Molecular characterization of β-1,3-glucanase gene isoform-3 (Gln3) from *Hevea brasiliensis,* clone RRIM-600

Dissertation submitted to
Mahatma Gandhi University
in partial fulfillment for the award of the degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

by

RABISHA V. P.
(Reg. No: 031027)

under the guidance of

Dr. A. THULASEEDHARAN
Deputy Director (Biotechnology)
Rubber Research Institute of India,
Kottayam- 686009, Kerala

SCHOOL OF BIOSCIENCES
MAHATMA GANDHI UNIVERSITY
KOTTAYAM-686560
KERALA

SEPTEMBER 2012

The Rubber Research Institute of India
Rubber Board, Ministry of Commerce and Industry, Govt. of India
Kottayam – 686 009, Kerala, INDIA

Dr. A. Thulaseedharan
Deputy Director (Biotechnology)

DD/ Biotech/2012 **September 3, 2012**

CERTIFICATE

This is to certify that the dissertation entitled "Molecular characterization of β-1, 3-glucanase gene isoform-3 (Gln3) from *Hevea brasiliensis*, clone RRIM-600" submitted by Ms. Rabisha VP, 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.

(A. Thulaseedharan)

Tele Phone: 91- 481- 2353311 to 2353320 Fax: 91- 481- 2353327
E-mail thulaseedharan@rubberboard.org.in

MAHATMA GANDHI UNIVERSITY

SCHOOL OF BIOSCIENCES

Prof. (Dr.) J.G.Ray
Director

Priyadarshini Hills P. O.
KOTTAYAM – 686 560
Kerala, South India
Phone: (0481) 2731035

CERTIFICATE

This is to certify that the dissertation entitled "**Molecular characterization of β-1,3-glucanase gene isoform-3 (Gln3) from *Hevea brasiliensis*, clone RRIM-600**" is an authentic record of the project work done by **Ms. RABISHA V P**, Mahatma Gandhi University, Kottayam at Rubber research Institute of India, Rubber board, Kottayam, kerala, under the guidance of **Dr. A. Thulaseedharan**, Deputy Director, Biotechnology Division, RRII, Kottayam, in Partial fulfillment 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.

6/9/12

Prof. (Dr.) J.G.Ray
Director

DECLARATION

I do hereby declare that the dissertation entitled **Molecular characterization of β-1,3-glucanase gene isoform-3 (Gln3) 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 Division, 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.

P.D. HILLS

SEPTEMBER 2012

RABISHA V. P.

ACKNOWLEDGEMENT

A fervent prayer and gratitude to The God Almighty for guiding me through every walks of my life and for His blessings showered upon me.

At the very outset, I can hardly find appropriate words to express my gratitude and obligation to my guide, Dr. A. Thulaseedharan, Deputy Director (Biotechnology), Rubber Research Institute of India, Kottayam for his excellent guidance, caring and patience which all providing me an excellent atmosphere for doing my project work at RRII. Above all I have benefited vastly from my association with him.

I acknowledge my sincere and heartfelt thanks to Prof. (Dr.) Jyothis Mathew, Former Director, School Of Bioscience, Mahatma Gandhi University, Kottayam and Dr. James Jacob, Director, Rubber Research Institute of India, Kottayam for giving me the permission to do the dissertation work at RRII, Kottayam.

I am grateful to Prof. (Dr.) J.G.Ray, Director, School of Biosciences, M.G University, Kottayam, for providing all the facilities for completing my project work.

I thank all the teachers who thought me right from kinder garden to Post Graduation for imparting their knowledge & sharing with me their valuable experiences. Especially I express my heartfelt gratitude to Dr. Jayachandran. K, Associate professor, School Of Biosciences, M.G.University. He has been a great source of inspiration to me.

Deepest gratitude to Dr. Keerthi T R, Dr. Linu Mathew and all other faculty members and non-teaching staffs of Biosciences department for their constant support and guidance during my Post Graduation.

I place in record my sincere thanks to, Ms. Suni Anie Mathew, Research Scholar, Biotechnology Division, RRII for her kind hearted support and encouragement throughout my work.

I extent my heartfelt regards to Ms. Vineetha Mohan & Ms. Supriya Radhakrishnan ,Research scholars, Biotechnology Division, RRII for their constant support and warm companionship extended to me in lab. They have stood by me through thick & thin giving me the confidence that I need.

With great respect, record my gratitude to Dr. R.G. Kala, K. Rekha, Mrs. P. Kumari Jayasree, Dr. S. Sobha, Mrs. R. Jayashree,(Scientists), Dr. S. Sushammakumari (Senior scientist) & Mrs. Lida Pavithran (Scientific assistant)., Biotechnology Division, RRII for their encouragement, timely help & advises rendered to me. I also thank all the staff, Research scholars & trainees at RRII for constant support & help.

I am grateful to library staffs of Central Library and Department library of M.G University and RRII for their valuable co-operation in providing reference materials and internet facility.

A special thanks to all my friends especially to Jyothi Lakshmi for the love & tremendous support during my project work. A word of thanks to all my classmates for their help & encouragement during my project work.

I bow my head before my family especially to Unni, for their love , support and encouragement with out which I could not have been able to purse my course of study.

Last but not least I express my sincere thanks to all who helped me for the successful completion of my work.

Rabisha V. P.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ALF	:	Abnormal leaf fall disease
BLAST	:	Basic Local Alignment Search Tool
CaMV	:	Cauliflower mosaic virus
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
LB	:	Luria-Bertani
NCBI	:	National Center for Biotechnological Information
PCR	:	Polymerase Chain Reaction
PR proteins	:	Pathogenesis Related proteins
RNA	:	Ribonucleic acid
RRII	:	Rubber Research Institute of India
RRIM	:	Rubber Reaerch Institute of Malasia
SignalP	:	Signal Peptide
T-DNA	:	Transfer DNA
TMV	:	Tobacco Mosaic Virus
UV	:	Ultra violet
UTR	:	Untranslated region
β- glu	:	β-1,3-glucanase

INTRODUCTION

Nature has not provided any other industrial raw material of plant origin as flexible as natural rubber. It is an elastomer derived from latex, a milky colloidal substance (Kush *et al.,* 1990) produced by plants like Para rubber tree (*Hevea brasiliensis*), gutta-percha (*Palaquium gutta*), rubber fig (*Ficus elastica*), Panama rubber tree (*Castilla elastica*), spurges (*Euphorbia* spp.), lettuce, common dandelion (*Taraxacum officinale*), Russian dandelion (*Taraxacum koksaghyz*), S*corzonera* and guayule (*Parthenium argentatum*). Among them, the commercial source is *Hevea brasiliensis,* due to the high yield and quality of rubber produced; accounting for more than 99% of the world's natural rubber production (Clement-Demange *et al.,* 1997). At present, more than 9.5 million hectares in about 40 countries are devoted to rubber tree cultivation with a production of about 6.5 million tons of dry rubber each year.

The native place of rubber plantation is in South America, discovered in 1500's. *Hevea brasiliensis* (Para rubber tree) belongs to the Euphorbiaceae family. The generic name is derived from a local word in the Amazon, 'heve' meaning rubber. The geographical distribution of *Hevea* include native locations like Bolivia, Brazil, Colombia, Peru, Venezuela and exotic locations like Cambodia, China, Ethiopia, India, Indonesia, Laos, Liberia, Malaysia, Myanmar, Philippines, Sri Lanka, Thailand, Uganda, Vietnam etc. The top three producers of rubber in the world are Thailand, Indonesia and Malaysia. In 2009 -'10, the production of natural rubber in India was 8.3 lakh tones making it fourth in ranking. More than 98% of rubber production is from southern part of India; especially in the valley of Western Ghats include Kerala, Tamil Nadu and Karnataka (Indian Rubber Statistics, 2010). This is because rubber is successfully cultivated in humid lowland tropical conditions, roughly between 15^0 N and 10^0 S, with comparatively little variation in temperature and high-rainfall with good internal drainage of the soil.

Hevea brasiliensis is a quick-growing tree, rarely exceeding 25 m in height in plantations, but wild trees of over 40 m have been recorded. Tree trunk is usually straight or tapered, branchless for 10 m or more, up to at least 50 cm in diameter (Fig.1.). Bark surface is smooth, grey to pale brown in colour with

abundant white latex; crown is conical and branches are slender. It has a well-developed taproot system with far-spreading laterals. Leaves are alternate, palmate and each leaf has 3 leaflets. Leaflets are elliptic petiolated with a basal gland pointed at the tip with lengths varying up to 45 cm; glabrous, with entire margin and pinnate venation. Inflorescence is in the form of pyramidal-shaped axillary panicles produced simultaneously with new leaves and arranged in cymose form. Flowers are small, greenish-white to yellow in colour, dioecious, female flowers usually larger than the male ones. In the female flower, gynoecium composed of 3 united carpels forming a 3-lobed, 3-celled ovary with a single ovule in each cell. Seeds are large, ovoid, slightly compressed, shiny, 2-3.5 x 1.5-3 cm, testa grey or pale brown with irregular dark brown dots, lines and blotches. Endosperm is white in viable seeds, turning yellow in older seeds. Seeds weigh about 2-4 g (Vinod *et al.*,2000).

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

The milky latex of *Hevea brasiliensis*, produced by laticiferous cells (specialized secretory system) in the phloem, is the raw material for natural rubber. It is a polymer of *cis*-isoprene (2-methyl-1,3-butadiene). The latex is a renewable resource that can be tapped. 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.

Rubber plantations supply raw materials for the production of many industrial goods required for automobiles, aircrafts, railways, textile industries, sports goods, engineering goods and even for building roads. 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 also of great economic importance. Seeds are source of rubber seed oil, recommended for manufacture of soap, paints, varnishes and is also 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 colour 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 (Wycherly., 1992).

Natural rubber cannot be totally replaced by synthetic rubber which is derived from petroleum and this synthetic rubber has certain limitations. It is not as elastic as natural rubber, does not have heat transfer properties and is obtained from a non-renewable source. Although synthetic rubber is often blended with natural rubber, various products, such as aero plane tires require 100% natural rubber. Hence, natural rubber is in high demand.

The manifold application of natural rubber earns it high economic value. But a remarkable imbalance between supply and demand calls for increasing productivity and area under rubber plantations. Traditional rubber growing belts in India are already saturated. Extending rubber plantations to non-traditional areas are challenging since they do not provide with favorable climatic conditions. Therefore, it is necessary to improve the quality of *Hevea* clones so as to withstand climate extremities, biotic stress and render high latex yield. Genetic

transformation through conventional breeding is certainly a promising tool for incorporation of agronomically important genes leading to improvement of existing *Hevea* genotype (Venkatachalem *et al.,* 2006). The major conventional method used for breeding in *Hevea* is bud-grafting. Extensive research is being conducted and various clones with improved yield and improved tolerance against either biotic or abiotic stresses have been released. But some of the shortcomings of conventional breeding are limited genetic diversity, long breeding cycle, chances of transfer of non-desirable traits along with the gene of interest, low fruit set, heterozygous nature, and insufficient availability of land and the absence of fully reliable early selection parameters. Therefore, new attempts to broaden genetic diversity by applying biotechnology should be done in parallel with prevailing techniques. Biotechnological interventions such as genetic transformation followed by rapid clonal propagation methods (like somatic embryogenesis) is an alternative for conventional crop improvement and commercial propagation technique.

Various diseases of rubber trees are responsible for considerable loss in latex yield. The major leaf diseases include abnormal leaf fall (ALF), powdery mildew disease, *Corenyspora* leaf disease etc. These are fungal in origin and can cause extensive damage to the rubber plantations when they not treated properly. Abnormal leaf fall disease caused by *Phytophthora* is the most destructive disease of rubber in the traditional rubber growing tract of South India necessitating crop protection every year. The incidence of abnormal leaf fall was observed in varying intensities every year during June to October. As the leaves are the source photosynthates for growth, the low leaf retention leads to poor girth. The girth is an indication of growth of trees. Girth in turn affects the volume of wood. The control of ALF can be achieved by micron spraying of copper oxychloride. Enhanced expression of defense genes through genetic engineering is another method of choice for combating the susceptibility of *Hevea* towards the disease.

β-1,3-glucanases are enzymes which are included in Pathogenesis related proteins (PR proteins) and are abundantly distributed in many dicotyledonous trees. β-Glucanases were proposed to be involved in plant defense as early as 1971 (Abeles *et al.*, 1971) as antifungal agents. It has been proposed that they can act at

least in two different ways: directly by degrading the cell walls of the pathogen (fungus), and indirectly by promoting the release of cell wall derived materials that can act as elicitors of defense reaction (Boller., 1993). The first β-1,3-glucanase gene from woody plants was isolated from *Hevea brasiliensis,* and codes for a basic vacuolar protein that has a C-terminal extension. This β-1,3-glucanase is highly expressed in latex rather than in leaf tissues (Chye and Cheung., 1995). Isolation and molecular characterization of β-1,3-glucanase gene and its role in combating abnormal leaf fall disease in *Hevea brasiliensis* Muell. Arg. has already been reported (Thanseem *et al.,* 2005). Recently it was observed that the genomic sequences encoding for β-1,3-glucanase is highly expressed in tolerant plants during diseased condition and that different genomic forms of β-1,3-glucanase controlled by separate promoters are present in *Hevea brasiliensis* .This gene can be a promising candidate for genetically engineering disease resistance in *Hevea.* It was observed that different genomic forms of β-1,3-glucanase controlled by separate promoters are present in the *Phytophthora*-tolerant clone RRII 105 of *Hevea brasiliensis* (Supriya and Thulaseedharan., unpublished). The sequences are reported in the NCBI database (NCBI Acc. Nos. Gln2- GU123623.2, Gln3- GU191335.1, Gln4- JN251022.1, Gln5- JQ650524.1, Gln6- JQ650525.1).

An understanding of the structure of promoters, function of their multiple components and the factors associated with their performance open up the possibility for modulation of gene expression. A promoter is a regulatory region of DNA typically located near the genes, which facilitates the expression of a particular gene. It is the site where the RNA polymerase enzyme binds and initiates transcription of proteins. In regard to abnormal leaf fall disease, another *Hevea* clone RRIM 600 is found to be relatively susceptible to ALF disease in the traditional areas. Hence, in this study an attempt has been made to study the gene and its partial promoter region of one of the isoforms of β-1,3- glucanase gene in this *Hevea* clone. Sequence identification and analysis may reveal any differences in regulation pattern of this gene. Also, promoter isolation of plant origin is important in the present era for transgenic crops because of the public concern due to the presence of viral origin of the commonly used CaMV 35S promoter.This will aid in future crop improvement programme in *Hevea brasiliensis* through genetic engineering.

OBJECTIVES

- Isolation and characterization of Gln 3 isoform of β-1,3-glucanase gene and its partial promoter region from *Hevea brasiliensis* clone RRIM-600.
- Compare the obtained sequence with earlier reported gene sequence from *Hevea brasiliensis* clone RRII-105.

REVIEW OF LITERATURE

The immune system evolved to protect multicellular organisms from pathogens. The immune system generates an enormous variety of cells and molecules capable of specifically recognizing and eliminating foreign invaders, all of which act together in a dynamic network. Protection by the immune system can be divided in to two related activities- recognition and response. The immune system is able to recognize molecular patterns that characterize groups of common pathogens and deal with these in a rapid and decisive manner. It can even detect subtle chemical differences that distinguish one foreign pathogen from another. A wide range of microorganisms including fungi, bacteria and viruses are also exploiting higher organisms as a source of food and shelter. Some of these can act as pathogens, which cause a variety of diseases. The higher organisms inclusive of both plants and animals have known to possess difference in defence mechanism. These defense mechanisms are completely based on their cellular organization.

It is known that plants are 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 pathogens. The disease is expressed when a susceptible plant is infected with virulent pathogen (compatible reaction) and vice versa. It will show no symptom if it is infected with an avirulent pathogen (incompatible reaction). In general, incompatible reaction occurs in the resistant host and compatible reaction causes diseases in the susceptible host as well. Among plant diseases, fungal diseases are rated as the most prevalent biotic stress contributing to yield loss (Grover and Gauthaman., 2003). Recent studies reveal that contribution of fungal diseases towards total yield loss of important crops in India is 18 – 31% (Grover and Pental., 2003).

Plants have developed diversified defense strategies to keep away pathogens. The developed resistance can be either pre-existing or induced. Resistance involving factors which are present in plants prior to the contact of pathogen are regarded as preexisting. This includes surface features of plants such as thickness of the cuticle, configuration of cuticular wax, topography of

leaf surface, degree of stomatal opening etc. Waxes on leaf and fruit surfaces form a water repellent surface, thereby preventing the formation of a thin layer of water on which, pathogen spores might be deposited and germinate. Thick, tough outer walls of epidermis prevent direct attack of fungal pathogens. All these structural features cause delay in pathogen penetration, thereby contributing significantly to plant defense by allowing time for the induction of more sophisticated defense mechanisms (Vinod et al., 2000).

Structural defense mechanisms which prevent an infection are histological and through 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 tissue resulting in localization of non-viable infected tissue. Abscission region are formed by tissue disruption creating intracellular space surrounding infected area, therefore it prevents normal, non-infected tissue from further infection. Tyloses formation commonly develops during the invasion of pathogens in to the xylem making it obstructed. Tyloses can be enormously and rapidly formed in the resistant plant whereas it is formed more slowly in the susceptible plant usually after infection was spread. Furthermore, the accumulation of gum intra-or intracellularly surrounding the infected area is also helpful in this regard. The rate of gum accumulation differs between different kinds of plant.

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 (HR). The HR is astereotypic response, that is, the nature of the response is similar for viral, bacterial and fungal pathogens. Some components of the HR appear to serve a general defense function independent of the inciting pathogen. Rapid cell death results in necrotic lesions at the site of infection. 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; fungi. Plant genes induced during the HR are commonly assumed to be part of the host's defense mechanisms. It is reported that β-1,3-glucanases are induced as part of the local–lesion response to viral infection in resistant hosts.

Natural rubber (*Hevea brasiliensis* Muell. Arg) is an important plant not only for world economic strategies but also for the use of living of humankind. The more social development, the more requirements of products made of rubber for utilization is increasing every day. Natural latex is one of important raw material available for making various kinds of products in heavy industries such as motor and vehicle industry, kitchenware and house ware. In our daily life, we are always involving with products made of rubber. Various diseases of rubber trees are responsible for considerable loss in latex yield. This will create a great loss in the rubber market thus leading to decrease in the economic status of the country. Some of the leaf diseases include powdery mildew disease, *Corenyspora* leaf disease, abnormal leaf fall disease etc. Most of the diseases that strike through the rubber plantations are fungal in origin. Fungus can cause extensive damage to the renewing bark of the tapping panel and persist in the bark for a long time if not treated adequately. The infected pods do not produce viable seeds. Loss of vigor, increase in plugging index, dieback of leaf bearing twigs and more weed growth are also some of the damages caused by the disease.

The Abnormal Leaf Fall Disease

The most destructive disease of rubber is the abnormal leaf fall (ALF) caused by different species of *Phytophthora.* Four species viz. *P. palmivora*, *P. meadii*, *P. nicotianae* var. *parasitica* and *P. botryose* have been isolated from infected specimens. However, the species most common in India is *P. meadii* (Edathil *et al.,* 2000). The disease has been observed in other rubber growing countries like Sri Lanka, Myanmar, Cambodia, Vietnam, Nigeria, Cameroon, Brazil, Costa Rica and Venezuela. Outbreak of the disease was also noticed in major natural rubber producing countries like Malaysia and Thailand (Chee., 1969). This 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. The incidence of abnormal leaf fall was observed in varying intensities every year during June to October. The disease incidence is severe in the clone RRIM 600.

Phytophthora is a remarkable genus of plant pathogenic fungi. The name *Phytophthora* is derived from Greek and literally means plant (phyto) destroyer (phthora). It belongs to the Kingdom Chromista, Class Oomycete and Family Phytiaceae. Its unique morphological, genetic and physiologic features combined with the wide range of diseases caused in large number of plant species, make *Phytophthora* one of the most fascinating subjects for investigation. The complicated life cycle with very distinct and strikingly different spore forms ranging from motile zoospores to thick walled oospores, make management of diseases caused by *Phytophthora* difficult and time consuming. Although, they are commonly referred to as fungi, *Phytophthora* and other Oomycetes have number of biological characteristics that are relatively uncommon in other fungi (Griffith *et al.*,1992). The cell walls of *Phytophthora* are composed of cellulose and β-1,3 linked glucan and not chitin, which is the common cell wall component of fungi outside Oomycetes (Bartnicki-Garcia and Wang., 1983). The cell wall is made principally of β-1,3 linked glucose polymers, which comprise about 80 – 90% of the cell wall dry weight. The cytoplasm of *Phytophthora* spp. contains mycolaminarin, a β-1,3 linked glucan, as the characteristic storage polysaccharide instead of glycogen (Wang and Bartnicki-Garcia., 1974). The recapitulation of the asexual cycle of the pathogen results in the rapid amplification from an initial infection. All the Oomycetes microorganisms have a short generation time and great reproductive capacity (Dick., 1992). For this reason, *Phytophthora* caused diseases of fruit, foliage, crowns and roots of plants are generally considered to be multi-cyclic that results in severe epidemics. There

are 67 recognized species of *Phytophthora*, that cause disease in a wide range of agricultural, horticultural and ornamental plants as well as causing devastating diseases in native ecosystems which account collectively for multibillion dollar losses in world cash crops. The most prominent examples are *P.infestans* and *P.palmivora.* Historically, their importance predate the development of the germ theory of diseases and the science of plant pathology has its origin in the analysis of the major disease epidemic of the 19th century, the Irish potato famine (1845–46) caused by *P. infestans* (Erwin., 1996).

Pathogenesis-related proteins

Several classes of proteins, called pathogenesis-related (PR) proteins, are induced in response to the infection of plants with microbial pathogens. Such proteins were first identified in tobacco, reacting hypersensitively to tobacco mosaic virus (TMV) (Van Loon and Van Kammen, 1970). Later, PR proteins were shown to be induced not only by pathogens, but also by abiotic stresses like wounding, ethylene treatment, light, heavy metals, *etc.* The PRs is accumulated in the infected tissues during HR and are also induced systemically with the development of systemic acquired resistance (SAR) against further infection by the pathogen. Induction of PRs has been found in many plant species belonging to various families (Van Loon and Van Strien ., 1999).

There is a family of PR-2 proteins, known as ß-1,3-glucanases (glucan endo-1,3-ß-glucosidases) are able to catalyze endo-type hydrolytic cleavage of the 1,3-ß-D-glucosidic linkages in ß-1,3-glucans. The ß-1,3-glucanases are abundant, highly regulated enzymes widely distributed in seed-plant species. Although the major interest in ß-1,3-glucanases stems from their possible role in the response of plants to microbial pathogens, there is strong evidence that these enzymes are also implicated in diverse physiological and developmental processes in the uninfected plant 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, and responses to wounding, cold, ozone and UV radiation.

β- 1,3-glucanases

β-glucanases are regarded as pathogensis related protein. According to IUBMB nomenclature they are listed in the enzyme catalog number under EC 3.2.1.# (1992). The first digit places them in class 3 hydrolases enzyme.The second digit (2) indicates that they hydrolyse glycosyl compounds and the third digit (1) indicates that they hydrolyse 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 enzyme nomenclature of glycosidic hydrolases is based on their substrate specificity and occasionally on their molecular mechanism, and such a classification does not reflect the structural features of these enzymes. Glycosyl hydrolases also classified based on amino acid sequence similarities (Henrissat., 1991). In this classification, β-1,3-glucanases constitute family 17 of glycosidic hydrolases, which comes under CAZy (Carbohydrate Active enzymes). The CAZy database describes the families of structurally related catalytic and carbohydrate binding modules of enzymes that degrade, modify or create glycosidic bonds. β-1,3-Glucanases are also classified as PR-2 proteins under pathogenesis-related protein families, which are able to catalyse endo-type hydrolytic cleavage of the 1,3-β-D-glucosidic linkage in β-I ,3-glucans. They are abundant highly regulated enzymes widely distributed throughout the plant kingdom (Simmons., 1994; Leubner-Metzger and Meins., 1999).

In plants, β-1,3-glucmases (β-glu) exist as multiple structural isoforms that differ in size, iso-electric point, primary structure, cellular localization and pattern of regulation. These isoforms are encoded by multi-gene families of considerable complexity in higher plants (Jin *et al.,* 1999). Such multiplicity could provide more flexibility for spatial and temporal regulation of diverse functions of the β-glu such as induced or tissue specific expression of pathogenesis related isoforms. Multiplicity of β-glu functions might confer advantages to plants by providing several lines of defense against invading microorganisms. Also the diversity of β-glu, as well as their organ specificity and developmental and differential expression patterns indicate that this enzyme has additional, as yet unidentified, biological functions in plant growth and

development. The most detailed sequence information of β-glu isoforms is available from cDNA and genomic DNA clones of tobacco. The various isoforms of the gene in *Nicotiana* have been classified in to 4 distinct structural classes. Similar structural isoforms have been reported from potato, tomato and other plant species also (Beerhues and Kombrink, 1994; Domingo *et al.,* 1994; Thirnrnapuram *et al.,* 2001). Class I β-glu, 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 class I genes encode proteins with basic iso-electric points and accumulate primarily in vacuoles. The class I enzymes are usually produced as a pre-protein with an N-terminal hydrophobic signal peptide which is co-translationally removed and a C-terminal extension, N-glycosylated at a single site. Glycosylation during processing is thought to facilitate targeting them to the vacuole. The vacuolar class I β-glu in *N. plumbaginifolia* were shown to be secreted into the medium in cultured tobacco cells via a novel pathway (Melchers et *al.,* 1993). The class I β-glu gene encoded protein Gn2 of shares 98 % amino acid identity with the intracellular vacuolar isoforms of β-glu isolated from N. *tabacum* earlier (Castresana., 1990). The class I basic isoforms isolated from potato are predominantly localized intracellularly, but are also found in considerable amounts in the extracelluar spaces of infected potato leaves (Schroder et *al.,* 1992). The potato class I basic β-glu also show structural features similar to tobacco class I β-glu. It contains a hydrophobic signal peptide of *25* amino acids and a putative C-terminal extension of 23 amino acids, including a potential glycosylation site. N and C-terminal processing results in a mature protein of 315 amino acids (Beerhues and Kombrink., 1994). The β-glu isolated from monocots *(Pisum sativum)* contains a long amino acid extension at the C-terminal end compared to the isoforms with a single glycosylation site (Buchner *et al,* 2002). It is expressed in young flowers and in the seed coat and is weakly expressed in the vegetative tissues during seedling development. The two basic vacuolar isofoms in *Arabidopsis,* BG1 and BG3, show significant structural homology to tobacco class I proteins except for the absence of C-terminal extension (Uknes *et al., 1992).*

In contrast to class I, the class I1 isoforms are acidic and are secreted into the extracellular space. The tobacco class I1 isoforms PR-2a, PR-2b and PR-2c are pathogen-induced acidic proteins without C-terminal extension with an apparent size ranging from 34 -36 kDa (Linthrost *et al.*,1990). The class I1 tobacco isoforms are at least 82% identical in amino acid sequence and differ from the class I enzymes at a minimum of 48.8% of the positions. Class I1 also includes two acidic 41 kDa stylar β-glu isoforms, Sp41 a and Sp41b, which are exclusively accumulated to higher levels in the transmitting tract of tobacco flowers (Ori *et al.*,1990). They do not appear to be pathogen-inducible, and hence are referred to as 'PR like' proteins. A pathogen-inductive acidic 35 kDa PR-2d (PR-Q') has been classified as class I11 β-glu as it differs at least 43% in amino acid sequence from the class I and class I1 enzymes (Payne et *al.*, 1490). They are also secreted proteins localized in the extracellular spaces. Two highly homologous cDNA clones for class III glucanase have been isolated from tomato plants infected with a viroid. Based on deduced amino acid sequences, TomPR-Q'a was found to be an acidic isofom, 86.7% identical to tobacco PR-Q,and TomPR-Q'b was found to be a basic isoforms, which shows 78.7% identity (Domingo, *et al.*, 1994). The fourth class is an acidic secreted β- glu that is not pathogen-induced; but is expressed in the anther tapetum, and is involved in the tetrad callose wall dissolution (Bucciaglia and Smith., 1994). As this 'Tag 1' is structurally divergent from other classes it is included as a novel class of tobacco β-glu. The mature Tag 1 is a 35 kDa protein with an N-terminal signal peptide, but without C-terminal extension and it shares absolutely conserved sequences found in all class of tobacco β- glu. It is 37 - 38% identical to class I, class I1 and class I11 tobacco β -glu. In addition to these four classes, two flower specific Arabidopsis genes encoding putative new isoforms (BG4 and BG5) of β -glu have also been identified (Delp and Palva., 1999).

β-1,3-glucanases and its defence mechanisms in plants

The role of β-glu in plant defense response have been well studied in several plant species. β-Glu were proposed to be involved in plant defense as early as 1971 (Abeles *et al.*, 1971). It has been first described in tobacco plant.

They generate a hyper sensitivity reaction (HR) against to TMV infection (Van Loon and Van Kammen., 1970). After this, β-glu were included as PR-2 family of proteins. There is now compelling evidence that β-glu, acting alone or in combination with chitinase, can help to defend against pathogen attack, especially against fungal pathogens. Since these reports, there is much evidence stating the role β-glu in pathogenesis. β-glu has anti-fungal activity *in-vitro* and β-1,3 linked glucan is found at relatively low concentrations in both monocots and dicots, but is a common component of the fungal cell walls. It has been proposed that they can act at least in two different ways: directly by degrading the cell walls of the pathogen, and indirectly by promoting the release of cell wall derived materials that can act as elicitors of defense reaction (Boller., 1993).

In healthy plants, β-1,3–glucanases have been found in higher concentrations in roots and lower leaves, relative to younger leaves (Felix and Meins., 1986; Vogeli-Lange *et al.,* 1994). The expression of β-1,3-glucanases is also regulated in response to hormones or pathogens. They are up-regulated in response to the stress hormone, ethylene (Felix and Meins 1987; Hart *et al.,* 1993)., and to ozone. The expression of β-1,3-glucanases is down regulated by the treatment with abscissic acid (ABA) (Leubner –Metzger *et al.,* 1995; Linthorst *et al* 1990; Van de Rhee *et al.,* 1993), and fungal infections (Chang *et al.,* 1992). β-l,3-Glucanase-deficient mutants show decreased susceptibility to necrotizing virus infection in tobacco plants. There are suggestions that β-l,3-glucanases defend plants against viruses. For example, a protein induced by TMV infection of tobacco reported to have antiviral activity and immunological similarity to human interferon-β is similar in sequence to class I β-1,3-glucanases (Edelbaum *et al.,* 1991). Fungal β-1,3-glucans, which might be generated by β-1,3-glucanase digestion of fungal cell walls, have been reported to have antiviral activity. On the other hand, Keamy and Wu (1984) have proposed that high β-l,3-glucanase levels promote virus infection. Although, they were unable to find a correlation between lesion and β-1,3-glucanase activity in several host plants, more recent studies based on measuring specific β-l,3-glucanases support their view., for example the virus-encoded movement protein is thought to promote TMV spread by increasing the effective aperture of plasmodesmata. Movement protein did not

modify secondary plasmodesmata of young tobacco leaves in which class I β-1,3-glucanases were not detectable (Felix and Meins, 1986; Ding *et al.*,1992).

In woody plants, a number of PR proteins have found to be induced in roots of Norway spruce seedlings following infection with *Pythium* spp. and two of these proteins have been reported to be acidic β-1,3-glucanases (Sharma *et al.*,1993). Karenlampi and co-workers (1994) showed that β-1,3-glucanases was also induced in stems and needless of 3-year old Norway spruce trees exposed to ozone. Investigation of the structure and expression of β-1,3-glucanases genes in peach may provide an understanding of the biology of defense-related genes in tree species., and eventually in other woody plants (Thimmapuram *et al.*, 2001).

The first β-1,3-glucanase gene from woody plants was isolated from *Hevea brasiliensis,* and codes for a basic vacuolar protein that has a C-terminal extension. This β-1,3-glucanase is highly expressed in latex rather than in leaf tissues (Chye and Cheung., 1995). Isolation and molecular characterization of β-1,3-glucanase gene and its role in combating abnormal leaf fall disease in *Hevea brasiliensis* Muell. Arg. has been reported (Thanseem *et al.*, 2005). It was observed that the genomic sequences encoding for β-1,3-glucanase is highly expressed in tolerant plants during diseased condition and that different genomic forms of β-1,3-glucanase controlled by separate promoters are present in *Hevea brasiliensis* (Supriya and Thulaseedharan., Unpublished). This gene can be a promising candidate for genetically engineering disease resistance in *Hevea.*

GENETIC ENGINEERING FOR DISEASE RESISTANCE

The excessive use of pesticides (includes fungicides, insecticides etc.) and chemicals contributes to soil degradation and water pollution. Up to the 1980s, maintenance and improvement of fertility and crop improvement was thought of chiefly in terms of addition of mineral fertilizers and use of pesticides. The uncontrolled use of many agro-chemicals is environmentally hazardous and it also increases the input cost of farmers. Conventional breeding procedures for disease resistance is time consuming and has to be a continuous process since

new races of pathogens evolve and crops become susceptible. Therefore, novel alternative strategies that would circumvent these problems are required to produce fungus resistant crop varieties. So genetic engineering is an alternative source for disease resistance in plants.

Genetic engineering is the manipulation of genes so that a particular trait can be changed. Simply it is the technology of bringing "evolution" with short span of time. Genetic manipulation could achieve easily among plant species with the aid of tissue culture. The era of plant genetic engineering in plants has begun with the discovery of the ability of a bacterium-the *Agrobacterium tumefaciens*- to transfer some of its DNA (T-DNA) into plant cells, "naturally". *Agrobacterium* is a soil bacterium that causes crown gall and hairy root disease in most dicots, gymnosperms and some monocots. T-DNA based vectors are created thereafter. Gene of interest is cloned in to the plant expression vectors that carry the right and left border sequences of T-DNA. The promise and eventual success of *Agrobacterium* as gene vector inspired the development of additional techniques for introducing DNA into plant cells. These include both chemical and physical methods like polyethylene glycol induced DNA uptake, microinjection, particle bombardment, electroporation, etc.

Normally genetic improvement of *Hevea* is very elaborate and time consuming. Crop improvement in *Hevea* has been achieved by incorporating genetic variability to maximize yield, vigor and also to develop disease tolerant varieties. In *Hevea* genetic transformation is a suitable tool to introduce various favorable characteristics in different clones. An understanding of the molecular mechanisms involved in disease resistance would be necessary for the development of any such alternative molecular tools for crop improvement. Molecular mechanism of disease resistance involves the structure, organization, expression and regulation of genes that govern plant-pathogen interactions. This is achieved with the aid of suitable vectors. A binary vector is a standard tool in the transformation of higher plants mediated by *Agrobacterium tumefaciens*. It is composed of the borders of T-DNA, multiple cloning sites, replication functions for *Escherichia coli* and *A. tumefaciens*, selectable marker genes, reporter genes,

and other accessory elements that can improve the efficiency of and/or give further capability to the system. For the suitable expression of these genes a promoter should be required.

Promoters

Regulation of gene expression is an art of DNA sequences which is upstream to the coding region by recruiting transcription factors, regulators and a RNA polymerase. The transcription of a gene is controlled by an intricate choreography of proteins binding to promoter, enhancer and repressor sites on the DNA sequence (Ptashne., 1988; Ptashne and Gann., 1997). The phenomenon "Transcription" is similar in both eukaryotes as well as in prokaryotes that leads to the generation of RNA by employing DNA as the template. As the name indicates "promoter" promotes gene expression. Usually contains a TATA box which serves to determine the start site of transcription (Dynan and Tjian, 1985). Transcription factors together with the catalyst RNA polymerase recognize a promoter by its structural features and associate with it to initiate transcription. In this process, the newly formed complex positions RNA polymerase at the transcription initiation site and activates transcription (Lewin., 2008). Promoters typically have a modular structure, consisting of multiple short sequence (5 to 20 nucleotides), called *cis acti*ng regulatory elements, most of which comprise transcription factor (TF) binding sites. These elements can be dispersed or can overlap and usually lie within the 1kb region upstream and surrounding a transcription start site (TSS). The combination of these regulatory elements is often unique for most genes involved in various pathways.

The promoter can simply be divided in two parts: a proximal part, referred to as the core, and a distal part. The proximal part is believed to be responsible for correctly assembling the RNA polymerase II complex at the right position and for directing a basal level of transcription (Nikolov *et al.,* 1996; Berk., 1999). It is mediated by elements, such as TATA and initiator boxes through the binding of the TATA box-binding protein, and other general TFs (Featherstone., 2002). The distal part of the promoter contains elements that regulate the spatio-temporal expression (Tjian and Maniatis., 1994; Fessele *et al.,* 2002). In addition to the

proximal and distal parts, somewhat isolated, regulatory regions have also been described, mainly in animals that contain enhancer and/or repressor elements (Barton *et al.,* 1997). The latter elements can be found from a few kilobase pairs upstream of TSS, in the introns or even at the 3' of the genes they regulate (Wasserman *et al.,* 2000). The promoters can be classified as constitutive, tissue-specific and inducible based on their activity. A constitutive promoter contains elements recognized by basal activators or transcription factors to initiate transcription in all tissues at all times. That means they initiate constitutive expression of particular gene for all times independent of specific conditions. However, inducible promoters are activated by one or more stimuli such as hormones (auxin, abscissic acid, gibberellic acid, ethylene, salicylic acid and/or methyl jasmonate), chemicals (tetracycline, dexamethasone, copper, nitric oxide), environmental conditions/stresses and biotic stresses, whereas tissue-specific promoters control gene expression in a tissue-dependent manner according to the developmental stage of the plant.

Constitutive promoters are the most common promoters used to drive the expression of various genes in plants. The most commonly used promoter for directing strong constitutive expression has been the Cauliflower Mosaic Virus (CaMV) 35S promoter (Odell *et al.,* 1985; Jefferson., 1987). Although a number of constitutive promoters have been isolated from plants and used for the generation of transgenic plants, most of them are protected by patents. Therefore, novel plant sequences that can function as promoters for the high-level expression of transgenes are to be identified and tested. A wider range of effective promoters would also make it possible to introduce multiple transgenes into plant cells while avoiding the risk of homology-dependent gene silencing (Schunmann *et al.,* 2003).

Eukaryotic promoters

Eukaryotic promoters are extremely diverse and are difficult to characterize. They typically lie upstream of the gene and can have regulatory elements several kilo bases away from the transcriptional start site. In eukaryotes, the transcriptional complex can cause the DNA to bend back on itself, which

allows for placement of regulatory sequences far from the actual site of transcription. Many eukaryotic promoters, contain a TATA box (sequence TATAAA), which in turn binds a TATA binding protein, which assist in the formation of the RNA polymerase transcriptional start site (often within 50 bp).

Types of promoters

Promoters are characterized according to the type or degree of control of gene expression, virtually in all tissues or depending on the tissues and the developmental stage of the plant. Additionally, promoters may operate in response to external and in some cases, controllable stimuli.

Constitutive promoters

Constitutive promoters are the most common promoters used to drive the expression of various genes in development of transgenics. A constitutive promoter might contain an element which responds to activators present in all tissues, all the time. Alternatively, there could be a transcription factor present in all the tissues, all the time, interacting with an element of a constitutive promoter. An example of such a promoter may be that of a housekeeping gene (homologous) or a gene derived from a plant pathogen with the ability to infect a wide range of host cells (heterologous) (Lewin., 1994). These promoters allow expression of the downstream coding region in all tissues irrespective of environmental and developmental factors (Benfey and Chua., 1990). There are number of constitutive promoters ,that are used in genetic transformation such as CaMV 35S promoters, ubiquitin promoters, actin promoters etc.

Tissue specific promoters

Spatial and developmentally controlled gene expression can be achieved using different 'tissue-specific' promoters. A wide range of promoters have been identified as tissue specific. They allow specific gene expression in virtually any desired cell type, tissue or organs. For instance, the *LEAFY* (*LFY*) promoter is activated during flower initiation and is therefore to some extent temporally regulated, but it will be active in a similar way in every flower whenever initiated (Deveaux *et al.*, 2003).Distinct *cis*-acting DNA sequences

confer spatially-regulated activation of gene expression within different regions (i.e. cotyledons, shoot apex and hypocotyls) and in different cell types of the same region (i.e. parenchyma and procambium) of the embryos. Many tissue specific promoters have been used to drive transgenes. For example, the promoter sequence of *Mec1* gene (coding for Pt2L4, a glutamic acid rich protein) isolated from *Cassava* plants used for transgenic expression in same species. Phaseolin gene encoding the major storage protein of bean (*Phaseolus vulgaris*) contains two upstream activating sequences (domains), UAS1 (-295 to -109) and UAS2 (-468 to -391). The activity of UAS1 was localized in the cotyledons and the shoot apex but expression in the hypocotyl required the presence UAS2. Additionally, both UAS1 and the 35S enhancer fragment directed GUS expression in the cotyledons and shoot apex, UAS1 yielded much higher activity in storage parenchyma cells and the 35S enhancer was mostly active in the pro cambial strands (Bustos *et al.*, 1991).

Inducible promoters

These promoters are activated by one or more stimuli such as hormones, chemicals, environmental conditions and biotic stresses. Inducible promoters are a very powerful tool in genetic engineering because the expression of genes operable linked to them can be regulated to function at certain stages of development of an organism or a particular tissue under defined conditions. There are virtually hundreds of inducible promoters that vary according to the organism source and cells or tissues where they regulate gene transcription (Singh *et al.*, 2002; Gurr and Rushton *et al.*, 2005; Nakashima and Yamaguchi-Shinozaki., 2006).

A high level of constitutive gene expression is usually needed for the production of important proteins for agronomical or commercial purposes in transgenic plants. To achieve this goal, we need to understand and exploit the mechanisms in plants developed to regulate expression levels of their various genes during evolution. Gene expression in eukaryotes is a multistage process controlled at transcriptional, post transcriptional, translational and post-translational levels (Lewin *et al.*, 2000).

The isolation and characterisation of plant promoter is required to understand the function of a gene completely. The regulation capacity and interaction of the promoter with transcription factors provide knowledge about how genes are expressed. Also functionally stable and constitutive/inducible promoters can be used in transgenic plants instead of the controversial viral promoters used presently.

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* clone 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.

Leaf sample weighing 1gm was frozen with liquid nitrogen and ground to a very fine powder using a mortar & pestle.

i. 10 ml of 2X CTAB extraction buffer was added and transferred to a 50 ml centrifuge tube.
ii. The tube was incubated at 55 °C for 30 minutes with occasional swirling.
iii. 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.
iv. Equal volume of Tris saturated phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed by gentle inversion.

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

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

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

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

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

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

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

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

xiii. 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.

xiv. DNA was washed twice in 70% ethanol.

xv. Suspended in sterile distilled water.

xvi. 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 micro liter 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 *Eco*R I and *Hind* III 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-3 (Gln 3)

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' AGT TTT AAT TTG GCG ATA G 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(10 X)	2 µl
dNTPs mix (dATPs, dGTPs, dCTPs, dTTPs [100µM each])	2 µl
Taq polymerase (3units /µl)	0.16 µl
Template DNA (10 ng / µl)	2 µl
Forward primer (250 nM)	1 µl
Reverse primer (250 nM)	1 µl
Total	**20 µl**

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:

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

The ligation mix was prepared as follows.

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

The ligation was performed overnight at 4°C 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. 100 µl 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 :

i) IPTG stock solution (0.1 M)

1.2 g IPTG

Add water to 50ml final volume.

Filter-sterilize and store at 4°C.

ii) 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.

iii) 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 readymade media powder (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.

iv) 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.

v) 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.

vi) 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 50mg/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).

vii) SOC medium (100ml)

2.0 g Bacto-tryptone

0.5 g Bacto-yeast extract

1 ml 1M NaCl

0.25 ml 1 M 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 2M Mg^{2+} stock and 2M glucose, each to a final concentration of 20mM. Made upto 100ml with sterile, distilled water. Filtered the complete medium through a 0.2µm filter unit. The final pH was 7.0.

viii) 2 M 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 of transformed cells

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.3.

Table.3. Colony PCR profile

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

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

7. Isolation of Recombinant 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.

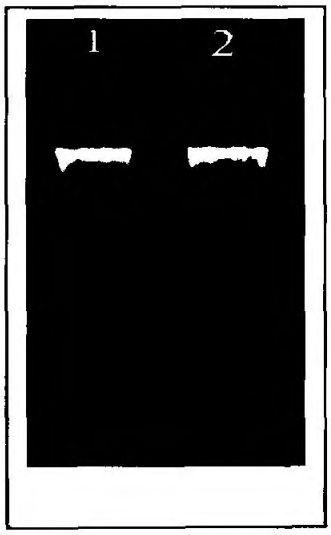
RESULTS AND DISCUSSION

Earlier studies conducted at Rubber Research institute of India, Kottayam on isolation and characterization of β-1,3-glucanase gene from the popular *Hevea brasiliensis* clone RRII 105 suggests that this gene is involved in conferring tolerance to abnormal leaf fall disease caused by *Phytophthora spp* (Thanseem *et al.*, 2005). Recent studies performed in *Hevea brasiliensis* clone RRII 105 revealed the existence of different isoforms of β-1,3-glucanase (Supriya and Thulaseedharan.,Unpublished). The *Hevea brasiliensis* clone RRII 105 is a relatively tolerant clone to abnormal leaf fall disease while RRIM 600 is a relatively susceptible clone. The present work was undertaken to characterize the β-1,3-glucanase gene isoform-3 (Gln 3) along with its partial promoter from the *Hevea brasiliensis* clone RRIIM 600 to compare with the sequence isoform from the *Hevea clone* RRII 105 , which must throw some light on the anti-fungal functions. For this DNA isolation, PCR amplification, cloning, sequencing and further sequence comparison of Gln 3 gene isoform has been done.

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 was of good quality without shearing and RNA contamination (Fig.2.). The concentration of extracted DNA from 1g of leaf sample which dissolved in 50 µl sterile distilled water was estimated to be about 1 µg/µl and further the concentration was adjusted to 20 ng/µl for using as template in PCR.

Fig.2. DNA Isolated from *Hevea* clone RRIM-600

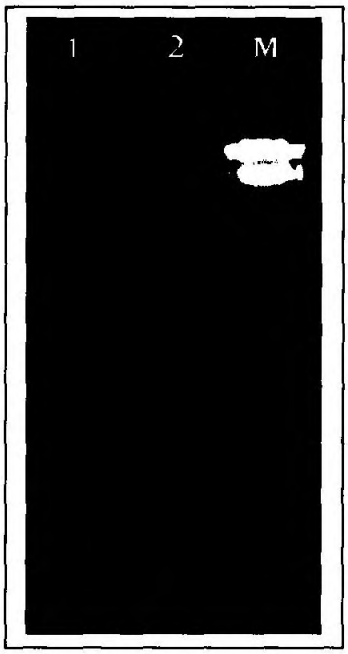


Lanes 1&2: DNA sample.

PCR amplification of β-1,3-glucanase gene isoform- 3 (Gln3)

With the specific primers for the β-1,3-glucanase gene isoform and its promoter a DNA amplicon of size ~1.4 kb was isolated through PCR. The observation made when samples were electrophoresed on 1.2% agarose gel. The products were distinct, single bands with no artifacts (Fig.3.).

Fig.3. PCR amplification of β-1,3-glucanase gene isoform-3 (Gln3).



Lanes 1&2: Amplified 1.4 kb Fragment.

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

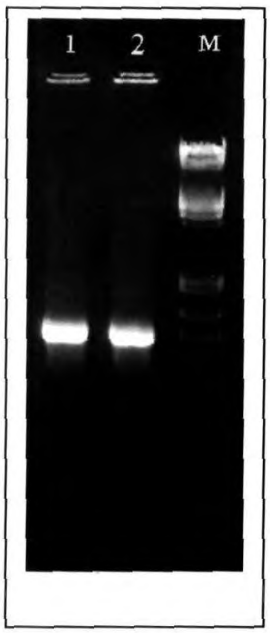
Cloning of β-1, 3-glucanase gene isoform-3 (Gln 3)

The specific DNA fragments amplified through PCR got eluted from the gel were cloned in the pGEMT-T Easy vector as per the manufacturer's instructions. After ligation, the ligation mixture was used to transform DHα competent cells. The transformed colonies were plated on LB medium containing antibiotic for selection and it is over layered with X-gal & IPTG for blue-white screening. On overnight incubation, good number of white colonies were developed indicating better transformation. From this transformed colonies few of them were used for plasmid isolation.

Colony PCR of transformed cells

To confirm the presence of insert in white colonies, a few of the colonies were directly used as template in PCR reactions using the specific primers. The products when electrophoresed on 1.2% agarose gel showed single bands in the exact size as in the initial PCR (Fig.4.)

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



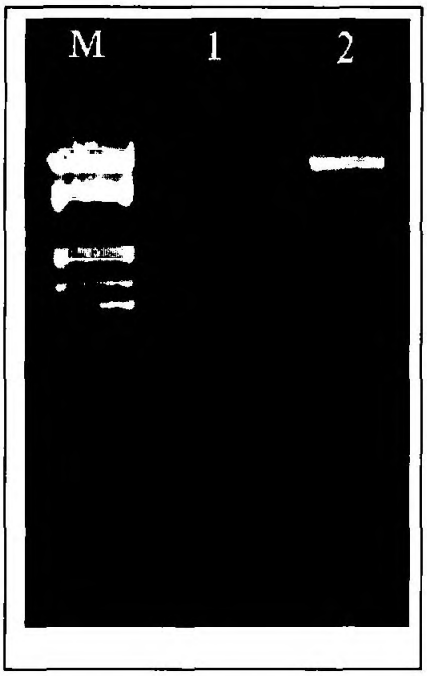
Lanes 1&2 : colonies transformed with ligation mixture.

M :Moleculamarker (*Eco*RI /*Hind*III lambda DNA double digest)

Recombinant Plasmid isolation

Recombinant plasmids isolated from transformed colonies were electrophoresed on 1% agarose gel. About three bands were observed in each lane showing the presence of different coil conformations of the plasmid (Fig.5.). The plasmids isolated was of good concentration. The presence of the insert in the recombinant plasmid has been confirmed as indicated by the higher molecular weight of the recombinant plasmid upon comparison with the insert-less control plasmid.

Fig.5. Recombinant plasmid DNA isolation



Lanes:

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

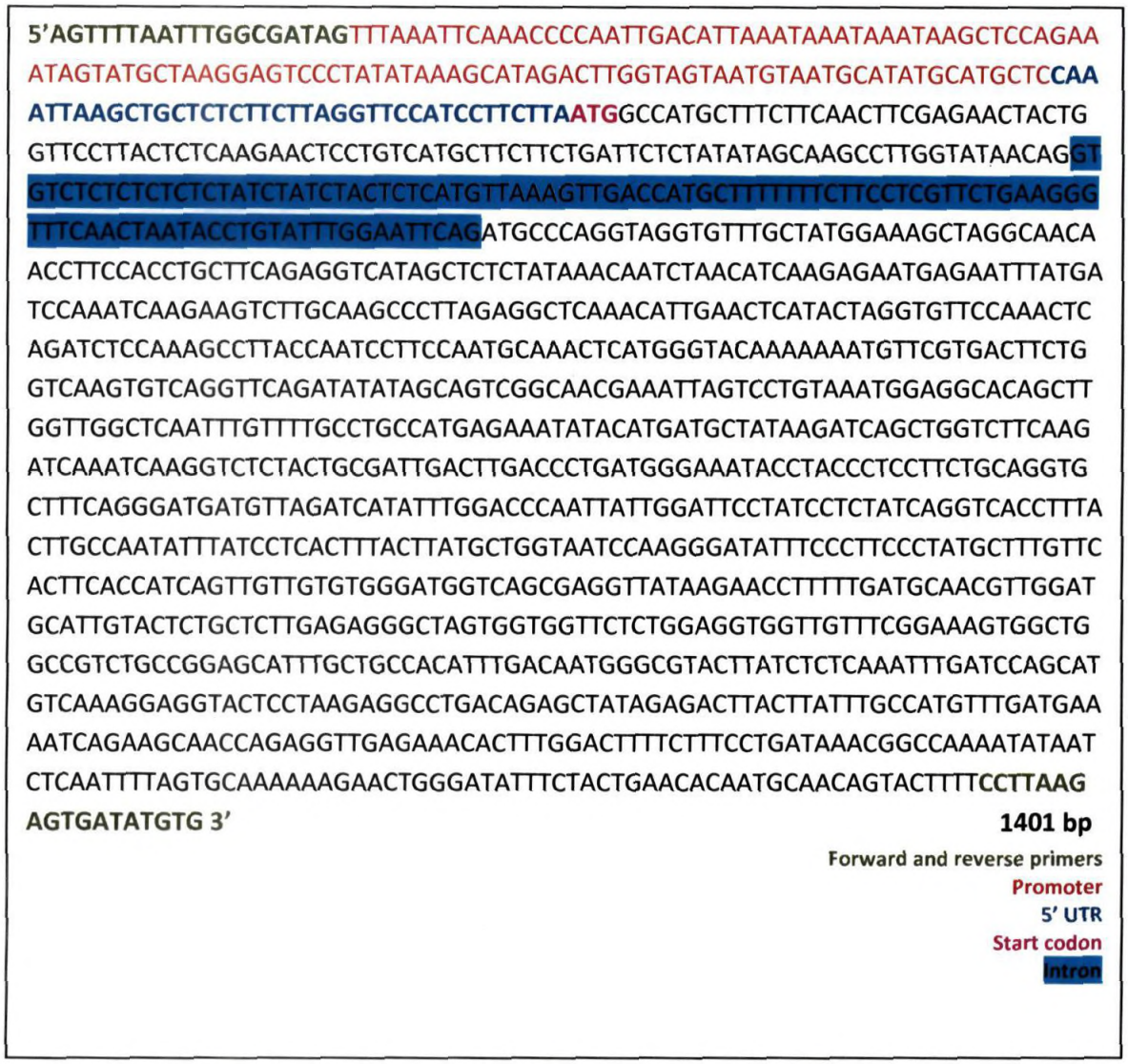
1: Control plasmid DNA without gene insert

2: plasmid with gene insert.

Nucleotide sequencing

The isolated plasmid vector carrying amplified fragments were then sequenced at M/S Macrogen, Korea. The nucleotide sequence of Gln 3 isoform of β-1,3-glucanase from RRIM 600 was analyzed using online bioinformatic tools like BLASTn, Clustal W etc. The BLASTn analysis showed that the the sequence is highly similar to the Gln 3 sequence reported earlier in the gene bank from *H. brasiliensis.* The sequence obtained has both promoter and gene sequence with a total of 1401 nucleotides within the given primer sequences (Fig.6.).

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



In the 1401 bp fragment, 173 bp constitute the promoter region.In the173 bp promoter region nucleotides 135 to 173 bp is 5'UTR region. The translation beginning codon or start codon (ATG) can be viewed after 5'UTR. There is an intron sequence with GT-AG splicing junction at 276 to 379 bp position. Other features of the sequence are given in table.4.

Comparison of Gln3 sequence with earlier reported Gln3 sequence from RRII-105

Using ClustalW online software tool, the sequence was aligned with that of Gln3 isoform of β-1,3-glucanase gene from RRII-105 (Fig.7.).

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

Start codon

Intron

Nucleotide variations

Stop codon

```
600     GTTTTAATTTGGCGATAGTTTAAATTCAAACCCCAATTGACATTAAATAAATAAATAAG 59
105     GTTTTAATTTGGCGATAGTTTAAATTCAAACCCCAATTGACATTAAATAAATAAATAAG 59
        ************************************************************

600     CTCCAGAAATAGTATGCTAAGGAGTCCCTATATAAAGCATAGACTTGGTAGTAATGTAAT 119
105     CTCCAGAAATAGTATGCTAAGGAGTCCCTATATAAAGCATAGACTTGGTAGTAATGTAAT 119
        ************************************************************

600     GCATATGCATGCTCCAAATTAAGCTGCTCTCTTCTTAGGTTCCATCCTTCTTAATGGCCA 179
105     GCATATGCATGCTCCAAATTAAGCTGCTCTCTTCTTAGGTTCCATCCTTCTTAATGGCCA 179
        ************************************************************

600     TGCTTTCTTCAACTTCGAGAACTACTGGTTCCTTACTCTCAAGAACTCCTGTCATGCTTC 239
105     TGCTTTCTTCAACTTCGAGAACTACTGGTTCCTTACTCTCAAGAACTCCTGTCATGCTTC 239
        ************************************************************

600     TTCTGATTCTCTATATAGCAAGCCTTGGTATAACAGGTGTCTCTCTCTCTCTATCTATCT 299
105     TTCTGATTCTCTATATAGCAAGCCTTGGTATAACAGGTGTCTCTCTCTCTCTATCTATCT 299
        ************************************************************

600     ACTCTCATGTTAAAGTTGACCATGCTTTTTTTCTTCCTCGTTCTGAAGGGTTTCAACTAA 359
105     ACTCTCATGTTAAAGTTGACCATGCTTTTTTTCTTCCTCGTTCTGAAGGGTTTCAACTAA 359
        ************************************************************

600     TACCTGTATTTGGAATTCAGATGCCCAGGTAGGTGTTTGCTATGGAAAGCTAGGCAACAA 419
105     TACCTGTATTTGGAATTCAGATGCCCAGGTAGGTGTTTGCTATGGAAAGCTAGGCAACAA 419
        ************************************************************

600     CCTTCCACCTGCTTCAGAGGTCATAGCTCTCTATAAACAATCTAACATCAAGAGAATGAG 479
105     CCTTCCACCTGCTTCAGAGGTCATAGCTCTCTATAAACAATCTAACATCAAGAGAATGAG 479
        ************************************************************

600     AATTTATGATCCAAATCAAGAAGTCTTGCAAGCCCTTAGAGGCTCAAACATTGAACTCAT 539
105     AATTTATGATCCAAATCAAGAAGTCTTGCAAGCCCTTAGAGGCTCAAACATTGAACTCAT 539
        ************************************************************

600     ACTAGGTGTTCCAAACTCAGATCTCCAAAGCCTTACCAATCCTTCCAATGCAAACTCATG 599
105     ACTAGGTGTTCCAAACTCAGATCTCCAAAGCCTTACCAATCCTTCCAATGCAAACTCATG 599
        ************************************************************

600     GGTACAAAAAAATGTTCGTGACTTCTGGTCAAGTGTCAGGTTCAGATATATAGCAGTCGG 659
105     GGTACAAAAAAATGTTCGTGACTTCTGGTCAAGTGTCAGGCTCAGGTATATAGCAGTCGG 659
        ****************************************** **** **************

600     CAACGAAATTAGTCCTGTAAATGGAGGCACAGCTTGGTTGGCTCAATTTGTTTTGCCTGC 719
105     CAACGAAATTAGTCCTGTAAATGGAGGCACAGCTTGGTTGGCTCAATTTGTTTTGCCTGC 719
        ************************************************************

600     CATGAGAAATATACATGATGCTATAAGATCAGCTGGTCTTCAAGATCAAATCAAGGTCTC 779
105     CATGAGAAATATACATGATGCTATAAGATCAGCTGGTCTTCAAGATCAAATCAAGGTCTC 779
        ************************************************************


600     TACTGCGATTGACTTGACCCTGATGGGAAATACCTACCCTCCTTCTGCAGGTGCTTTCAG 839
105     TACTGCGATTGACTTGACCCTGATGGGAAATACCTACCCTCCTTCTGCAGGTGCTTTCAG 839
        ************************************************************
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600     GGATGATGTTAGATCATATTTGGACCCAATTATTGGATTCCTATCCTCTATCAGGTCACC 899
105     GGATGATGTTAGATCATATTTGGACCCAATTATTGGATTCCTATCCTCTATCAGGTCACC 899
        ************************************************************

600     TTTACTTGCCAATATTTATCCTCACTTTACTTATGCTGGTAATCCAAGGGATATTTCCCT 959
105     TTTACTTGCCAATATTTATCCTTACTTTACTTATGCTGGTAATCCAAGGGATATTTCCCT 959
        ********************* ************************************

600     TCCCTATGCTTTGTTCACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAA 1019
105     TCCCTATGCTTTGTTCACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAA 1019
        ************************************************************

600     GAACCTTTTTGATGCAACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGG 1079
105     GAACCTTTTTGATGCAACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGG 1079
        ************************************************************

600     TTCTCTGGAGGTGGTTGTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCAC 1139
105     TTCTCTGGAGGTGGTTGTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCAC 1139
        ************************************************************

600     ATTTGACAATGGGCGTACTTATCTCTCAAATTTGATCCAGCATGTCAAAGGAGGTACTCC 1199
105     ATTTGACAATGGGCGTACTTATCTCTCAAATTTGATCCAGCATGTCAAAGGAGGTACTCC 1199
        ************************************************************

600     TAAGAGGCCTGACAGAGCTATAGAGACTTACTTATTTGCCATGTTTGATGAAAATCAGAA 1259
105     TAAGAGGCCTGACAGAGCTATAGAGACTTACTTATTTGCCATGTTTGATGAAAATCAGAA 1259
        ************************************************************

600     GCAACCAGAGGTTGAGAAACACTTTGGACTTTTCTTTCCTGATAAACGGCCAAAATATAA 1319
105     GCAACCAGAGGTTGAGAAACACTTTGGACTTTTCTTTCCTGATAAACGGCCAAAATATAA 1319
        ************************************************************

600     TCTCAATTTTAGTGCAAAAAAGAACTGGGATATTTCTACTGAACACAATGCAACAGTACT 1379
105     TCTCAATTTTAGTGCAAAAAAGAACTGGGATATTTCTACTGAACACAATGCAACAGTACT 1379
        ************************************************************

600     TTTCCTTAAGAGTGATATGTG 1401
105     TTTCCTTAAGAGTGATATGTG 1401
        *********************
```

The deduced sequence showed 98% sequence homology with the earlier reported Gln3 isoform of RRII-105. The promoter sequences as well as the 5'UTR sequences did not show variations. There are some variations observed for a few nucleotides in the gene portion. The sequence shows nucleotide substitutions at three different positions- 640, 645 and 922 in the exon portion (Table.5.). From sequence alignment it is evident that the sequences are similar. They may also have the same open reading frame and amino acid sequence. As reported earlier, the coding sequence is obtained by joining the 173..275 and 380..1401.

Amino acid sequence predicted for Gln 3 isoform from RRIM-600

The reading frame was analysed for sequence obtained from RRIM 600 using ExPASY tool and the obtained amino acid sequence is given in Fig .8.

Fig.8. Amino acid sequence predicted for Gln3 isoform from RRIM-600

MAMLSSTSRTTGSLLSRTPVMLLLILYIASLGITDAQVGVCYGKLGNNLPPASEVIALYK
QSNIKRMRIYDPNQEVLQALRGSNIELILGVPNSDLQSLTNPSNANSWVQKNVRDFWSSV
RFRYIAVGNEISPVNGGTAWLAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLMGNTY
PPSAGAFRDDVRSYLDPIIGFLSSIRSPLLANIYPHFTYAGNPRDISLPYALFTSPSVVV
WDGQRGYKNLFDATLDALYSALERASGGSLEVVVSESGWPSAGAFAATFDNGRTYLSNLI
QHVKGGTPKRPDRAIETYLFAMFDENQKQPEVEKHFGLFFPDKRPKYNLNFSAKKNWDIS
TEHNATVLFLKSDM

ClustalW of amino acid sequences comparing RRII-105 and RRIM-600

The amino acid sequences from both the clones were compared using ClustalW sequence alignment tool (Fig.9.).

Fig. 9. ClustalW of amino acid sequences comparing RRII-105 and RRIM-600

```
105     MAMLSSTSRTTGSLLSRTPVMLLLILYIASLGITDAQVGVCYGKLGNNLPPASEVIALYK 60
600     MAMLSSTSRTTGSLLSRTPVMLLLILYIASLGITDAQVGVCYGKLGNNLPPASEVIALYK 60
        ************************************************************

105     QSNIKRMRIYDPNQEVLQALRGSNIELILGVPNSDLQSLTNPSNANSWVQKNVRDFWSSV 120
600     QSNIKRMRIYDPNQEVLQALRGSNIELILGVPNSDLQSLTNPSNANSWVQKNVRDFWSSV 120
        ************************************************************

105     RLRYIAVGNEISPVNGGTAWLAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLMGNTY 180
600     RFRYIAVGNEISPVNGGTAWLAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLMGNTY 180
        *.************************************************************

105     PPSAGAFRDDVRSYLDPIIGFLSSIRSPLLANIYPYFTYAGNPRDISLPYALFTSPSVVV 240
600     PPSAGAFRDDVRSYLDPIIGFLSSIRSPLLANIYPHFTYAGNPRDISLPYALFTSPSVVV 240
        ************************************:***********************

105     WDGQRGYKNLFDATLDALYSALERASGGSLEVVVSESGWPSAGAFAATFDNGRTYLSNLI 300
600     WDGQRGYKNLFDATLDALYSALERASGGSLEVVVSESGWPSAGAFAATFDNGRTYLSNLI 300
        ************************************************************

105     QHVKGGTPKRPDRAIETYLFAMFDENQKQPEVEKHFGLFFPDKRPKYNLNFSAKKNWDIS 360
600     QHVKGGTPKRPDRAIETYLFAMFDENQKQPEVEKHFGLFFPDKRPKYNLNFSAKKNWDIS 360
        ************************************************************

105     TEHNATVLFLKSDM 374
600     TEHNATVLFLKSDM 374
        **************
```

The protein sequence showed 98% homology with reported RRII 105 sequence when analyzed with BLASTp tool. In the sequence obtained from RRII 105, at position 122 it is leucine and for RRIM 600 it is phenyl alanine while at 216th position tyrosine is replaced by histidine in RRIM 600 (Table.6.). Whether these replacements affect the function of the protein molecule needs to be functionally validated, although these kind of replacements are common and compatible (Betts and Russell., 2003).

Table.4. Features of DNA and amino acid sequence of Gln3 isoform from RRIM-600

Clone	Characteristics	
	DNA	Protein
RRIM-600	Total number of bases – 1401 bp %A = 27.7 [388] %T = 31.3 [446] %G = 19.4 [272] %C = 21.1 [295] Base count 388a, 446t, 272g, 295c	Total number of amino acids – 374 Molecular weight of protein – 41343.0 Strongly basic amino acids - 35 Strongly acidic amino acids - Total number of negatively charged residues (Asp + Glu): 32 Total number of positively charged residues (Arg + Lys): 35 pI value – 8.76 4.1 charge at pH 7.0

The nucleotide and amino acid sequence variations between the two clones are compared in table.5 and table.6.

Table.5. Difference in nucleotides between the two clones

Point of variation in DNA sequence	RRII-105	RRIM-600
640	C	T
645	G	A
922	T	C

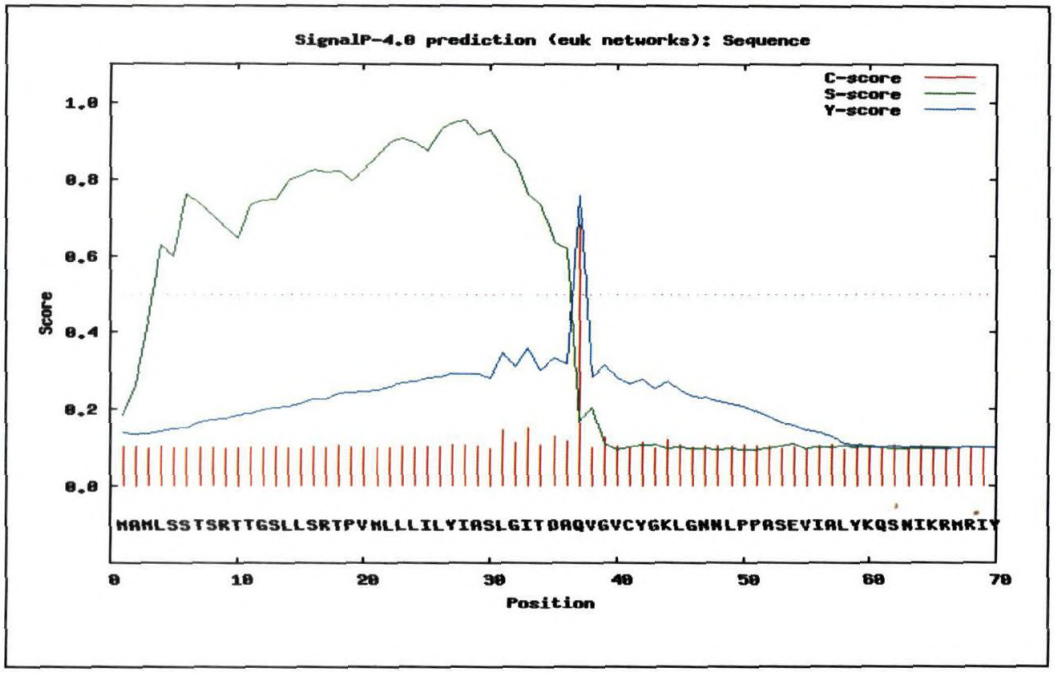
Table.6. Difference in amino acids between the two clones

Point of variation in amino acid sequence	RRII-105	RRIM-600
122	Leucine	Phenyl alanine
216	Tyrosine	Histidine

Prediction for Signal peptides:

The method incorporates prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks and hidden Markov models. Targeting signals are the pieces of information that enable the cellular transport machinery to correctly position a protein inside or outside the cell. This information is contained in the polypeptide chain or in the folded protein. The continuous stretch of amino acid residues in the chain that enables targeting are called signal peptides or targeting peptides. A signal peptide is a short peptide chain (3-60 amino acids long) that directs the transport of a protein towards a particular organelle/ secretory pathway. The predicted signal peptide of the isolated sequences showed a signal peptide probabilty of 0.987 with cleavage sites between positions 36 and 37 (Fig.10.).

Fig.10. Signal peptide prediction of Gln3 from the *Hevea* clone RRIM-600

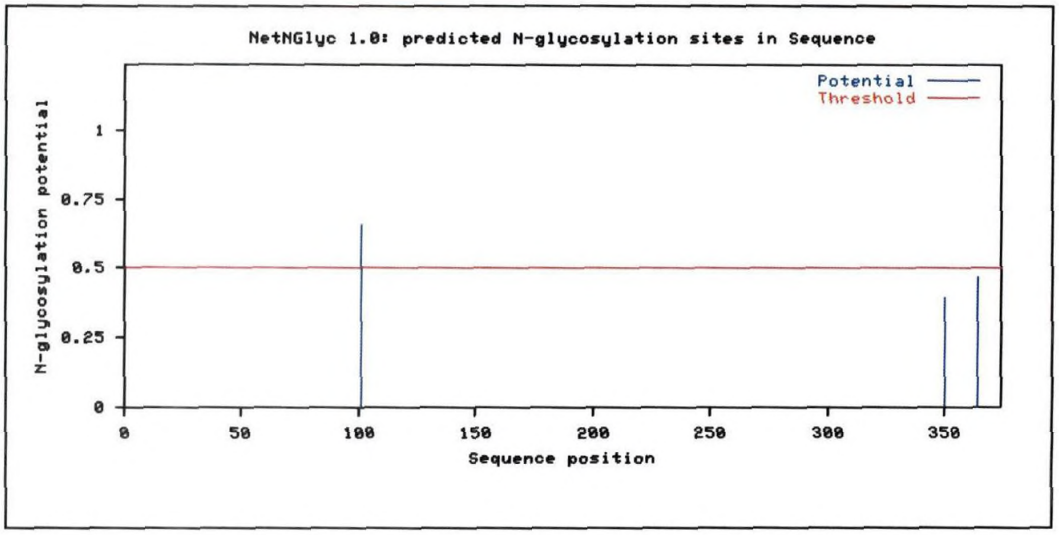


As reported earlier, these basic β-1,3-glucanases (as indicated by the iso-electric point) are often vacuolar and can be excreted through the secretory pathway. The peptide usually contains a C-terminal extension with N- glycosylation which acts as a signal anchorage. The prediction of C-terminal glycosylation through NetCglyc server showed no probability of anchorage of these proteins in the memberane. Therefore it can be a secretory protein.

NetNGlyc prediction:

The NetNGlyc software attempts to distinguish glycosylated sequons from non-glycosylated ones in the polypeptide chain. The software predicts a high potential for glycosylation for Gln-3 peptide at position 103 which crossed the threshold value of 0.5 (Fig.11). Two other less potential sites at positions 350 and 364th asparagine were also noticed.

Fig. 11. NetNGlyc prediction of Gln3



Majority of the proteins in the plant system residing in the extracellular compartment and the endomembrane system are glycosylated by N-linked oligosaccharides. The N-glycosylation of proteins has a great impact both on their physico chemical properties and on their biological functions (Rayon et al., 1998). N- linked glycosylation is important for the folding of some eukaryotic proteins and can also serve for protein transport towards the cell surface.

N- glycosylation usually occurs in the asparagine which occur in the Asn-Xaa-Ser/Thr stretch (where Xaa is any amino acid except Proline). Most *N*-linked oligosaccharides begin with addition of a 14-sugar precursor to the asparagine in the polypeptide chain of the target protein in eukaryotes. The structure of this precursor is common to most eukaryotes and contains 3 glucose, 9 mannose, and 2 *N*-acetylglucosamine molecules. A complex set of reactions attaches this branched chain to a carrier molecule called dolichol and then it is transferred to the appropriate point on the polypeptide chain as it is translocated into the ER lumen. After attachment, once the protein is correctly folded, the three glucose residues are removed from the chain and the protein is available for export from the ER. The glycoprotein thus formed is then transported to the Golgi where removal of further mannose residues may take place. Whether an oligosaccharide is high-mannose or complex is thought to depend on its accessibility to saccharide-modifying proteins in the Golgi. If the saccharide is relatively inaccessible, it will most likely stay in its original high-mannose form. If it is accessible, then it is likely that many of the mannose residues will be cleaved off and the saccharide

will be further modified by the addition of other types of group as discussed above. The result shows that the isolated Gln-3 glucanase from the *Hevea* clone RRIM 600 is also a protein which will be glycosylated in the *invivo* conditions.

SUMMARY AND CONCLUSION

Nature has not provided any other industrial raw material of plant origin as flexible as natural rubber. *Hevea brasiliensis* is the primary source of natural rubber and this crop grown in several parts of the world. Natural rubber has multifarious uses in automobiles, aircrafts, railways, textile industries, sports goods. Natural rubber cannot be totally replaced by synthetic rubber. Various challenges faced to rubber plant is responsible for considerable loss in latex yield. This include both biotic and abiotic stresses. Major biotic stess are pathogenic organisms. It is known that plants are infected by many pathogens including bacteria, virus, fungi and nematodes. The most destructive disease of rubber in India is the Abnormal leaf fall (ALF) caused by different species of *Phytophthora.* This causes a decrease in yield upto 40%. *Phytophthora* is a remarkable genus of plant pathogenic fungi. Therefore methods are developed to protect plant from severe loss. Conventional breeding procedures for disease resistance is time consuming and has to be a continuous process since new races of pathogens evolve and crops become susceptible. Therefore, novel alternative strategies that would circumvent these problems are required to produce fungus resistant crop varieties. Therefore, genetic engineering is an alternative source for disease resistance in plants. For this purpose the most appropriate gene for the interested trait in to be identified and characterized.

β-1,3-glucanases are enzymes which are included in Pathogenesis related proteins (PR proteins) and are abundantly distributed in many dicotyledonous trees. β-Glucanases were proposed to be involved in plant defense as early as 1971 as antifungal agents. It has been proposed that they can act at least in two different ways: directly by degrading the cell walls of the pathogen (fungus), and indirectly by promoting the release of cell wall derived materials that can act as elicitors of defense reaction. The role of β-1,3-glucanase (Gln 1) in controlling abnormal leaf fall disease caused by P*hytophthora* is well studied. Recently it was observed that different genomic forms of β-1,3-glucanase controlled by separate promoters are present in the *Phytophthora*-tolerant clone RRII 105 of *Hevea brasiliensis.*

In the present work the β-1,3-glucanase isoform-3 (Gln 3)from an abnormal leaf fall disease susceptible *Hevea* clone, RRI-600 was characterized and compared with an already reported sequence from an abnormal leaf fall disease tolerant clone RRII-105. For this, initially DNA was isolated from the selected clone and a 1.4 kb sized glucanase gene was amplified using PCR technique. It was then purified cloned and transformed to competent DH5∞ cells. After cloning, DH5∞ cells were plated in LB medium for antibiotic selection supplemented with IPTG and X-Gal. The developed colonies selected through blue white screening. White transformed colonies containing the inserts were observed. Colony PCR was done to ensure the presence of insert and suitably grown for plasmid isolation. Recombinant plasmids were isolated and again PCR amplified and good quality plasmids were selected for sequencing. The obtained sequence was characterized with the help of online bioinformatic tools like BLASTn and ClustalW. A 1401 bp sequence of Gln 3 along with the partial promoter was characterized. The deduced sequence showed 98% homology with the earlier reported Gln 3 isoform of RRII 105, and more than 95% homology with other isoforms isolated from RRII 105. There were three nucleotide variations in the gene portion and two amino acid sequence variations between the two clones. The promoter sequences as well as the 5'UTR sequences do not show any variations.

The SignalP analysis also showed a signal peptide probability of 0.987 with a cleavage site in between the 36^{th} and 37^{th} amino acid position, so that the mature protein starts from the 37^{th} amino acid residue. In general, basic β-1, 3-glucanases are often vacuolar and can be excreted through the secretory pathway. It usually contains a C-terminal extension with N- glycosylation which acts as a signal anchorage. The prediction of C-terminal glycosylation through NetCglyc server showed no probability hence there is no anchorage of these proteins in the membrane. Therefore it could be a protein which can be secreted into the surrounding medium(cytoplasmic) without anchorage.

For the full understanding of the structural and functional aspect of this gene isoform further studies are required in future. The clonal variations found

with the protein sequence even if it is minute, can lead to changes in structural conformations and their properties. Site directed mutagenesis can be performed in order to change the properties of the enzyme so that favourable characters can be developed. There is a possibility for nucleic acid variations in those portion; may be that leads to disease resistance in plants. Expression studies based on glucanase gene during diseased condition in both tolerant and susceptible clone will bring out further evidence.

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