND CONCLUSION

Hevea brasiliensis is an economically important crop. It accounts for more than 99% of the world's natural rubber production. The world consumption of rubber is increased day by day. So it is necessary to increase the yield and protect the tree from various diseases. Many of the high yielding clones of *Hevea brasiliensis* are susceptible to abnormal leaf fall disease which is caused by various *Phytophthora* species and results in a yield loss of up to 40% in highly susceptible clones.

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. 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 hydrolyzing β-1,3- glucans contributing to pathogen death. They can also release fungal cell wall fragments that in turn can act as elicitors of active host defense response. But in the case of *Hevea*, some clones are susceptible and some other clones are tolerant to abnormal leaf fall disease. Earlier, it was found that after infection with *Phytophthora meadii* in tolerant clones, the gene expresses continuously for four days but in susceptible clones, the expression of gene stopped after 48 hours (Thanseem *et al.*, 2005). Later different genomic forms of β-1,3-glucanases have been isolated form a single *Hevea* clone RRII 105 (Supriya & Thulaseedharan, unpublished data). These different forms were found to be controlled by different promoters also. Since the gene expression is regulated by their respective promoters, here a study was done to find whether any sequence variations exist between the promoter region of this gene in tolerant and susceptible clones and whether these sequence variations contributes to the property of tolerance or susceptibility to abnormal leaf fall disease of *Hevea.* For that Gln 5- glucanse gene form for which 847 bp promoter region has been isolated (Supriya *et al.*, 2012 (NCBI Acc. No. JQ650524) was chosen.

Present study was done by selecting two clones of *Hevea*, RRII 105, a moderately tolerant clone and a suceptible clone, RRIM 600 towards the abnormal leaf fall disease. DNA was isolated from these clones and a 2 kb glucanase gene was PCR amplified using

Gln 5 gene specific primers. The forward primer was chosen from the promoter region and reverse primer was chosen from the 3' end of the gene containing the stop codon. The amplified product was then purified and cloned in pGEM®T easy vector. After this chemically competent DH5∞ cells were transformed with the cloned vector and plated in antibiotic selective LB medium with IPTG and X-Gal. The developed colonies were selected through blue white screening. White transformed colonies containing the inserts were observed. Colony PCR was carried out to identify positive colonies carrying the cloned genomic DNA with gene specific forward and reverse primers. Then plasmids were isolated from the recombinant bacteria and good quality plasmids were selected for sequencing. The sequencing was carried out with the M13 forward and reverse primers. The obtained sequence was analyzed with the help of Bioinformatics tools like, BLASTn, ClustalW, and PLACE prediction software. The sequence showed only minor differences with the earlier reported sequence. On comparison with the reported sequence from RRII 105, the promoter region showed variations in four nucleotides only. The *cis*-elements present in the promoter region of the deduced sequence were predicted using the PLACE software. Promoter sequence show the presence of *cis* acting element WRKY at position 281 & 727 (+), WBOX at 726 (+). The presence and the number of *cis*-elements are exactly similar with that of the report. No new *cis*- elements or absence of *cis*- elements were found in the Gln 5 glucanase gene form isolated from RRIM 600.

Promoter region of β-1,3-glucanane gene can also contribute to the tolerance or susceptibility to abnormal leaf fall disease. Variations in the promoter regions can regulate the transcription factors binding to promoter and thereby preventing the binding of RNA polymerase. The present work is a comparative analysis of a single isoform of β-1,3- glucanase gene with promoter sequence of 847 bp from tolerant clone RRII 105 and susceptible clone RRIM 600. The result showed that there are only minor differences between the two clones. Therefore, further studies are required to prove the reason which acts as the main factor contributing to tolerance or susceptibility.

Plant promoter element studies have been conducted world wide. It is still an area of novelty and active research. In order to study the role and efficiency of a particular protein it is necessary to understand the initial factors of transcription. An outlook on the

type of proteins that bind onto *cis*-elements in the promoter region gives an idea about the type of protein produced. Pathogen - inducible plant promoters contain multiple *cis*-acting elements, only some of which may contribute to pathogen inducibility. Recent studies reported that defence signaling is highly conserved at *cis*-acting element, thus promoters are valuable tools for the study of signaling and transcriptional activation during plant- pathogen interaction.

Since the result revealed that there is no difference in the presence and number of the *cis* – elements in the promoter region of Gln 5 gene in the *Hevea* clone RRIM 600 and the sequence of RRII 105, it could be concluded that though cis- elements play some role in the defence against ALF, there can be other reasons for the susceptibility of RRIM 600. Preliminary studies like this are needed to find out the mechanism behind the differential expression of the glucanase genes in the tolerant and the susceptible clones of *Hevea*. It can be concluded that, though cis-elements are present in the gene, studies should be extended to find out the presence or absence of the suitable transcription factors in the tolerant and the susceptible *Hevea* clones to bind to these cis-elements inorder to regulate the gene expression. This in turn could provide new insights into the cause behind the differential glucanase gene expression in these *Hevea* clones with varying degrees of tolerance and susceptibility.

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